Toxicological effects of metal oxide nanoparticles in combination with microbial components and inflammatory mediators on intestinal epithelial cells

by

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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TABLE OF CONTENTS

ABSTRACT

Inflammatory bowel disease (IBD) are inflammatory disorders with multi-factorial etiologies and many contributing factors (e.g., genetic disposition, luminal antigen, gut microbiota, and environment) that occur in about 1 in 300 persons in the United States and other western countries, but its etiology is still not well understood. Research has shown that maintenance of epithelial barrier plays an important role in mucosal homeostasis. Intestinal epithelial cells (IECs) play a critical role in formation and maintenance of the intestinal epithelial barrier which functions as a protective barrier from microbial components, environmental pollutants, food particles, and other luminal content and xenobiotics. As environmental factors can contribute to the onset or exacerbate the effects of IBD, there are not many studies investigating the impact of environmental factors, more specifically metal oxide nanoparticles found in the environment and food products, on the maintenance of the intestinal epithelial barrier. Furthermore, there are no studies investigating the impact of metal oxide nanoparticles in combination with microbial components or host inflammatory mediators on the health of intestinal epithelial cells.

Metal oxide nanoparticles, specifically manganese oxide nanoparticles (MnO NP) and titanium dioxide nanoparticles (TiO_2 NP), are used in everyday life in a variety of applications, including welding, magnetic resonance imaging, drug delivery, and food products. Despite the gastrointestinal (GI) tract being the major route of excretion of metal oxide nanoparticles, studies that assess the toxicity of these nanoparticles on intestinal epithelial cells (IECs) and their interaction with components of the GI tract, such as the microbiota and host inflammatory mediators, are virtually nonexistent.

In this research, we examine the toxicity of MnO NP in conjunction with bacterial lysate on IECs and TiO_2 NP in conjunction with host inflammatory mediators on IECs. In the studies in this dissertation, MODE-K cells, mouse intestinal epithelial cells, were exposed to nanoparticles (10 µg/ml MnO NP or 50 µg/ml TiO_2 NP) alone or nanoparticles plus bacterial lysate or nanoparticle plus host inflammatory mediators for the duration of the experiment. Taken together, data suggests that toxicological effects of metal oxide nanoparticles (MnO NP and TiO_2 NP) on intestinal epithelial cells were exacerbated in the presence of presence of microbial components and host inflammatory mediators.

CHAPTER 1. GENERAL INTRODUCTION

Inflammatory bowel disease (IBD) are inflammatory disorders with multi-factorial etiologies that occur in about 1 in 300 persons in the United States and other western countries, but its etiology is still not well understood. IBD describes a group of chronic intestinal inflammatory disorders that consist of complex interactions between gastrointestinal microbiota, genetics, immune system and the environment with the environment being the least studied of the four contributing factors. Research has shown that maintenance of epithelial barrier plays an important role in maintaining mucosal homeostasis. Intestinal epithelial cells (IECs) play a critical role in formation and maintenance of the intestinal epithelial barrier which functions as a protective barrier from microbial components, environmental pollutants, food particles, and other luminal content and xenobiotics. As environmental factors can contribute to the onset or exacerbate the effects of IBD, there are not many studies investigating the impact of environmental factors on the maintenance of the intestinal epithelial barrier.

With the increasing use of metal oxide nanoparticles in everyday life in a variety of applications, including welding, magnetic resonance imaging, drug delivery, and food products, there needs to be more studies assessing the impact of these nanoparticles on the intestinal epithelial barrier as the gastrointestinal (GI) tract is the major route of excretion of metal oxide nanoparticles. Together the research in this dissertation evaluates the impact of metal oxide nanoparticles in combination with microbial components or host inflammatory mediators on intestinal epithelial cells. By investigating the impact of metal oxide nanoparticles in combination with microbial

components and host inflammatory mediators, we are adding a level of complexity to the assessment of nanoparticle toxicity on intestinal epithelial cells that represents a more realistic representation of *in vivo* situation as intestinal epithelial cells do not interact with these nanoparticles in isolation.

Dissertation Organization

This dissertation is composed of 5 chapters, two of which will be submitted to journals. After this introductory chapter, Chapter 2 is a review of the literature relevant to this dissertation. Chapter 3 is a manuscript that will be submitted to the journal, Environmental Toxicology and Pharmacology. Chapter 4 is a manuscript to be submitted to Food and Chemical Toxicology. This dissertation also contains a general conclusions and discussion chapter that discusses the conclusions from Chapters 3 and 4 and future directions for research. This dissertation also contains an appendix that is a partial manuscript and it will be used as pilot data for future studies and grant proposals.

CHAPTER 2. LITERATURE REVIEW

Inflammatory Bowel Disease (IBD)

An increase in the occurrence of inflammatory bowel disease (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), has been observed in the western societies, making IBD one of the most common chronic inflammatory diseases in this population in recent years [(1)]. Epidemiological studies of IBD are important because various epidemiological data, such as differences in occurrence between different age groups, geographic distribution and particularly changes in incidence over time within one area, can offer important insight into pathogenesis, disease associations, health burden, and natural history of disease [(2)]. Therefore, epidemiologic and genetic research is crucial to understand the role of genetics and environmental factors in the development of IBD [(3)]. As genetic changes in the population does not explain the increase in GI disease seen in Western societies, it is necessary to start including the contribution of environmental factors as they are constantly and rapidly being altered and incorporated in everyday life. Smoking, oral contraceptives, diet, and appendectomy have all been studied as environmental factors that contribute to the risk or onset of IBD. However, the results from these studies do not completely explain the prevalence or increases in the incidence of IBD [(4)]. Many genes, such as NOD2 and TNFSF15, linked to IBD play critical roles in the normal function of the immune system. Additionally, genes such as tight junction proteins linked to epithelial integrity may play a role in the pathogenesis of inflammatory bowel disease as well, but this relationship is not well understood. One of the major pathological indicators of IBD includes an increased permeability of the intestinal epithelial barrier and chronic inflammation.

Cytokines play a critical role in the immune response and act as mediators of inflammation, with some being pro-inflammatory and others anti-inflammatory. In the inflamed intestinal mucosa, IBD patients were shown to produce elevated levels of pro-inflammatory cytokines such as IL-1ß, IL-6, IL-8, and TNFα [5]. Studies have shown that pro-inflammatory cytokines, including TNFα, IL-1β, and IFN-γ, cause an increase in the permeability of the intestinal epithelial barrier [6]. The understanding of these effects in IBD patients will contribute to the overall knowledge of IBD and the identification of intervention strategies.

Ulcerative colitis and CD, the primary disease etiologies associated with IBD, are precipitated by a complex interaction between environmental, genetic, and immunoregulatory factors [7]. Higher rates of IBD are seen in northern, industrialized countries, with the greater prevalence among Caucasians and Ashkenazic Jews [8-10]. Regardless of the underlying genetic predisposition, a growing body of data implicates a dysfunctional mucosal immune response to resident bacteria in the pathogenesis of IBD, especially CD. Possible triggers include a chronic inflammatory response precipitated by infection with a particular pathogen or virus or a defective mucosal barrier [5,6]. The characteristic inflammatory response results in an infiltration of neutrophils and macrophages, after the release of chemokines and cytokines from the intestinal epithelial cells. In this regard, the epithelial barrier plays an important role in the maintenance of mucosal immune homeostasis by controlling and preventing the immune response from decaying into unmanageable inflammation. There has been a growing body of data that have identified genes associated with defects in epithelial barrier function and epithelial permeability and disorders that are well recognized and

contribute to early onset IBD [11]. A study performed in mice showed that mice deficient in guanylate cyclase 2C (GUCY2C$^{-/-}$) exhibited intestinal barrier hyperpermeability associated with reduced expression of tight junction proteins. Conversely, activation of GUCY2C in mice reduced intestinal barrier permeability associated with increased expression junctional proteins [12]. In an *in vitro* model of polymorphonuclear leukocyte (PMN) transepithelial migration in the intestinal mucosa of CD patients, the expression and regulation of ADAM17 activity was higher both in the early phase of PMN transepithelial migration and in active CD [13]. This data indicates that genetic predisposition can alter the intestinal epithelial barrier and play a role in causing deleterious effects that can lead to the impairment of barrier function, induction of inflammatory cytokines and development of chronic inflammation and diseases caused by chronic inflammation such as IBD.

Immune Response and Cytokines

Cytokines, which can be pro-inflammatory or anti-inflammatory, are small secreted proteins released by cells that have a specific effect on the interactions and communications between cells. Cytokines are involved in a variety of biological processes, including cell activation, replication, and differentiation, and they are central to the development of inflammation and immunity [5]. The imbalance between pro-inflammatory and anti-inflammatory cytokines that occurs in IBD patients impedes the resolution of inflammation and leads to disease perpetuation and tissue destruction [14]. Inflammatory bowel disease, which includes CD and UC, represents a group of chronic disorders characterized by inflammation of the gastrointestinal tract (GIT), typically with a relapsing and remitting clinical course of disease. The intestinal inflammation in IBD is

controlled by a complex interplay of innate and adaptive immune mechanisms. Cytokines are key signals in the intestinal immune system, and are known to participate in the disruption of the so-called normal state of controlled inflammation (physiological inflammation of the gut) [15]. Activated macrophages are thought to be major contributors to the production of inflammatory cytokines in the gut, and an imbalance of cytokines contributes to the pathogenesis of IBD [15,16]. Cytokine responses are the key pathophysiologic elements that govern the initiation, evolution and, ultimately, the resolution of GI inflammation that characterize IBD. Cytokines are small peptide proteins produced mainly by immune cells that facilitate communication between cells, stimulate the proliferation of antigen specific effector cells, and mediate local and systemic inflammation via autocrine, paracrine, and endocrine pathways [17].

In IBD, the innate immune response plays a critical role. Activated dendritic cells (DC) and macrophages secrete cytokines that actively regulate the inflammatory response in UC and CD [18]. Once secreted by antigen presenting cells (APC) such as DCs and macrophages, these cytokines activate and induce the differentiation of T cells that results in the activation of adaptive immunity. T cell dysregulation is often a characteristic of IBD associated with the failure to clear overreactive and/or autoreactive T cells that may result from an imbalance between Treg, Th1, Th2 and Th17 cells populations in the affected tissue [16]. Proinflammatory cytokines are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions. There is abundant evidence that certain pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 are involved in the process of pathological pain [15,16,18,19]. IL-6 has been shown to play a crucial role in several processes essential

to intestinal epithelial cells. The intestinal epithelial barrier is composed of intestinal epithelial cells which are connected by tight junction proteins, adherens junctions, gap junctions, and desmosomes. All these components interact and regulate intestinal epithelial permeability which plays an important role in regulation of gut homeostasis. In particular, IL-6 produced by epithelial cells and intraepithelial lymphocytes at the onset of mucosal injury has been shown to be crucial for epithelial cell proliferation and wound healing [20]. Results from an *in vitro* study using Caco-2 cells showed that IL-6 modulation plays a role in regulating intestinal epithelial tight junction permeability is by decreasing the expression of claudin-2 [21]. There have also been studies in which IL-6 is required for survival of intestinal epithelial cells and development of colitis-associated cancer [22]. This shows that IL-6 is an important cytokine that plays a role in the function and response of the intestinal epithelial barrier and a major regulator of several functions during colonic inflammation.

Intestinal Epithelial Barrier

Intestinal mucosa is composed of different types of epithelial cells with specific functions (Figure 1).

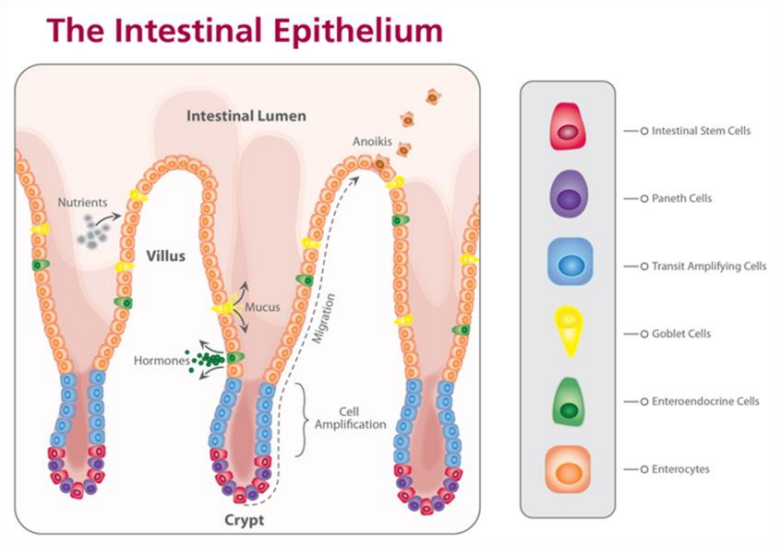


Figure 1. The intestinal epithelium and various intestinal epithelial cell types

The intestinal epithelium is populated by distinct types of cells derived from stem cells, such as the absorptive cells (enterocytes) and a variety of secretory cells including goblet cells, hormone-secreting enteroendocrine cells, Tuft cells, and antimicrobial peptides-secreting Paneth cells [23]. All but the Paneth cells mature and differentiate as they migrate from the crypts to the tips of the villi in the small intestine [24,25]. This continuous process of replication and differentiation replenishes the intestinal epithelium every 3-7 days and is important for the maintenance of epithelial function, integrity, and health [23, 26, 27]. As the GIT is constantly exposed to microbes and microbial antigens, the intestinal epithelium must be capable of rapid regeneration in the event of tissue damage. Disruption of the intestinal barrier have been shown to lead to or be present in the early onset of several immune-mediated diseases including IBD, food allergy, and celiac disease [28-30]. Epithelial cells control association of bacterial populations with the mucosal surface without disrupting the intestinal microbiota and are also capable of

modulating mucosal immune responses indicating their essential role in maintaining gut homeostasis. Consequently, the regulation of intestinal epithelial homeostasis is crucial for the maintenance of the structure and barrier function of the intestinal mucosa. As the gut mucosa is continuously facing a barrage of biological challenges including local and foreign antigen, environmental toxicants, and biological toxins, the intestinal epithelial barrier is the first line of physical defense in the intestine (Figure 2).

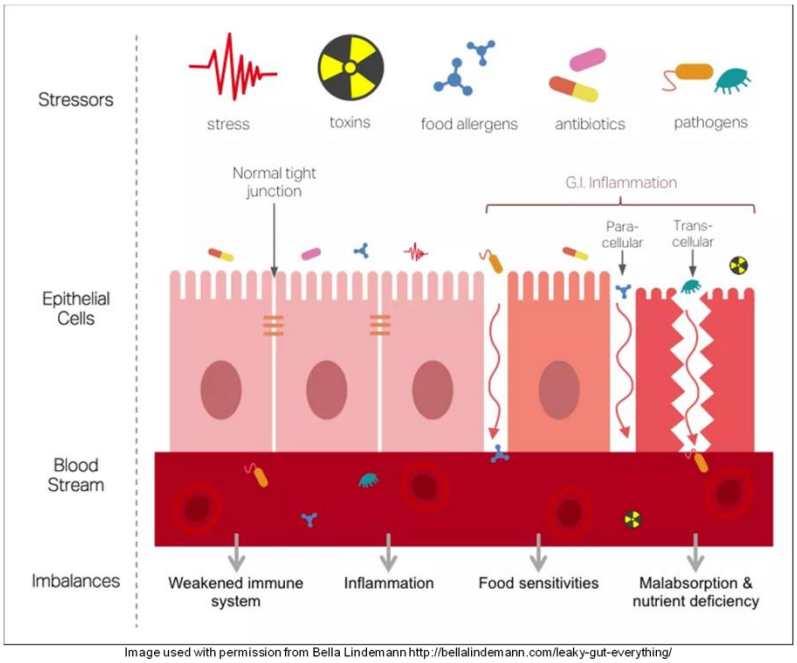


Figure 2. The intestinal epithelial barrier facing biological challenges

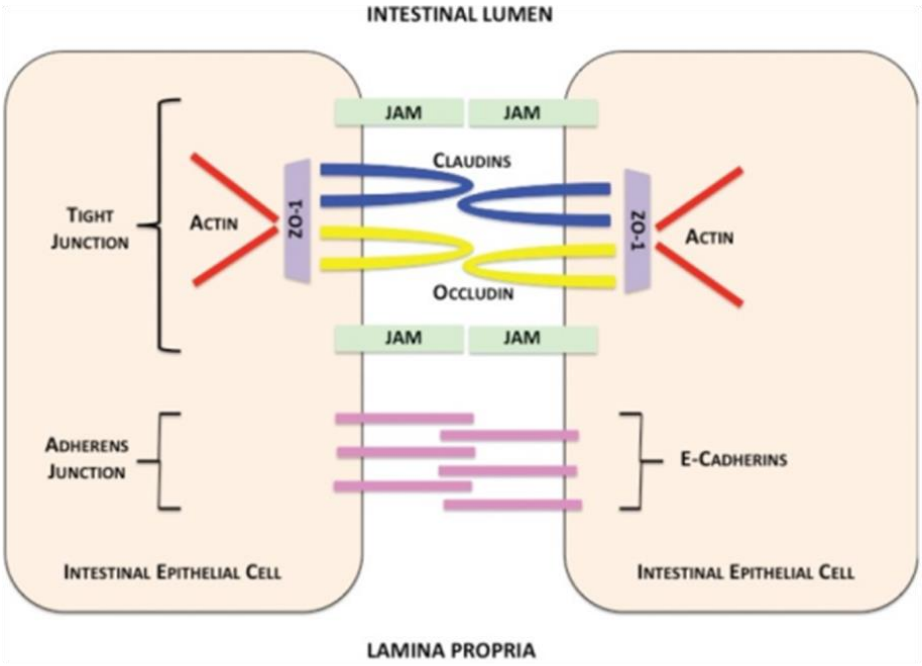
The intestinal epithelium and its permeability is regulated by the presence of tight junctions (TJs), comprised of claudins, occludins, and zona occludens (ZO), which ultimately regulates molecule passage through the paracellular spaces [(31)]. Factors that contribute to the integrity and proper function of the intestinal epithelial barrier includes, but are not limited to, resilient innate immune responses, intestinal epithelial cell

integrity, and epithelial paracellular permeability that is regulated by tight junction proteins [31]. Intestinal epithelial cells recognize foreign and indigenous matter and accordingly interact with mucosal immune cells to establish and create an immunologically balanced mucosa eventually establishing intestinal homeostasis [32]. Consequently, intestinal epithelial cells are at the forefront of the formation and continuance of a physiologically and immunologically balanced intestinal environment. In intestinal epithelial cells, the expression of tight junction proteins are critical to maintaining a healthy mucosal environment and important to an effectively functioning intestinal barrier [33]. As some of the challenges that the intestinal epithelium faces are going to come from unknown influences, there will also be some advantages and disadvantages that will be presented by microbiota both resident and transient. For example, resident, non-pathogenic members of the microbiota play a role in several intestinal epithelial cell processes, including intestinal epithelial cell turnover, promotion of epithelial restitution, and reorganization of tight junction proteins [34]. However, there are also disadvantages that can be presented by microbial products. Microbial products have been shown to interact with and interfere with tight junction processes, which then negatively affect the integrity of the intestinal epithelial barrier by compromising tightness of the intestinal epithelial barrier [35]. HT-29 cells exposed to staphylococcal enterotoxin B and cholera toxin for 48 hours resulted in an increase in claudin-2 which resulted in increased epithelial permeability [35]. As increased chronic inflammation is one of the main pathological indicators of IBD, there also seems to be a deleterious effect on the integrity of the intestinal epithelial barrier suggesting that there may be

polymorphism in genes linked to expression of proteins involved in maintaining intestinal epithelial barrier and tight junctions.

Tight Junction Proteins

The intercellular junctional complex, which consists of TJs, adherens junctions, gap junctions, and desmosomes, is critical for the maintenance of the intestinal barrier (Figure 3).



Hammer AM, Morris NL, Earley ZM, Choudhry MA. The First Line of Defense: The Effects of Alcohol on Post-Burn Intestinal Barrier, Immune Cells, and Microbiome. Alcohol Res. 2015;37(2):209-22.

Figure 3. Intercellular junctional complexes

TJs, which are composed of claudins, occludins, and zona occludens (ZO) proteins, are a multifunctional complex that create a seal between adjacent epithelial cells near the apical surface [36]. Due to interactions with external stimuli, such as food residues as well as pathogenic and resident bacteria, TJs are not static but are highly

active structures that are continuously being modified [37]. They can regulate the entry of nutrients, ions, and water while restricting microbial entry and, thus, are the central gate keepers of the paracellular spaces. The intestinal epithelium consists of mainly absorptive enterocytes (over 80%) but also include enteroendocrine, goblet, and Paneth cells [38]. These epithelial cells are tightly bound together by intercellular junctional complexes that regulate paracellular permeability and are crucial for the integrity of the epithelial barrier. Adherens junctions are located beneath the TJ and are involved in cell-cell adhesion and intracellular signaling [39]. Both TJ and adherens junctions (together known as the apical junctional complex) are associated to the actin cytoskeleton [39, 40]. Desmosomes and gap junctions are involved in cell-cell adhesion and intracellular communication, respectively [41, 42]. Claudins are tetraspan transmembrane proteins of TJs that determine the barrier properties of cell–cell contact and paracellular tightness and the selective ion permeability existing between the plasma membranes of two neighboring cells [43]. Claudins can completely tighten the paracellular cleft for solutes, and they can form paracellular ion pores [43, 44].

Claudins, specifically claudin-2 which has a pore radius of 3.25–4 angstroms (Å), can affect and modify the uptake of water and other solutes [44-46]. Claudins are mainly responsible for the “pore” pathway, and the two extracellular loops work as an “electrostatic selective filter” to select the size and charge for small pores whereas an increase in the expression of pore forming claudins often leads to barrier dysfunction [44]. Claudins play a role in forming pores that make the intestinal epithelial barrier permeable while they also play a role in creating seals which makes the intestinal barrier impermeable. Claudins 2, 7, 10, and 15 have been identified as pore forming

claudins while claudins 4, 5, 8, 11, and 14 selectively decrease the paracellular cation permeability through TJ therefore identified as seal forming claudins [44]. The regulation of TJs is very critical to barrier maintenance and barrier dysfunction can lead to a change in expression of TJs and an influx of toxic agents crossing the intestinal epithelium resulting in tissue damage and/or inflammation with detrimental effects to gut homeostasis.

As the intestinal epithelial barrier function is an important aspect in gut homeostasis, there are several agents that can interfere with and affect transepithelial electrical resistance (TEER) and expression of TJ proteins, ultimately affecting intestinal epithelial barrier permeability. Included in many of these toxic agents are nanoparticles, such as silver nanoparticles. Using T84 cells as an *in vitro* model of the human GI epithelium, results showed that, silver nanoparticles between 10 nm and 110 nm in size had significant effects on intestinal permeability by altering TJ function as measured by a decrease in TEER [47]. Without being toxic to cells, exposure to silver nanoparticles has been shown to significantly affected gene expression of NOTCH1 and ZO-2 which are involved in anchoring TJs, cellular proliferation, and signaling [47]. In another study using both *in vitro* (Caco-2, human intestinal epithelial model) and *in vivo* (mouse) models, results provided direct evidence that intestinal epithelial tight junctions can be opened transiently and reversibly by chitosan nanoparticles that affected the trans-membrane junctional adhesion protein (JAM-1), which directly associate with the zona occludens [48]. Such effects have the potential to compromise the integrity of the intestinal epithelium and a disruption of gastrointestinal homeostasis [47].

In contrast, some nanoparticles do not possess cytotoxicity characteristics; however, even without the adverse cytotoxic effects on intestinal epithelial cells, nanoparticles can alter or downregulate the expression of TJ proteins critical for maintenance of the intestinal epithelial barrier [47, 48]. By impairing the intestinal epithelial barrier, toxic agents from the lumen will be able to freely cross the epithelial barrier resulting in an adverse response with downstream consequences including inflammation and oxidative stress which can then lead to more serious health consequences, such as IBD and other forms of chronic disease.

Gut Microbiota

Through a complex interaction with the host, gut microbiota influences several essential functions of the GI epithelium including functional maturation of the physical barrier, enhancing and maturation of the mucosal immune responses, and detoxifying harmful intestinal content [49-51]. In addition, the gut microbiota enhances the host's metabolic capacity for processing nutrients and drugs and modulate multiple metabolic pathways in a variety of organ systems [51]. Because a disruption to the balance of the microbiota (e.g., dysbiosis) is associated with an increased risk of developing inflammatory diseases, allergic diseases, and metabolic disorders, it is very important to understand how changes in microbiota affect GI disease conditions, facilitate the clinical interpretation of treatments with xenobiotics, and assess the relationship between the environment and disease susceptibility [52-54].

In vivo studies performed in germ-free mice have shown that intestinal inflammation and developmental aspects of the adaptive immune system are influenced by intestinal bacterial colonization [52, 55-57]. Furthermore, there is bidirectional

communication between the microbiota and the host's epithelial cells via secretion of quorum-sensing molecules or miRNA that may have direct effects on the epithelium [52, 58]. Microbiota plays a role in metabolizing nonabsorbed dietary carbohydrates, exfoliated epithelial cells, and mucus to produce metabolites, such as butyrate, that greatly influence intestinal epithelial energy needs and regulation of immune response (e.g., Treg cells) [58-60].

Intestinal Epithelial Cells and Cell Culture Models

The abundance of innate and adaptive immune cells that exist together with the microbiota in the mammalian GIT requires barrier and regulatory mechanisms that maintain a balance between host–microbial interactions. This homeostasis depends on the diverse functions of intestinal epithelial cells (IECs) including the physical segregation of resident bacteria in the lumen from the host and the integration of microbial signals such as angionenins [61, 62]. Consequently, the various types of IECs present in the GI epithelium are critical in mediating intestinal homeostasis that permits the development of an immunological environment tolerant to colonization by a diverse microbiota.

The intestinal epithelium is populated by distinct types of cells derived from stem cells, such as the absorptive cells (enterocytes) and the secretory cells (mucus-secreting goblet cells, hormone-secreting enteroendocrine cells, Tuft cells, and antimicrobial peptides-secreting Paneth cells) [23]. Enterocytes and colonocytes are the most abundant cell type in both small and large intestines, respectively. Their primary function is to absorb nutrients apically and export them basally [63]. Another type of cell present in the intestinal epithelium is the M (membranous or microfold) cell, which are

critical for sampling of intestinal microbiota and for transferring pathogens across the epithelial barrier for and facilitate presentation of microbes to underlying lymphocytes, macrophages and dendritic cells [64]. Another type of cell present in the intestinal epithelium are goblet cells, which are the most abundant secretory cells in intestinal epithelia. Goblet cells comprise about 10-15% of the small intestinal epithelium and up to 50% of the colonic epithelium and their main function is to produce and secrete mucus that provides the epithelial cells with protective shield against toxic luminal contents [63, 65]. Paneth cells are another intestinal epithelial cell type located in the base of the crypts that produce and secrete antimicrobial peptides and proteins into the lumen. Paneth cells are unique in several respects compared to other secretory cells of the intestine [63]. Paneth cells also secrete factors that help sustain and modulate the epithelial stem and progenitor cells that cohabitate in the crypts and rejuvenate the small intestinal epithelium [66]. Another cell type found in the intestinal epithelium is the enterochromaffin cell. Enterochromaffin cells are responsible for the release of many hormones, mainly serotonin, that play a critical role in the regulation of intestinal motility [67]. Enteroendocrine cells, which consists of at least 15 different subtypes and comprise approximately 1% of the small and large intestinal epithelium, are scattered throughout the mucosa as individual cells that produce and secrete multiple hormones including motilin and secretin which elaborately control physiological and homeostatic functions in the digestive tract such as GI motility and digestion [68, 69].

With the varying cell types present in the intestinal epithelium, there are many *in vitro* cell culture models that are used to study and assess various functions of the intestinal epithelium. Caco-2 cells, which are derived from a heterogenenous human

epithelial colorectal adenocarcinoma, are a well characterized intestinal *in vitro* model widely used to evaluate the ability of chemicals to cross the intestinal barrier and their transport mechanisms [70]. Permeability values estimated with this model correlate well with human in vivo absorption data for many drugs and chemicals [70]. Another cell type used as an *in vitro* model to study epithelial membrane permeability is MDCK (Madin−Darby canine kidney) cells. MDCK cells are used as possible tool for assessing the membrane permeability properties of early drug discovery compounds due to their absorption properties and rapid cell replication properties [71]. Another cell type frequently used in *in vitro* intestinal studies are T84 cell line. The T84 colonic adenocarcinoma cell line, which has been used extensively as a model for studies of epithelial chloride secretion, also produces mucin and secretes it in culture and is used as a model to assess the extent to which regulatory mechanisms involved in the secretion of mucin from goblet cells [72]. One more cell type frequently used in *in vitro* intestinal studies are HT-29 cells. HT-29 cells, which originate from a human colon adenocarcinoma, represent a useful model system to study epithelial differentiation in vitro because they are highly unpolarized and undifferentiated making them a useful to study the events leading to the polarization of epithelial cells in a monolayer. [73]. Another cell line that exists that is a suitable *in vitro* model to study function of intestinal epithelial cells are MODE-K cells. MODE-K cells, which are SV40 virus transformed, are derived from normal small intestinal cells of mice is another cell line. MODE-K cells express MHC class I and/or class II molecules and retain most of the phenotypic and functional properties of normal enterocytes making them a suitable model for studying the molecular basis of epithelial cell interactions [74].

Nanotoxicology

With the introduction of the nanotechnology field and nanoparticles, nanoparticles possess the potential to revolutionize the biomedical field because of their capability to impact organs and tissues at the molecular and cellular levels. Nanomaterials have been promoted as a revolutionary technology for cell and tissue engineering, medical device development, and the encapsulation and delivery of drugs, vaccines, diagnostic reagents, and genes [75]. However, current research is fixated on the therapeutic applications of nanoparticles while disregarding many of the potential adverse environmental impacts. For example, despite the prevalent usage and generation of nanoparticles, information regarding cytotoxic effects and possible health hazards associated with nanoparticles use is extremely limited. In fact, toxicity issues related to nanomaterials used in nanomedicine are often ignored [76]. Thus, along with the development of novel nanomaterials, experts in related scientific fields are calling for a simultaneous assessment of the toxicological and environmental effects of nanoparticles as studies have shown the use of medical products containing nanoparticles and nanomaterials may lead to chronic health risks [75, 77, 78]. Patients with chronic diseases, such as chronic obstructive pulmonary disease, asthma, and cardiovascular disease might be helped by nanomedicine but it is just such patients whose health is adversely affected by nanoparticles in the environment [77]. This shows that nanoparticles possess the capability to target and penetrate the barrier of specific organs and cells may contribute to their associated toxicity highlighting the need for more studies to assess their toxicological and biological effects.

Nanotechnology is a rapidly growing field that has many potential advantages in various fields. With the goal to improve magnetic resonance imaging and appearance of foods, manganese oxide nanoparticles (MnO NP) and titanium dioxide nanoparticles (TiO_2 NP), have played in important role in advancing these fields [79-84]. MnO NP is a better T1 contrast agent than the current contrast agent that is used to capture and develop images in magnetic resonance imaging (MRI) while TiO_2 NP enhances the appearance of food products. The increasing interest in nanoparticles, such as MnO NP for advanced technologies and biomedical applications including MRI, drug delivery, and oncology, has led to great excitement about potential benefits but also concern over the potential for adverse human health effects [85]. With the growing concerns that have arisen about unintentional environmental and biological effects of nanoparticles, there needs to be further reevaluation of the toxicity that these MnO NP may have on biological systems [86]. For example, metal-based nanoparticles could contribute to adverse health conditions including concentration dependent alterations in gene expression and other critical physiological processes, such as mitochondrial function and inflammatory response, due to improper intracellular trafficking and accumulation [87].

More complete understanding of the basis of metal based nanoparticles toxicity is essential to elucidate the occupational, environmental, and health exposure risk-assessments related to NP toxicity. Understanding underlying process and mechanisms from administration to excretion must be completely assessed before large-scale production and use of NPs are safely and efficiently applied in their respective field of medicine. Regardless of whether it is direct and intentional ingestion or administration,

the GIT has been shown to be a possible route of entry or excretion for some of these nanoparticles including MnO NPs [88, 89]. However, studies that take into consideration the interaction between gut microbiota and environmental factors (e.g., nanoparticles) are extremely limited. Furthermore, disruption of the gut microbiota has been linked with GI conditions such as inflammatory bowel disease [90]. Therefore, assessing the effects of nanoparticles on the intestinal epithelium in the presence of gut microbiota and bacterial components presents a holistic and more realistic approach to assess the effects of NPs on intestinal epithelial cells as dysbiosis or cytotoxic effects on intestinal epithelial cells caused by nanoparticles could potentially worsen GI inflammation.

Manganese Oxide Nanoparticles

Manganese (Mn) is found in all organisms and has critical roles in cell survival and death mechanisms by regulating Mn-containing enzymes such as manganese superoxide dismutase (MnSOD) [91]. Experimental studies using human B cells and human neuroblastoma cells show that excess manganese exposure leads to accumulation of manganese in mitochondria and cell death occurs with features of an apoptotic mechanism that included the activation of caspase-8 [92, 93]. However, with the goal of aiming to improve several areas in the biomedical and environmental fields, manganese based nanoparticles have been studied for many various applications. Manganese oxide nanoparticles (MnO NP) are promising materials used as contrast agents for magnetic resonance imaging (MRI), drug delivery, an ionization-assisting reagent in mass spectroscopy, and waste water treatment [79-84, 94]. In a study using breast cancer cells, the use of MnO NP in magnetic resonance imaging results in better images because they possess enhanced image signal making them better T_1 contrast

agents than the current use of gadolinium-based nanoparticles [79-83]. Furthermore, MnO NP have also been used for intracellular delivery of siRNA while simultaneously being used as a T_1 contrast agent [79, 83]. However, research has shown that these MnO NP can negatively affect and alter functions of many organs.

Results from an *in vivo* study performed in rats that were intracerebrally injected with MnO NP showed negative changes in the locomotor and spatial memory abilities that were associated with dopaminergic neuronal impairment and inflammation [95]. In a separate study evaluating the acute MnO NP effects on liver cells, results revealed increased levels of lactase dehydrogenase, an indicator of cell damage, in rat liver cells [96]. Furthermore, an *in vivo* study performed in rats dosed with MnO NP for 30 days revealed decrease levels of antioxidant activity, damaged hepatocyte membranes, and protein synthesizing abnormalities in the liver [97]. Deleterious effects on liver cells revealed by those studies suggest that MnO NP can acutely and chronically affect an organ with critical metabolic functions including the detoxification of various xenobiotics. In a different study assessing oral administration of MnO NP to rats for 28 days, it was shown that MnO NPs were recovered in feces showing the GI tract as a route of excretion. Furthermore, this study also indicated that prolonged exposure to MnO NP resulted in genetic damage, biochemical alterations and histological changes in the kidney and spleen [89]. However, while this study investigated systemic effects, they did not investigate the effects of MnO NP on the GI tract.

Results from these various *in vitro* and *in vivo* studies have shown that MnO NP can cause deleterious effects across multiple cellular components (e.g., mitochondria), tissues, and organ systems including the brain, liver, kidney, and spleen. However, as

the GIT has been shown to be a major route of exposure for MnO NP, no study has assessed the effects of MnO NP on the intestinal epithelial cells, which is crucial in gut health and formation of the of intestinal epithelial barrier.

Titanium Oxide Nanoparticles

Titanium dioxide nanoparticles (TiO_2 NP) less than 100 nm in diameter are commonly found in many consumer food products with a ranging daily intake between 15-37.5 mg per day for a 75 kg adult [98-100, 104]. Despite being found in many food products consumed, little is known about the fate, toxicity, and mechanism by which TiO_2 NP affect cellular functions of intestinal epithelial cells and the maintenance of the epithelial barrier. Because of novel properties offered by their small size, TiO_2 NP are introduced in a rising number of food products (www.nanotechproject.org) as an authorized food colorant additive [100]. However, TiO_2 NP has been shown to have deleterious effects. TiO_2 NP pretreated with digestive fluids inhibit the growth of undifferentiated Caco-2 cells [101]. In addition, uptake of TiO_2 NP by enterocytes and paracellular passage across intact epithelia have been demonstrated [102]. This process occurs diffusely along the length of the GIT and total uptake may be important due to the large surface area of the epithelium [103]. As nanoparticles can affect cellular components, such as the mitochondria, cellular uptake and accumulation of nanoparticles derived from heavy metal toxicants can cause toxicity including apoptosis. Reactive oxygen species (ROS) production by cells exposed to TiO_2 NP has been evidenced and considered as the main mechanism of cellular toxicity in various cell types [104-106].

Several studies established the consequential effects of TiO_2 NP exposure on cells and tissues, such as ROS induction and inflammatory cytokine production [104-108]. TiO_2 NP have also been shown to be absorbed from the GIT [99,109]. Results of an *in vivo* study proposed that TiO_2 NP uptake across the GIT to take place via the Peyer's patches, due to the high presence of TiO_2 NP found in the underlying lymphoid tissues [99, 109, 110]. TiO_2 NPs could translocate across both the regular intestinal epithelium and through Peyer's patches resulting in NP persistence in gut cells with the potential to induce chronic damage [103]. In a study using Caco-2 cells and mice, consequences of chronic damage induced by TiO_2 NP included changes in expression of genes necessary for cell survival, up-regulation of genes involved in inflammation, apoptosis and cell cycle, and with down-regulation of genes involved in energy metabolism [104]. Understanding the effect of TiO_2 NP on epithelial cells and intestinal barrier and absorption of these nanoparticles is essential and vital in order to fully elucidate the safety of orally administrated TiO_2 NP [101].

Organoids

Intestinal epithelial cells play a role in the maintenance the intestinal epithelial barrier that acts as a physical barrier between our body and exogenous substances. Cytotoxic environments in the GI tract can cause impairment and death of the epithelial cells which results in the breakdown of this barrier function which can lead to uncontrollable inflammation increasing the risk of developing inflammatory diseases such as IBD. Until recently, intestinal epithelial-damaging activities of environmental toxicants, drugs, and treatments could be tested only in vivo in animal models because of the poor survival rate of primary IECs ex vivo [111]. The three-dimensional culture and

outgrowth of intestinal crypt stem cells into organoids have offered new possibilities to culture and study IECs ex vivo. Organoids represent an important bridge between traditional 2D cultures and *in vivo* mouse/human models, as they are more physiologically relevant than monolayer culture models and are far more amenable to manipulation of niche components, signaling pathways and genome editing than *in vivo* models [112]. The use of stem cells has allowed researchers to intensively study the processes that govern embryonic development, lineage specification and tissue homeostasis, as well as the mechanisms that contribute to the onset and manifestation of disease. Furthermore, organoids can be used for modeling host–microbe interactions [113]. It also delivers invaluable mechanistic insight into the development of stem cells and their niches, while providing an opportunity to monitor their differentiation into mature functional lineages such as enterocyte, goblet cells, and enteroendocrine cells [114]. Organoids ultimately provide an opportunity to investigate the effects of environmental toxicants, drugs, and treatments on the development of the intestinal epithelial barrier during normal and disease conditions while incorporating a more holistic approach that can be manipulated to mimic the intestinal environment that includes host-microbial interactions.

Mitochondrial Function in Intestinal Cells and Maintenance of Intestinal Epithelial Barrier

Intestinal epithelial cells play a critical role in formation and maintenance of the intestinal epithelial barrier which functions as a protective barrier from xenobiotics and foreign substances and function as a key regulator of intestinal homeostasis. Balanced and dynamic interactions among mucus layers, intestinal epithelial cells, microbiota, and

host immune defense is essential for the maintenance of the intestinal mucosal homeostasis [115, 116]. The disruption in the intestinal homeostasis (e.g. excessive inflammation, impaired intestinal epithelial barrier) results in the defective mucus barrier with increased permeability that results in inflammation and injury of the intestinal mucosal cells [117,118]. Therefore, the lining of the intestine, which is composed of intestinal epithelial cells, is renewed at an extraordinary rate, outpacing all other tissues in the vertebrate body [119]. Basement membrane flow, mitotic pressure and active flow here have been the three general mechanisms identified in the proliferation of intestinal epithelial cells, renewal of the intestinal epithelial barrier, and movement of the epithelium [120]. Disturbances in epithelial function are implicated in inflammatory and neoplastic diseases of the GIT. Control of intestinal epithelial stemness is crucial for tissue homeostasis and the mitochondrial function plays a critical role in maintaining intestinal epithelial stemness, which is the self-renewing capacity of the stem cells which are within the base of the crypt to migrate upward towards the lumen of the gut as they mature [121]. Furthermore, mitochondrial function is a key player in maintaining the luminal redox status of the glutathione/glutathione disulfide (GSH/GSSG) and cysteine/cystine (Cys/CySS) couples, which support luminal nutrient absorption, mucus fluidity, and a diverse microbiota crucial for maintaining intestinal epithelial integrity [122-124].

Mitochondria play an important role during the interaction of gut microbiota with the host cell and this mitochondrial activity may be an important factor that modulates microbiota diversity and quality, probably due to the role of mitochondria during the inflammatory and immune responses [125]. Mitochondria are the major source of cellular

reactive oxygen species (ROS) and can induce cytokine release depending on the ROS levels in the cell [125]. Interestingly mitochondrial ROS may also be involved in the regulation of the gut epithelial barrier. A study performed *in vitro* using colonic biopsy samples showed that induced mitochondrial dysfunction allowing transepithelial movement of bacteria across the intestinal epithelial barrier [126]. Furthermore, an example of possible mitochondria–microbiota functional interaction was shown when modification of mitochondrial energy metabolism in male rats, via diet, was associated with increased production of butyrate which have been known to be produced by gut microbiota [127]. The health and function of the mitochondria plays a crucial role in intestinal epithelial cells and ultimately the function and maintenance of the intestinal epithelial barrier and gut homeostasis. Therefore, there is a need for studies investigating the effects of xenobiotics and environmental toxicants that can affect mitochondrial function in intestinal epithelial cells while also interrogating the role of microbial components in disease pathogenesis as this would be a better representation of the holistic interactions occurring in the intestinal environment.

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CHAPTER 3. CYTOTOXIC EFFECTS OF MANGANESE OXIDE NANOPARTICLES IN COMBINATION WITH MICROBIAL COMPONENTS ON INTESTINAL EPITHELIAL CELLS

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Abstract

Manganese oxide has been shown to cause toxicity often referred to as manganism and is associated with occupational-related disease symptoms (e.g., welders). In addition, manganese oxide nanoparticles (MnO NP) are used in a variety of medical applications including drug delivery and magnetic resonance imaging (MRI) to obtain high resolution anatomical images of tumors and gastrointestinal (GI) inflammation. Along with the benefits, there are adverse consequences associated with the use MnO NPs as previously published. Additionally, GI research has shown that maintenance of the epithelial barrier plays a critical role in mucosal homeostasis and immunity as evidenced in a number of GI disorders including inflammatory bowel disease, which occurs in about 1 in 300 persons worldwide. However, studies assessing the interactions between MnO NP, the microbiota, and host inflammatory cytokines on mucosal homeostasis are virtually nonexistent. Using an in vitro model of GI homeostasis and epithelial wound healing, we examined the adverse effects of MnO NP alone or in combination with a bacterial lysate derived from *E. coli.* We hypothesized that treatment with MnO NP in combination with a bacterial lysate will synergistically

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induce adverse and deleterious effects on intestinal epithelial cells. Specifically, MODE-K cells, a murine intestinal epithelial cell line, were exposed to MnO NP alone or in combination with the bacterial lysate and/or inflammatory cytokines for 12, 24, or 48 hours depending on experimental procedure. Compared to MnO NP alone, these studies demonstrated that the addition of MnO NP plus bacterial lysate resulted in 1) exacerbated deleterious effects of MODE-K cells 2) severely inhibited cell migration and wound healing 3) enhanced secretion of inflammatory cytokines such as IL-6, and 4) induced mitochondrial dysfunction as evidenced by in mitochondrial respiration. While the bacterial lysate had no deleterious effect on the MODE-K cells, results demonstrated that there is a synergistic interaction between MnO NP and microbial components on intestinal epithelial cells that adversely affect epithelial integrity which can lead to a negative impact on mucosal homeostasis.

Introduction

As the gut mucosa is continuously facing a barrage of biological challenges including host-derived and foreign antigen, environmental toxicants, and biological toxins, the intestinal epithelial barrier is the first line of physical defense in the intestine. The epithelial barrier plays an important role in the maintenance of mucosal immune homeostasis by regulating inflammation [(1-4)]. The permeability of the intestinal epithelium is regulated by the presence of tight junctions (TJs) that ultimately regulate molecule passage through the paracellular spaces [(1)]. Intestinal epithelial cells (IECs) recognize foreign and indigenous matter and accordingly interact with mucosal immune cells to establish and create an immunologically balanced mucosa resulting in intestinal homeostasis [(2)]. Consequently, intestinal epithelial cells (IECs) are at the forefront of the

physiological barrier separating the host from the phlogistic components present in the intestinal lumen. In IECs, tight junction proteins are critical to maintaining a healthy mucosa and important to an effectively functioning intestinal barrier [3]. As the intestinal barrier is constantly faced with steady insult from microbial and host influences, there are going to be many challenges that have to be surmounted in the maintenance of the intestinal epithelial integrity that can potentially affect the mucosal homeostasis. As some of these challenges are going to come from multiple influences, there will also be some advantages and disadvantages that will be presented by the gut microbiota. For example, commensal microbiota, that inhabit our gut, plays a role in several IEC processes, such as the rate of intestinal epithelial cell turnover, promotion of epithelial restitution, and reorganization of tight junction proteins, that are critical for maintaining a healthy intestinal epithelial barrier [3]. However, there are also deleterious outcomes that can be induced by microbial products. Microbial products (e.g., enterotoxin) have been shown to interact and interfere with tight junction processes, which then negatively affect the integrity of the intestinal epithelial barrier by compromising the integrity of the intestinal epithelial barrier and induce antigen-related intestinal inflammation [4]. As chronic inflammation is one of the main pathological indicators of inflammatory bowel disease (IBD), the intestinal epithelial barrier and tight junction proteins play a major role in maintaining a balance between host–microbial interactions that can affect susceptibility to and the pathogenesis of IBD [5].

Disruption of the intestinal barrier and tight junction proteins (TJs), along with increased levels of proinflammatory cytokines, have been shown to lead to or be present in the early onset of intestinal diseases and disorders including IBD [39-41].

During normal homeostasis, proinflammatory cytokines in a normal, healthy human are not a problem because cytokines are involved in a variety of biological processes, including cell activation, growth, differentiation, and are essential to the regulation of inflammation and immunity [6]. However, the imbalance between pro-inflammatory and anti-inflammatory cytokines that occurs in IBD is what impedes the resolution of inflammation and maintenance of the intestinal epithelium which eventually leads to disease perpetuation and tissue destruction [7].

Inflammatory bowel disease, which includes Crohn's disease (CD) and ulcerative colitis (UC), represents a group of chronic disorders characterized by inflammation of the gastrointestinal tract (GIT). The intestinal inflammation characteristic of IBD is controlled by a complex interplay between innate and adaptive immune mechanisms [8]. Cytokines are key signals in the intestinal immune system, and are known to participate in the regulation of the controlled inflammation (physiological inflammation of the gut) [8]. Various cell types have been studied to evaluate toxicological effects and sensitivity of the intestinal epithelium to proinflammatory cytokines. In a study performed using Caco-2 intestinal cells, interferon gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) synergize to induce intestinal epithelial barrier dysfunction [9]. In a different study using MODE-K intestinal epithelial cells, TNF-α decreased cellular respiration and increased mitochondrial dysfunction which resulted in apoptosis of these epithelial cells [10].

Inflammatory mediators, such as host cytokines like TNF-α, can induce production of proinflammatory cytokines in IECs. In a study performed using IEC-6 intestinal epithelial cells, TNF-α was found to enhance both interleukin-6 (IL-6) mRNA

expression and protein secretion [11]. IL-6 is a pleiotropic, pro-inflammatory cytokine which plays an important role in promoting inflammatory response in the gut and in the systemic circulation [7, 8]. Elevated IL-6 serum levels have been detected in acute and chronic inflammation. Moreover, elevated IL-6 production has been associated with CD and UC [7]. As elevated levels of localized IL-6 in the intestine may be a characteristic feature of active IBD, intestinal epithelial cells are one of the major cell types responsible for the production of IL-6 [12]. Modulation of IL-6 levels in the GIT can undermine the integrity of the intestinal epithelial barrier by decreasing the expression of tight junction proteins and increasing intestinal barrier permeability [13, 14].

Nanotechnology is a rapidly growing field that has been shown to provide many advantages in various areas of medicine. With the goal of aiming to improve several technological areas, such as drug delivery and magnetic resonance imaging, the usage of nanoparticles, specifically metal nanoparticles including manganese oxide-based nanoparticles (MnO NP), have played in important role in advancing these fields [15-20]. The increasing interest in nanoparticles, such as MnO NP, for advanced technologies and biomedical applications has led to great excitement about potential benefits but also concern over the potential for adverse human health effects [21]. With the growing concerns regarding the unintentional environmental and biological effects of nanoparticles on the GIT, there needs to be further reevaluation of the toxicity that MnO NP may have on biological systems [22]. For example, metal-based NPs could contribute to adverse health conditions resulting from alterations of gene expression, dysregulation of physiological processes due to improper intracellular trafficking, and accumulation of NPs at toxic concentrations [23]. More complete understanding of the basis of metal-

based NPs toxicity is essential to elucidate the occupational, environmental, and health risk-assessments. Regardless of whether it is intentional or unintentional ingestion, the GIT has been shown to be the primary route of entry for metal nanoparticles including MnO NP which one can be exposed to during occupation (e.g. welding) or biomedical field (e.g. magnetic resonance imaging (MRI)) [24, 25]. Assessing the effects of nanoparticles on intestinal epithelial cells in the presence of bacterial components presents a more holistic, realistic model of exposure when assessing the effects of MnO NP on gut epithelial cells as the gut microbiota can influence cellular physiology, metabolism, and immune function [26]. However, studies that take into consideration the interaction between host epithelial cells, nanoparticles and microbiota are extremely limited. Because IECs are exposed to both microbial components as well as pro-inflammatory cytokines *in vivo*, we hypothesize that the combination of these factors will result in more realistic evaluation of the toxicity of environmental pollutants, such as MnO NP. Therefore, to mimic the *in vivo* environment, we examine the adverse effects of MnO NP in conjunction with bacterial lysate on intestinal epithelial cells. Results indicated that the health of intestinal epithelial cells was adversely affected when they were exposed to a combination of MnO NP and whole cell bacterial lysate than when they were treated with either stimulant alone. These results indicate that it is important to integrate the contributions of multiple stimuli on IEC health and ultimately intestinal barrier integrity in order to obtain a more representative impact of metal-based nanoparticles on the health and homeostasis of the GIT.

Materials and Methods

Chemicals

Manganese oxide nanoparticles (MnO NP (US Research Nanomaterials, Inc**.**, Houston, TX, USA)) used in all experimental procedures were kindly provided by Dr. A. Kanthasamy. According to product sheet associated with this product, range of MnO NP that were used were under 200nm in diameter.

Cell Culture and Treatment

The MODE-K intestinal epithelial cell line generated from the small intestine of a C3H mouse was used in all experimental procedures described below (kind gift from Dr. C. Elson). In this study, MODE-K cells were grown in high glucose (4.5 g/L) DMEM medium containing 10% FBS, 2 mM L-glutamine, 50 units of penicillin, and 50 µg/mL streptomycin. In general, cells were inoculated into a tissue culture plate (six to 96 well) depending on the experimental requirements and were cultured overnight in a humidified atmosphere of 5% CO_2 at 37 °C. The length of each experiment and MODE-K cell density for each experiment has been provided in the methods section for each experimental procedure. The four treatment groups for experimental procedures were: control group, 1 µg/mL bacterial lysate alone, 10 µg/mL MnO NP alone, and 10 µg/mL MnO NP plus 1 µg/mL bacterial lysate group. The culture medium was switched from high glucose medium to low glucose medium when cells were being prepared to be used in an assay the following day. All experiments were conducted in low glucose DMEM medium. Dose titration experiments were performed with the bacterial lysate and the MnO NPs and minimal dose (that did not affect cell viability after 24 hours) were

chosen in order to assess the interactions between the MnO NPs and the bacterial lysate.

Preparation of Bacterial Antigen

An adherent invasive *E. coli* (AIEC) strain LF82 that has been associated with ileal Crohn's disease was cultivated in BHI broth and incubated at 37 °C in an aerobic environment. Bacteria were harvested after overnight growth, washed with PBS, frozen, lyophilized, and stored at -20 °C until used. Bacterial cells were weighed and resuspended in sterile PBS (2 mg/mL) prior to being subjected to two freeze-thaw cycles, followed by sonication at 50 amps, 75 amps, and 100 amps for 30 seconds at each amperage. This sonication cycle was repeated three times. The lysates were sterilized by ultraviolet irradiation and sterility confirmed bacteriologically. With minor modifications, this method was used as previously described [27].

MTS Cell Viability Assay

Cell viability was measured using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation (MTS assay kit from Promega Corporation, Madison, WI). MODE-K cells were inoculated into 24-well plates with 120,000 cells/mL per well one day prior to treatment to achieve complete monolayer. The next day, MODE-K cell monolayers were washed twice with low glucose DMEM before addition of the respective stimulants diluted in low glucose DMEM medium for 48 hours. Following treatment, 20 µL of MTS solution reagent mix was added to each well and incubated at 37 °C for 30 minutes. At the end of incubation, readings were taken at a wavelength of 490 nm and another reference reading for each well was taken at 670 nm to eliminate background. This method was previously described and adopted to fit our experimental procedure [28].

Cytokine Release Analysis

Prior to assessing cell viability, supernatants were collected from MODE-K cell cultures at 24 and 48 hours after addition of the MnO NP or LF82 bacterial components and analyzed for the presence of cytokines. A multiplex assay was used to detect the presence of IL-6, TNFα, and IFNγ. The cytokines in each sample were measured using multiplex bead analysis. Standard curves were generated using Bio-Plex Manager Software Version 6.0 (Bio-Rad Laboratories, Inc., Carlsbad, CA, USA)

In vitro Wound Healing Assay

For one-dimensional migration assays, 300,000 MODE-K cells per well were suspended in DMEM (low glucose, 1.0 g/L) inoculated into 12 well plates and incubated at 37 °C in 5 % CO_2 overnight. The next day a (+) shaped scratch (i.e., monolayer wound) was created in each well using a P-200 pipet tip creating a cell free gap that, in time, would be covered by replicating/migrating MODE-K cells. Treatments were then added and cells were then incubated for 24 hours. Images were taken at 0 and 24 hours post-wounding. The four treatment groups were: medium alone (i.e., control), 1 µg/mL bacterial lysate, 10 µg/mL MnO NP, and 10 µg/mL MnO NP plus 1 µg/mL bacterial lysate. After 24 hours, experiments were terminated. Images were taken using a 5x lens on an inverted microscope Leica DMi1 (Leica Microsystems, IL, US) using the software Leica Application Suite 4.5.0 (Leica Microsystems, IL, US) from 10 representative wounded areas per treatment. Images and gap spacing were then measured and analyzed with an automation and image analysis software Metamorph 5.0.7 (Molecular Devices LLC, CA, US). Gap closure was quantified by determining the mean distance of closure (i.e., healing) of the gap. This was done by measuring the area of the cell-free

space separating the two edges of the cellular monolayer at 24 hours after addition of the treatments, and then subtracting this value from starting value that was measured at the 0-hour time point. This method was previously described and adopted to fit our experimental procedure [29].

Mitochondrial Superoxide Generation

Mitochondrial superoxide generation was measured using MitoSOX™ Red Mitochondrial Superoxide Indicator (Thermo Scientific). The four treatment groups were: medium alone, 1 µg/mL bacterial lysate, 10 µg/mL MnO NP, and the 10 µg/mL MnO NP plus 1 µg/mL bacterial lysate. MODE-K cells were plated in 96-well plates with 8,000 cells/well one day prior to treatment and allowed to attach overnight. The next day cells were washed 2 times with low glucose DMEM medium before addition of the respective treatments suspended in low glucose DMEM medium for a period of 24 hours. Following treatment, MitoSox reagent was added to each plate well at a 1:1000 concentration. Relative fluorescence intensity was then acquired using a microplate reader 24 hours post-treatment.

Mitochondrial Respiration

Mitochondrial respiration was measured using a Seahorse XF24e Extracellular Flux analyzer (Seahorse Bioscience, North Billerica, MA). The Seahorse XF24e Extracellular Flux analyzer is a high-throughput instrument that yields real-time measurements of cellular respiration rates. In our studies, we were interested in assessing the effects of four treatments on mitochondrial respiration and their effects on oxygen consumption rates (OCR). MODE-K cells were seeded at a density of 300,000/mL into a T-25 culture flask and incubated overnight in 5% CO_2 at 37.8ºC. The

next day, cells were treated with respective treatments for 24 hours. Stimulated cells were recovered from the plates by scraping and added to the Seahorse plates using CellTac at 60,000 cells per well and incubated for 1 hour to allow the cells to adhere. At this time, the mito-stressor reagents Oligomycin (1 µM), FCCP (2 µM) and Rotenone/Antimycin A (0.5 µM) were loaded into the corresponding injection ports of the Seahorse FluxPak cartridge and placed in the Seahorse analyzer to be equilibrated. After equilibration, the plate containing treated cells was then placed in the Seahorse analyzer covered with the Flux Pak cartridge. The analyzer was then programmed to measure the basal OCR readouts at five specified time intervals before progressing to inject the mito-stressors every three cycles of measuring OCR. Measuring mitochondrial respiration with the Seahorse analyzer allowed the measurement of the effect of each treatment at the three critical stages of cellular respiration.

Statistical Analysis

All statistical data analyses were performed using Prism 4.0 (GraphPad Software, San Diego, CA). Data were analyzed using one-way ANOVA with the Tukey post-test for comparing all treatment groups with that of the control. Multiple comparisons test was also performed to analyze differences between treatment groups. Differences with $p < 0.05$ were considered significant. All data is representative of two independent experiments containing six wells per treatment.

Results

Mitochondrial Superoxide Generation

The presence of reactive oxygen species (ROS) may negatively impact the crucial barrier function of the intestinal epithelium. Reactive oxygen species

are increased in the inflamed mucosa of IBD patients and may contribute to loss of intestinal barrier function [30]. Furthermore, during inflammation oxidative stress can lead to the induction of cell injury and apoptosis in intestinal epithelial cells [31].

To assess for mitochondrial ROS, we used MitoSOX assay. After 24 hours of incubation with the specific treatments, measurements were taken and data was normalized to percent control (Figure 1). Although there was no significant difference, there was approximately a 15% increase in mitochondrial superoxide generation in both treatment groups containing MnO NP (Figure 1). This data suggests that mitochondrial superoxide generation in intestinal epithelial cells appears to be an effect driven by the presence of MnO NP.

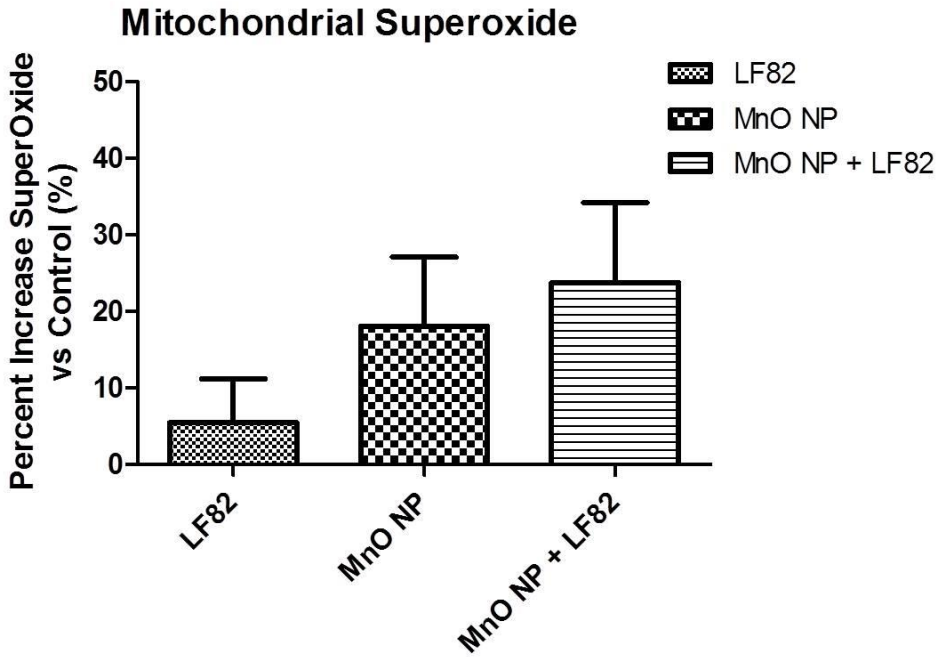

Figure 1. Mitochondrial superoxide generation was measured from MODE-K intestinal epithelial cells 24 hours post-treatment. Data was normalized to percent control. Data presented were obtained from 2 different experiments. This data suggests that mitochondrial superoxide generation in intestinal epithelial cells appears to have an effect driven by the presence of MnO NP. All data is representative of two independent experiments containing six wells per treatment.

Cytokine Release

Damage and impairment of the intestinal surface barrier may result in an increased permeability and absorption of toxic and phlogistic factors leading to inflammation and disequilibrium of GI epithelial homeostasis [34]. This inflammatory response can lead to the induction of cell injury and apoptosis of intestinal epithelial cells [31, 34].

After 24 hours of incubation, supernatants were harvested from MODE-K cell cultures to measure total cytokine secretion. However, IL-6 was the only cytokine detected. A significant effect was seen between treatment groups ($p < 0.05$) (Figure 2a). Post-hoc analysis (Tukey) indicated significantly ($p < 0.05$) higher IL-6 production when the 10 µg/mL MnO NP + 1 µg/mL of bacterial lysate Treatment group was compared to the control group. Furthermore, analysis revealed a significant increase in IL-6 secreted when the 10 µg MnO NP + 1 µg/mL LF82 treatment group was compared to the 10 µg/mL MnO NP alone treatment. Compared to NP or bacterial lysate alone, the results revealed a synergistic IL-6 release when MnO NP was combined with the *E. coli* bacterial lysate. Similar results were also seen after 48 hours of incubation (Figure 2b). Further comparison between the 24 hours and 48 hours samples revealed a threefold increase in the 10 µg/mL MnO NP + 1 µg bacterial lysate group suggesting that Mode-K intestinal epithelial cells were even more affected after extended exposure.

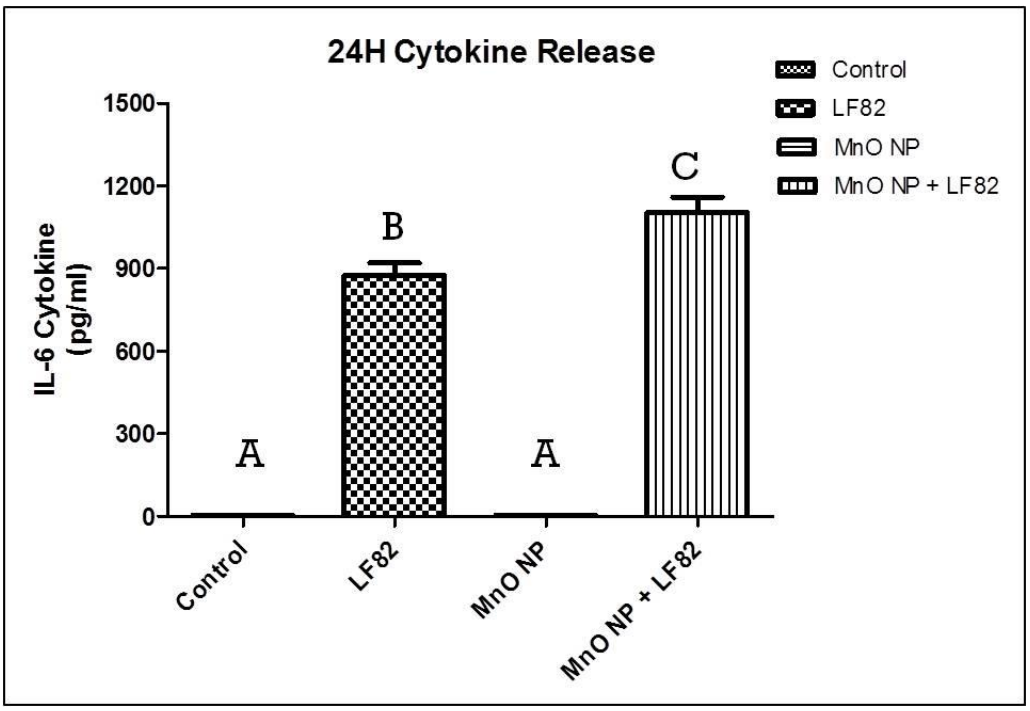

Figure 2A. Supernatants from each well were taken at 24 hours post-treatment and individually measured for IL-6 cytokine release from MODE-K intestinal epithelial cells.

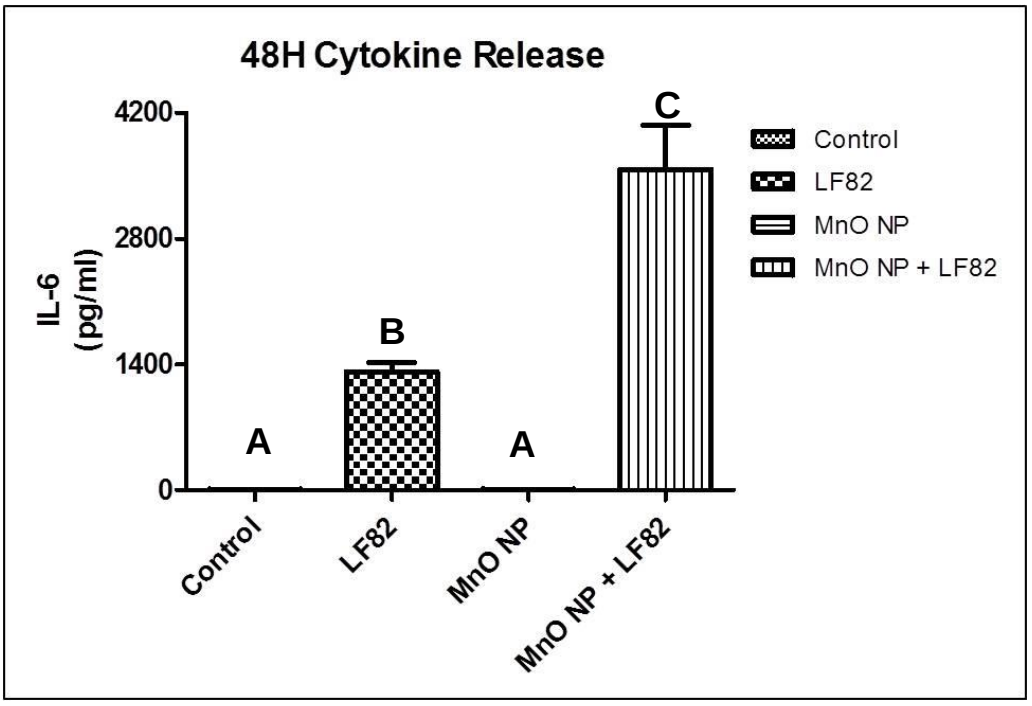

Figure 2B. Supernatants from each well were taken at 48 hours post-treatment and individually measured for IL-6 cytokine. IL-6 levels showed at three fold increase at 48 hour time point. All data is representative of two independent experiments containing six replicates per treatment. Different letter denotes difference in significant ($p < 0.05$) change.

Wound Healing

Wound healing is a crucial function of the intestinal epithelium as it can be injured by toxic luminal substances, inflammation, interactions with microbes, oxidative stress, and pharmaceuticals [30, 32]. Upon injury, the intestinal epithelium undergoes a wound healing process which is dependent on the balance of three cellular events; restitution, proliferation, and differentiation of immature epithelial cells adjacent to the wounded area [33]. Therefore, using a scratch wound assay, the effects of MnO NP on the wound healing process of MODE-K cells was assessed. Using a one-way ANOVA, a significant effect was observed between treatment groups and controls ($p < 0.05$) (Figures 3A and 3B).

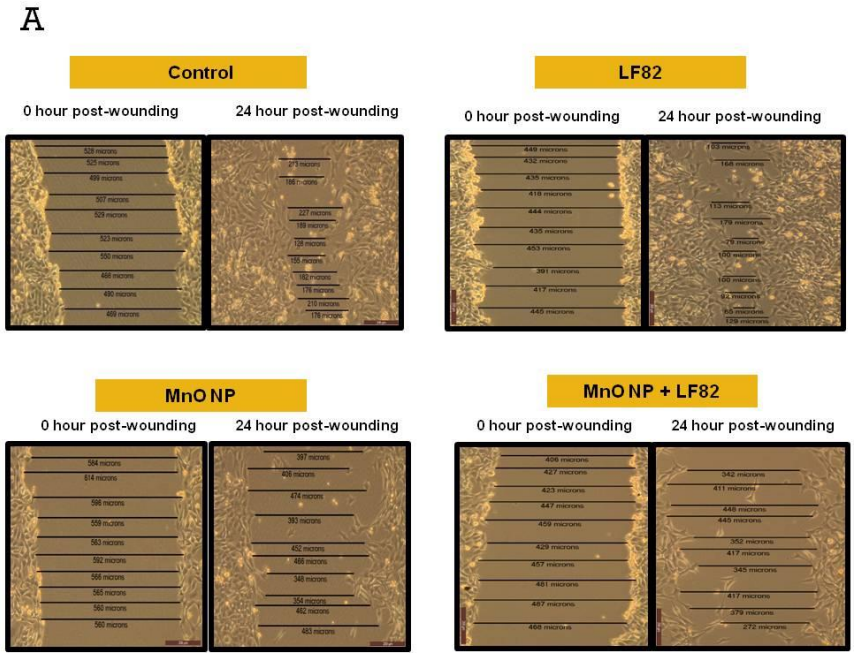

Figure 3. Wound healing assay served as an analysis of the effects of MnO NP on cell migration in MODE-K intestinal epithelial cells. MODE-K cells were incubated with treatment for 24 hours. Measurements were taken at 0 hours and 24 hours post-treatment (Figure 3A). Values were plotted as the difference between the timepoints (Figure 3B). Results are a representation of two independent experiments containing six replicates per treatment. Different letter denotes difference in significant ($p < 0.05$) change.

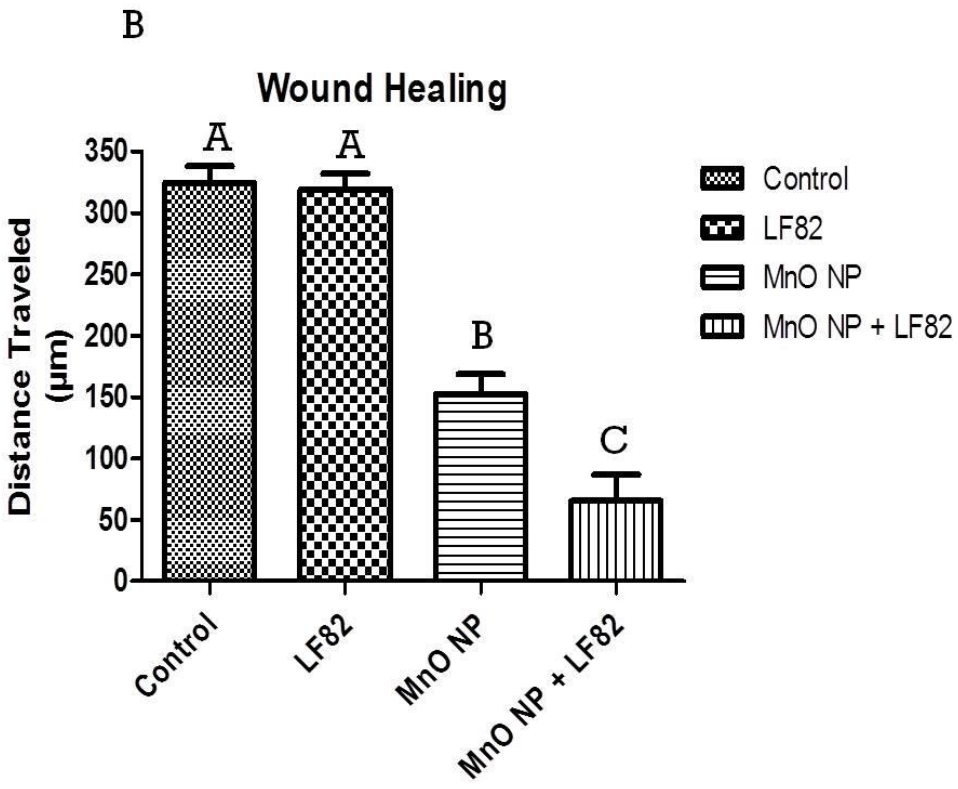

Figure 3 (continued) . Wound healing assay served as an analysis of the effects of MnO NP on cell migration in MODE-K intestinal epithelial cells. MODE-K cells were incubated with treatment for 24 hours. Measurements were taken at 0 hours and 24 hours post-treatment (Figure 3A). Values were plotted as the difference between the timepoints (Figure 3B). Results are a representation of two independent experiments containing six replicates per treatment. Different letter denotes difference in significant ($p < 0.05$) change.

Post-hoc analysis (Tukey) indicated a significantly ($p < 0.05$) slower wound healing response when the control group was compared to the 10 µg/mL MnO NP alone and the 10 µg/mL MnO NP + 1 µg/mL LF82 treatment groups. In addition, analysis indicated a significant difference in wound healing process when the 1 µg/mL LF82 treatment group was compared to the 10 µg/mL MnO NP alone and the 10 µg/mL MnO NP + 1 µg/mL LF82 treatment groups. Furthermore, analysis revealed a significant ($p < 0.05$) decrease in wound healing when the 10 µg/mL MnO NP + 1 µg/mL LF82 treatment group was compared to the 10 µg/mL MnO NP alone treatment group. An even more severe and significant ($p < 0.05$) decrease in distance traveled across gap

was seen when MODE-K intestinal epithelial cells were treated with MnO NP + LF82 compared to MnO NP alone suggesting a synergistic negative effect in cell migration (i.e., wound healing process).

Mitochondrial Respiration

Mitochondrial function plays a critical role in maintaining intestinal stemness and homeostasis [(35)]. As mitochondrial dysfunction is associated with a loss of proliferative capacity by intestinal epithelial cells, the effects on crucial factors of mitochondrial respiration were measured using a mitostress test. Utilizing the Seahorse XFe24 Analyzer, changes in basal respiration, maximal respiration, proton leak, ATP production, and spare respiration capacity were measured (Figure 4).

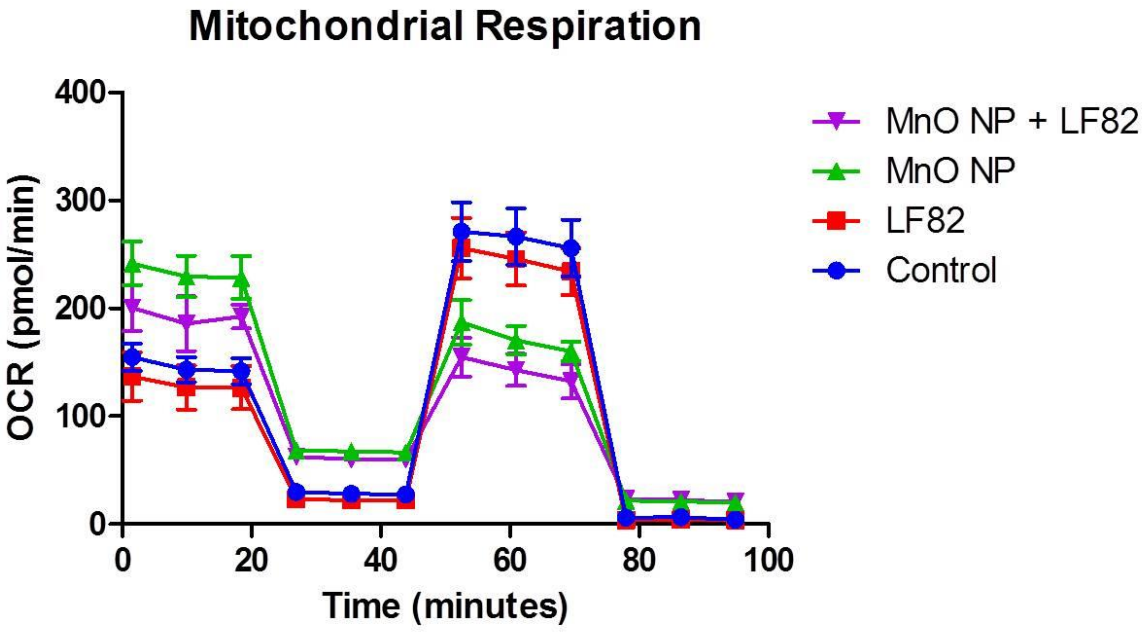

Figure 4. Mitochondrial respiration provides a robust test for analyzing the key parameters of mitochondrial function. Functional mitochondrial measurements are key to understanding cellular activation, proliferation, differentiation, and dysfunction. Any group containing MnO NP had higher oxygen consumption rates and impairment of mitochondrial dysfunction after 24 hours. Data signifies that dysfunction of intestinal epithelial cell mitochondria is a MnO NP induced effect.

Basal respiration is a measurement that shows the energetic demand of the cell under baseline conditions. Analysis showed significantly ($p < 0.05$) elevated levels of basal respiration in both treatments groups containing MnO NP when compared to medium and bacterial lysate alone, with the highest increase coming in the MnO NP (Figure 5).

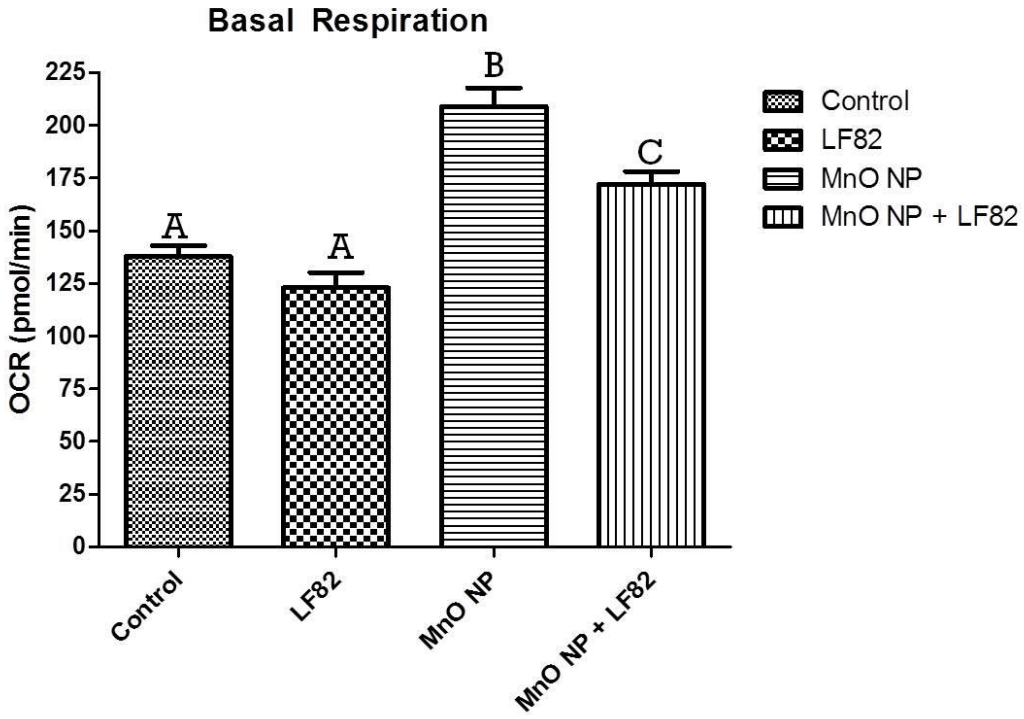

Figure 5. Basal respiration is a measurement that shows the energetic demand of the cell under baseline conditions. Basal respiration of MODE-K cells were measured after 24 hours of treatment incubation. Data shown is a representation of five independent wells. Significant ($p < 0.05$) difference is denoted by difference in letter.

Another parameter measured is maximal respiration, which is a measurement of the maximum rate of respiration that cells can achieve when operating at maximum capacity. Significantly ($p < 0.05$) lower levels of maximal respiration were seen in cells

treated with the MnO NP alone and even more severe effects were observed in the MnO NP + LF82 treatment group (Figure 6).

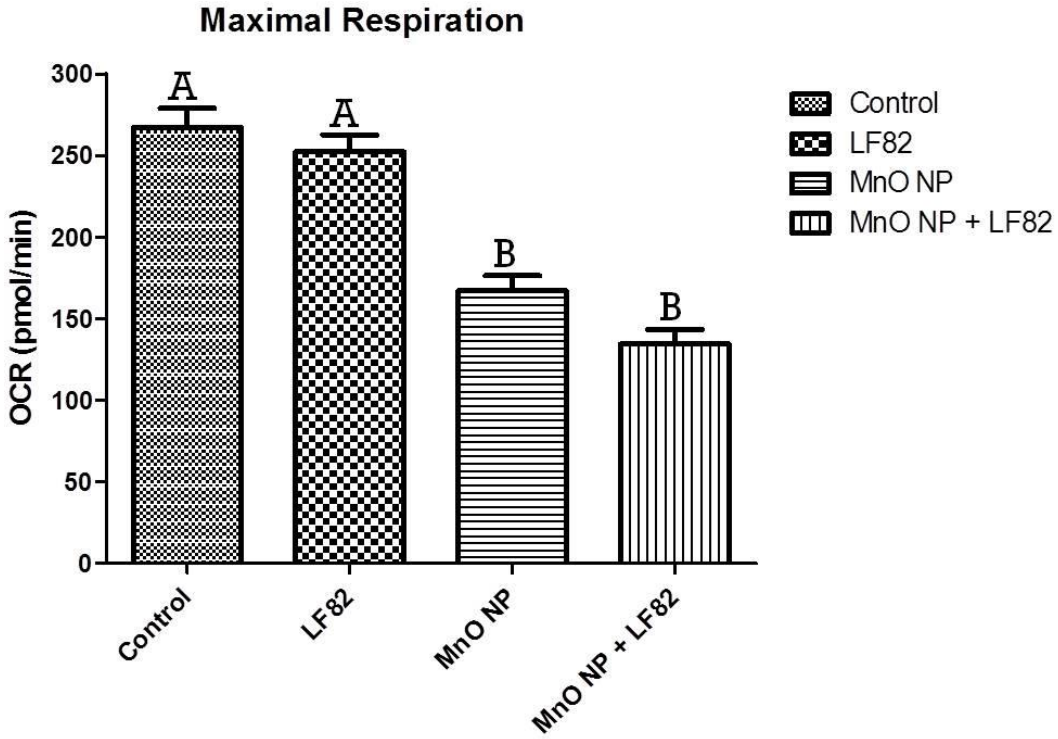

Figure 6. Maximal respiration is a measurement of the maximum rate of respiration that cells can achieve when operating at maximum capacity. Maximal respiration of MODE-K cells were measured after 24 hours of treatment incubation. Data shown is a representation of five independent wells. Significant ($p < 0.05$) difference is denoted by difference in letter.

However, in the presence of MnO NP + bacterial lysate, cells experienced a greater decrease in maximal respiration, although not statistically significant, indicating that the presence of microbial components further impacted mitochondrial respiration. Another parameter measured in the mitostress test is mitochondrial proton leak. Mitochondrial proton leak can have a major impact on mitochondrial oxidative phosphorylation coupling efficiency and production of reactive oxygen species. MnO NP caused a significant effect ($p < 0.05$) in mitochondrial metabolic activity as evidenced by

the increase in proton leak in both groups containing MnO NP suggesting that this is a MnO NP driven effect (Figure 7).

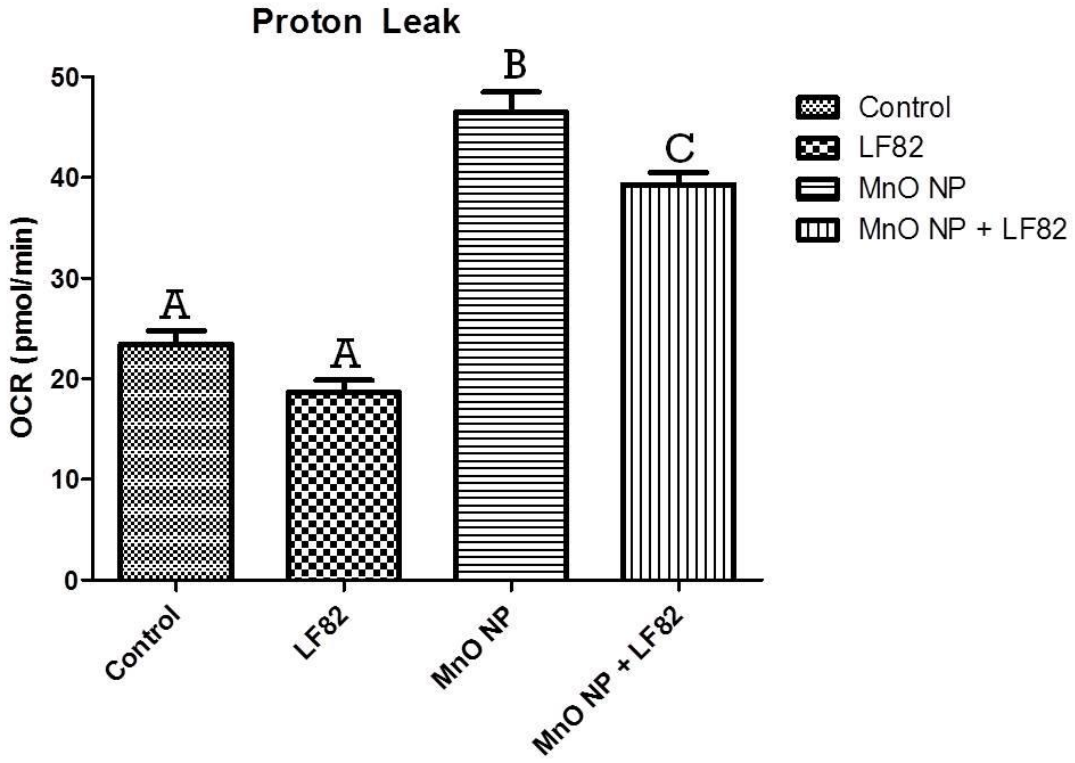

Figure 7. Mitochondrial proton leak has a major impact on mitochondrial coupling efficiency and production of reactive oxygen species. Proton leak of MODE-K cells were measured after 24 hours of treatment incubation. Data shown is a representation of five independent wells. Significant ($p < 0.05$) difference is denoted by difference in letter.

ATP production was another parameter that is measured during the mitostress test. MnO NP induced higher oxygen consumption rates as evidenced by the significant ($p < 0.05$) increase in ATP production when compared to control and bacterial lysate alone, with even more significant seen in the MnO NP alone treatment group when compared to MnO NP + bacterial lysate (Figure 8).

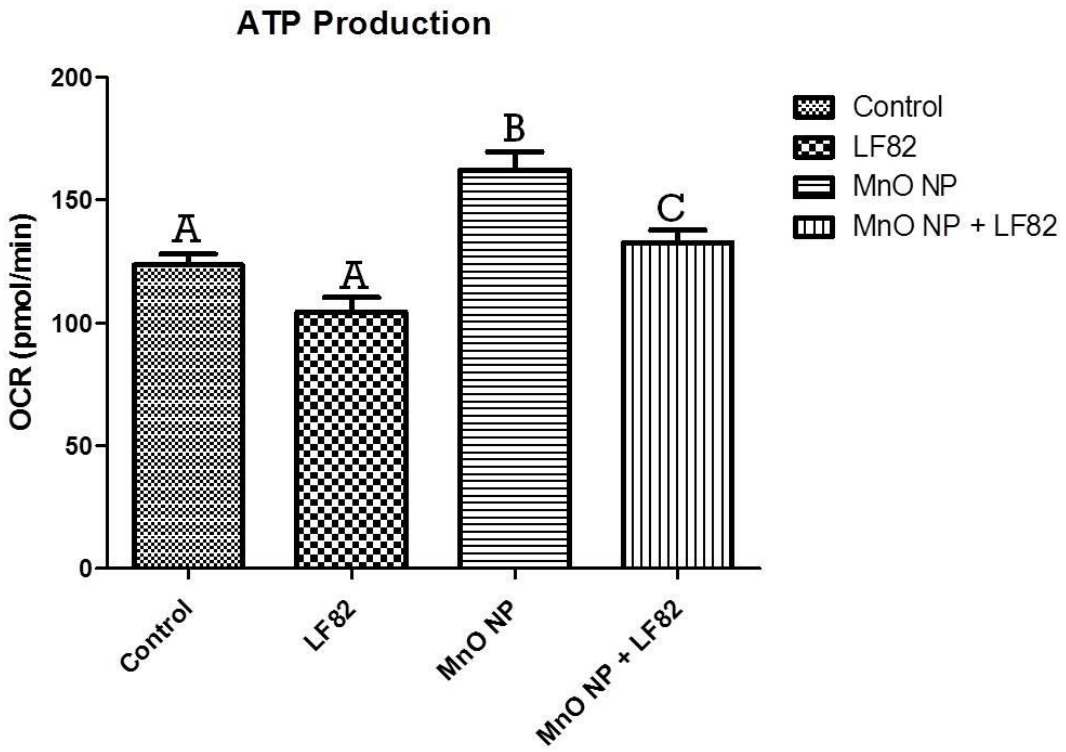

Figure 8. ATP production of MODE-K cells was measured after 24 hours of treatment incubation. Data shown is a representation of five independent wells. Significant ($p < 0.05$) difference is denoted by difference in letter.

Any group containing MnO NP experienced an increase in ATP production suggesting this to be a MnO NP driven effect. The final parameter measured in the mitostress test was spare capacity, which measures the capability of the cell to respond to an energetic demand, which can be an indicator of the cell's fitness or flexibility. When compared to controls, any group treated with MnO NP had a significant ($p < 0.05$) decrease in the spare respiratory capacity suggesting that a decrease in respiratory spare capacity is a MnO nanoparticle driven effect independent of the presence of the bacterial lysate (Figure 9).

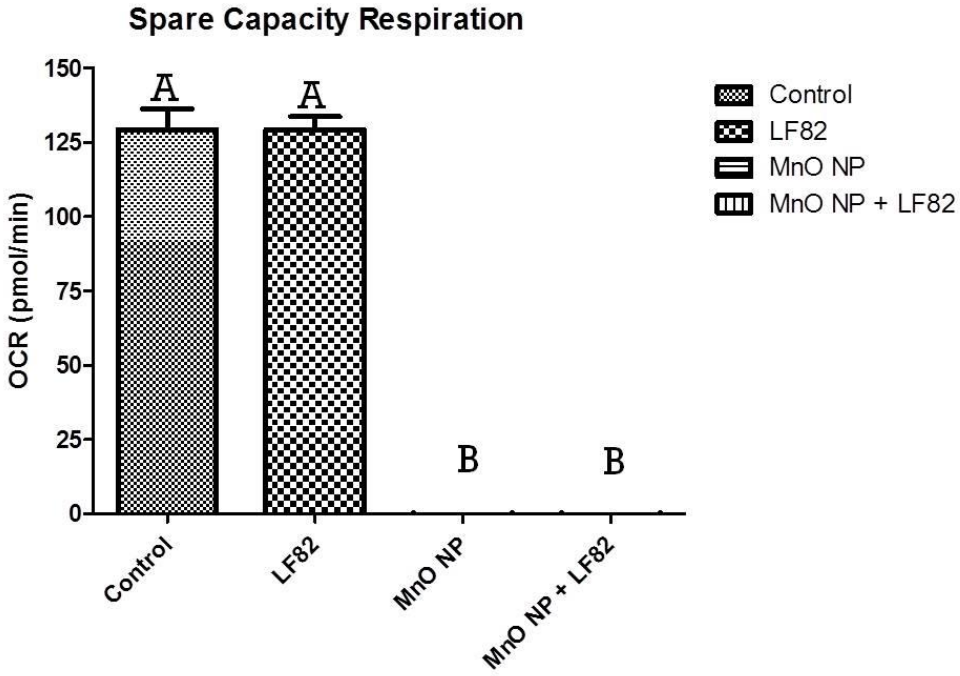

Figure 9. Spare capacity measures the capability of the cell to respond to an energetic demand, which can be an indicator of the cell's fitness or flexibility. Spare capacity of MODE-K cells were measured at 24 hours post-treatment initiation. Data shown is a representation of five independent wells. Significant ($p < 0.05$) difference is denoted by difference in letter.

This data shows that dysfunction of mitochondrial activity and mitochondrial respiration is an effect driven by the presence of MnO NP. In addition, data suggested that when Mode-K intestinal epithelial cells were treated with MnO NP + bacterial lysate, the effects were more severe than when cells were treated with MnO NP alone in some of the parameters analyzed. This data suggests that in the presence of microbial components, mitochondrial activity suffered the most severe insult of all treatment groups suggesting the presence of the bacterial lysate greatly contributes to the already deleterious effects presented by MnO NP when assessing some of the parameters mitostress test. Collectively, these results show that impairment of mitochondrial function is an effect driven by the MnO NP but in the presence of microbial components, there is a greater capability to impair some aspects of the mitochondrial respiration in

intestinal gut epithelial cells as evidenced by the difference seen in some of the parameters measured in the mitostress test.

MTS Assay

Mitochondrial dysfunction is associated with a loss of proliferative capacity in intestinal epithelial cells [35]. As mitochondrial function is associated with health and maintenance of gut epithelial barrier, MTS assay provided crucial insight about cell viability and health. After 48 hours of incubation, a significant decrease was seen in the MnO + bacterial lysate treatment group ($p < 0.05$) (Figure 10).

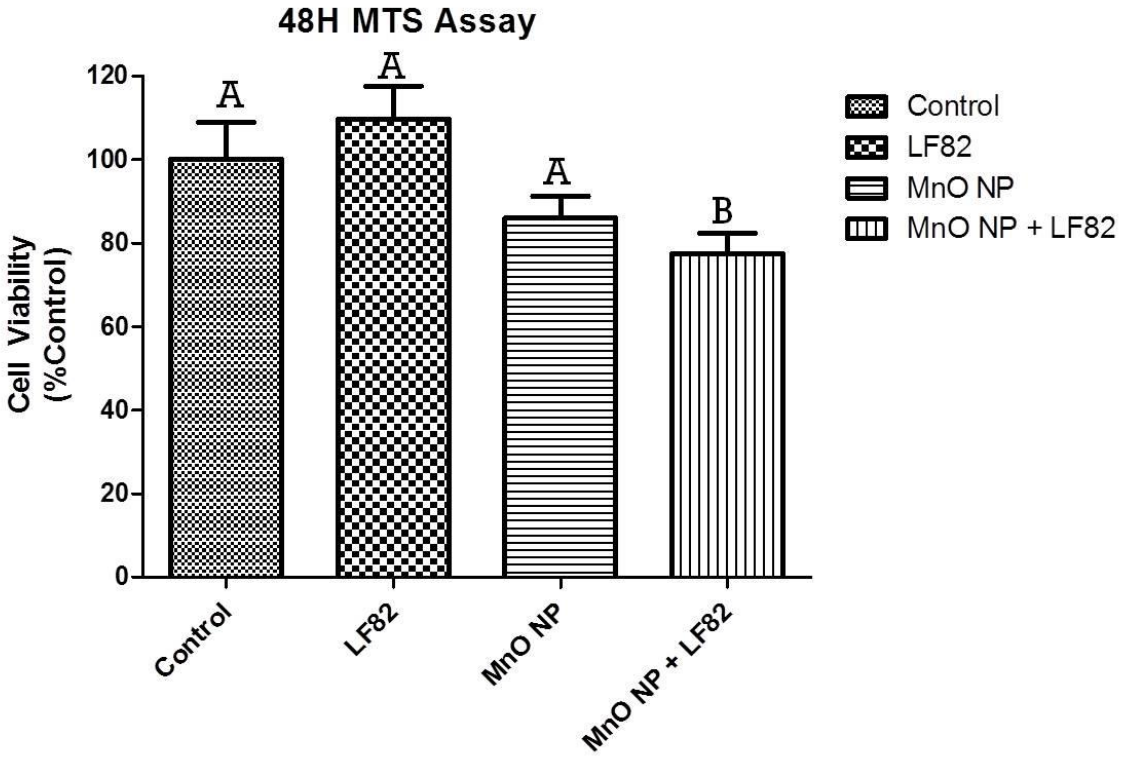

Figure 10. Cell viability was measured using MTS assay. As MTS assay takes into account three parameters (cytotoxic effect, cytostatic effect, and antiproliferative effect), any decrease in viability may be a result from either one or a combination of these three parameters. After 48 hours of treatment incubation, viability of MODE-K cells was measured. Data shown is a representation of two independent experiments containing 6 replicates per treatment with significant ($p < 0.05$) difference denoted by difference in letter.

Discussion

The intestinal epithelial barrier, which consists of several elements including intestinal epithelial cells, tight junctions, and adherens junctions, prevents microorganisms such as viruses, as well as undesirable solutes, toxins, and luminal antigens from entering the body [36, 37]. It is suggested that disruption of the intestinal barrier function and repeated intestinal epithelial damage are key features of IBD, as well as other intestinal disorders [34, 8]. As IBD has multifactorial etiology and is an interaction between factors such as genetics, microbiota, immune response and environment, there needs to be more studies to investigate the contribution of these nanoparticles and environmental factors. The occupational environment in manganese ore processing, metallurgy, metalworking, and welding is a potential source of increased exposure to MnO NP via inhalational and ingestion [47]. MnO NP have been shown to cause impairment of dopaminergic neurons and induce inflammation in the brain [48]. Addtionally, MnO NP have been shown to cause an increase in oxidative stress and apoptosis in alveolar epithelial cells [47]. However, not much studies have looked at the effects of MnO NP toxicity on intestinal epithelial cells. This study gives new insights regarding the toxicity of MnO NP for intestinal epithelial cells especially in combination with phlogistic components from the GI lumen as most studies evaluate MnO NP alone which underestimates their *in vivo* toxicity. By themselves, MnO NP negatively affected the MODE-K epithelial cells but in the presence of bacterial lysate, synergistic effects are seen in several biological functions including inflammatory cytokine production and wound healing. Therefore, we can conclude that a combination of MnO NP and a bacterial lysate induced cytotoxic effects more severely than MnO NP alone.

Because manganese ions were detected in culture supernatants (data not shown), it is possible that excessive Mn^{++} ions in the culture medium could impact other mitochondrial functions [38]. Mn has an essential role in the survival and death mechanisms in cells of all organisms because it regulates Mn-containing enzymes such as manganese superoxide dismutase (SOD2) and regulates expression and activity of caspases. Accumulation of Mn to excessive levels has been shown to cause cellular dysfunction and cell death [38]. The effects seen in mitochondrial dysfunction are associated with deleterious effects seen in cellular migration results shown in the wound healing assay suggesting possible correlation between mitochondrial dysfunction and the wound healing response. Data suggest the presence of MnO NP led to an accumulation and excess level of Mn^{++} in IECs that disrupted the balance of the redox interaction and caused mitochondrial dysfunction. This led to deleterious effects in wound healing response that we saw in this study. Control of intestinal epithelial stemness is crucial for tissue homeostasis and the mitochondrial function plays a critical role in maintaining intestinal stemness [42]. Dysfunction of the mitochondria was accompanied by impairment in ATP production which resulted in reduced proliferation of the intestinal epithelium and loss of stemness [42]. As our study shows that MnO NP (exacerbated with LF82) can cause impairment of mitochondrial respiration of MODE-K cells, we predict that the presence of MnO NP in the gut *in vivo* will affect the mitochondrial energy capacity of IECs and consequently the stemness of the intestinal epithelium and lead to subsequent impairment in barrier formation that will result in a leaky gut.

Mitochondria play an important role during the interaction of gut microbiota with host cells and mitochondrial activity may be an important factor that modulates microbiota diversity and quality [43]. A study performed using colonic biopsy samples showed that induced mitochondrial dysfunction affected epithelial barrier function, which allowed transepithelial flux of bacteria across the intestinal epithelial barrier [44]. An extraordinary amount of energy is required and being turnover during the inflammatory and immune responses [45]. For example, the first contact of the gut microbiota with the intestinal epithelium stimulates toll-like receptors in IECs which results in the recruitment of immune cells that subsequently produce IgA to limit the over-colonization of gut microbiota [46]. Based on the described in vitro studies, it is predicted that MnO NP will dysregulate the in vivo mitochondrial function leading to a loss in epithelial barrier integrity and the infiltration of gut microbes subsequently inducing an elevated proinflammatory response [46]. Based on our study, we predict that MnO NP, which have been shown to induce mitochondrial dysfunction and cause synergistic inflammatory response, this process will be dysregulated leading to an infiltration of gut microbes across the intestinal epithelium and subsequently an elevated proinflammatory response.

The GI tract has been shown to be the primary route of ingestion and excretion for metal nanoparticles. The results of these studies show that the addition of bacterial components to intestinal epithelial cells treated with MnO NP might be predictive of the cellular response seen *in vivo* as this represents a realistic exposure. Currently, the toxicity of nanoparticles is evaluated with various approaches that include both *in vitro* and *in vivo* methods of assessment but rarely do they take into consideration the

presence of the microbial community that exist in the body. Additionally, nanoparticle studies that take into consideration the interaction between nanoparticles and environmental components (e.g., microbiota) are extremely limited. The results of the present study demonstrate that there is a necessity to integrate the contribution of other stimuli present in the gut, such as microbial components and/or inflammatory cytokines, on IEC health and ultimately intestinal barrier integrity as this represents a more holistic approach that more closely mimics the gut environment.

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CHAPTER 4. CYTOTOXIC EFFECTS OF TITANIUM DIOXIDE NANOPARTICLES IN COMBINATION WITH INFLAMMATORY MEDIATORS ON INTESTINAL EPITHELIAL CELLS

A paper to be submitted to *Food and Chemical Toxicology*

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Abstract

Titanium dioxide nanoparticles (TiO_2 NP) are commonly found in many food products such as donuts, chewing gums, milk-based products, chocolate, and products with white icing or powdered sugar toppings. Despite being found in many consumed food products, little is known about the fate, toxicity, and potential deleterious effects of TiO_2 NP to affect cellular functions of intestinal epithelial cells critical to the maintenance of the epithelial barrier along the gastrointestinal (GI) tract. Furthermore, TiO_2 NP has been shown to be aggregated in the ileum, which is the region of the GI where the onset of inflammatory bowel disease (IBD) has been shown to develop. GI research has shown that maintenance of the epithelial barrier plays a critical role in mucosal homeostasis and immunity as evidenced in a number of GI disorders including IBD, which occurs in about 1 in 300 persons worldwide. Despite this data, there is no study assessing the direct effects of TiO_2 NP in combination with host inflammatory mediators on intestinal epithelial cells. Using an in vitro model of GI homeostasis and epithelial wound healing, we examined the effects of TiO_2 NP alone or in combination with inflammatory mediators (TNFα/IFNγ). We hypothesized that treatment with TiO_2 NP in combination with a bacterial lysate will induce adverse and deleterious effects on

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intestinal epithelial cells. Specifically, MODE-K cells, a murine intestinal epithelial cell line, were exposed to TiO_2 NP alone or in combination with TNFα/IFNγ for 24 hours. Compared to TiO_2 NP alone, these studies demonstrated that the addition of TiO_2 NP plus TNFα/IFNγ resulted in 1) exacerbated deleterious effects of MODE-K cells 2) potentiated mitochondrial superoxide generation 3) negatively affected cell migration as evidenced by wound healing assay, and 4) enhanced secretion of inflammatory cytokines such as IL-6. While the TNFα/IFNγ had no deleterious effects on the MODE-K cells data suggests that in the presence of TNFα/IFNγ, TiO_2 NP had potentiating effects on intestinal epithelial cells that adversely affect intestinal epithelial barrier which can lead to a negative impact on mucosal homeostasis.

Introduction

Titanium dioxide nanoparticles (TiO_2 NP) are commonly found in many food products such as donuts, chewing gums, milk-based products, chocolate, and products with white icing or powdered sugar toppings [1-3]. Despite being found in many consumed food products, little is known about the fate, toxicity, and potential deleterious effects of TiO_2 NP to affect cellular functions of epithelial cells critical to the maintenance of the epithelial barrier along the gastrointestinal tract (GIT). Because of novel properties offered by their size, nanoparticles (NP) are being introduced into a rising number of food products (www.nanotechproject.org) as an FDA approved additive used as a food colorant [4]. However, data that have shown that TiO_2 NP can be toxic to epithelial cells and to induce deleterious biological effects [5]. For example, TiO_2 NP pretreated with digestive fluids, which included saliva, gastric, duodenal, and bile fluid, inhibited cell growth in undifferentiated Caco-2 cells [5]. In addition to inhibiting growth,

these TiO_2 NP can be taken up by IECs resulting in translocation across the intestinal epithelium [6, 7]. Compared to uptake by M cell overlying the Peyer's patches, translocation occurs diffusely along the entire length of the GIT and, given the amount of TiO_2 NP that may be consumed, the total uptake of ingested TiO_2 NPs may be important given the large surface area of the GI epithelium [7]. Collectively, these previous studies point out that TiO_2 NP possess the ability to have adverse cytotoxic effects on intestinal epithelial cells (IECs). Despite this data, there are virtually no studies assessing the direct effects of TiO_2 NP on IECs and the mechanisms by which these effects occur. It has been suggested that ROS production is the main cytotoxic mechanism affecting various cell types [8-10]. However, there have been no studies found investigating these mechanisms of action of TiO_2 NP in IECs.

In vitro studies have identified several biochemical changes indicative of oxidative stress and inflammation induced by exposure of keratinocytes to TiO_2 NP [11, 12]. In keratinocytes, TiO_2 NP have been shown to affect the glucuronidation pathway, which is a major pathway for metabolizing xenobiotics, by decreasing the two major cofactors, UDP-glucuronate and glucuronate [12]. Several studies established the consequential effects of TiO_2 NP exposure on cells and tissues, such as ROS induction and inflammatory cytokine production, often associated with carcinogenesis [8-12]. TiO_2 NP have also been shown to be taken up from the lumen of the GIT [3, 13]. An *in vivo* study revealed TiO_2 NP uptake from the GIT via the Peyer's patches. The sampling proficiency of M cells leads to a relative high concentration of TiO_2 NP in the lymphoid tissues of mice [3, 13, 14]. TiO_2 NP translocating across the epithelial barrier would induce a loss of epithelial integrity and linger in gut cells or interstitial spaces where they could

become toxic and potentially trigger impairment of GIT function [7]. Consequences of the chronic damage associated with the presence of TiO_2 NP may include, but is not limited to, changes in expression of genes necessary for cell survival, inflammation, apoptosis and cell cycle, and with down-regulation of genes involved in energy metabolism [7]. Understanding the effect of TiO_2 NP on epithelial cells, intestinal barrier function, and absorption of these nanoparticles is essential and vital for assessing the safety of orally administrated or consumed TiO_2 NP [5].

The increasing interest in the use of nanoparticles, including TiO_2 NP, in the food industry has led to immense enthusiasm about possible advantages, such as improving the appearance of foods, but also worry over their potential to cause cytotoxic effects [15]. With the growing concerns that have arisen about unintentional biological effects of nanoparticles, there needs to be more in-depth evaluation of the effects that TiO_2 nanoparticles may have on biological systems [16]. For example, metal-based nanoparticles can be present at low levels but then accumulate to concentrations that disrupt intracellular trafficking leading to deleterious effects on gene expression in genes, such as gluthathione peroxidases and superoxide dismutase 3 (SOD3), which are responsible for antioxidant activity in cells [17]. More complete understanding of the basis of metal-based nanoparticle toxicity is essential for the complete risk-assessment of NP exposure on occupational, environmental, and human/animal health. Understanding underlying process and mechanisms from administration to excretion must be completely assessed and understood because nanoparticles, such as TiO_2 NP, are currently being used in the food industry. As a result, the GIT will be the primary route of entry and excretion for some of these nanoparticles TiO_2 NP [18, 19]. However,

there are currently no studies performed that assessing the effects and interactions between TiO_2 NP and inflammatory mediators on IECs.

Assessing the effects of nanoparticles on IECs in the presence of host inflammatory cytokines and/or phlogistic components from the GI lumen presents a more holistic and realistic cellular response when assessing effects of metal NPs on gut epithelial cells knowing that immune responses influence physiological, metabolic, nutritional, and microbiological functions that have been linked with GI conditions such as inflammatory bowel disease [20]. Based on this information, we hypothesize that the of TiO_2 NP in conjunction with host inflammatory cytokines will have deleterious effects on the health of intestinal epithelial cells. We showed that some of the cellular functions necessary for the maintenance of the intestinal epithelial barrier were inhibited after treatment with TiO_2 NP and inflammatory mediators.

Materials and Methods

Nanoparticles

Titanium dioxide nanoparticles (TiO_2 NP) used in all experimental procedures were purchased from US Research Nanomaterials, Inc. (Houston, TX, USA). Particles were suspended in low glucose (1 g/L) DMEM at a stock concentration of 2 mg/mL prior to addition to the cells. TiO_2 NP used in this study were less than 25nm in diameter.

Cytokines

Murine tumor necrosis factor-alpha (TNFα) and interferon-gamma (IFNγ) were used in all experimental procedures and were purchased from Peprotech (Rocky Hill, NJ, USA). TNFα and IFNγ cytokines were suspended in complete low glucose DMEM

culture medium at 10 µg/mL and 100 µg/mL, respectively, and then stored frozen at -80° C until used.

Cell Culture and Treatment

MODE-K epithelial cells derived from the small intestine of a C3H mouse was used in all experimental procedures described below (kind gift from Dr. C. Elson). In this study, MODE-K cells were grown in high glucose (4.5 g/L) DMEM medium containing 10% FBS, 2 mM L-glutamine, 50 units of penicillin, and 50 mg/mL streptomycin (complete medium). In general, cells were seeded in a tissue culture plate in accordance to the experimental requirements and were cultured overnight in a humidified atmosphere of 5% CO_2 at 37 °C. The length of each experiment and cell density used for each experiment has been provided in the methods section for each experimental procedure. The four treatment groups for experimental procedures were: medium alone, 5 ng/mL TNFα/IFNγ (2.5 ng/mL of each cytokine), 50 µg/mL TiO_2 NP, and 50 µg/mL TiO_2 NP + 5 ng/mL TNFα/IFNγ. All experiments were conducted in low glucose (1.0 g/L) DMEM medium. To optimize the amount of cytokine used in these studies, dose titrations were performed with TNFα/IFNγ to find doses that had minimal effects on the MODE-K cells (e.g., viability) and these minimal doses were chosen in order to facilitate the ability to assess the interactions between the TiO_2 NP and these host inflammatory mediators.

Mitochondrial superoxide generation

Mitochondrial superoxide generation was measured using MitoSOX™ Red Mitochondrial Superoxide Indicator following the manufacturer's instructions (Thermo Scientific). The four treatment groups for experimental procedures were: medium alone,

5 ng/mL TNFα/IFNγ (2.5 ng/mL of each cytokine) only group, 50 µg/mL TiO_2 NP only group, and 50 µg/mL TiO_2 NP + 5 ng/mL TNFα/IFNγ group. MODE-K cells were plated in 96-well plates with 8,000 cells/well one day prior to treatment and allowed to attach overnight. The next day cells were washed twice with low glucose DMEM medium before being plated with respective treatments in low glucose DMEM medium for a period of 24 hours. Following treatment, MitoSox reagent was added to each plate well at a 1:1000 concentration. Relative fluorescence intensity was then acquired 24 hours post-treatment using a microplate reader.

Cytokine Release analysis

Prior to assessing cell viability, supernatants were collected from MODE-K cell cultures at 24 and 48 hours after addition of the TiO_2 NP or other stimulants and analyzed for the presence of cytokines. A multiplex assay was used to detect the presence of IL-6, TNFα, and IFNγ. The cytokines in each sample of the supernatant were measured using multiplex bead analysis for immunoassays. Standard curves were generated using Bio-Plex Manager Software Version 6.0 (Bio-Rad Laboratories, Inc., Carlsbad, CA, USA) and the amount of cytokine in each sample was determined.

Wound Healing Assay

For one-dimensional cellular migration assays, 300,000 MODE-K cells were resuspended in DMEM (low glucose, 1.0 g/L) inoculated into 12 well plates and incubated overnight. Before the experiment, cells were confluent and quiescent in low glucose DMEM (the overnight medium). At the beginning of the experiment, a (+) shaped gap was created in each well using a P-200 pipet tip creating a cell free gap that, in time, would be covered by replicating/migrating MODE-K cells. Cells were then

incubated. Images were taken at 0 and 24 hours post-wounding. The four treatment groups for experimental procedures were the same as described above. After 24 hours, experiments were terminated. Images were taken using a 5x objective on an inverted microscope Leica DMi1 (Leica Microsystems, IL, US) using the software Leica Application Suite 4.5.0 (Leica Microsystems, IL, US) from 10 representative wounded areas per condition. Images and gap spacing were then measured and analyzed with an automation and image analysis software Metamorph 5.0.7 (Molecular Devices LLC, CA, US). Gap closure was quantified by determining the mean distance of closure of the gap. This was done by measuring the area of the cell-free space dividing the length of the gap at 24-hours, and then subtracting this value from starting value that was measured at 0-hour. This method was previously described and adopted to fit our experimental procedure [21].

Mitochondrial Respiration

Mitochondrial oxygen consumption rates were measured using a Seahorse XF24e Extracellular Flux analyzer (Seahorse Bioscience, North Billerica, MA). The Seahorse XF24e Extracellular Flux analyzer is a high-throughput instrument that yields real-time measurements of cellular respiration rates. In our studies, we were interested in assessing the effects of our treatments on mitochondrial respiration and the effects of our treatments on oxygen consumption rates (OCR). Cells were seeded at a density of 300,000/ml into a T-25 culture flask and incubated overnight in 5% CO_2 at 37.8 ºC. The next day cells were treated with respective treatments for 24 hours. Stimulated cells were recovered from the plates by scraping and added to the Seahorse plates using CellTac at 60,000 cells per well and incubated for 1 hour to allow the cells to adhere. At

this time, the mito-stressor reagents Oligomycin (1 µM), FCCP (2 µM) and Rotenone/Antimycin A (0.5 µM) were loaded into the corresponding injection ports of the Seahorse FluxPak cartridge and placed in the Seahorse analyzer to be equilibrated. After equilibration, the plate containing treated cells was then placed in the Seahorse analyzer covered with the Flux Pak cartridge. The analyzer was then programmed to measure the basal OCR readouts at five specified time intervals before progressing to inject the mito-stressors. Measuring mitochondrial respiration with the Seahorse analyzer allowed us to determine the effect of each treatment on the three critical stages of respiration. The first stage involves the addition of an ATP synthase inhibitor, oligomycin, which suddenly decreases the OCR. The next stage is the addition of FCCP, which acts as an uncoupler that increases mitochondrial oxygen consumption by disinhibiting the flow of protons across the mitochondrial inner membrane due to uncoupling of the electron transport chain. The final stage is the addition of rotenone/antimycin A, which is a respiratory chain inhibitor that completely shuts down mitochondrial activity.

Statistical Analysis

All statistical data analyses were performed using Prism 4.0 (GraphPad Software, San Diego, CA). Data were analyzed using one-way ANOVA with the Tukey post-test for comparing all treatment groups with that of the control. Multiple comparisons test was also performed to analyze differences between treatment groups. Differences with $p < 0.05$ were considered significant. All data is representative of two independent experiments. Data is representative of two independent experiments containing six wells per treatment.

Results

Mitochondrial Superoxide Generation

The presence of reactive oxygen species (ROS) may negatively impact the barrier function of the GI epithelium. An increase of ROS in the inflamed mucosa of inflammatory bowel disease (IBD) patients has been reported and may contribute to loss of intestinal barrier function [22]. Furthermore, during inflammation, oxidative stress can lead to the induction of cell injury and apoptosis of intestinal epithelial cells [23]. As an intestinal epithelial cell model, murine MODE-K cells were used to evaluate the effect of TiO_2 NP on critical cell functions associated with formation and maintenance of epithelial homeostasis. After 24 hours of incubation with each treatment, mitochondrial superoxide generation was measured. Data was normalized to percent control.

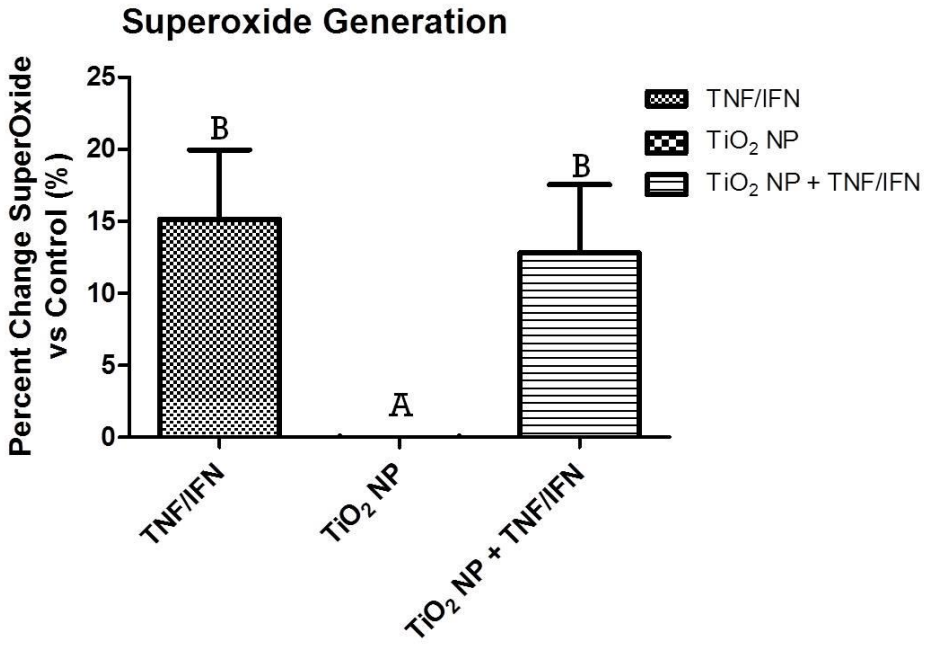


Figure 1. Mitochondrial superoxide generation was measured from MODE-K intestinal epithelial cells 24 hours post-treatment. Data was normalized to percent control. Data presented is a representative of two independent experiments containing six replicates per treatment. Different letter denotes difference in significance ($p < 0.05$).

Significant ($p < 0.05$) difference was seen when any treatment group containing TNFα/IFNγ, whether TNFα/IFNγ alone treatment group or TiO_2 NP + TNFα/IFNγ group, was compared to the TiO_2 NP alone treatment group (Figure 1). There was at least a 12% increase in mitochondrial superoxide generation in both treatment groups containing TNFα/IFNγ indicating that generation of mitochondrial superoxide generation was cytokine driven and not related to the presence of the TiO_2 NP. Interactions between TiO_2 NP and a bacterial lysate generated from *E. coli* LF82 were also evaluated but bacterial lysate did not exacerbate any deleterious effects when added in combination with the TiO_2 NP (data not shown).

Wound Healing Assay

Wound healing is a crucial function of the intestinal epithelium as it can be injured by toxic substances from the lumen, normal digestion, inflammation, interactions with microbes, oxidative stress, and/or pharmaceuticals [22, 24]. Upon injury and loss of epithelial cells, the intestinal epithelium undergoes a wound healing process which is dependent on the balance of three cellular events; proliferation, differentiation, and restitution of epithelial cells adjacent to the wounded area [25]. Therefore, to address the impact to TiO_2 NP on cellular proliferation, we used a scratch wound assay [21]. After "wounding" the MODE-K cell monolayer, measurements of the gap were taken at 0 hours post-wounding and then again at 24-hours post-treatment.

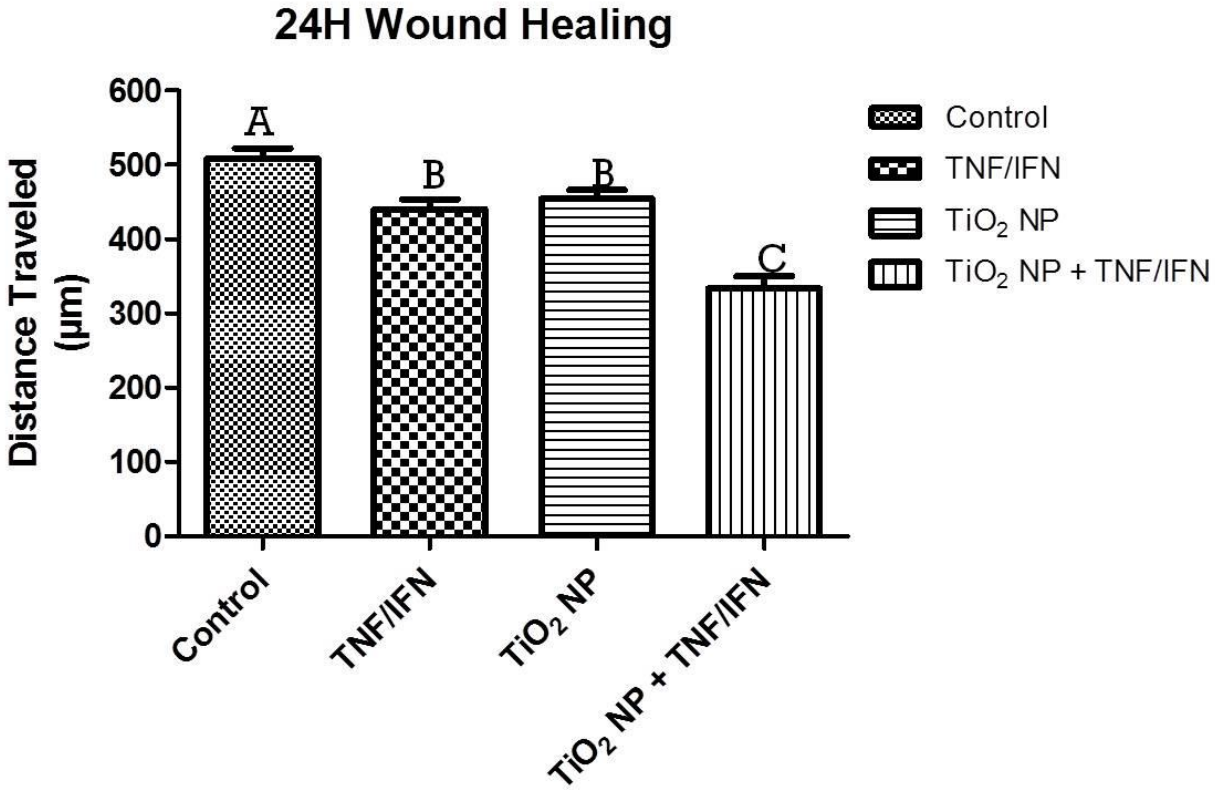


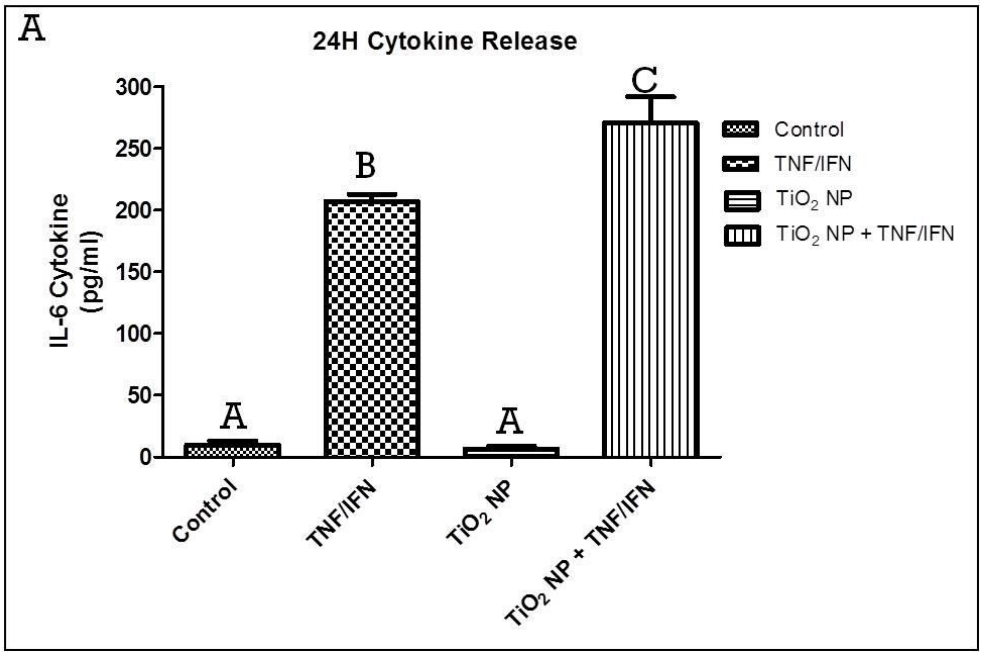
Figure 2. Wound healing assay served as an analysis of the effects on cell migration in MODE-K intestinal epithelial cells. Measurements were taken at 0 hours and 24 hours post-treatment. Values were plotted as the difference between the timepoints. Results are representative of two independent experiments containing six replicates per treatment. Different letter denotes difference in significant ($p < 0.05$) change.

A significant effect was observed between treatment groups and controls ($p < 0.05$). Furthermore, there was significantly ($p < 0.05$) slower wound MODE-K cell replication in the TiO_2 NP + TNFα/IFNγ treatment group when compared to the TiO_2 NP only treatment group (Figure 2). There was no significant difference seen when the TNF/IFN treatment group and the TiO_2 NP only group were compared to each other. As immune stimulatory molecules are continually present in GI tissue, the epithelial barrier will face persistent interactions from both the microbiota and innate and adaptive

immune response. As cellular migration is an important component in the turnover process of epithelial cells in order to maintain integrity of the epithelial barrier, the results in the wound healing assay shows that TiO_2 NP alone can hinder epithelial replication and/or migration. Furthermore, in the presence of host inflammatory cytokines, the deleterious effects on MODE-K cells induced by TiO_2 NP are exacerbated.

Cytokine Release

Damage and impairment of the intestinal surface barrier may result in an increased penetration and absorption of toxic and phlogistic components across the epithelium leading to inflammation, uncontrolled innate immune response, and disequilibrium of mucosal homeostasis [26]. This inflammatory response can lead to the induction of cellular injury and apoptosis of intestinal epithelial cells [23, 26]. Therefore, cytokine analysis was performed to address the ability of TiO_2 NP to stimulate MODE-K cells. After 24 hours of incubation, supernatants were harvested and cytokine levels were measured. A significant ($p < 0.05$) induction of IL-6 was observed between treatment groups ($p < 0.05$) (Figure 3A).



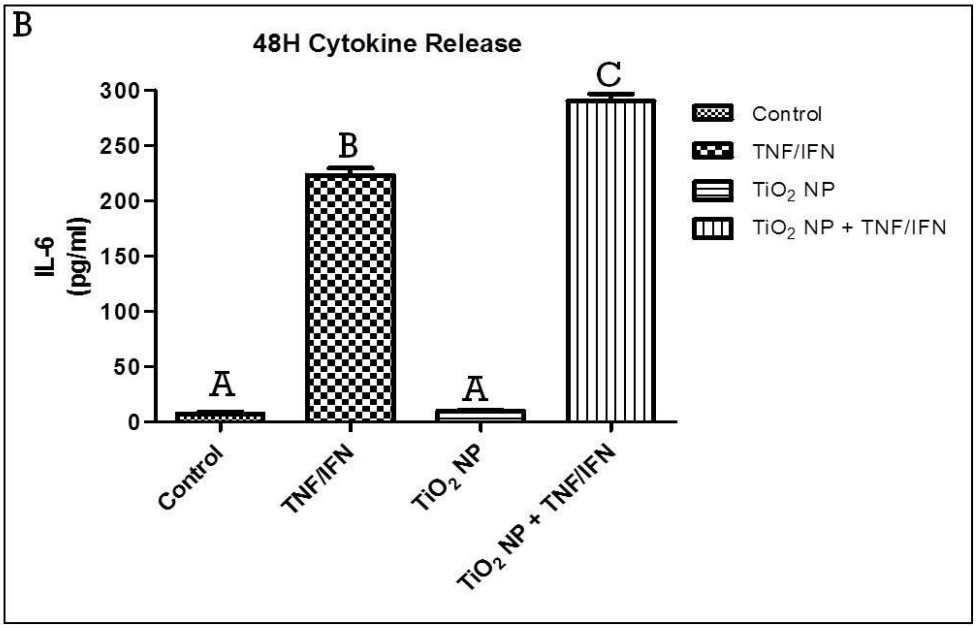


Figure 3. Supernatants from each well were taken at 24 hours post-treatment and individually measured for IL-6 cytokine release from MODE-K intestinal epithelial cells (Figure 3A). Then, supernatants from each well were taken at 48 hours post-treatment and individually measured for IL-6 cytokine (Figure 3B). Data shown is representative of two independent experiments containing six replicates per treatment. Different letter denotes difference in significant ($p < 0.05$) change.

Post-hoc analysis indicated significantly ($p < 0.05$) higher IL-6 production when MODE-K cells were stimulated with TiO_2 NP in the presence of TNFα/IFNγ compared to the control group. In addition, analysis indicated a significant ($p < 0.05$) increase in IL-6 secretion from MODE-K cells treated with TNFα/IFNγ alone when compared to cells treated with TiO_2 NP alone. Furthermore, analysis revealed a significant ($p < 0.05$) increase in IL-6 secretion at both 24 hours (Figure 3A) and 48 hours (Figure 3B) post-stimulation when the TiO_2 NP + TNFα/IFNγ treatment group was compared to cells treated with TiO_2 NP alone. The results of cytokine release revealed a synergistic effect on IL-6 secretion release when TiO_2 NP was combined with TNFα/IFNγ.

Mitochondrial Respiration

Mitochondrial function plays a critical role in maintaining intestinal stemness and homeostasis [27]. As mitochondrial dysfunction is associated with a loss of proliferative capacity in intestinal epithelial cells, the effects on crucial stages of mitochondrial respiration was measured as described in Materials and Methods. Utilizing the Seahorse XFe24 Analyzer, the parameters measured to assess mitochondrial stress included basal respiration, maximal respiration, proton leak, ATP production, and spare respiratory capacity (Figure 4).

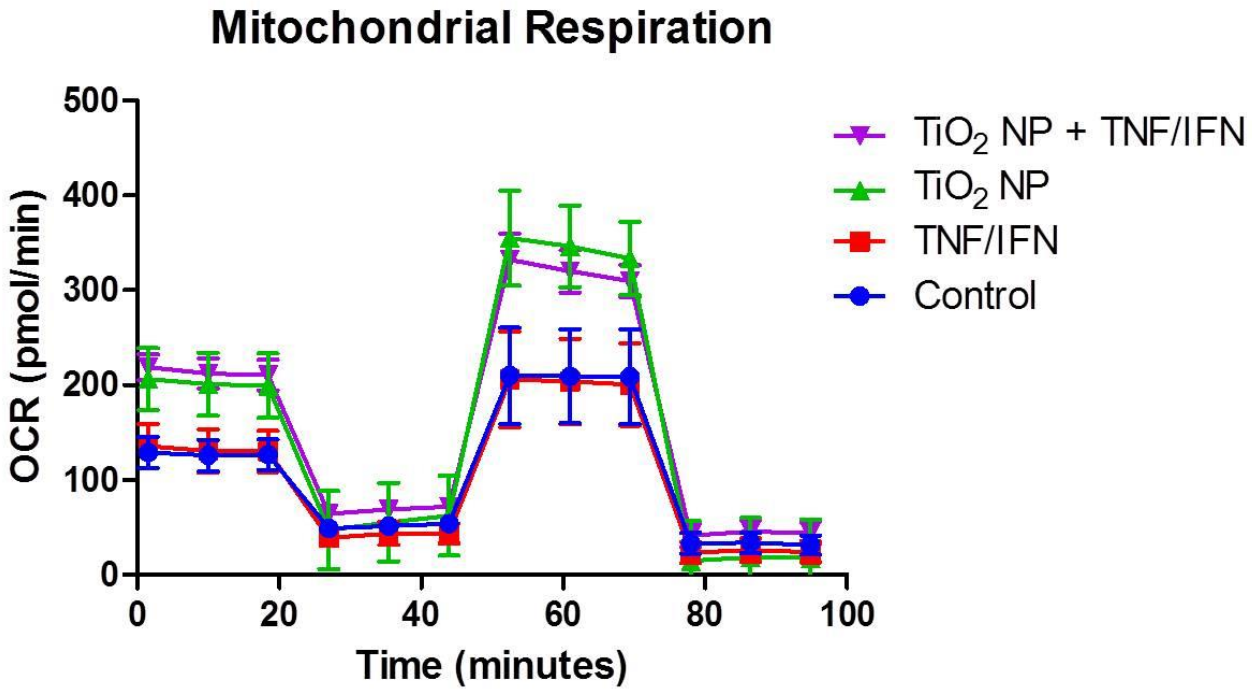


Figure 4. Mitochondrial respiration provides a robust test for analyzing the key parameters of mitochondrial function. Functional mitochondrial measurements are key to understanding cellular activation, proliferation, differentiation, and dysfunction. Mitochondrial function of MODE-K cells were measured after 24 hours of treatment incubation. The parameters measured were basal respiration, maximal respiration, proton leak, ATP production, and spare capacity.

Basal respiration is a measurement that shows the energetic demand of the cell under baseline conditions. Analysis showed significantly ($p < 0.05$) elevated levels of basal respiration in both groups of MODE-K cells treated with TiO_2 NP when compared to control and TNFα/IFNγ groups (Figure 5).

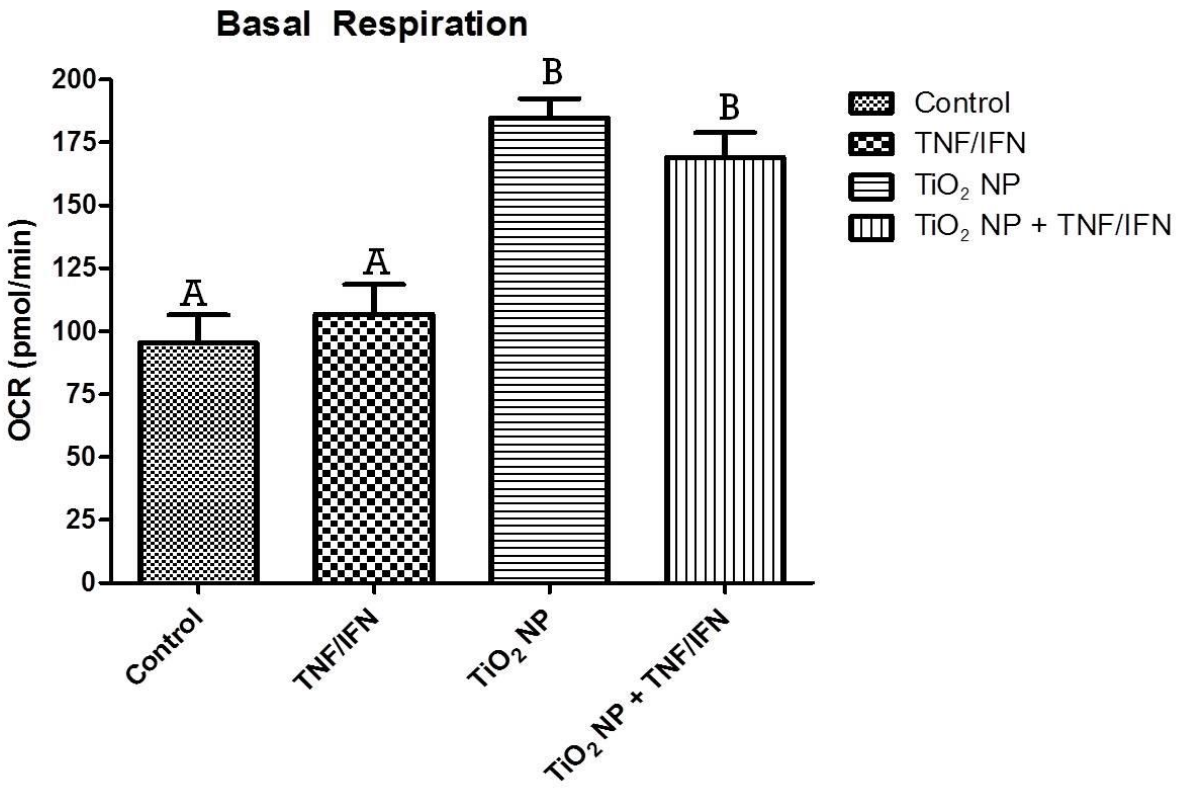


Figure 5. Basal respiration is a measurement that shows the energetic demand of the cell under baseline conditions. Basal respiration of MODE-K cells were measured after 24 hours of treatment incubation. Data shown is a representative of six independent experimental wells. Significant ($p < 0.05$) difference is denoted by difference in letter.

Maximal respiration was another parameter measured which represents the maximum rate of respiration cells can achieve when operating at maximum capacity. Significantly ($p < 0.05$) higher levels of maximal respiration were observed in all treatment groups receiving TiO_2 NP when compared to control and TNFα/IFNγ treatment groups (Figure 6).

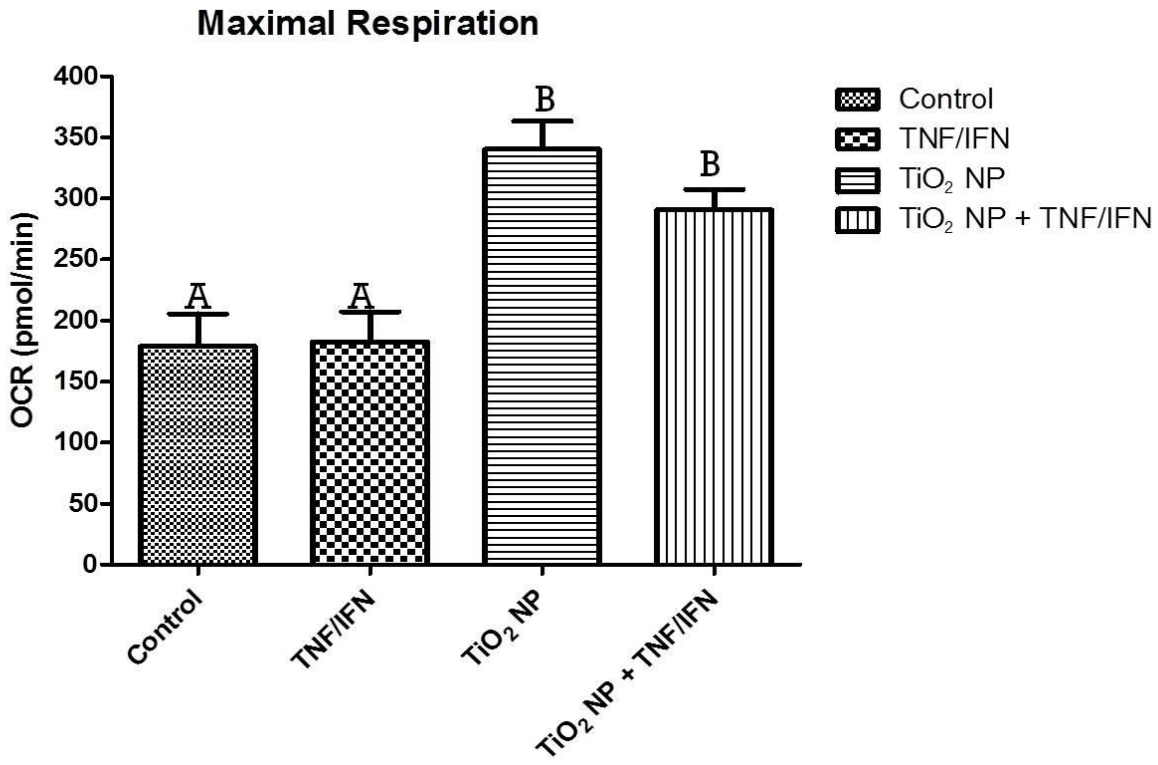


Figure 6. Basal respiration is a measurement that shows the energetic demand of the cell under baseline conditions. Basal respiration of MODE-K cells were measured after 24 hours of treatment incubation. Data shown is a representative of six independent experimental wells. Significant ($p < 0.05$) difference is denoted by difference in letter.

Another functional parameter measured was mitochondrial proton leak which can have a major impact on the coupling efficiency of Complexes I through IV in the mitochondrial membrane. While significant differences ($p < 0.05$) were induced in MODE-K cells treated with TiO_2 NPs in comparison to the other treatment groups (Figure 7).

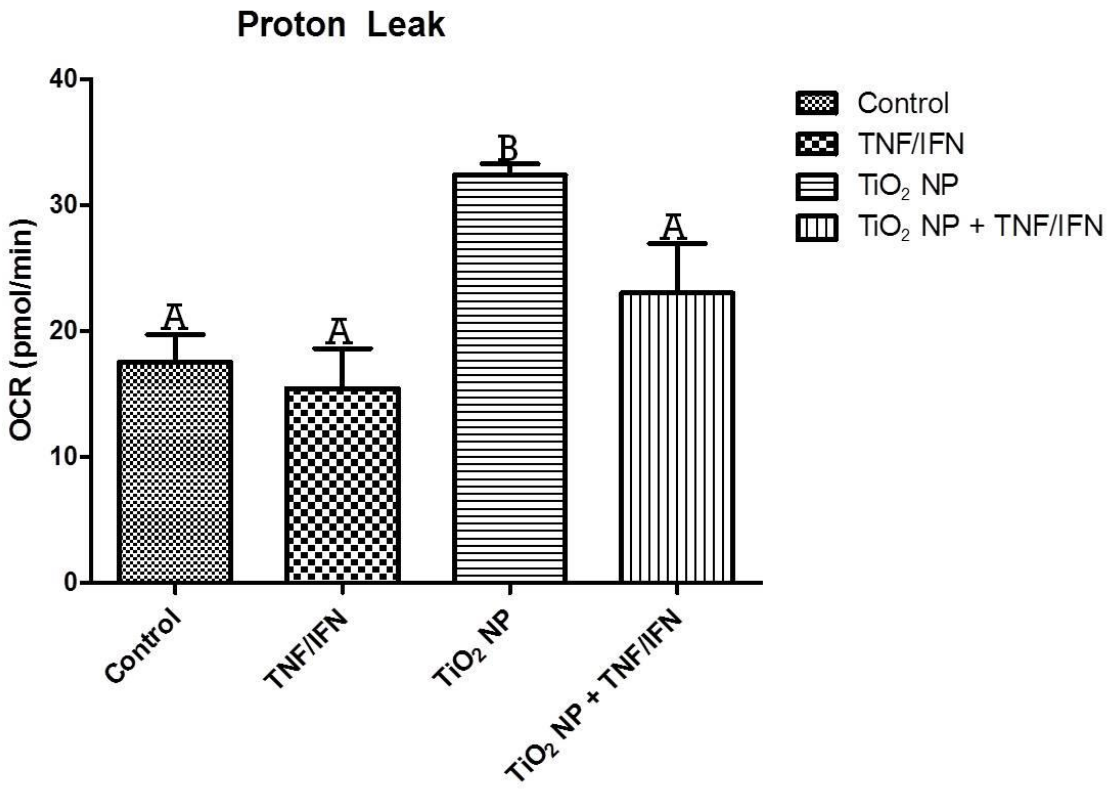


Figure 7. Mitochondrial proton leak has a major impact on mitochondrial oxidative phosphorylation coupling efficiency and production of reactive oxygen species. Proton leak of MODE-K cells were measured after 24 hours of treatment incubation. Data shown is a representative of five independent experimental wells. Significant ($p < 0.05$) difference is denoted by difference in letter.

ATP production, which measures ATP produced by the mitochondria that contributes to meeting the energetic needs of the cell, was another parameter that was measured following the addition of the TiO_2 NPs. Incubating MODE-K cells in the presence of TiO_2 NP or TiO_2 NP + TNFα/IFNγ treatment group caused a significant ($P < 0.05$) increase in ATP production when compared to control treatment group, with the highest increase seen in the TiO_2 NP only group (Figure 8).

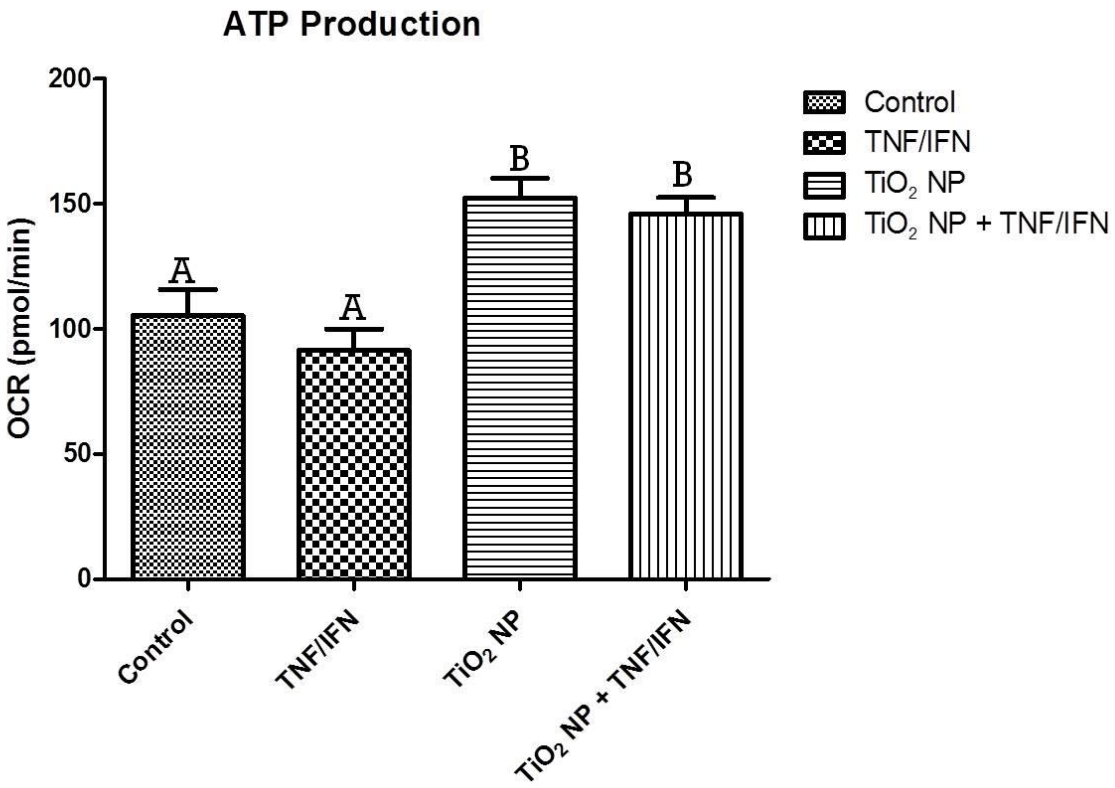


Figure 8. ATP production of MODE-K cells was measured after 24 hours of treatment incubation. Data shown is a representative of five independent experimental wells. Significant ($p < 0.05$) difference is denoted by difference in letter.

The presence of TiO_2 NP, alone or in combination with TNFα/IFNγ, result in at least a 45% increase in oxygen consumption in MODE-K cells suggesting an increase in metabolic activity.

Lastly, respiratory spare capacity was measured which approximates the capability of the cell/mitochondria to respond to an energetic demand and is used as an indicator of the cell's fitness or flexibility. When compared to control treatment group, any group treated with TiO_2 NP had higher levels of spare capacity. This suggests that changes to spare capacity appears to be a TiO_2 NP driven effect (Figure 9).

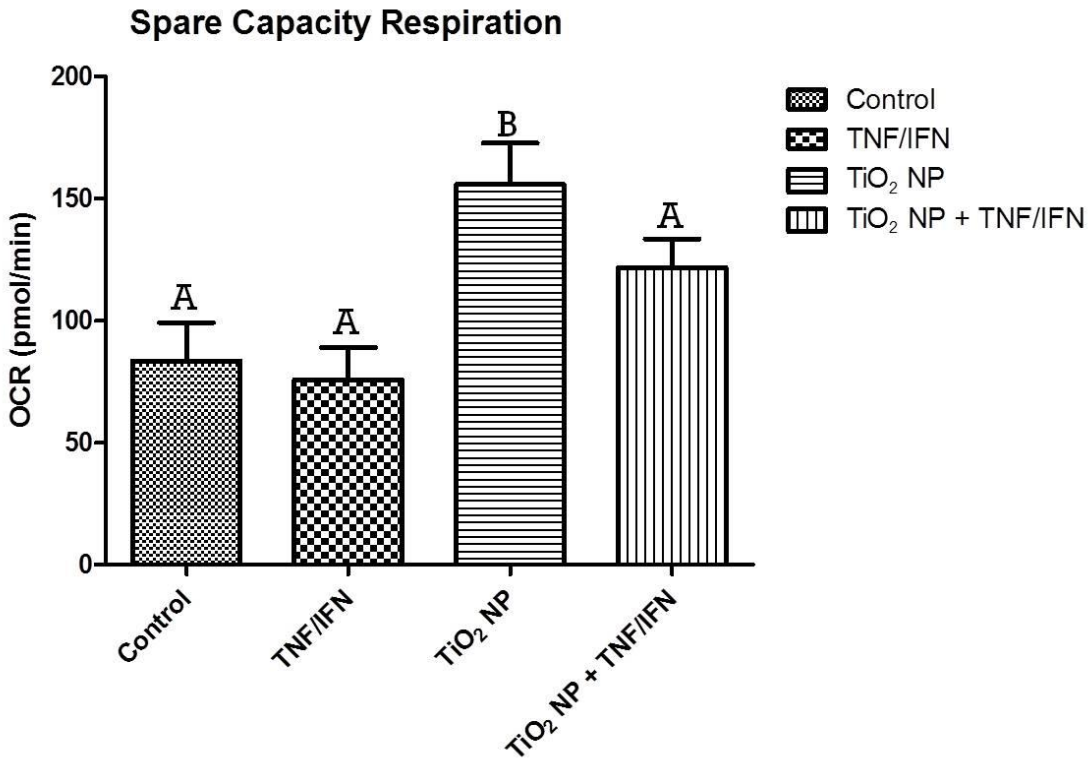


Figure 9. Spare capacity measures the capability of the cell to respond to an energetic demand, which can be an indicator of the cell's fitness or flexibility. Spare capacity of MODE-K cells were measured at 24 hours post-treatment initiation. Data shown is representative of five independent experimental wells. Significant ($p < 0.05$) difference is denoted by difference in letter.

In addition, data suggested that when MODE-K cells were treated with TiO_2 NP + TNFα/IFNγ, the magnitude of the spare capacity was less robust than when cells were treated with TiO_2 NP only suggesting that host inflammatory response plays a role in ameliorating mitochondrial respiration. Collectively, these results show that changes in mitochondrial function in MODE-K cells exposed to the TiO_2 NPs is an effect driven by the presence of the TiO_2 NPs as opposed to TNFα & IFNγ.

Discussion

The intestinal epithelial barrier, which consist of several elements including intestinal epithelial cells, tight junctions, and adherens junctions, prevents undesirable solutes such as microorganisms, viruses, toxins, and luminal antigens from entering the body [28, 29]. It is suggested that disruption of the intestinal barrier function and repeated/chronic intestinal epithelial damage are key features of IBD, as well as other intestinal disorders [26, 29]. This study gives insight into potential deleterious effects associated with GIT exposure to TiO_2 NPs in combination with inflammatory cytokines on the maintenance of intestinal epithelial barrier. By themselves, TiO_2 NP did not prove to be cytotoxic to MODE-K intestinal epithelial cells. However, when MODE-K were treated with TiO_2 NP in combination with TNFα/IFNγ, data showed that this combination negatively affected biological functions such as epithelial restitution which play major roles in the health, formation, and maintenance of the intestinal epithelial barrier.

This study concluded that inhibition in cellular function, as evidenced by MODE-K cell replication, were observed and were more severe as early as 24 hours after exposure to TiO_2 NP + TNFα/IFNγ than cells treated with TiO_2 NP alone. As mitochondrial reactive oxygen species may also be involved in the regulation of the gut epithelial barrier, induced mitochondrial dysfunction can affect epithelial barrier and the permeability of the GI epithelium. [30]. Due to the substantial amount of energy necessary and used during the inflammatory and immune responses, mitochondrial activity has a crucial role in the interaction between gut microbiota and host epithelial cells [31, 32]. Based on the results herein, it is hypothesized that IECs treated with TiO_2

NP in the presence of microbial components and host inflammatory mediators will demonstrate adverse responses such as wound healing and cellular migration as well as actin and cytoskeleton rearrangement.

With the goal to improve the appearance of foods, the usage of nanoparticles specifically TiO_2 NPs, the GIT will be exposed to moderate to excessive levels of these nanoparticles. While TiO_2 NPs are found in powdered-sugar donuts, the highest levels of TiO_2 NP are found in products such as chewing gum, milk-based products, chocolate, tablets of dietary supplements, and products with white icing [(1)]. Microparticles of TiO_2 have been shown to amass in macrophages within the mucosal associated lymphoid tissue (MALT) of the human GIT. In addition, sites of the greatest uptake of TiO_2 NPs are similar to the tissue locations (ileum) where the initial lesions of Crohn's disease are seen [(33)]. Although levels of TiO_2 NPs tested in the present study did not induce cytotoxic effects, in the presence of host inflammatory cytokines, such as TNFα and IFNγ, the presence of TiO_2 NPs were shown to impair biological functions and processes that would be necessary for formation and maintenance of the intestinal epithelial barrier. it is estimated that daily human intake is within the range of 15-37.2 mg/day for a 75-kg adult [(7)]. Because the GIT is the primary route of intake and excretion of TiO_2 NP, the intestinal epithelial barrier will be consistently exposed to TiO_2 NP beyond the concentration and time range used in the present in our vitro studies. Furthermore, the inclusion of pro-inflammatory cytokines in the experimental design provided a more holistic and realistic model to assess the impact of TiO_2 NP exposure on IECs. Currently, the toxicity of nanoparticles is evaluated with various approaches that include both *in vitro* and *in vivo* methods but rarely do they take into consideration

the presence of microbial contributions and/or the host innate immune response (e.g., inflammatory cytokines).

Lastly, proper studies and screening techniques will need to be carefully considered and tailored for each cell of interest when assessing toxicity of nanoparticles. For example, as microbial components and host inflammatory mediators are present in the gut, these co-stimulatory mediators can change how a cell responds, which in turn can change the physiological outcome and cellular metabolism. Therefore, when assessing toxicological effects of nanoparticles on the GIT, it is necessary to factor in the above-mentioned components in order to mimic the *in vivo* environment of IECs and assess the interactions between those factors and the NPs. A failure to consider these factors might downplay level the toxicity of these NPs. Furthermore, *in vitro* studies utilizing nanoparticles give a limited view of deleterious effects due to finite number of cells in experiments. For nanoparticles, such as TiO_2 NP which are widely used as a food colorant, there needs to be more in depth and long-term *in vivo* studies that can account for extended exposure to TiO_2 NPs in order to fully assess the toxicological effects on the GIT and intestinal epithelium associated with chronic consumption.

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CHAPTER 5. GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

This section presents an overview of the results and findings of this dissertation, with a special emphasis on overall implications as they relate to: 1) assessment of the intestinal epithelial barrier when evaluating toxicological effects of nanoparticles and 2) the need to consider more holistic approaches when assessing toxic effects of nanoparticles *in vivo*. A discussion of the major findings for each research chapter included in this dissertation are covered in the respective Discussion section.

Importance of Assessing the Intestinal Epithelial Barrier and the Importance of Holistic Approaches When Assessing the Toxicity of Nanoparticles

Intestinal epithelial cells (IECs) play a critical role in formation and maintenance of the intestinal epithelial barrier which functions as a protective barrier from xenobiotics and foreign substance. Rather than focus on the response of inflammatory cells (e.g., macrophages, PMNs), it is necessary to include an assessment of the intestinal epithelial barrier in toxicity studies assessing cellular and/or molecular interactions with nanoparticles. Collectively, the results presented in the Chapters of this dissertation highlighted the fact that different effects were manifested on IECs by different metal oxide nanoparticles. This points out that there is a need to assess the impact of nanoparticles based on their composition and not on the size of the particle. Additionally, there is a need to include an assessment of the bioenergetics, metabolic effects and mitochondrial function of IECs as well as our studies have shown that an impairment in the mitochondrial respiratory capacity negatively affected IECs and cellular functions that play a critical role in the formation of intestinal epithelial barrier such as wound healing.

In Chapter 2, MnO NPs severely impacted mitochondrial function as evidenced by impairment of mitochondrial respiration. As manganese (Mn) plays an essential role in biological functions (i.e., antioxidant enzymes) important to the function of the mitochondria, the presence of MnO NP, which were shown to release Mn^{++}, severely impairs mitochondrial function. Mitochondrial respiratory chain consists of four complexes (Complex I, II, III, and IV) with functions in the electron transport chain that include ATP production via oxidative phosphorylation that is crucial for basal respiration, maximal respiration, and spare respiratory capacity which are all key factors in the metabolic function of cells. Impairment of these complexes have been shown to negatively affect oxidative phosphorylation resulting in effects ranging from the release of reactive oxygen species to the induction of apoptosis. Our studies show that MnO NP interferes and disrupts the function of these complexes in the mitochondrial electron transport chain. In addition, when MnO NP are in the presence of microbial components (i.e., TLR ligands), these effects are exacerbated as the interaction between the two have been overlooked.

Increase in GI disease has risen much more rapidly in Western societies than can be accounted for by genetic changes alone; however, environmental factors, such as metal oxide particles, are continuously being introduced into multiple aspects of everyday life. As IECs are exposed to antagonistic, environmental cofactors, mitochondrial impairment and inflammatory mediators, the toxic effects of these metal oxide nanoparticles have the potential to have synergistic deleterious effects that affect processes necessary for maintenance and formation of the intestinal epithelial barrier. As microbial components stimulate cells via Toll-like receptors, the additional presence

of MnO NP impair mitochondrial function resulting in IECs becoming biologically overwhelmed leading to their impairment. As mitochondrial electron transport can be impaired, this can potentially affect other functions of the mitochondria such as intracellular calcium absorption which is critical to health of cells.

In Chapter 3, the effects of TiO_2 NP on the IECs were also investigated. TiO_2 NP are found in many foods such as powdered-sugar donuts, chewing gum, milk-based products, chocolate, tablets of dietary supplements, and products with white icing. Studies. However, studies assessing the toxicity of TiO_2 NP on IECs are virtually nonexistent. When added to IECs in combination with microbial components, TiO_2 NP did not show any changes. However, when paired with inflammatory mediators such as IFNγ and TNFα, TiO_2 NP induced deleterious changes in wound healing, increased mitochondrial superoxide generation, and increases in mitochondrial activity (see Chapter 3). In contrast to MnO NPs which exacerbated deleterious effects in the presence of bacterial components, TiO_2 NPs in the presence of bacterial components did not exacerbate deleterious effects. Although Ti^{4+} (Titanium) does not have any role in biological functions, when in the presence of host inflammatory mediators, TiO_2 NP were able to change the physiological and cellular metabolic function of MODE-K cells. This suggests that when assessing the effects of nanoparticles, it is necessary to consider all factors that that a cell will encounter in an *in vivo* environment to fully assess the toxicity and deleterious effects.

In vitro studies of nanoparticles give a limited view of deleterious effects due to finite number of cells in experiments and acute studies. For nanoparticles such as TiO_2 NP, which are present in many foods currently on the market, there needs to be more *in*

vivo chronic studies that can account for the extended and accumulated level of exposures to fully assess the toxicological effects on the GI tract and intestinal epithelium associated with chronic consumption. As accumulation of genetic changes in the population do not explain the increase in GI disease seen in Western societies, it is necessary to start including the contribution of environmental factors as they are constantly and rapidly being altered and included in everyday life, including the use of metal oxide particles in foods such as TiO_2 NPs which have been shown accumulate in regions of the GIT where IBD often initiates.

For future studies, the use of primary 3D *ex vivo* organoid culture models for screening, investigating, and assessing chronic toxicological effects of nanoparticles could be beneficial. Using primary 3D *ex vivo* organoid culture models will allow for evaluation of primary cells while providing the opportunity for extended surveillance and assessment of tight junction proteins necessary for formation of the intestinal epithelial barrier. These tissue culture models can be passaged continuously and give insight as to the changes occurring at the molecular level, including epigenetic changes, after every passage by assessing time dependent changes between primary 3D *ex vivo organoid* culture models from different timeframes.

When designing an experimental approach and screening techniques assessing the toxicity of nanoparticles, it is necessary to consider the composition of the nanoparticles, choose the appropriate cell line or tissue culture model and design each experiment to test a hypothesis. For example, IECs in the gut will either encounter microbial components and host inflammatory mediators or both that can change the physiological outcome and cellular metabolism based on how a cell responds.

Therefore, when investigating the toxicological effects of consumed or ingested nanoparticles, such as MnO_2 NP and TiO_2 NP, on the GI tract and gut homeostasis, it is necessary to factor in those components, such as the microbial interaction and the interaction with host inflammatory mediators, to achieve more realistic assessments. In conclusion, the work presented in this dissertation highlight the importance of including biological components that a given tissue type may encounter *in vivo* when designing an experimental approach for assessing the toxicological effects of nanoparticles so that the most holistic assessment of what transpires *in vivo* is obtained.

It is necessary to include primary cultures of 3D organoids in this assessment as they can give insight to chronic and long-term effects of the NP toxicity on IECs. As the GI epithelium is constantly active to prevent overcolonization of gut microbes, maintain a clinically anti-inflammatory environment, and constantly turning over every 3-7 days, it is necessary to also factor in chronic effects of the environmental impacts (e.g. NPs) on IECs and formation of the intestinal epithelium. As we have taken initial steps towards adding a level of complexity to the assessment of NP toxicity on IECs, our studies have shown that the addition of either microbial components or host inflammatory mediators increase the toxicity of NPs and that IECs do not interact with these NPs in isolation. The next step of assessment is to use animal models that integrate both microbial components and host inflammatory mediators while assessing NPs toxicity to achieve an even more complete representation of *in vivo* situation than seen in our studies.

APPENDIX: CYTOTOXIC EFFECTS OF MANGANESE OXIDE NANOPARTICLES IN COMBINATION WITH MICROBIAL COMPONENTS IN INTESTINAL ORGANOIDS

Introduction

Intestinal epithelial cells (IECs) play a role in the maintenance the intestinal epithelial barrier that acts as a physical barrier between our body and exogenous substances. Cytotoxic environments in the GI tract can cause impairment and death of the epithelial cells which results in the breakdown of this barrier function. This breakdown of the intestinal epithelial barrier can then lead to an uncontrollable inflammation which increases the risk of developing diseases such as IBD and other inflammatory diseases. Until recently, intestinal epithelial-damaging activities of environmental toxicants, drugs, and treatments could be tested only in vivo in animal models because of the poor survival rate of primary IECs ex vivo [(1)].

The three-dimensional culture and outgrowth of intestinal crypt stem cells into organoids have offered new possibilities to culture and study IECs ex vivo. Organoids represent an important bridge between traditional 2D cultures and *in vivo* mouse/human models, as they are more physiologically relevant than monolayer culture models and are far more amenable to manipulation of niche components, signaling pathways and genome editing than *in vivo* models [(2)]. The organoid system allows researchers to intensively study the processes that govern embryonic development, lineage specification and tissue homeostasis, as well as the onset and manifestation of disease. Furthermore, organoids can be used to for modeling host– microbe interactions [(3)]. It also delivers invaluable insight into the development of stem cells and their niches, while providing an opportunity to monitor their differentiation into mature functional

lineages [4]. Organoids ultimately provide an opportunity to investigate the effects of environmental toxicants, drugs, and treatments on the development of the intestinal stem cells during normal and disease conditions while incorporating a more holistic approach that mimics the intestinal homeostatic environment that includes the host-microbial interaction. Since intestinal stem cells *in vivo* are exposed to both microbial components that play a role in their development and maturation, we hypothesize that the combination of these factors will result in the enhanced toxicity of environmental pollutants, such as MnO NP. Therefore, to mimic the *in vivo* environment where intestinal stem cells are exposed to both environmental pollutants and microbial components and create a more realistic assessment, in the present study we examine the adverse effects of MnO NP in conjunction with bacterial lysate on intestinal organoids.

Materials and Methods

Chemicals

Manganese oxide nanoparticles (MnO NP (**US Research Nanomaterials, Inc.**, Houston, TX, USA)) used in all experimental procedures were provided by Dr. A. Kanthasamy.

Organoid Culture and Treatment

Organoids used in this study was provided by Dr. Qun Wang. The four treatment groups for the experimental procedure were: control group, 1 µg/mL bacterial lysate only group, 30 µg/mL MnO NP only group, and 30 µg/mL MnO NP and 1 µg/mL bacterial lysate group. Dose titration was performed and minimal dose was used.

Preparation of Bacterial Antigen

An adherent invasive *E. coli* (AIEC) strain LF82 that has been associated with ileal Crohn's disease) was cultivated in BHI broth and incubated at 37 °C in an aerobic environment. Bacteria were harvested after overnight growth, washed with PBS, frozen, lyophilized, and stored at -20 °C until used. Bacterial cells were weighed and resuspended in sterile PBS (2 mg/ml) prior to being subjected to two freeze-thaw cycles, followed by sonication at 50 amps, 75 amps, and 100 amps for 30 seconds at each amperage. This sonication cycle was repeated three times. The bacterial lysate was then sterilized by ultraviolet irradiation and sterility confirmed bacteriologically. With minor modificatons, this method was used as previously described [(5)].

MTS Cell Viability Assay

Cell viability was measured using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation (MTS assay kit from Promega Corporation, Madison, WI). Organoids were plated in 24-well plates one day prior to treatment with low glucose DMEM. The next day cells were plated with respective treatments in low glucose DMEM media for 48 hours. Following treatment, 20 µL of MTS solution reagent mix was added to each plate well and incubated at 37 °C for 30 minutes. At the end of incubation, readings were taken at a wavelength of 490 nm and another reference reading for each well was taken at 670 nm to eliminate background. This method was previously described and adopted to fit our experimental procedure [(6)].

Results

MTS Cell Viability

Mitochondrial dysfunction is associated with a loss of proliferative capacity in intestinal epithelial cells [7]. As mitochondrial function is associated with health and maintenance of gut epithelial barrier, MTS proliferation and cytotoxicity assay is crucial for insight about cell viability and health of cell population. The four treatment groups for the experimental procedure were: control group, 1 µg/mL bacterial lysate only group, 30 µg/mL MnO NP only group, and 30 µg/mL MnO NP and 1 µg/mL bacterial lysate group. After 48 hours of incubation with treatment, at least a 21 % decrease in mitochondrial activity was seen in the treatment group 30 µg/mL MnO NP and 1 µg/mL bacterial lysate group when it was compared to any other group (Figure 1).

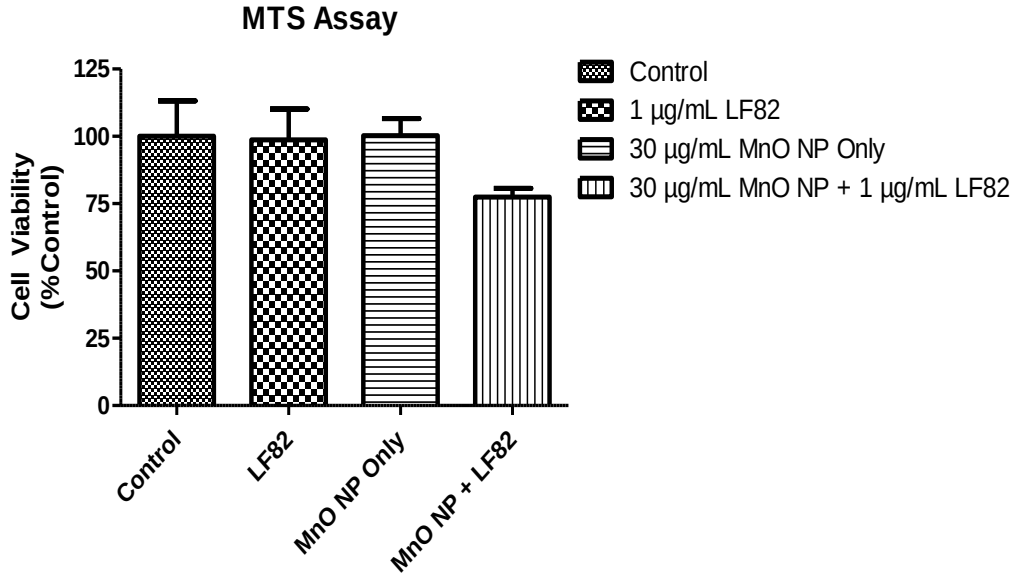

Figure 1. Cell viability was measured using MTS assay. As MTS assay takes into account three parameters (cytotoxic effect, cytostatic effect, and antiproliferative effect), any decrease in viability may be a result from either one or a combination of these three parameters. After 48 hours of treatment incubation, viability of MODE-K cells was measured. There was at least a 21% decrease in viability of organoids in MnO NP + LF82 group when compared to any other treatment groups.

The results of MTS revealed that the presence of MnO NP with bacterial lysate induced cytotoxicity and decreased cell viability more than any of the other treatment groups. As MTS assay takes into consideration three parameters (cytotoxic effect, cytostatic effect, and antiproliferative effect), any decrease in viability may be a result of either one or a combination of these three parameters that may affect health of intestinal organoids.

Discussion

The intestinal epithelial barrier, which consists of several elements including intestinal epithelial cells, tight junctions, and adherens junctions, prevents undesirable solutes such as microorganisms, viruses, toxins, and luminal antigens from entering the body [8, 9]. It is suggested that disruption of the intestinal barrier function and repeated intestinal epithelial damage are key features of IBD, as well as other intestinal disorders [9, 10]. As organoids is a close representation of intestinal stem cells, they can give insight into the development of IECs and their developmental process during disease [4]. These organoids also can be beneficial in providing details on the development of the intestinal epithelial barrier after encountering environmental pollutants. Results from this study show that treatment with MnO NP only did not affect the viability of organoids. However, viability of organoids in the MnO NP and bacterial lysate treatment group were at least 21% less when compared to any of the other treatment groups. This data suggests that in a more holistic environment that contains other components that are present in vivo, environmental pollutants, such as MnO NP, have an even more deleterious effect.

As environmental pollutants may not have effects by themselves, the presence of microbial components can induce other factors such as an inflammatory response, that in combination with effects of environmental pollutants, induce toxicity that exacerbates effects and ultimately leads to detrimental consequences. These results imply that there is a necessity to incorporate other stimuli present in the gut, such as microbial components and inflammatory cytokines, when investigating effects on IEC health and ultimately intestinal barrier integrity as this represents a more holistic approach that more closely mimics the gut environment. In addition. Furthermore, these results suggest that organoids can provide information into the development of intestinal stem cells in gut disorders and diseases as they present us with an ability to monitor and track the differentiation of stem cell into mature functional lineages that eventually develop to form the intestinal barrier.

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