Interaction of TiO₂ and ZnO NPs with human skin cells

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

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**INTERACTION OF TiO_2 AND ZnO NANOPARTICLES
WITH HUMAN SKIN CELLS**

ARCHANA GAUTAM

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2018

**INTERACTION OF TiO₂ AND ZnO NPs WITH
HUMAN SKIN CELLS**

ARCHANA GAUTAM

**SCHOOL OF MATERIALS SCIENCE AND
ENGINEERING**

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement
for the degree of Doctor of Philosophy

2018

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Authorship Attribution Statement

This thesis contains material from a paper submitted in the following peer-reviewed journal where I am the first author.

Chapter 5 is submitted as Gautam, A., Rakshit, M., Nguyen, K.T., Kathawala, M.H., Nguyen, L.T.H., Tay, C.Y., Wong, E. and Ng, K.W., 2019. Understanding the implications of engineered nanoparticle induced autophagy in human epidermal keratinocytes in vitro. *NanoImpact*, 15, p.100177. <https://doi.org/10.1016/j.impact.2019.100177>

The contributions of the co-authors are as follows:

- Gautam, A. designed and performed the experiments, analyzed the data, performed statistical calculations and wrote the manuscript.
- Rakshit, M. and Wong, E. contributed to the autophagy experiments, analyzed the data and helped to write the manuscript
- Nguyen, K.T. and Tay, C.Y. designed and helped with the apoptosis experiments.
- Kathawala, M.H. was responsible for dosimetry assessment and evaluation.
- Nguyen, L.T.H. helped to supervise the experiments and contributed to data analysis.
- Ng, K.W. conceived the project, helped to design the study, supervised the team and edited the manuscript.

23 Aug 2018

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Abstract

Nanotoxicology encompasses the study of the toxicity of nanoparticles (NPs) in order to better understand the adverse effects of NPs on human health and evaluate the health risks associated with the use of NPs. With the influx of large numbers of nanomaterials in many applications such as cosmetics, paints, and medicine, nanotechnology has been gaining immense attention in recent years. Titanium dioxide (TiO₂) and Zinc oxide (ZnO) NPs are produced abundantly and are among the most ubiquitously used NPs in various consumer products, especially sunscreens. Penetration of these NPs through the intact skin barrier has been shown, albeit at low levels. Concerns over their increased penetration across the skin in cases of the compromised or altered skin barrier and the consequent physiological influences are real. However, understanding of nanoparticle-mediated toxicity is still limited. Therefore, the goal of this study is to assess the physiological effects of TiO₂ and ZnO NPs interactions with primary human epidermal keratinocytes and dermal fibroblasts, with attention to accurate dosimetry measurements and particle solubility considerations. Since the current findings on NPs interaction with skin cells are mostly based on 2D monolayer cell culture and animal models, in this study, 3D human skin explants have also been used to better understand the effects of NPs in the skin tissue. Based on 2D monolayer studies, it was observed that both TiO₂ and ZnO NPs were toxic to cells at higher concentration, inducing various cellular responses such as oxidative stress, apoptosis, inflammation, and autophagy. The cellular response was dependent on the dose and exposure period. TiO₂ NPs induced lower cytotoxicity even at higher doses, whereas ZnO NPs caused acute cell death at a much lower dose. Comparing the cellular responses of two cell types, it was observed that both NPs were comparatively more toxic to keratinocytes than to fibroblasts. In keratinocytes, the onset of autophagy was observed at much lower/non-toxic doses. At higher/sub-toxic doses, both autophagy, and inflammation were induced as NPs mediated toxicity. This study, for the first time, shows that autophagy induction at higher doses of TiO₂ NPs blocked p65 phosphorylation in keratinocytes, thereby blocking the inflammatory pathway. However, this phenomenon was not observed in keratinocytes exposed to ZnO NPs. In contrast to this, NPs induced autophagy was not

observed in dermal fibroblasts. Furthermore, from the studies performed on human skin explants, it was observed that TiO₂ and ZnO NPs did not cause any significant effects on skin physiology and wound healing. Although these NPs proved to be toxic on cells based on 2D monolayer culture, their significant effects on cells in 3D human skin explants were not observed. Both NPs were internalized by the keratinocytes at the wound edge, and notably, penetrated into the nuclei of the keratinocytes. The penetration and uptake of NPs were also dependent on the dose and exposure period. Although higher amounts of TiO₂ NPs penetrated into the nuclei, they did not cause significant DNA damage to the keratinocytes. On the other hand, ZnO NPs caused DNA damage to the keratinocytes located at the wound edge and migrating tongue of the wound. In summary, this study revealed that lower doses of TiO₂ and ZnO NPs induced autophagy in keratinocyte acts as a pro-survival mechanism and protects the cell against oxidative stress and inflammation. At higher doses, they caused toxic effects to the cells, however, they did not significantly affect skin physiology or wound healing.

Lay Summary

Moderate amount of sun rays is beneficial for good health, however, too much sun exposure could cause harmful effects. It has been well known for many years that excessive sun exposure could increase the risk of skin cancer. Sunscreens have been used for decades to protect from the harmful rays of the sun. The main ingredients in the sunscreen responsible for blocking the sun rays are TiO₂ and ZnO NPs. These ingredients, although invisible to the human eye, could cause serious health effects. Numerous studies have reported that these NPs can harm living cells when exposed to higher doses. Since sunscreens are topical application products, human exposure to these NPs can be via the skin. Healthy human skin has a very strong barrier function preventing any foreign materials to penetrate into the body. The potential ability of these NPs to penetrate via healthy skin is reported to be very low. However, there can be situations in which the barrier function of the skin is compromised, such as skin diseases, wounds or cuts. In these scenarios, the NPs could penetrate into the skin and reach the viable cells layer, where they can interact with the skin cells and cause adverse effects. Therefore, this study is focused on studying the interaction of these NPs with viable skin cells. It has been observed that these NPs could adversely affect the skin cells at high administered doses. However, these studies were done by direct exposure of NPs onto the monolayer culture of skin cells. Monolayer cultures may not accurately represent the accurate cellular condition of the human body. Therefore, studies were also performed on human skin explants that were obtained from patients undergoing abdominal reduction surgeries. Wounds were made on the skin biopsies, and NPs were administered over the wound. It was observed that NPs did not significantly affect the skin physiology or wound healing process. However, they could be internalized by the cells and can cause DNA damage. Although more studies need to be done in order to confirm the risks associated with the exposure of skin to these NPs, this study demonstrates the potential physiological impact of direct cellular exposure to TiO₂ and ZnO NPs, and the possibility of these NPs being uptaken by skin cells through an open wound.

Abbreviations

NPs	Nanoparticles
TiO ₂	Titanium Dioxide
ZnO	Zinc Oxide
UV	Ultraviolet
FDA	Food and Drug Administration
GRAS	Generally Regarded As Safe
ISO	International Organization for Standardization
SPF	Sun Protection Factor
OCD	Occupational Contact Dermatitis
IL	Interleukin
VEGF	Vascular Endothelial Growth Factor
PDGF	Platelet-Derived Growth Factor
KGF	Keratinocyte Growth Factor
ECM	Extracellular Matric
bFGF	Basic Fibroblast Growth Factor
FGF	Fibroblast Growth Factor
IGF	Insulin-like Growth Factor
ROS	Reactive Oxygen Species
MSO	Mitochondrial Superoxide
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B
IKK	IκB kinases
Hsp90	Heat shock protein 90
TNF	Tumor Necrosis Factors
ATP	Adenosine Triphoshpate
PLA2	Phospholipase A2
MAPK	Mitogen-activated protein kinase
PI3-K	Phosphoinositide 3-kinase
ATG	Autophagy Related Gene

LC3-II	Lipidated microtubule-associated Light Chain 3
MHC	Major Histocompatibility Complex
TWAD	Time Weighted Average Dosage
ISDD	Invitro Sedimentation Diffusion and Dosimetry
TEM	Transmission Electron Microscope
EDS	Energy Dispersive Spectroscopy
EELS	Electron Energy Loss Spectroscopy
XRD	X-ray Diffraction
BET	Brunauer–Emmett–Teller
DLS	Dynamic Light Scattering
PDI	Poly Dispersity Index
ICPOES	Inductively Coupled Plasma Optical Emission Spectrometry
PCV	Packed Cell Volume
TPP	Techno Plastic Products
DMEM	Dulbecco's modified eagle medium
PBS	Phosphate buffer solution
Cm-H ₂ DCFDA	Chloromethyl derivative of 2',7'-Dichlorofluorescein diacetate
DMSO	Dimethyl sulfoxide
NAC	N-acetyl cysteine
MDC	Monodansylcadaverine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ECL	Enhanced chemiluminescence
NL	NH ₄ Cl and leupeptin
IRB	Institutional Review Board
HRP	Horse radish peroxidase
FBS	Fetal Bovine Serum

Chapter 1

Introduction

This chapter begins with a brief introduction to nanotechnology and its impact on human life. Nanotechnology, more precisely, engineered NPs, are being used in a wide range of applications today. As such, the wide availability of products based on engineered NPs in markets has drawn serious attention towards the safety and risks of these NPs. Out of many NPs, inorganic metal oxide NPs: TiO_2 and ZnO are focused in this thesis. Their application in sunscreen and cosmetic products, the potential toxic risks of using them, and hence the need for further investigation of their toxicity is discussed. The hypothesis and objective of this research work are listed, along with the scope of the work and the novelty achieved. This chapter ends with a brief overview of each chapters in this thesis.

1.1. Research Background

Nanotechnology, today, is the most influential force changing human existence significantly with its capabilities to produce nano-sized materials. Although nanotechnology is relatively a recent development in the field of scientific research, its history dates back to 1950's when American Physicist Richard Feynman (1918-1988) discussed about nano-sciences in 1959 in his speech "There's Plenty of Room at the Bottom" where he speculated the possibility of direct manipulation of individual atoms [1]. However, his talk went unnoticed at that time and only over a decade later, the term "nanotechnology" was first used in 1974 by Norio Taniguchi [2]. Since then, the emergence of nanotechnology accelerated rapidly with its endless possibilities in all aspects of life. Nanotechnology could cure cancer, alleviate world hunger, solve the energy crisis, provide clean drinking water, and provide better air quality [3]. It could benefit society in unexpected ways, but it could also concoct unimaginable weapons of destruction: it could be the new asbestos.

Today, nanotechnology is widely used in medicine, industry, cosmetics, life sciences, and research. Incredibly, the biggest utilization of nanotechnology is in consumer-based everyday products with more than 1300 different products based on nanotechnology [4]. However, a debate regarding the safety and risks of nanotechnology-related products and applications to human health has arisen along with public awareness. As the information on safety and potential hazards of NPs is still not very clear, it has led to a number of controversies. The safety evaluation of these NPs has, therefore, become a serious concern. As such, "nanotoxicology", an important sub-discipline of nanotechnology has been established with the aim of developing a thorough understanding of "nanosafety". Identification of nanotechnology derived potential hazard will benefit human health and also help to advance nanotechnology as a whole. To this end, this study is designed to study the potential toxicity effects of engineered NPs TiO_2 and ZnO in human skin.

Inorganic metal oxide micro-particles TiO_2 and ZnO , have been widely used in sunscreens for decades due to their large benefits as excellent UV filters [5, 6]. With the help of

nanotechnology, these particles were reduced to nano sizes, further augmenting their optical properties [7]. The nano-size makes them appear transparent on the skin; a highly favourable property for use in cosmetic products [8]. As such, many cosmetic companies have started using copious amounts of nanomaterials in their products. According to the US Food and Drug Administration (FDA), they are generally regarded as safe (GRAS) materials, thereby allowing for incorporation of TiO_2/ZnO in sunscreens up to 25%, alone or in combination [9]. It is well-known that with the decrease in particle size, the specific surface area of the particle increases, making them more reactive than their bulk form [10]. Therefore, despite the significant benefits of TiO_2 and ZnO NPs, their advantages can be potentially outweighed by the risk of toxicity if these NPs are able to cross the skin barrier and interact with the underlying viable cells [6]. The NPs may come in contact with the skin through environmental or occupational exposure or through deliberate application of sunscreens or any other formulations containing NPs. Their ability to interact with the viable skin by penetrating through the stratum corneum into the epidermis is still questionable.

Being a common ingredient in sunscreen and other topical application products, TiO_2 and ZnO NPs effect on the human skin cells are undoubtedly important. The matter of safety and toxicity of these NPs have been an issue of interest to the public. Although they possess minimal ability to penetrate through the skin layers [11], their potential penetration in the skin with altered barriers such as diseased skin, or even UV burnt skin has rarely been studied. Additionally, the possibility of these NPs being directly exposed to viable skin layers through cuts or wounds cannot be neglected. Therefore, numerous studies have been taken up for toxicity studies of these NPs in various cell types. TiO_2 and ZnO NPs-mediated cytotoxic and genotoxic effects have been shown in many in vitro studies [11]. The underlying mechanisms in NPs-induced toxicity, including apoptosis, necrosis, oxidative stress, and autophagy has also been explored in many cell types. Among these mechanisms, autophagy is one of the recently emerged and its cellular and molecular mechanisms in NPs-induced toxicity with context to human skin cells have not been well understood yet. Our group has previously shown that TiO_2 and ZnO NPs can induce autophagy in human epidermal keratinocytes at a very low administrative dosage [12]. However, the mechanism

of autophagy induction has not been studied in detail. Additionally, numerous recent evidence has demonstrated the role of autophagy in inflammation [13]. Inflammation is one of the important cellular response to NPs. Therefore, this research work aims to study the mechanism of NPs mediated toxicity in context with autophagy and inflammation. Furthermore, to date, the assessment of NPs mediated toxicity has mostly been done using 2D in vitro cell culture models. Although 2D models are largely well suited for toxicological assessments, they may not accurately model the functions of 3D tissue having cell-cell and cell-matrix interaction. Despite providing important and valuable information, tests performed on 2D cell culture models may not accurately predict in vivo toxicity and other biological responses. Thus, in this study, we aim to bridge this gap by using a more physiologically relevant model, ex-vivo skin explants as a promising and valuable approach to further the knowledge on NPs toxicity.

A key factor in evaluation toxicity of TiO₂ and ZnO NPs is whether they are able to penetrate into the skin. According to current evidence, both TiO₂ and ZnO NPs are not able to penetrate into the viable layers of the skin, but rather remain on the skin surface or stratum corneum [11]. However, most of these studies were performed on healthy skin. In a real-world scenario, NPs can be exposed to compromised skin barrier such as skin with wounds or cuts. In the absence of stratum corneum barrier, there is a potential for these NPs to penetrate and reach the viable layers of the skin or even reach the systemic circulation from where they can translocate into the various organ through the bloodstream. Therefore, although the previous findings reporting that NPs are not able to penetrate into the skin may be valid, there remains a concern of the risk of penetration if the skin barrier has been altered. Thus, the particles interaction when they come in contact with the cells residing in the skin tissue needs to be done. Lastly, the effects of these NPs in wound healing also needs to be investigated - this is one of the motivations for this research. Therefore, the aim of this study is to fulfil this gap with a better understanding of the biological effects of these NPs' interaction with skin cells.

1.2. Hypothesis

It was hypothesized that:

1. TiO₂ and ZnO NPs induce autophagy in epidermal keratinocytes and dermal fibroblasts which acts as a pro-survival response to oxidative stress and inflammation.
2. TiO₂ and ZnO NPs can be internalized by epidermal keratinocytes through an open wound.
3. TiO₂ and ZnO NPs can affect normal wound healing processes through direct interaction with skin cells.

1.3. Objectives

In order to prove the hypothesis stated above, this study is divided into three parts with corresponding objectives as under.

- 1) *NPs characterization and dosimetry:*
 - a) To characterize materials properties of TiO₂ and ZnO NPs.
 - b) To compute the actual dose of NPs experienced by cells through dosimetry.
- 2) *2D studies on individual cell types:*
 - a) To carry out accurate and physiologically relevant exposure studies of TiO₂ and ZnO NPs on two major cells types of skin: human epidermal keratinocytes and human dermal fibroblasts.
 - b) To understand the effects of exposure studies as part of hazard identification for risk assessment purposes.
 - c) To propose the mechanism that brings about the effects found in point 2a, especially with regards to the roles of autophagy and inflammation.
- 3) *3D studies on human skin explants:*
 - a) To understand and compare the cell physiological effects of direct interaction of TiO₂ and ZnO NPs with viable skin cells via exposure through an ex-vivo open wound model.

- b) To correlate the cell physiological effects of TiO₂ and ZnO NPs influences on re-epithelialization of an open wound.

1.4. Scope

Considering dermal exposure of NPs, this study was conducted on two major types of skin cells: human epidermal keratinocytes and human dermal fibroblasts, for investigating cellular toxicity. Although a combination of TiO₂ and ZnO NPs are mostly used in sunscreen, in this research work, individual NPs effects are focused. The simulation of sunscreen ingredients is not within the scope of this project. Thorough characterization of NPs and computation of dosimetry simulation was employed to calculate the actual mass of NPs experienced by the cells at a particular time. The focus was on investigating the toxic effects of NPs on the cells with an emphasis on the actual dose of NPs received by the cells at a particular time. Oxidative stress, autophagy, and inflammation induced by NPs were investigated with the aim of revealing the mechanism of NPs induced toxicity. Furthermore, human skin explants were used to investigate the direct interaction of NPs with the skin cells and their effects on wound healing. Histological, immunohistochemistry and immunofluorescence techniques were employed to investigate NPs' effects in wound healing. Lastly, bio-transmission electron microscopy was used to observe NPs uptake, localization, and ultrastructural changes in the skin tissue. The penetration of TiO₂ and ZnO NPs through the normal intact skin is not within the scope of this work.

1.5. Novelty

Numerous reports on safety aspects of sunscreen NPs mainly focus on various kind of toxicological works on 2D cell culture models. Till date, NPs' effects on direct interaction with cells residing inside the human skin tissue have not been studied yet. Therefore, in this study, human skin explants with an open wound have been used to understand the interaction of NPs with skin cells. This ex-vivo model is also used to study the effects of NPs on wound healing.

1.6. Dissertation Overview

This thesis addresses the interaction of commercial NPs: TiO₂ and ZnO in human skin by investigating their effects on individual cell types and on human skin explants.

Chapter 1 provides a rationale for the research and outlines the hypothesis, objective, scope, and novelty of the work.

Chapter 2 provides a review of the literature on nanotechnology and potential exposure to nanomaterials, focusing on the dermal route of NPs. This is followed by a brief introduction of the human skin and the process of wound healing. Likewise, the current understanding of NPs' toxicity to skin and the biological mechanisms of toxicity is discussed.

Chapter 3 comprises of the experimental methodology, materials used, analysis and approaches used in this study. The rationale behind using the equipment and their working principle is also discussed.

Chapter 4 contains a thorough characterization of NPs used in this study and computation of NPs dose by dosimetry.

Chapter 5 presents the findings and discussions on TiO₂ and ZnO interaction with human epidermal keratinocytes based on 2D cell culture.

Chapter 6 presents the findings and discussions on TiO₂ and ZnO interaction with human dermal fibroblasts based on 2D cell culture.

Chapter 7 presents the findings on the direct interaction of TiO₂ and ZnO NPs with skin cells through exposure in an open wound, and their effects on wound healing based on human skin explants.

Chapter 8 summarizes the overall findings of this study followed by the future outlook of this thesis.

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Chapter 2

Literature Review

This chapter provides a detailed review of the literature on nanotechnology and the necessity of nanotoxicity. It begins with an introduction to nanotechnology and NPs, with the main focus on TiO₂ and ZnO NPs and their use in sunscreen. This is followed by the various routes of exposure to these NPs and their abilities of skin penetration. The chapter progresses with an introduction on the anatomy/physiology of skin and the process of wound healing. Furthermore, the recent findings on the adverse effects of these NPs on human skin cells are presented along with the biological mechanism of NPs mediated toxicity. Finally, it ends with the current state of knowledge on NPs induced toxicity and the necessity of toxicity studies for safety and risk evaluation of NPs.

2.1. Nanomaterials and Nanotechnology

The term nanomaterial refers to “a material with any external dimensions in the nanoscale or having internal structure or surface structure in the nanoscale” (International Organization for Standardization (ISO) 2008). In October 2011, European Union (EU) redefined nanomaterials as “a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1nm-100nm”. This definition is most widely used to establish whether a material is a nanomaterial or not. Among the various types of NPs, natural NPs are present in biological and geological systems, such as viral capsids or some crystal structures. They are naturally produced and have a specific use or give special properties to the material. Engineered NPs or manufactured nanomaterials are intentionally produced for commercial production and have been used for a few decades in a variety of products: paints, cosmetics, pharmaceuticals, food products, electronic compounds, and so on. Other applications such as imaging, surface coating and functionalization for a biological use have also been developed using engineered NPs. This type of NPs includes fullerenes, carbon nanotubes, as well as metal-based NPs. Incidental NPs are produced during an industrial or mechanical process.

At the nanoscale, the surface-to-volume ratio of NPs is increased exponentially, which gives them very unique physical, chemical, and biological properties, making them dramatically different in comparison with the bulk form of the similar chemical composition. The materials are thought to have ultrahigh reactivity, high specific surface areas, special electronic characteristics and quantum effects at the nanoscale [1]. Exploitation of these unique physical, chemical, and electrical properties gave rise to nanotechnology. Nanotechnology is the manipulation of NPs or nanomaterials in order to achieve the desired properties. The fabrication of engineered nanomaterials and the application of nanomaterials in different fields started rapidly. The market of nanotechnology is growing and reached an estimated generation of revenues of a trillion dollar in 2015, with the development of nanoelectronics, nanomaterials, nanobiology, and

nanomedicine [2]. The widespread of nanotechnology potentially increased the risk of intentional or unintentional human exposure and environmental release [3]. The exposure of NPs to the human or other living organisms may be through dermal contact, exposure through water, inhalation or ingestion. This accidental human exposure and release of NPs into the environment possess unknown health risk. Therefore, the concern regarding the safety and risk evaluation of NPs has been raised. To address the adverse health effects caused by NPs, a new field of bioscience, nanotoxicology, emerged [4]. Nanotoxicology is the branch of science which deals with the study of the toxicity of nanomaterials. Nanotoxicity is crucial to determine whether/or to what extent there is the risk associated with the NPs. Nanotoxicity related studies are being increased exponentially since early 2000 to 2017 and are still increasing (figure 2.1).

Figure 2.1. The number of PUBMED listed publications from 1 January 2000 to 8 July 2018 on “nano toxicity”, “nano toxicology” and “nano safety”, generated from PUBMED database on 8/9/2018.

2.1.1. NPs in sunscreen

The use of nanotechnology in consumer products started in the early 2000s and today many

products relying on nanotechnology are readily available in the market and have gained huge commercial success. Sunscreen is one of those products which contains insoluble, inorganic mineral based engineered NPs TiO_2 and ZnO as effective ingredients. These NPs have been widely popular due to their stability and low allergic reaction under UV exposure [5]. However, the limitation of these NPs was the natural opaqueness of the micro-sized particles making it appear white on the skin accompanied by grainy feeling. With the advancement of nanotechnology, these shortcomings were addressed by reducing the particle size to the nano range [6]. At the nanoscale, a scattering of visible light by the particles was reduced, making them invisible to the naked eye without compromising, if not even further enhancing their optical properties as UV filters [7].

Sunscreens are widely used on a daily basis to protect the skin from harmful UV rays. Consumers apply a thick coat over large areas of the body and reapply frequently. Although NPs exposure through the skin is generally perceived as less hazardous than other routes of exposure such as inhalation or ingestion, there still remains significant knowledge gaps in this topic. According to current evidence, penetration of NPs through intact skin barrier is extremely unlikely. However, such penetration may be increased when the physical barrier of the skin is compromised. There are possible scenarios that a person's skin barrier can be damaged. For instance: if a person applies a heavy coat of sunscreen and then suffers an injury on his arms/legs, the applied sunscreen might come in contact with the open wound and penetrate into the deeper layers of the skin. Many studies on the interaction of NPs with biological systems including skin cells have been reported. These NPs have been shown to be capable of exerting a variety of toxicological influences including cell death, oxidative stress, inflammation and DNA damage. Therefore, the main focus of this work is placed on NPs in sunscreen to provide a concise perspective on the understanding of nanotoxicology in the skin.

TiO_2 exists in two states: anatase and rutile, which are of the highest commercial importance representing 90% and 10% of the market respectively. The band gap energy of rutile and anatase TiO_2 is 3.2 eV and 3.0 eV respectively, corresponding to the wavelength of 385 nm and 400 nm respectively [8]. Likewise, the most common form of ZnO is Zincite

which has a broadband spectrum with a band gap energy of 3.2 eV, with an ability to absorb both UVA and UVB rays [9, 10]. These properties make TiO₂ and ZnO excellent UV filters providing Sun Protection Factor (SPF) more than 30. In addition to providing a broad spectrum of protection, they are also irritation and sensitization free, which makes them an ideal material for use in sunscreens [6, 7].

2.2. Potential exposure to NPs

Due to the high magnitude of NPs-based application, the risk of exposure to NPs has increased. Exposure is an important variable which needs to be examined before reviewing the context of relevant toxicology and human health outcomes. The risk of exposure to NPs exists at numerous scenarios within the duration of NPs lifecycle, starting from synthesis to disposal. The levels of exposure, the amount, the duration of exposure and the type of exposure to nanomaterials varies in all these scenarios [11]. It is estimated that 2 million new workers will be exposed to engineered NPs over the next 15 years [12]. Workers in the industrial situation are at the highest risk of exposure during the production, handling, transferring, packaging, and mixing of high volumes of NPs. The general population may get exposed to NPs once the NPs move into commercial production, through the use of NPs based consumer products. Additionally, the release of NPs into the environment is also possible through industrial waste or accidental release from NPs based products. For instance, TiO₂ and ZnO NPs may be released into the water through the use of commercial sunscreens. TiO₂ may also be released into the air through commercial sprays. Humans may be indirectly exposed to these particles in the air, water or food chain [13].

The possible routes of human exposure to NPs include i) inhalation, ii) ingestion and iii) dermal uptake. Besides these, another potential route of exposure is an injection of NPs for medical purposes. Inhalation of NPs may be by the use of commercial powders, sprays and cleaning products. Nano-sized particulates in the polluted air such as that derived from vehicle exhaust, demolition, spray paints, and waste combustion also create a major exposure threat. Likewise, NPs exposure through ingestion could be by consuming food products containing NPs such as silver and TiO₂ NPs, which are used in flavor enhancers,

food pigments or health supplements [14]. Additionally, consuming contaminated food and water, and the use of sun protective lip balms may also result in exposure to NPs. Inadvertent ingestion of NPs by hand-to-mouth transfer is also possible.

NPs uptake by the skin is mostly due to topical application of consumer products such as sunscreen, cosmetics, sprays and other drug treatments [13, 15]. Additionally, paints, coating, and sealants may also contain NPs which can be uptaken by the skin. NPs may also be exposed to the skin by the use of NPs based wound dressings and surgical sutures [16]. Skin integrity is an important factor in determining the risk of exposure. Skin diseases such as occupational irritant contact dermatitis (OCD) is very common in the industrial sectors. Loss of skin integrity, in the case of OCD or any other skin diseases, could enhance NPs penetration and permeation. The risk of exposure gets higher when skin damage and enhanced exposure is combined together [17]. Airborne NPs may also lead to dermal exposure by deposition of NPs on to skin or clothing through inertia or gravitational settling [18].

2.2.1. Exposure from topical application of sunscreen

Numerous studies have been performed to assess the toxicity and risks of engineered NPs. In addition to toxicological data, the estimation of particles exposure is also essential for risk assessment. Lorenz et al. modeled potential exposure to NPs by the application of cosmetic products and personal care products. According to the exposure assessment analysis, exposure of NPs on the skin of female teenagers could amount to as much as 1.61 mgday⁻¹kg⁻¹ or 23.5 g per year for an average 40 kg individual from the application of sunscreens alone [19]. Numerous reports have shown that these NPs can penetrate the stratum corneum and reach the viable cell layers, especially when the skin barrier is compromised such as via UV irradiation [20, 21]. Even though most studies report very low penetration of NPs, e.g. Miguel-Jeanjean reported that 0.19% of the TiO₂ NPs applied on skin reached the epidermis and dermis combined [22], considering the high amounts which might be exposed to the skin, the payload which penetrates the skin might still be

significant. Considering the small size, even low masses could still mean millions of NPs which further substantiates the concerns.

2.2.2. Dermal penetration of TiO₂ and ZnO NPs

A key factor in evaluating toxicity is whether NPs can penetrate the skin layers. Dermal penetration of NPs is an extensive issue which needs to be better understood. Based on the physiochemical properties of the particles, three pathways of penetration have been reported: intercellular, transcellular and transappendageal which includes through hair follicles and sweat glands [23]. Although only a small portion of appendages is present on the skin, the impact of NPs penetration through it is higher due to the depth it can reach. It has been well reported that lipophilic molecules lesser than 600 Da can passively penetrate through the skin. Till date, numerous in vitro and in vivo studies investigating NPs penetration across the skin and have shown conflicting results.

Majority of studies on humans and animals have found that TiO₂ and ZnO NPs do not have the potential ability to penetrate through the skin and the underlying tissues [20]. Kimura et al. investigated NPs skin penetration by topical application of commercial sunscreen with TiO₂ and ZnO concentrations 3.4% and 8.1% respectively to excised porcine ear skin and they found the NPs in grooves and hair follicles only [24]. Similarly, Kuo et al. applied ZnO alone to the excised human skin and showed that less than 0.03% of the total applied amount was able to penetrate into the skin but no particles were detected at the deeper layers [25]. On the other hand, relatively deep penetration of TiO₂ NPs into hair removed skin of micro pigs was observed by Senzui et al. The particles were found in hair follicle pocket, 1mm from the surface, but not in the viable epidermis or dermis [26]. Wu et al. investigated absorption and toxicity of TiO₂ followed by chronic dermal exposure for 30 days on hairless mice and porcine skin. They showed that various sizes of TiO₂ NPs, including P25, were able to penetrate through the skin and translocate into various organs of mice. TiO₂ 10 nm and 25nm caused severe damage in the skin. Atrophy in the dermis and excessive keratinization were observed in association with oxidative stress and collagen depletion. These conditions of the skin closely relate to skin ageing [27]. These

results were consistent with the results showed by Adachi et al. on subchronic exposure of TiO₂ NPs on the dorsal skin of hairless rat for 56 consecutive days. Although they did not find any evidence of NPs skin penetration, focal parakeratosis and spongiosis were observed in the epidermis of the skin [28]. Ryu et al. also showed the formation of skin crusts with hyperkeratosis and papillomatosis in rat skin in vivo after 90 days of repeated dose [29].

Gulson et al. performed a study on dermal penetration of ZnO from sunscreen by applying the sunscreen on the healthy human skin. They detected zinc in blood and urine of the subjects and concluded that a small amount of zinc can be absorbed through the skin exposed to sunlight. Although the amount of zinc detected was not significantly higher than the level of zinc normally present in the body, it still suggested the possibility of penetration of zinc ions through healthy skin. Another finding is that absorption of zinc ions dependent on skin thickness as the level of zinc obtained from female patients were higher than male [30]. These observations were also confirmed by Megan et al. in which they detected a significant amount of zinc in blood and major organs of virgin mice after topical application of sunscreen containing ZnO NPs [31].

Since the skin is exposed to the sun after application of sunscreen, the behavior of NPs on exposure to sunlight should also be considered. Bennet et al. observed that sunlight caused disaggregation of TiO₂ aggregates which facilitated in penetration of TiO₂ NPs through the entire dermal profile. ICPMS analysis of full dermal profile yielded 370 mg TiO₂ per kg in the skin after 5 hours of sunlight exposure whereas only 130 mg TiO₂ per kg was present in unirradiated samples. The penetration also increased with exposure time [32].

There are possible scenarios where a person's skin barrier is compromised such as UV burnt skin, cuts/wounds, and skin diseases. The penetration of NPs could be enhanced in these scenarios. Monterio-Riviere et al. investigated the penetration of TiO₂ and ZnO NPs into the UVB burnt pigskin and showed enhanced penetration into the stratum corneum. TiO₂ penetration was shown to have greater penetration ability than ZnO but no evidence of systemic absorption was found [33]. More recently, Crosera et al. studied in vitro skin absorption of TiO₂ NPs on damaged and intact full thickness human abdominal skin. They

detected NPs only in the epidermal layer but not in the dermal layer after 24 hours of exposure to both in intact and damaged skin, suggesting the low toxic potential of these NPs at skin level [34]. In addition to this, NPs may also be directly exposed to viable skin cells through wounds and cuts.

The current evidence shows that the penetration of metal oxide NPs through the intact human skin under normal condition is extremely unlikely due to the strong barrier function provided by the stratum corneum. However, impaired or altered stratum corneum may lead to direct exposure of viable skin cells to the NPs and also might enhance penetration into the deeper skin layers. For instance, if a person gets cuts or wounds during their outdoor activity after application of a heavy coat of sunscreen, it is more likely for the NPs to directly reach deeper layers of the skin. The NPs penetration in this type of real-life scenarios needs to be investigated. NPs penetration through impaired skin and interaction with cells and tissues have rarely been studied. It is important to ascertain how the viable cells interact with the NPs, given the cells are directly exposed to the particles through an open wound. To increase the knowledge in this area, this study focuses on the interaction of NPs with viable cells through open wounds in a full thickness skin explants. This is one of the motivations for this research.

2.3. The skin

The skin is the first line of defense against any external hazards, injuries or pathogens. Besides protection from external injuries, it also prevents excess water loss from the body and plays a role in thermoregulation. It covers the area of about 1.5 to 2m² of an adult human body and contributes around 15% of the body weight. The thickness of human skin ranges from 1.5 mm to 4.0 mm depending upon age, gender or body parts. It is composed of three layers: the epidermis, the dermis, and the hypodermis or subcutaneous tissue [35]. The integumentary system of skin is shown in figure 2.2.

Figure 2.2. The integumentary system of skin and its derivative structures.

2.3.1. The epidermis

The epidermis is the outermost layer of skin which is a stratified, squamous epithelium layer composed of keratinocytes, melanocytes, Langerhans cells and Merkel cells. The epidermis is divided into four layers, based on keratinocyte morphology and position: basal cell layer (stratum germinativum), squamous cell layer (stratum spinosum), granular cell layer (stratum granulosum), and cornified or horny cell layer (stratum corneum) [36, 37].

2.3.2. The dermis

The dermis is subdivided into two regions: papillary dermis and reticular dermis. The papillary dermis is the uppermost layer of dermis consisting of loose elastic and collagen fibrils within a significant amount of ground matrix. Unlike the epidermis, the dermis is vascularized and contains nerves, sweat glands, and hair follicles. Fibroblasts, macrophages, and mast cells are accommodated in the dermis. Other cells such as lymphocytes and leukocytes enter the dermis during inflammation [37].

2.4. Wound healing

Keratinocytes are a major cell type in the epidermis which is responsible for maintaining barrier function. It also plays a major role in maintaining epidermal homeostasis and restoring the epidermis on an event of injury. The epidermis is continually renewing to maintain epidermal homeostasis by the differentiation of basal keratinocytes. The process by which a keratinocyte differentiates and migrate from the basal layer to the surface of the skin is called keratinization. The keratinocytes at the basal layer are attached to the basement membrane with the help of hemidesmosomes. These undifferentiated keratinocytes undergo mitosis and proliferate. As they divide, they detach from the basement membrane and migrate towards suprabasal layers and eventually become corneocytes in stratum corneum. During this phase, the cell cycle of keratinocyte ceases and the expression of various epidermal markers is initiated. They build up keratin intermediate filaments which serve as cells' cytoskeleton. The keratin filaments converge to form desmosomes at the plasma membrane. Desmosomes are specific to keratinocytes which helps them to attach with each other. During this degradative phase, the cells lose their cellular organelles and merges together appearing as cell envelopes. These are known as the horny layer or corneocytes. This process of keratinocytes maturation and degradation is known as terminal differentiation [37]. Any disturbance in this dynamic equilibrium can result in skin diseases such as psoriasis.

On the event of cutaneous injury, keratinocytes and fibroblasts play a critical role in re-establishment of the skin barrier. This process of restoration is known as epithelialization. In response to skin injury, keratinocytes release interleukin-1 (IL-1), which activates and increases keratinocytes migration and recruits inflammatory cells at the injury site. Growth factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), secreted by the keratinocytes induces migration of endothelial cells in the wound bed. IL-1 is a double paracrine factor which also activates and promotes fibroblast migration [38] [39]. It up-regulates production of Keratinocyte Growth Factor (KGF) by the fibroblasts, which simulates keratinocyte proliferation and migration. Likewise, PDGF secreted by keratinocyte promotes fibroblast proliferation and ECM production.

Fibroblasts also secrete paracrine factors such as basic fibroblast growth factor (bFGF/FGF-2), keratinocyte growth factor (KGF/FGF-7), vascular endothelial growth factor A (VEGF-A) and insulin-like growth factor-1 (IGF1). These growth factors signal the adjacent keratinocytes [39]. In response to the paracrine factors from keratinocytes and the signals from inflammatory cells, the fibroblasts synthesize collagen, ECM proteins and differentiate into myofibroblast which facilitates wound closure. Growth factors and cytokines secreted by fibroblasts and keratinocytes are critical to maintaining homeostasis at normal conditions whereas, during cutaneous injury, they are necessary for wound repair and closure.

The activation of keratinocytes in the wound healing process is characterized by expression of keratin intermediate filaments, K6 and K16. The expression of K16 could be triggered by cytoskeletal changes. It is important for wound healing as it provides plasticity and flexibility to the tissues while maintaining the resilience of the intracellular scaffold. The keratinocytes at the wound edge start migrating to the denuded area. Meanwhile, the keratinocytes behind the migrating tongue start proliferating and migrating towards the wound edge. Keratinocytes at the basal layer are characterized by mitotically active cells consisting of keratin filaments, K5 and K14. As they move towards the upper layers, their differentiation process is characterized by the substitution of K5 and K14 by K1 and K10 (figure 2.3).

Figure 2.3. Schematic representation of the epidermis with the expression of different keratin markers, the arrow represents the direction of keratinocyte migration and differentiation during normal epidermal renewal.

Change in expression of the keratins is specifically important in wound healing as well as epidermal homeostasis [40]. These keratin pair provide specific functional requirements to keratinocytes. A wrong activation of any of this keratin can lead to the formation of a chronic wound edge and induce the non-healing of the wound. During the influence of hyperproliferative stimuli in certain disorder, including cancer, the expression of K1 and K10 is drastically reduced [41]. Chronic wounds display a permanent inflammation state and the mechanisms that block wound healing. The keratinocytes are able to proliferate but not migrate [42-45]. This process is common in the case of diabetes or microvascular diseases, and in elderly people. Wound healing rate also has a correlation with age [46], as it has been shown that the migration time of keratinocytes from the basal layer to the surface is 50% slower for aged individuals [47]. Less tight contact between the dermis and epidermis is also responsible for the slower healing process in elders [48, 49]. Various NPs such as silver, gold, copper, as well as TiO_2 and ZnO , have shown potential therapeutic effects in wound healing. However, the impacts of accidental or unintentional exposure of NPs to the skin have not been studied so far.

2.5. Toxicity studies

Due to increasing concerns over NPs safety, modest research work has been devoted in assessing adverse effects of these NPs on the various biological system including human and animal cell lines, animal models, human volunteers and other biological organisms. The main issue with the application of engineered NPs in consumer products is their potential toxic properties. In the context of skin, if the NPs are able to reach the viable skin layers, the question will be whether these NPs are able to interact with the underlying cells and tissues to cause adverse effects. The cellular response depends on the diversity of physiochemical parameter of NPs such as the type of particles, size, shape, surface area, charge, formulations, dose, and exposure time, including the type of the cells. Extensive studies of NPs toxicity has been done concurrently on various in vitro (animal and human skin cells) and in vivo (animal models and human volunteers) models.

2.5.1. 2D in vitro skin models

Numerous studies have alluded that TiO₂ and ZnO NPs impart toxicity in various cell lines. Preceding knowledge, corroborated by our previous studies, have demonstrated that individually, TiO₂ and ZnO NPs are both able to cause dose-dependent extents of toxicity ranging from DNA damage to causing apoptosis and cell death in human epidermal keratinocytes [50-55]. TiO₂ NPs has also shown to be rapidly internalized by the keratinocytes [56]. Other harmful effects such as reduction in keratinocyte proliferation and disturbances in calcium homeostasis have also been observed [57, 58]. One of the major concerns is that these NPs can induce elevated levels of intracellular Reactive Oxygen Species (ROS) which have been shown to irreversibly oxidize DNA, mitochondria and other organelles and cause a variety of genotoxic effects in cells [59-61]. Increased oxidative stress caused by ROS induction has also been reported to bring about a series of toxic events such as inflammation, genotoxicity, fibrosis, and carcinogenesis in various cell types [62-64]. Studies on human epidermal keratinocytes have revealed that TiO₂ and ZnO NPs impart adverse effects on cells due to the intracellular formation of free radicals causing changes in cellular morphology, mitochondrial dysfunction, DNA damage and cell cycle disruption [65]. Acute cytotoxic and genotoxic effects caused by direct oxidative damage triggered by the release of singlet oxygen by sun-activated TiO₂ was observed in keratinocytes [66]. Mitochondrial damage was also observed in human HaCaT keratinocytes exposed to TiO₂ 10 µgml⁻¹ for 72 hours [67]. TiO₂ at a concentration of 0.7 µgcm⁻² and higher induced membrane damage regardless of exposure time, 24 hours to 7 days of exposure in HaCaT cells [34]. These effects depend on size, concentration, and length of exposure to the NPs.

ZnO NPs have been known to cause more pronounced effects than TiO₂ due to the solubility of the ZnO NPs in the cell culture medium which results in cells being exposed to aqueous Zn²⁺ ions [68]. Most of the in-vitro studies show a drastic reduction of cell viability within a small concentration range of ZnO NPs. ZnO NPs of around 20 nm have shown to cause apoptosis with an up-regulation of p53 and phosphor-38 in human dermal fibroblast [50]. ZnO NPs, 100 nm were found to be internalized and induced dose and time-

dependent cytotoxicity in HaCaT cells with a 50% reduction in cell viability at ZnO concentration $200 \mu\text{gml}^{-1}$ for 24 hours and $50 \mu\text{gml}^{-1}$ for 48 hours or 72 hours. Apoptosis-related genes such as BAX, PUMA, and NOXA were expressed by these cells along with ROS, DNA damage and cell cycle arrest at the G2/M checkpoint, leading to cell death by apoptosis [69]. A mixture of TiO₂ NPs (anatase and rutile, 4:1) exposure to HaCaT cells for 24 hours also induced oxidative stress, activated pro-apoptotic proteins, down-regulated anti-apoptotic proteins and caused cell death by apoptosis [70].

In addition to the toxic effects, keratinocytes also respond to TiO₂ and ZnO NPs by upregulation of autophagy [56, 71]. In our previous study, we have demonstrated that TiO₂ NPs are able to induce autophagy in keratinocytes at very low administered doses [56]. Several cell lines have demonstrated upregulation of autophagy through ZnO NPs exposure as well [65, 72, 73]. Autophagy regulates the degradation of various components of the inflammatory pathway, and there is numerous evidence of the interplay between the two pathways. For instance, under the influence of Heat shock protein 90 (Hsp90) inhibitors, autophagy degrades the I κ B kinases (IKK): IKK α , IKK β , and IKK γ which can prevent activation of the p65 NF κ B [74, 75]. In another study, it was shown that p62, a cargo receptor for autophagic degradation, activates p65 NF κ B in response to different stimuli like tumor necrosis factors (TNF) or interleukins (IL) [76]. The activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) in turn can also regulate p62 expression in certain tumor cell lines [77].

2.5.2. 3D models: human skin explants and organotypic skin cultures

At present, the understanding of the cellular effects of NPs and mechanism of toxicity is mainly based on the studies performed in 2D monolayer cell culture models. However, the findings obtained from these studies might overestimate the toxic effects of the NPs as these models have very low anatomical and physiological relevance to the actual tissue or organ. The cells in 2D monolayers lose their original tissue organization and protein-protein interactions limiting their ability to recapitulate the appropriate levels of in vivo responses. Therefore, despite providing valuable information, studies based on 2D

monolayer cultures do not accurately determine in vivo toxicity responses and biological interactions [78]. Addressing this issue, significant progress has been made in developing various skin models such as co-culture models, 3D organotypic skin culture models and in vitro and in vivo models for toxicological screening of NPs. Among these, in vivo models represent the most physiologically relevant models [79]. However, the legal and ethical issues associated with these models add further complications, limiting its use. The other alternative, in vivo animal models, is widely used for nanotoxicity studies but the animal skin tissue differs from that of human skin in terms of histology and physiology. Also, there exists ethical problems as well as the policy on animal welfare. Organotypic cultures provide a great alternative to in vivo models as it can provide 3D structure, including inter-cellular interactions between keratinocytes and fibroblasts. Nonetheless, there exist certain limitations such as lack of blood circulation, nerve innervation and other cell types present in vivo models. Therefore, of all these models, skin explant is an ideal model which can achieve physiological outcomes due to their distinct characteristics [80].

There are not many studies on NPs toxicity performed in human skin explants models. In a study on human abdominal full thickness skin explant, no permeation of TiO₂ NPs in either intact or damaged skin was observed. TiO₂ NPs were found at the epidermal layer but not in the dermal layer, suggesting the low toxic potential of TiO₂ NPs at skin level [81]. The study of copper II oxide in the intact epidermis of human skin organ culture showed that the NPs could induce inflammation with the secretion of IL-1 α , IL-6, and TNF- α without any evidence of penetration [82]. Further studies simulating real-world scenarios on the skin with impaired barrier needs to be done for the safety evaluation of TiO₂ and ZnO NPs. NPs penetration through the skin and direct interaction with the viable skin cells have also rarely been studied. It is important to find out how the viable cells of the skin interact with the NPs when the cells are directly exposed to the NPs through an open wound. Therefore, in this study, human abdominal skin explants are used to study the interaction of these NPs with viable skin cells.

In addition to these methods, various new methods of nanotoxicity assessment have been developed which allows researchers to better understand biophysical responses of cells in

presence of nanoparticles. Some of the recent nanotoxicity approaches are Atomic Force Microscopy (AFM), molecular force spectroscopy, cytoindenter, magnetic twisting cytometry, microfluidics, magnetic tweezers, microplate manipulation, optical tweezers or laser traps and optical stretcher. These technologies have facilitated quantitative experimental and computational studies at the nanoscale on how the mechanical properties of a cell are altered by organizational or molecular changes occurring within itself due to nanoparticles exposure [83]. For example, AFM is a powerful force sensitive technique, which can be used to assess toxicity by collecting information on cell topography, membrane nanostructures, cytoskeleton organizations, alterations in plasma membrane potential and biomechanics (adhesion force, elasticity) of cells, providing qualitative and quantitative results at nanoscale resolutions. Assessment of nanotoxicity from a biomechanics perspective can lead to a better understanding of the body's response to nanoparticles because the changes occurring at the molecular and cellular level can be correlated to changes occurring at the macroscopic level. In addition to this, micro-chip-based biosensors are also widely used for monitoring cellular nanotoxicity. Nowadays, nanotoxicity assessment studies have been more shifted towards measuring a single cell behavior. The use of cell-on-a-chip with a microfluidic system is recently in practice for nanotoxicity assessment [84].

2.6. Biological mechanisms of toxicity

The potential toxic effects mediated by NPs include oxidative stress, inflammation, autophagy, genetic damage or genotoxicity, inhibition of the cell division and cell death [62, 85].

2.6.1. Oxidative stress

There are various mechanisms by which NPs can cause toxic effects in the biological system. Oxidative stress has been reported as one of the major route of NPs induced toxicity by the generation of ROS [86]. ROS are highly reactive chemical compounds such as

hydrogen peroxide, superoxide, hydroxyl radicals, which are produced by the cells during normal biological processes such as inflammation and mitochondrial respiration [62]. ROS are needed for the cell signaling mechanism and for the defense mechanism of the immune system as well as for maintaining homeostasis. However, when the ROS generation from the NPs exceeds the amount of ROS generated for the normal cellular activity, there is an imbalance in the redox state of the cells due to which the cells come in stress [87]. This oxidative stress may cause severe effects by causing irreversible damage to proteins and lipids in the cells leading to DNA damage which may further result in cell death [88]. Oxidative stress is also reported to damage the cells, causing membrane damage, mitochondrial dysfunction, apoptosis, and genotoxicity. DNA damage may occur by chromosomal fragmentation, breakage of DNA strands and the induction of gene mutations [87].

ROS can be classified into primary or secondary. Primary ROS, superoxide anion (O_2^-) are generated during the process of cell metabolism. This radical is not able to trigger protein or DNA damage. But when they are able to react with redox-active transition metals (M), they produce secondary ROS, $\cdot OH$ radical by the process called Fenton reaction:

The secondary ROS ($\cdot OH$ radical) is the primary mediator of DNA damage [89]. Oxidative stress is also caused by overproduction of superoxide in the mitochondria of the cells. The mitochondria superoxide (MSO) are often generated as a by-product of the oxidative mechanism of the cells. Mitochondria, the powerhouse of the cell, is where ATP is synthesized by the reduction of molecular oxygen. During this process, some of the oxygen molecules do not get completely reduced, forming superoxide anion radicals, and subsequently other oxygen-containing radicals [90]. In addition, excess ROS generated in the cells during oxidative stress can also damage mitochondria by disturbing mitochondrial membrane potential and permeability [91]. Since mitochondria are involved in the production of adenosine triphosphate (ATP) and provide energy for normal functioning of

the cells, loss of mitochondrial function is another cause of nanotoxicity as cells need the energy to survive and maintain biological function.

2.6.2. Inflammation

Inflammation is a natural defense mechanism of the body which is triggered by a range of factors: irritants, toxins, foreign materials, damaged tissue or pathogens. This complex process involves the recruitment of immune cells to the injury site by the production of various pro-inflammatory cytokines. Keratinocytes are a primary component of the epidermal barrier and hence play a major role in the innate immune response. When the skin is exposed to any irritants or harmful pathogens, keratinocytes produce inflammatory cytokine IL-1 α that serves as a mediator for inflammatory and immunologic reactions. The cytokines influence the migration of inflammatory cells and migration, and proliferation of keratinocytes and triggers production of other cytokines including IL-6, IL-8, Tumor Necrosis Factor-alpha (TNF- α) and phospholipase A2 (PLA2). Fibroblasts are highly sensitive to IL-1 α and thus amplifies cytokine production and contribute to the recruitment of inflammatory cells which propagates inflammatory response in skin [92] [93]. Prolonged inflammation can trigger the release of toxic-by products such as ROS which has been reported to cause direct toxicity to the cells by promoting cell death [94]. In addition to this, oxidative stress can also result in the release of pro-inflammatory mediators of various inflammatory pathways such as NF- κ B, mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3-K) [95]. Numerous studies have reported that NPs induce inflammation when introduced into the physiological system.

2.6.3. Autophagy

Autophagy has recently emerged as a novel biological effect of NPs on cells and not much is known about its toxicological influences [56]. Autophagy is a normal physiological process of maintaining homeostasis and normal functioning of the body by removing damaged cell organelles, misfolded or aggregated proteins as well as intracellular pathogens from the body by a strictly regulated lysosomal pathway. In fact, it can be called

as a survival mechanism of the body by getting the body rid of damaged, excess or abnormal organelles and proteins [85]. On the basis of the delivery route of cytoplasmic material to the lysosome, the process of autophagy can be differentiated into three different routes: 1) macro-autophagy, 2) micro-autophagy, and 3) chaperone-mediated autophagy [96].

Macro-autophagy, or simply autophagy is a highly complex process consisting sequential steps: induction of autophagy, the formation of autophagosome precursor, the formation of autophagosomes, fusion with lysosomes, degradation of cytoplasmic cargo, transportation of degraded products to cytoplasm and lysosome formation [97]. Figure 2.4. shows the cellular processes during autophagy [98]. In mammalian cells, induction of autophagy is due to stress conditions, including starvation. The autophagosome precursor, phagophore is an isolation membrane of varied organs. The phagophore gets elongated and encloses some portion of cytoplasm within itself. The structure thus formed is called an autophagosome. Autophagosome is formed at random places in the cytoplasm. The formation of autophagosome requires autophagy-related (ATG) proteins along with insertion of lipidated microtubule-associated light chain 3 (LC3-II) protein. LC3-II is originated from its parent molecule LC3-I. During the formation of autophagosome, cytosolic LC3-I is converted to LC3-II which attaches to the autophagosome's membrane [99]. The outer membrane of autophagosome is then fused with the lysosomal constituents of the cells and the enclosed cytoplasmic constituents are degraded [100]. Autophagosome can also fuse with late endosomes, multivesicular bodies and major histocompatibility complex (MHC)-class-II-loading compartments [101].

Since autophagy is able to degrade an entire cell, the unregulated activation of autophagy can be harmful as it may lead to cell death. Normally, autophagy is activated during stress conditions such as nutrient deprivation as well as during physiological and pathological processes such as infection, neurodegenerative diseases and cancer [96]. Many studies have reported that autophagy can crosstalk with various cellular processes like apoptosis and necrosis. Therefore, it is considered an essential part of NPs mediated toxicity [102].

Figure 2.4. Schematic representation of cellular processes during autophagy.

2.7. Summary

The unique physical and chemical properties of NPs have brought about numerous novel and beneficial applications which has improved our lives. However, the same properties have enabled them to interact with the biological system and cause unexpected toxic effects. Inorganic NPs like TiO_2 and ZnO are being widely used in sunscreen and other cosmetic products. However, information about the negative health implications and toxicological impacts of these nanoparticles is still incomplete. Hence, this work has identified the following areas as major knowledge gaps and the research efforts are focused to clarify the uncertainties in the field.

- i. Numerous studies have provided insights into NPs induced cellular toxicity, yet the underlying biochemical mechanisms have not been fully characterized.
- ii. Specific to dermal exposure, more profound understanding about NPs induced autophagy in relation to oxidative stress, apoptosis and inflammation are needed in order to better appreciate the real hazards of such exposure.
- iii. According to the current literature, NPs penetration through healthy intact skin is very low. However, such penetration might be increased if the skin's barrier is compromised.

- iv. Most of the cellular responses observed till date has been based on studies performed in 2D monolayer cell culture models. The 2D monolayer is too simplistic to capture the complexities of skin tissue, therefore, NPs-cells interactions need to be studied using 3D skin models.
- v. There also remains confusions and contradictions regarding the mechanism of NPs mediated toxicity due to the complexity of studying cell-NPs interaction.

Therefore, in this current study, the interaction of commercial TiO₂ and ZnO NPs with primary human epidermal keratinocytes and dermal fibroblasts is investigated, based on physiologically relevant amounts of NPs penetration across the skin as supported by current literature.

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Chapter 3

Experimental Methodology

This chapter presents the experimental methodology and materials used in this research study. The first section explains the method used in the physical characterization of the NPs. The working principle of the equipment used for characterization is explained briefly. The second section explains the process of computing the actual dose of NPs experienced by the cells at a particular time (represented as TWAD), by using dosimetry and ISDD simulation. The third section comprises of materials, methods, and assays used for various toxicity assessment experiments based on 2D cultures of skin cells. This is followed by the method and assays used for the toxicity studies on human skin explants. The assumptions made in the use of human skin explants are also listed. Lastly, it ends with the statistical analysis used to present the data.

The overview of the sequence of the experiments and the chosen methodology is summarized in the flow chart below (figure 3.1).

Figure 3. 1. A flow chart representing the sequence of experiments and chosen methodology.

3.1. NPs Characterization

The aim of toxicological studies is to provide a critical understanding of the relationship between NPs and the biological system which are an essential component of risk assessment. The behavior of a nanoparticle is dependent on their physical and chemical properties including their size, shape, morphology, surface area, structure, surface chemistry and crystallinity [1]. Thus, characterization of NPs is an indispensable requirement for correlating its properties with biological responses measured from any in vitro or in vivo studies. In absence of adequate information on NPs characterization, the conclusion drawn from toxicological studies could have limited value as they are generic to NPs chemical composition only. These findings will not suffice as the properties of NPs of similar composition may differ with its size, shape, crystal structure, specific surface area and its behavior in the medium. Therefore, in order to ensure reliable and reproducible

findings for establishing risk assessment on a particular nanoparticle, it is crucial to identify the NPs type. For this, sufficient NPs characterization needs to be performed. Some of the principal characteristics of NPs which needs to be characterized before performing toxicity studies are size, shape, state of dispersion, chemical composition, surface area and surface chemistry [2].

In this toxicological study, commercially available NPs were used. P25 TiO₂ and ZnO and NPs were purchased from Evonik Degussa (Essen, Germany) and Melorium Technologies (USA) respectively. The characterization of these NPs and the equipment used are briefly described as follows.

3.1.1. Size and morphology

Transmission Electron Microscope (TEM) is a highly sophisticated instrument developed to obtain higher magnification to the levels where the conventional optical microscopes are not able to reach. Unlike the conventional optical microscopes whose ability is limited by the wavelength of visible light, TEM works on fast moving electron beam whose wavelength is lesser than 1 Å [3]. The transmission of the electron beam depends on the sample being imaged. The denser sample will allow fewer electrons to transmit whereas thinner samples will allow more electrons to transmit. Thus allowing imaging of non-uniform sample (less than 200 nm) [4]. There are a series of electromagnetic lenses below the sample positions which helps to deliver the signals to the detector. This signal transmission is accompanied by magnification from 50 times to the factor of 10⁶ [5]. This extraordinary magnification of the incident electrons results in unique capabilities and widespread application across many scientific disciplines.

Figure 3.2. a) Digital image of the Carl Zeiss Libra 120 plus, b) Schematic representation of TEM.

The digital image of TEM is shown in figure 3.2a and the schematic representation of the path of electrons in a TEM column is shown in figure 3.2b. The fast-moving electrons are emitted from the point source of the filament (cathode) when they are heated. The filament is usually made up of lanthanum hexaboride (LaB₆) or Tungsten (W). This accelerated beam of electrons is then focused into a small coherent beam with the use of condenser lenses. A condenser aperture is used to filter out the unwanted scattered electrons before the formation of the image. The beam coming from the condenser aperture falls on the sample and three different electron-sample interaction takes place, i) unscattered electrons or transmitted beam, ii) elastically scattered electrons or diffracted beam and iii) inelastically scattered electrons. The objective lens focuses the transmitted beam and forms an image of the sample. Objective aperture can be used to enhance the contrast of the image by selecting the electrons contributing to the image formation. The intermediate lens then allows controlling if the image will be based on the electron diffraction or imaging [4, 5]. Finally, the projector lens expands the beam into the phosphor screen and inverts it for proper orientation. The image on the phosphor screen can be directly viewed by the user.

A charged-coupled device (CCD) helps to transmit the image to a computer screen where it can be used for further analysis.

In addition to high magnification imaging, the interaction of energetic electron beam with the sample produces characteristic radiation which can be measured for characterizing materials properties using techniques such as Energy Dispersive Spectroscopy (EDS), Electron Energy Loss Spectroscopy (EELS), backscattered and secondary electron imaging [4]. EELS is an analytical technique which can be used to study all the elements in the periodic table [5]. It is absorption spectroscopy which measures the changes in the kinetic energy and angular distribution of the electrons after interaction with the specimen. The schematic representation of the interaction of the electron beam with the samples is shown in figure 3.3a.

As the electrons pass through the thin sample, they interact with the atoms of the sample. Some electrons get scattered as secondary electrons, some pass through the sample without any loss in their energy while some undergo inelastic scattering with the loss in energy as they pass through the sample. This causes the sample to be in the excited state and therefore to return to its original state, loss of energy in the form of X-rays, visible photons or Auger electrons occurs. A detector detects these scattered incident electrons and gives rise to electron energy loss signal. A typical EELS spectrum is shown in figure 3.3b. The first peak occurring at 0 eV represents the electrons which did not undergo inelastic scattering (zero-loss peak), remaining peaks result from the electrons which have interacted with the atoms in the specimen [5, 6].

Figure 3.3. a) Schematic illustration of TEM specimen-electron beam interactions, b) EELS spectrum of Titanium.

The approximate size of the NPs used in this research is in the range of 20-25nm. Therefore, TEM was used to observe the size and morphology of the particles. Additionally, TEM was also used to locate the NPs uptaken by the cells in the skin tissue due to its high resolution and magnification. Lower kV (120 kV) TEM was used in order to protect the samples during imaging. At higher kV, the tissue samples might get burnt due to the strong electron beam. NPs samples for TEM imaging were prepared by making a well-dispersed mixture of $1 \mu\text{gml}^{-1}$ of each NPs in 100% pure methanol with ultra-sonication for 30 minutes. A drop of this well-dispersed mixture was dropped onto carbon-coated copper grids and allowed to dry fully at room temperature. TEM, Carl Zeiss Libra 120 Plus with an accelerating voltage of 120 kV was used to determine the size and morphology of the NPs. Multiple images were taken for each NPs and were analyzed for determining NPs size and shape. ImageJ software was used to measure the size of each nanoparticle. The longest distance across each particle was taken as the diameter. The average diameter which was obtained by measuring at least 50 NPs, was presented as the pristine size of the NPs.

3.1.2. Crystal Structure

X-ray diffraction (XRD) is a non-destructive analytical technique used for the identification of various crystal structures. X-rays are electromagnetic waves of wavelength in the range of 0.1-100 Å and energies in the range of 120 eV to 120 keV. When the incident X-ray beam interacts with the sample, different phenomena occur, i) part of the X-ray beam gets transmitted through the sample, ii) part of the X-ray beam is absorbed by the sample, iii) part of the X-ray beam is scattered and iv) part of the X-ray beam is diffracted. The diffracted beam satisfies Bragg's law ($n\lambda=2d\sin\theta$) where n is a positive integer representing the order of diffracted beam, λ is the wavelength of the incident ray, d is the distance between adjacent planes of atoms (the d -spacing) and θ is the angle of incidence of the x-ray beam. The schematic representation of Bragg diffraction is shown in figure 3.4.

Figure 3.4. Schematic representation of Bragg's diffraction.

Like a fingerprint, the d -spacing is a unique characteristic of every crystalline solid. Therefore, by scanning the sample through a range of 2θ angles, d -spacing from all randomly oriented atoms in the powdered sample is obtained. The d -spacing is compared with a standard reference pattern in the database which helps in identification of the material.

An X-ray diffractometer consists of three basic elements, i) an X-ray tube, ii) goniometer and iii) X-ray detector. The filament inside the X-ray tube is heated by electric current. The number of electrons emitted from the tube depends on the applied electric current. High

voltage, around 50 to 60 kV applied within the tube causes the electrons to accelerate and hit the target, usually made of copper. When the accelerating electrons hit the target, X-rays are generated. The X-rays pass through a set of Soller slits and divergence slit and is directed on the sample at goniometer. The function of Soller slits is to reduce the axial divergence of the waves which increases the resolution. The divergence slit is also known as an anti-scatter slit which reduces the height divergence of the waves. After interaction with the sample, the beam passes through the same set of slits before passing through receiving slits and finally reaching the detector [7, 8]. The digital image of XRD and schematic representation of XRD setup is shown in figure 3.5.

Figure 3.5. a) Digital image of the Bruker D8 Advance XRD, b) Schematic representation of XRD setup.

Crystal structure of the NPs was investigated using Bruker D8 Advance X-ray diffraction (XRD). A generous amount of unmodified NPs powder was loaded onto the hollow space of the powder sample holder of XRD and glass slide was used to press the powder so that the surface was smooth. The excess powder was removed from the holder. The XRD analysis was done with Cu-K α X-rays of wavelength (λ)= 1.540598 Å with 2θ scan from 10° to 70° with a step size of 0.19 for both TiO₂ and ZnO NPs because most of the characteristic peaks of TiO₂ and ZnO NPs falls within this range. The diffraction patterns

obtained were compared to a database containing reference patterns in order to identify the phases present in the NPs using Match! Software.

3.1.3. Specific surface area

The specific surface area is a material property of solids defined as surface area per unit mass or volume. NPs, in particular, possesses a large surface area to volume ratio which causes them to exhibit unique physical and chemical properties compared to their bulk form [9]. Therefore, the measurement of the specific surface area of NPs is critical.

Figure 3.6. Digital image of Tristar 3020 ASAP model.

The surface area measurement is based on the principle of static adsorption of nitrogen gas around the surface of the particles under very low temperature. The equipment consists of a degasser unit to degas the samples, a vacuum pump, and a control module. The gas flowing in the degassing unit removes contaminants adsorbed on the surface and pores of the sample. The degassed sample is kept in an evacuated sample tube and exposed to analysis gas at a series of precisely controlled pressures. The number of gas molecules adsorbed onto the pores and surface of the samples increase with the increasing pressure. The quantity of gas molecules adsorbed is recorded at each equilibrated pressure. After the

adsorption process, the entire surface of the sample is completely covered with gas and the thickness of the adsorbed film increases. After it reaches to the point where condensation of gas molecules begins on the surface, the desorption process begins in which the pressure reduces systematically in the liberation of the adsorbed molecules. The change in the quantity of gas on the surface of the sample at each decreasing equilibrium pressure is quantified. The two sets of data, adsorption isotherm and desorption isotherm are obtained. The number of molecules adsorbed or desorbed as a function of relative pressure is called as adsorption and desorption isotherms from which the information on surface and pore of the samples is yielded by the help of BET model. BET surface area (m^2g^{-1}) is calculated by the equation below [10].

$$SA_{\text{BET}} = \frac{\text{CSA} \times (6.023 \times 10^{23})}{(22414 \text{ cm}^3 \text{ STP}) \times (10^{18} \text{ nm}^2 / \text{m}^2) \times (S + Y_{\text{INT}})}$$

Where,

SA_{BET} = BET surface area.

CSA = analysis gas molecular cross-sectional area (nm^2)

STP = volume of gas monolayer

S = slope

Y_{INT} = y-intercept

Brunauer–Emmett–Teller (BET) surface area was determined by using ASAP Tri-Star II 3020 surface area and porosity analyzer from Micromeritics (figure 3.6). Fine powder of unmodified TiO_2 and ZnO NPs powder were kept in sample holder tubes. In order to prevent the adhesion of NPs powder to the walls of the tubes, straw was used to load the samples into the tube. The weight of the empty tube and the tube with the sample was noted. The samples were then degassed under vacuum at a temperature of 200°C for 2 hours. The degassing temperature and time were chosen after careful optimization. The degassing process removes the contaminants attached on to the surface and pores of the sample. After degassing, the samples were allowed to cool down by placing the tubes in the cooling chamber. The tubes with the degassed samples were again measured to calculate the sample mass before and after degassing. The tubes were then installed into the sample ports for

analysis. Tristar 3020 analysis software was used to create the method file for standard analysis.

3.1.4. Hydrodynamic size

Different phenomena occur when the NPs are dispersed in a biological medium or buffer solution. The aggregation and agglomeration of NPs and adsorption of proteins onto the surface of the NPs result in the formation of larger complexes. The agglomeration and aggregation of NPs depend largely on the type of biological medium or buffer in which they are dispersed. Therefore, it is crucial to characterize the hydrodynamic size of NPs in various test medium used for the study. Malvern Zetasizer, working on the principle of Dynamic Light Scattering (DLS) was used to characterize the behavior of NPs in the medium.

The size measurement by DLS depends on the Brownian motion of NPs. Brownian motion is the random diffusion or movement of particles in the fluid due to continuous bombardment by their surrounding molecules. As per Stokes-Einstein's equation, the diffusion coefficient is inversely proportional to the particle size:

$$d(H) = \frac{kT}{3\pi\eta D}$$

Where,

$d(H)$ = hydrodynamic diameter

D = diffusion coefficient

K = Boltzmann's constant

T = absolute temperature

H = viscosity

The larger molecules or particles will have a slower Brownian motion and vice versa. When the laser light is passed through the particles dispersion, light is scattered in all directions. As the particles are continuously moving or diffusing, the scattered light intensity fluctuations are random. The small faster moving particles will result in more fluctuation

of the scattered light whereas the larger slower moving particles will have lesser fluctuations. The scattered light is detected by the detector and analyzed to give the size distribution of particles present in the sample. The schematic illustration of the hydrodynamic size of particles and DLS is shown in figure 3.7.

Therefore, the hydrodynamic diameter of particles correlates to the effective particle movement within a liquid. The hydrodynamic diameter measured by DLS is defined as “the size of a hypothetical hard sphere that diffuses in the same fashion as that of the particle being measured” [11]. The hydrodynamic diameter calculated from the diffusional property of particles indicates the size of the particles plus electric double layer due to hydration/solvation of particles.

Figure 3.7. Schematic representation of a) particle with an electric double layer, and b) basic components of DLS.

To measure the hydrodynamic size, the powder NPs were dispersed well in PBS to make a stock solution. The stock solution was then exposed to UV for 15 minutes. The UV step is optional and was done to follow the same process by which NPs dispersion was prepared to treat the cells. The dispersion was then ultra-sonicated in a bath sonicator for 10 minutes. After which it was diluted into required working concentration followed by ultra-sonication

for further 10 minutes. Immediately after sonication, the dispersion was analyzed by the Zetasizer. The hydrodynamic size, zeta potential, polydispersity index (PDI) was noted.

3.1.5. Solubility of ZnO NPs

Inductively coupled plasma optical emission spectrometry (ICPOES) is an analytical technique used to determine the elemental composition of the samples (mostly dissolved in water) by the use of plasma and a spectrometer. The sample to be analyzed is conducted by a peristaltic pump through a nebulizer into a spray chamber. The aerosol thus produced is lead into an argon plasma. The argon plasma is generated by the interaction of an RF field and ionized argon gas. The plasma is extremely hot and can reach up to 10,000°K, which allows complete atomization of the elements in the sample. This helps to minimize potential chemical interferences during elemental analysis. The electrons in the aerosol take up thermic energy and reach a higher excited state. When the electrons come back to ground state, energy is liberated as photons. Each element has its own characteristic emission spectra. The spectrometer separates individual wavelength of light and focuses the desired wavelengths to the detector. The detected light intensities are compared to the intensities of standards of known concentration to obtain an elemental concentration in the sample [12]. Schematic representation of basic components of an ICPOES is given in figure 3.8.

Figure 3.8. Schematic representation of basic components of an ICPOES.

ZnO NPs were dispersed in cell culture medium and PBS at different concentrations, in the same way as it was prepared to treat the cells and was incubated for 24 hours at 37°C. The suspension was then centrifuged at 2000g for 15 minutes, and the supernatant was collected. The supernatant was filtered with the 0.22µm filter in order to minimize the presence of any ZnO NPs. ICP-OES (Perkin Elmer, Optima 2000 DV) was used for measurement of soluble Zn^{2+} in the supernatant.

3.2. Dosimetry

The cellular response to NPs is proportional to the concentration of the NPs at the site of action. Therefore, it is indispensable to accurately determine the quantity of NPs received by the cells at a particular time. The NPs in the biological medium have a high tendency to aggregate and agglomerate. The transformation and kinetic properties of NPs agglomerates/aggregates play a vital role in their mass transport (sedimentation/diffusion) within the biological medium. The particles are less than 10 nm transport principally by diffusion. The rate of transport increases as their size reduces. The particles greater than 200 nm transports relatively faster by sedimentation. The particles between 10 nm and 100 nm transport via both sedimentation and diffusion [13]. The rate of mass transport is governed by the NPs pristine and hydrodynamic diameter, NPs density, effective density, media density, media viscosity, and temperature. The effective density represents the density of NPs agglomerate which consists of both NPs and the biological medium trapped in the agglomerates.

Figure 3.9. Schematic representation of administered dose versus delivered dose to the cells in vitro.

The effective density of NPs was calculated by the method of volumetric centrifugation [14]. 1 ml of 1 mgml⁻¹ suspension of TiO₂ and ZnO NPs in complete EpiGRO medium and DMEM was kept in packed cell volume (PCV) tubes (figure 3.10) (Techno Plastic Products (TPP), Trasadingen, Switzerland). The tubes were centrifuged at 2000g for 1 hour in a bench-top swing out rotor centrifuge. From the pellet obtained, the volume of the agglomerate pellet (V_{pellet}) was measured with the help of slide-rule like easy measure device which was also provided by the PCV tube manufacturers. In the case of ZnO NPs, ICPOES was performed on the supernatant to measure the amount of soluble Zn²⁺. The V_{pellet} was then used to calculate the effective density of the agglomerate unit using the equation below.

$$\rho_{\text{EV}} = \rho_{\text{media}} + \left[\left(\frac{M_{\text{ENM}} - M_{\text{ENMsol}}}{V_{\text{pellet}} SF} \right) \left(1 - \frac{\rho_{\text{media}}}{\rho_{\text{ENM}}} \right) \right]$$

Where,

ρ_{media} = density of media,

M_{ENM} = mass of NPs,

M_{ENMsol} = solubilized mass,

V_{pellet} = volume of the pellet,

SF = stacking factor,

ρ_{ENM} = density of NPs.

The effective density thus obtained was used to calculate the fraction of administered TiO₂ and ZnO NPs deposited over time on the cell culture well plate (48 well plate, 96 well plate, T75 flask) with the help of ISDD model provided by Dr. Justin Teeguarden (Pacific National Laboratory, Oregon State University Joint Faculty). Media height, volume, temperature, viscosity, density, particle diameter, density, concentration, agglomerate diameter (hydrodynamic size of the particle) and effective density was input in ISDD model in MATLAB program and the fraction of the NPs deposited over time was simulated. The fraction of NPs deposited was plotted against time and the area under the curve obtained from the profile gave the time-weighted average (TWA) factor which was converted to time-weighted average dosage (TWAD) in terms of surface area (mgcm⁻²) by

multiplying it with the administered dose in terms of mass. Both administered dose (μgml^{-1}) and TWAD per surface area ($\text{mgcm}^{-2}\text{hr}$) have been reported wherever necessary.

Figure 3.10. PCV tubes used for volumetric centrifugation of NPs.

3.3. 2D In vitro study

To understand the NPs effects on skin, firstly the NPs effects on individual cell types: human epidermal keratinocytes and human dermal fibroblasts were studied based on in vitro 2D cell culture models.

3.3.1. Cell culture and maintenance

Human cell lines: keratinocytes and fibroblasts were used in this project because they are the major type of cells found in skin. In an event of NPs penetration into the skin, they are the primary cells which will be exposed to the particles. Primary Epidermal Keratinocytes; normal human adult foreskin (ATCC® PCS-200-010™) was cultured in serum-free EpiGRO™ Human Epidermal Keratinocyte medium (Chemicon, SCMZ-BM) supplemented with growth factors (Chemicon, SCMK001-S). Primary human dermal fibroblasts, purchased from ATCC (PCS-201-010™) were cultured in Dulbecco's Modified Eagle Medium (PAN Biotech, Germany) supplemented with 10% FBS, 5% antibiotic and 4mM L-Glutamine. The cells were maintained to grow in a humidified

environment at 37°C and 5% CO₂ for three to four days until 70 to 80% confluence was reached. After the cells reached confluency, they were trypsinised with 0.05% trypsin and collected by centrifuging at 250g for 5 min. The cell pellet was re-suspended in complete media and counted by hemocytometer. The fibroblasts and keratinocytes were then seeded into the required well plate with the density of 5000 cells per square cm and 10,000 cells per square cm respectively. They were incubated for 24 hours to allow complete attachment before exposing to NPs.

3.3.2. Preparation of NPs suspension and exposure to cells

The stock solution (5 mgml⁻¹) of NPs was prepared in 1X PBS (PAN Biotech, Germany) followed by UV sterilization for 15 minutes. The stock solution was then ultra-sonicated for 10 minutes and then diluted with the complete medium to get required working concentrations. The working solution of required concentration was again ultra-sonicated for 10 minutes prior to exposure to the cells. The cell medium was replaced with the medium containing NPs and incubated for the required time point (4 hours or 24 hours). The nanoparticle suspension was always prepared immediately before exposure to the cells. To observe the toxic effects contributed by aqueous Zn²⁺ in ZnO NPs dispersion, ZnCl₂ was dissolved in DI water and diluted in culture medium to obtain equimolar amounts of dissolved Zn²⁺ ions (measured by ICPOES).

3.3.3. Cell viability assay

Cellular metabolic activity and proliferation were measured to detect the viability of the cells exposed to NPs. The regulation of cell metabolism is the mechanism by which growth signals control cell growth and death. The metabolic status of a cell is intimately tied to its ability to successfully grow, proliferate, and survive. Likewise, cell proliferation increases in the number of cells and is defined by the balance between cell divisions and cell loss through cell death or differentiation. Therefore, cellular metabolic activity and proliferation were measured to monitor cell health. To measure the cell viability, the cells were seeded

in a 48 well plate. Two-time points: 4 hours and 24 hours were selected in order to study time and dose-dependent effects to NPs.

3.3.3.1. Metabolic activity assay

Alamar blue metabolic assay (Invitrogen) was used to quantify cellular metabolic activity. After each time point of NPs exposure: 4 hours and 24 hours, the culture media was removed and the cells were washed thrice with PBS and replaced with 10% Alamar blue reagent in pure medium (without supplements). The cells were incubated at 37°C, 5% CO₂ for 30 to 60 minutes (unless a slight change in color of the reagent was observed) for the reduction of the dye. The absorbance of the reagent was measured with a microplate reader (Infinite M200, TECAN Inc., Maennedorf, Switzerland) at wavelengths 570 nm and 600 nm which was then correlated with cellular metabolic activity according to manufacturer's protocol.

3.3.3.2. DNA quantification assay

PicoGreen, DNA quantification assay (Molecular Probes) was done to evaluate cell proliferation. After each time point of NPs exposure: 4 hours and 24 hours, the culture media was removed and the cells were washed thrice with PBS and chilled Cell lysis buffer (1X in DI water) (Cell Signaling Technology) was added in each well. Thaw and freeze cycle was used twice to prepare a homogenous solution of the lysate. The cells were kept at -80° for 15 minutes and thawed at room temperature. 100 µl of the cell lysate was mixed with 100 µl of the picogreen solution, prepared according to the manufacturer's protocol and incubated for 5 mins at room temperature. Fluorescence reading was taken with the microplate reader (Infinite M200, TECAN Inc., Maennedorf, Switzerland) at emission and excitation wavelengths of 480 nm and 520 nm respectively. A standard DNA curve was prepared by serial dilution of the standard DNA and the amount of DNA was calculated against the standard curve.

3.3.4. Oxidative stress

Generation of reactive oxygen species (ROS) is a major event during NPs induced toxicity. Most of the metal-based NPs generate free radicals via Fenton reaction. Excessive production of free radicals causes oxidative stress which can lead to potentially damaging biological responses. The cells were seeded in a black 96 well plates with clear bottom and incubated with NPs for required time points: 4 hours and 24 hours. Oxidative stress in the cells was assessed by measuring intracellular ROS and MSO using Chloromethyl derivative of 2',7'-Dichlorofluorescein diacetate (Cm-H₂DCFDA) and MitoSOX™ Red mitochondrial superoxide indicator respectively. Cm-H₂DCFDA is a straightforward technique which is mostly used to detect redox state of the cells. The dye passively diffuses into the cells, which makes helps to detect ROS and is highly sensitive to changes in the redox state of the cells. Mitochondria are a major source of cellular superoxide and superoxide is the origin of ROS leading to oxidative stress. Therefore, MitoSOX Red reagent was used to detect ROS generated in the mitochondria. The dye permeates into the live cells where it specifically targets to mitochondria. It is rapidly oxidized by superoxide and not by other free radicals.

3.3.4.1. Intracellular ROS measurement

Cm-H₂DCFDA (Molecular Probes) probe was used as an indicator of intracellular ROS. A stock solution of the dye (10M) was prepared by mixing one vial of the dye with 8.65µl of Dimethyl sulfoxide (DMSO) and was diluted with PBS to get 10 µM working solution. The cells were washed thrice with PBS to remove unbounded NPs from the cells. 100 µl of the working solution of the dye was replaced in each well and incubated at 37°C, 5% CO₂ for 30 minutes. After incubation, the dye was replaced with 1X PBS and fluorescence reading was taken with the microplate reader (Infinite M200, TECAN Inc., Maennedorf, Switzerland) at emission and excitation wavelengths of 492 nm and 527 nm respectively. The reading of blank wells with PBS was taken to subtract background. The dye is very much sensitive to air and light, hence the experiment was conducted in dark.

3.3.4.2. Mitochondrial superoxide measurement

MitoSOXTM Red mitochondrial superoxide indicator (Molecular Probes) was used to detect the superoxide generated in the mitochondria of the cells. The stock solution (5M) of the dye was prepared by mixing 1 vial of the dye with 13 μ l of DMSO and the working solution was prepared with further dilution to 5 μ M with PBS. The cells were washed thrice with warm PBS and 100 μ l of dye was kept in each well. The cells were allowed to incubate for 15 minutes. After incubation, the dye was replaced with 100 μ l of PBS and reading was taken with a microplate reader (Infinite M200, TECAN Inc., Maennedorf, Switzerland) with emission and excitation wavelengths of 510 nm and 580 nm respectively. The reading of blank wells with PBS was taken to subtract background.

3.3.5. Antioxidant treatment to cells

N-acetyl cysteine (NAC) (Sigma Aldrich) was added to the cells 1 hour before exposing the cells to NPs. The cells were incubated with 10 mM NAC prepared in complete medium. After incubation, it was replaced with NPs suspension which was also prepared in complete cell medium containing NAC.

3.3.6. Apoptosis assay

Metal oxide nanoparticles including TiO₂ and ZnO have been shown to cause apoptosis in cells. NPs induced inflammation and ROS can cause direct toxicity and cell death via apoptosis. Annexin V was used to detect apoptosis in cells exposed to NPs as it can detect apoptosis at an early stage. Annexin V provides an advantage regarding sensitivity and specificity compared to other methods. It directly attaches on the surface of the apoptotic cells which is a simple and easily applicable method to detect apoptosis. After exposure to NPs, the cells were collected by using trypsin and was stained with Annexin V & Dead Cell Assay Kit (Merk Millipore) for 20 minutes and was analyzed according to the manufacturer's protocol using Muse® Cell Analyzer .

3.3.7. Monodansylcadaverine (MDC) staining for autophagy

MDC staining kit obtained from Cayman, USA was used to detect autophagic vacuoles induced in the cells by NPs. MDC is a biochemical marker specific to autophagic vacuoles due to its ability to incorporate into multilamellar bodies via an ion trapping mechanism and the interaction with membrane lipids. The cells were seeded in 96 clear bottom black well plate and treated with NPs for 24 hours. For positive control, the cells were treated with 4 μ M tamoxifen provided in the assay kit. The working solution of the reagent was prepared by diluting it 1:1000 times in cell-based assay buffer. After 24 hours' incubation, the cells were washed thrice with Cell-based Assay buffer prepared according to the manufacturer's protocol. The cells were allowed to incubate for 10 minutes at 37°C. After incubation, the cells were washed two times with cell-based assay buffer and they were analyzed at emission and excitation wavelengths of 510 nm and 580 nm respectively using a plate reader (Infinite M200, TECAN Inc., Maennedorf, Switzerland). The fluorescence signal obtained was normalized to the cell number.

3.3.8. Cytokine/Growth factor array

Various cytokines are secreted by the cells during the onset of inflammation. Therefore, the inflammatory cytokine array was used to detect the cytokines secreted by keratinocytes exposed to NPs. Likewise, the fibroblast is responsible for secretion of growth factor arrays which help in keratinocyte and fibroblast migration during wound healing. Therefore, growth factor array was used in order to observe the effects of NPs in cells migration and wound healing. The levels of cytokines secreted by the keratinocytes exposed to different concentration of NPs were measured using Human Inflammation Antibody Array Membrane (Abcam 134003) according to the manufacturer's protocol. The array membranes were blocked for 30 mins with the blocking solution, provided in the array kit. The supernatant of the cells exposed to TiO₂ and ZnO NPs were collected and incubated overnight at 4°C with the membranes. The membranes were washed with Wash buffer I for three times and Wash buffer II for two times and were incubated for 2 hours at room temperature with Biotin-Conjugated Anti-Cytokines. After incubation, the membranes

were again washed with Wash buffer I and II as mentioned earlier and incubated with HRP-Conjugated Streptavidin. Finally, after washing, the membranes they were transferred onto the plastic sheet with Detection buffer for chemiluminescence detection. The detection was done in an imaging system with 2 to 10 minutes exposure to CCD camera. The background signals were subtracted, and each array was normalized with the corresponding DNA number of the cells treated to NPs before comparing the cytokine expression levels among different groups.

3.3.9. Western Blot analysis

To study the changes in total protein levels, total cellular proteins were extracted by lysing the cells in ice-cold RIPA lysis buffer (150mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 8). Protein concentration was determined by Bradford protein assay (Bio-Rad Laboratories, Inc., USA) using bovine serum albumin (BSA) as a standard. 30-70 µg of proteins per sample were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto a nitrocellulose membrane (Whatman, Millipore, USA). Membranes were subjected to reversible Ponceau S staining (0.5% (w/v) Ponceau S in 1.0% (v/v) acetic acid) to visualize total amounts of proteins resolved in each lane prior to blocking with 5% milk in TBST buffer (22.4 g/l Tris base, 80 g/l sodium chloride and 0.05% Tween 20 (BioRad, USA) pH 7.5). Proteins of interest were detected by specific antibodies and visualized using enhanced chemiluminescence (ECL) Western Blot detection reagent (GE Healthcare, USA) and autoradiography with Kodak films (USA) or hyperfilms (GE Healthcare, USA). Densitometric analysis was performed by quantifying the intensity of the bands using ImageJ software (NIH, USA). Either actin or tubulin was used as the loading control for analysis of total cell homogenates and RIPA lysates while LAMP1 was used for normalizing the total amounts of proteins in the lysosome-enriched fractions.

3.3.9.1. Antibodies and chemicals

The following primary antibodies were used for immunoblotting at the following concentrations: anti-Actin (1:10,000) (Abcam, UK); anti-LC3 (1:1000, Cell Signaling Technology and Novus Biologicals, USA), anti p62, NF- κ B and pNF- κ B (1:1000; Cell Signaling Technology, USA) and anti TRAF6 (1:2000). Secondary antibodies used for immunoblotting were goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated IgG (1:10000, Sigma-Aldrich, USA). The following different chemical inhibitors were used: leupeptin (100uM working, Enzo Life Sciences) and Ammonium Chloride (20mM working, Sigma-Aldrich).

3.3.9.2. Calculation of LC3-II flux

To study the activity of autophagy, LC3-II flux was calculated. During autophagy, LC3 II is degraded in the autophago-lysosome, as such the rate of turnover of LC3-II was studied under different conditions to calculate autophagy flux. Hence, the cells were treated with or without lysosomal inhibitors (NH₄Cl and leupeptin; NL) for 4 hours. Dividing the amount of LC3 II that accumulates upon lysosomal inhibition, with the amount of LC3 II in cells without lysosomal inhibition helps us to estimate the rate of autophagic turnover. The cells were harvested and lysed using RIPA buffer and subjected to immunoblotting. The LC3-II flux was calculated using the following formula;

$$LC3II\ FLUX = \frac{\text{Amount of LC3 II in NL treated condition}}{\text{Amount of LC3 II in untreated condition}}$$

3.4. 3D study on human skin explants

Skin explants were used to investigate the interaction of NPs with the skin cells in a complex 3D environment because 2D monolayer studies are not able to recapitulate the complexities of skin tissue. The critical concentration which showed significant effects on cells in 2D studies were chosen for further studies in skin explants.

3.4.1. Human skin explants and wound generation

Human skin explants were obtained from patients undergoing plastic surgeries. Abdominal skin explants from anonymous female donors (41-64 years old) were obtained from a local hospital with ethical approval from the NTU Institutional Review Board (IRB-2017-06-019). The skin was collected as soon as possible after the surgery. It was rinsed with PBS, 70% ethanol, followed by PBS containing 2% antibiotic-antimycotic. The subcutaneous fat and tissues were removed from the skin. Biopsy punches of 8 mm were made, and a wound was created by holding a tiny portion of the epidermis with a tweezer and cutting the skin below with sharp scissors (figure 3.11). Immediately after making the wounds, the biopsies were exposed to NPs by adding 10 μ l of NPs suspension in PBS at various concentrations over the wound. It was made sure that the NPs solution remains on top and do not come in contact with the culture medium. The control groups were exposed to PBS only without any particles. The skin biopsies were maintained at the air-liquid interface until the desired timepoint: 1 day, 3 days and 7 days in DMEM supplemented with 10% FBS, 1% antibiotic-amphotericin and 4mM L-Glutamine at 37°C and 5% CO₂. Deep well plates and inserts were purchased from Falcon (Falcon plate 6W Deep-Well; Falcon insert 6W 3 μ M). After a certain time point (day 1, day 3 and day 7), the biopsies were fixed with 4% paraformaldehyde (Sigma Aldrich) diluted in PBS. For TEM analysis, they were fixed with a mixture of 4% PFA and 2.5% glutaraldehyde (Sigma Aldrich).

Figure 3.11. Biopsies of skin explants with wound maintained in 6-well plate culture inserts.

The overall assumptions made with the use of skin explants are as follows:

- i. The explant cultures are most physiologically representative compared to 2D and organotypic cultures.
- ii. The re-epithelialization capability of the epidermis is retained during the course of culture.
- iii. All cell types present retain their native phenotype during the course of culture.

3.4.2. Histological analysis

After fixation, the biopsies were processed for histological analysis for the microscopic study of wound healing. The biopsies were dehydrated with a series of ethanol: 70%, 80%, 90%, two changes of 100% for one hour each and left overnight at 100% ethanol. The next day, they were followed by two changes of Xylene for 3 hours and left overnight in Xylene. Then they were infiltrated with paraffin wax, overnight before embedding into cassette blocks. 5 μ m thin sections of the biopsies were made by microtome and the sections were attached to the glass slides. The slides were kept overnight at 37°C for the proper attachment of the tissue section.

3.4.2.1. Hematoxylin and Eosin (H&E) Staining

The glass slides were dewaxed with two changes of xylene and rehydrated with a descending series of ethanol (100% to 70%), 3 minutes each. The slides were rinsed in tap water for 5 mins and stained with hematoxylin dye for 5 to 10 minutes, followed by 30 seconds wash in running tap water. The slides were then dipped in acid alcohol (70% ethanol, 1% Hydrochloric acid (37%), 29% DI water) for 15 seconds and again washed for 30 seconds. They were then placed in Scott's tap water (2g Sodium Bicarbonate, 20g Magnesium sulfate in 1L DI water) for 5 minutes followed by 30 seconds wash. Finally, they were stained with Eosin dye for 5 to 10 minutes and again washed. After staining, the slides were dehydrated following the ascending series of alcohol (70% to 100%). They

were mounted with DPX mounting medium (Sigma Aldrich) and allowed to dry overnight before microscopic analysis. H&E dye was purchased from ThermoFisher Scientific.

3.4.2.2. Immunoperoxidase staining

The glass slides were dewaxed in xylene and rehydrated in ascending series of ethanol. The sections were heat exposed in the appropriate antigen retrieval (pH 6 or pH 9) with 1X Dako Target Solution diluted in ultra-pure water. The slides were allowed to cool down before washing two times in PBST (PBS 1 X with 0.5% Tween 20). Quenching of endogenous peroxidase was done for 30 mins with 1 drop of Dako Real Peroxidase Blocking solution. The slides were then washed in running tap water and rinsed in PBST, 5 minutes each. Unspecific binding sites were blocked using 10% goat serum (Merk Millipore) in PBS for 1 to 2 hours. Primary antibody diluted in 10% goat serum was added on the sections and incubated overnight at 4°C for the antibody to bind properly. The next day, the slides were rinsed in running tap water for 10 minutes and PBST for 5 minutes. 1 drop of secondary mouse/rabbit antibody Envision+/Horse Radish Peroxidase (HRP) was added for 30 minutes and washed again with running tap water and PBST. DAB reagent was prepared immediately before use by mixing 1 drop of chromogen in 1 ml of substrate solution. 100 µl of DAB was applied over the sections and development of color was observed. As soon as the brown color was observed, the reaction was stopped by placing the slides in tap water. The slides were washed in running tap water for 5 minutes and counterstained with hematoxylin. After which they were rehydrated and mounted with DPX mounting medium. All the reagents were purchased from Thermofisher Scientific.

Human cytokeratin 14 antibody (LL001) MAB3164 was purchased from RnD systems and diluted in PBS to obtain a stock solution of 500 µgml⁻¹. The rest of the keratin antibodies were purchased from Abcam: Anti-cytokeratin 1 antibody (LHK1) antibody ab81623, Anti-cytokeratin 10 antibody (DE-K10) ab9026, Anti-cytokeratin 16 antibody (LL025) ab80574, Anti-cytokeratin 5 antibody (XM26) ab17130. Anti-Collagen Type IV Antibody, clone 24.12.8 (PHM-12) MAB1430 was obtained from Merk Millipore. The dilution and incubation time for the primary antibody is mentioned in the table below.

Table 3. 1. Optimized parameters for immunoperoxidase staining.

<i>Keratin antibody</i>	<i>Target retrieval solution's pH</i>	<i>Dilution</i>	<i>Primary antibody incubation</i>	<i>Secondary antibody</i>
<i>K1</i>	9	1:500	Overnight, 4°C	Mouse
<i>K5</i>	6	1:75	30 min, RT	Mouse
<i>K10</i>	6	1:200	30 min, RT	Mouse
<i>K14</i>	6	1:100	Overnight, 4°C	Mouse
<i>K16</i>	6	1:500	30 min, RT	Mouse
<i>Collagen 4</i>	6	1:50	Overnight, 4°C	Mouse

3.4.2.3. Immunofluorescence staining

The glass slides were dewaxed in xylene and rehydrated in ascending series of ethanol. The sections were heat exposed in pH 6 with 1X Dako target solution mentioned above. The slides were washed with PBST and blocked for 1 hour with 10% goat serum. γ H2AX primary antibody diluted in 10% goat serum at 1:500 times was used to stain the slides overnight at 4°C. After overnight incubation, the slides were washed with running tap water and PBST for 10 minutes and 5 minutes respectively. The sections were then probed with Alexa Fluor 488 conjugated secondary anti-mouse IgG (Thermofisher Scientific) at 1:250 times for 30 minutes. The slides were washed with PBS and stained with Hoechst dye for nuclear staining at 1:1000 times for 15 minutes. The slides were again washed with PBS and coverslips were mounted using the anti-fade mounting medium (Thermofisher Scientific). The images were taken using a Zeiss fluorescence microscope.

3.4.3. Methylene Blue analysis

Figure 3.12. Schematic representation of a methylene blue stained skin biopsy with the measured parameters.

Methylene blue is a cationic dye staining used for determining morphology and viability of eukaryotic cells. The presence of negatively charged molecules in the cells such as polyphosphates like DNA, RNA attracts the positively charged dye causing the staining phenomena. In the skin explants, the viable keratinocytes at the wound area took up the stain. Loeffler's methylene blue (Merck Millipore) was used to stain the wound area of the biopsies. The biopsies were washed with DI water and fully immersed in methylene blue solution for 5 minutes. They were again rinsed with DI water and dipped in acid alcohol for 10 seconds, followed by a rinse in DI water. Stereomicroscope images were taken and the images were analyzed using ImageJ software. The area and perimeter of healed and unhealed areas were measured by manual selection. The length and width of the healed and unhealed area were measured as shown in figure 3.12.

3.4.4. TEM analysis of skin explants

The fixed biopsies were cut into 1mm cubes and washed three times with PBS. They were postfixated using 2% Osmium tetroxide (OsO_4 , Sigma Aldrich) at room temperature for 2 hours. They were further washed with DI water to fully remove the fixative. Dehydration of the tissues was done with an ascending series of ethanol (25%, 50%, 75%, 95% and 100%) and pure acetone, for 40 minutes each at room temperature. The infiltration with

the resin (Araldite 502 kit, Ted Pella) was started at room temperature with the mixture of acetone and resin at the ratio 1:1, 1:3 and 1:6 for 2 hours, 1 hour and overnight respectively. The next day, tissues were transferred to the fresh resin at room temperature and incubated for 1 hour. They were then transferred again to another fresh resin and incubated for 1 hour at 40°C. The same process was repeated at 45°C and 50°C, using fresh resin each time. The tissues were finally embedded into a polyurethane mold at incubated at 60°C for at least 48 hours. The tissue embedded capsules were sectioned at thickness 80 to 100 nm though ultra-microtome using glass knife and collected on copper grids (EMS). The sections on copper grids were stained with 2% uranyl acetate and lead citrate for 8 minutes each at room temperature. The sections were viewed using TEM, Carl Zeiss Libra with an accelerating voltage of 120 kV.

3.5. Statistical Analysis

All the results are shown as a mean $\pm$ standard deviation. Three samples were taken in each sample as replicates, n=3. One-way analysis of variance (ANOVA) was performed to find out differences among groups. Tukey's test was carried out to see if there was equal variance across all groups. All the statistical analysis was performed using the OriginPro software. $P < 0.05$ was considered to be statistically different in all statistical testing.

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Chapter 4

Characterization of Physical Properties of NPs

In this chapter, a comprehensive characterization of TiO₂ and ZnO NPs used for this toxicological study are presented. The most important characteristic of NPs such as size, morphology, crystal structure, specific surface area and hydrodynamic properties have been characterized and is presented in the first section of this chapter. In addition, the solubility issue of ZnO NPs is also attributed. The second section emphasizes on computing NPs dose considering the sedimentation and/or diffusion behavior of NPs and the solubility of ZnO NPs in the biological medium used for cell culture. Lastly, it ends with the discussion on the summary of the importance of characterization and dosimetry and the findings obtained from the study.

4.1. NPs Characterization

4.1.1. Size and morphology

Particle shape and size was investigated by TEM which revealed that both TiO₂ and ZnO NPs possessed similar shape and size. TEM photomicrographs of the NPs showed that TiO₂ NPs were either spherical or polygonal in shape whereas ZnO NPs were mostly spherical (figure 4.1), with mean particle diameters of 22 ± 4 nm and 20 ± 2 nm, respectively. The shape and sizes of these NPs were similar to the ones commercially used in sunscreen [1].

Figure 4.1. Representative TEM micrographs of a) TiO₂ and b) ZnO NPs, Scale bar = 100nm.

4.1.2. Crystal structure

XRD patterns were acquired to determine the crystal properties of unmodified NPs powder. NPs exist in various crystal structures and many studies have suggested the effects of crystal structures in NPs toxicity. TiO₂ NPs in sunscreen and cosmetic products are usually present as a mixture of anatase and rutile crystal structures. Rutile structure is more stable and suggested to be less toxic than the anatase structure [2]. TiO₂ NPs used in this study was a mixture of anatase and rutile crystal structure as confirmed by the XRD profile in figure 4.2 a. The sharp diffraction peaks at 25.31° , 37.90° , and 47.96° correspond to its

anatase structure and sharp peaks at 27.46° , 36.19° , 41.32° and 62.7° corresponds to its rutile structure [3]. Similarly, figure 4.2 b shows the profile of ZnO NPs where the peaks between 31° to 69° confirmed the polycrystalline Zincite structure of ZnO NPs [4].

Figure 4.2. XRD diffraction peaks of a) TiO₂ and b) ZnO NPs.

Calculation of crystal size from XRD:

According to Scherrer's equation,

$$\text{Crystallite size} = (0.9 \lambda) / (d \cos \theta)$$

Where,

$$\lambda = \text{wavelength of Cu-K}\alpha 1 = 1.540598 \text{ \AA}$$

d = full width at half maximum intensity of peak (calculated from origin software) for TiO₂, $d = 0.50598^\circ$ and for ZnO, $d = 0.48782^\circ$

θ = Bragg's angle; for TiO₂, $\theta = 12.67^\circ$ and for ZnO, $\theta = 18.14^\circ$

Putting these values in the Scherrer's equation, the crystallite size of TiO₂ NPs and ZnO NPs were 16 nm and 17 nm respectively.

4.1.3. Specific surface area

At the nanoscale, the surface area to volume ratio of NPs is exceptionally high which increases their chemical reactivity and biological activity [5]. Therefore, it is essential to compare the surface area of NPs. BET is extensively used to measure surface area and porosity of fine powders and porous solids. The BET surface area of TiO₂ and ZnO NPs measured was $52.33 \pm 1.70 \text{ m}^2\text{g}^{-1}$ and $23.22 \pm 2.42 \text{ m}^2\text{g}^{-1}$ with the correlation coefficient 0.9988 and 0.99978 respectively. The similar surface area of TiO₂ and ZnO NPs was reported by previous studies [1, 6].

4.1.4. Hydrodynamic properties

It is essential to characterize the hydrodynamic size of NPs as they may significantly differ from their pristine size due to agglomeration of NPs when they are dispersed in media. The size distribution may also not be similar when they are transferred from deionized water to test media (cell culture media) during experiments. Such changes may alter the toxicity results. Therefore, size measurements were done in all biological medium used in this study. Table 4.1 shows the hydrodynamic sizes and zeta potential of the NPs in the cell culture medium and water.

Table 4. 1. Hydrodynamic size of TiO₂ and ZnO NPs in the cell culture medium.

<i>Hydrodynamic Properties</i>	<i>TiO₂</i>			<i>ZnO</i>		
	<i>Size (nm)</i>	<i>Zeta potential (mV)</i>	<i>PDI</i>	<i>Size (nm)</i>	<i>Zeta Potential (mV)</i>	<i>PDI</i>
<i>Epigro</i>	1914	-11.0	0.45	1471	-11.1	0.41
<i>DMEM</i>	1748	-12.6	0.35	1243	-12.4	0.44
<i>Ultrapure H₂O</i>	408	29	0.37	351	25	0.45

The hydrodynamic size of both NPs was observed greater than their pristine sizes which suggested agglomeration of NPs in water and biological medium. Higher aggregation of NPs was observed in cell culture medium compared to pure water. Additionally, the

hydrodynamic size of NPs dispersed in the Epigro medium was measured higher compared to DEMEM medium. Figure 4.3 and 4.4 shows the representative histograms of the size distribution of NPs by intensity measurement. The larger peak represents that most of the NPs fall under the range specified in the table. The smaller peaks may be due to the presence of some larger or smaller aggregates in the medium or due to dust or any other things in the solvent. The NPs were observed to be more stable in ultrapure water than in medium which was represented by higher Zeta potential (29 and 35 mV) of both NPs in ultrapure water. Negatively charged zeta potential was observed for the NPs dispersed in medium and positively charged zeta potential was observed for NPs dispersed in ultrapure water. This is because the pH of ultrapure water is lesser than the pH of the medium. Additionally, the polydispersity index (PDI), an indicator of aggregation of NPs in the medium was also measured. The higher values of PDI suggests a more polydisperse system and vice versa. The PDI values obtained lesser than 0.5 indicated reliable measurement.

Figure 4.3. Representative plot of size distribution by the intensity of TiO₂ NPs dispersed in a) Epigro medium, b) DMEM, and c) ultra-pure water.

Figure 4.4. Representative plot of size distribution by intensity of ZnO NPs dispersed in a) Epigro medium, b) DMEM, and c) ultra-pure water.

4.1.5. Solubility of ZnO NPs

The degree of metal ion release is another important toxicity parameter. Unlike TiO_2 , ZnO NPs are partially soluble in cell culture media and therefore, Zn^{2+} released from ZnO NPs suspension is commonly accepted as an important contributor of toxicity. Hence, it is crucial to determine the amount of Zn^{2+} ions dissolved from a certain concentration of ZnO NPs in the medium.

Different concentrations of ZnO NPs dispersed in the biological medium were incubated for 24 hours at 37°C and the levels of Zn^{2+} ions present in the solution was measured by ICP-OES (Table 4.2). As seen in table 4.2, a dose-dependent increase of Zn^{2+} ions with the increase in ZnO NPs concentration was observed. The slightly higher amount of aqueous Zn^{2+} was observed in DMEM compared to Epigro medium.

Table 4. 2. Concentration of Zn^{2+} ions dissolved from ZnO NPs dispersed in cell culture medium.

<i>ZnO NPs (μgml^{-1})</i>	<i>Dissolved Zn^{2+} (μgml^{-1})</i>	
	<i>Epigro medium</i>	<i>DMEM</i>
100	9.336 ± 0.14	10.138 ± 0.06
50	7.144 ± 0.18	7.927 ± 0.04
10	3.337 ± 0.08	3.712 ± 0.037
5	1.868 ± 0.04	2.01 ± 0.02
1	0.371 ± 0.04	0.48 ± 0.003
0.1	0	0.053 ± 0.0005
0	0	0
10 (in PBS)	0	0

4.2. Dosimetry

In suspension, NPs experience two driving forces: isotropic diffusion caused by Brownian motion and anisotropic sedimentation caused by gravity. The ISDD model developed by Hinderliter et al simulates the particle kinetics using a key parameter called effective density (ρ_{EV}) of NP agglomerates [7]. The effective density (ρ_{EV}) was calculated by measuring the effective volume of NPs agglomerates using the in vitro dosimetry characterization developed by DeLoid et al [8]. Figure 4.5 shows the pellet of TiO_2 NPs obtained after volumetric centrifugation and measurement of the pellet volume using slide ruler. The effective density (ρ_{EV}) of TiO_2 and ZnO agglomerates in the Epigro medium used was calculated to be 1.370 gcm^{-3} and 1.731 gcm^{-3} , respectively. Similarly, in DMEM, ρ_{EV} of TiO_2 and ZnO NPs was calculated to be 1.346 gcm^{-3} and 1.65 gcm^{-3} , respectively. These values are much lower than the density of pristine TiO_2 and ZnO (4.23 gcm^{-3} and 5.61 gcm^{-3} , respectively). This is because agglomerates contain lots of empty spaces filled with the medium, which is much less dense than the particulates and brings down the overall average density of the agglomerate.

Figure 4.5. Measurement of volume of TiO_2 NPs agglomerate in DMEM a) a packed cell volume tube with TiO_2 agglomerate, and b) Slide rule like easy measure device used to measure the volume of pellet.

The ISDD model was used to simulate the sedimentation profile of the NPs in suspension. Figure 4.6 and 4.7 shows the plot of the fraction of NPs sedimented as a function of time in the different cell culture medium. Due to the low effective density, the time taken for the NPs to sediment is higher in media suspensions. Comparing the two NPs suspension in 96 well plates (100 μl media volume), it was noted that ZnO NPs would sediment completely between 4 to 5 hours, whereas complete deposition of TiO_2 NPs took place after 9 hours. Likewise, even in 48 well plates and 150 cm^2 cell culture flasks, ZnO deposited faster than TiO_2 due to higher effective density.

Figure 4.6. Simulated transport rate of NPs in Epigro medium calculated using ISDD model. a) Fraction of administered dose of TiO_2 NPs deposited over a given time period, and b) Fraction of administered dose of ZnO NPs deposited over a given time period.

Figure 4.7. Simulated transport rate of NPs in DMEM calculated using ISDD model. a) Fraction of administered doses of TiO_2 NPs deposited over a given time period, and b) Fraction of administered dose of ZnO NPs deposited over a given time period.

Comparing the deposition rate between the two media, it was observed that both NPs deposited faster in the Epigro medium than in DMEM. For example, if we look at the fraction deposited in 96 well plate, it was observed that complete deposition of NPs in Epigro medium took place within 4 to 8 hours, whereas in DMEM, complete deposition was observed within 8 to 12 hours. This difference in deposition rate was due to the difference in the effective density of NPs agglomerate in different media. In addition to

this, the viscosity of DMEM is higher than Epigro medium, leading to slower sedimentation of NPs in DMEM.

Considering that only particles deposited can contribute to cellular responses, a time-weighted average dose (TWAD) parameter was calculated using the area under ‘the fD against incubation time (t)’ curve. The table of a fraction of AUC for different experimental conditions is listed in table 4.3. Wherever applicable, both administered dose (μgml^{-1}) and TWAD ($\text{mgcm}^{-2}\text{hr}$) are reported to correlate cellular response results. The term “administered dose” used represents the concentration of NPs in the cell culture medium. For example, an administered dose of volume 250 μl and concentration 25 μgml^{-1} of ZnO NPs in the Epigro medium in 48-well plates incubated for 4 hours and 24 hours would equal a TWAD of 0.0142 $\text{mgcm}^{-2}\text{hr}$ and 0.157 $\text{mgcm}^{-2}\text{hr}$ respectively.

Table 4. 3. Fraction of area under curve values obtained from ISDD simulation for Dosimetry.

<i>Culture condition</i>	<i>Epigro medium</i>				<i>DMEM</i>			
	<i>TiO₂</i>		<i>ZnO</i>		<i>TiO₂</i>		<i>ZnO</i>	
	<i>4 hr</i>	<i>24 hr</i>	<i>4 hr</i>	<i>24 hr</i>	<i>4 hr</i>	<i>24 hr</i>	<i>4 hr</i>	<i>24 hr</i>
<i>96 well plate</i>	0.926	19.650	1.822	21.777	0.5427	16.5499	0.6861	18.1115
<i>48 well plate</i>	1.013	20.021	1.991	21.977	0.5936	17.1878	0.7504	18.6151
<i>150 cm² flask</i>	1.447	21.205	2.592	22.566	0.8479	19.2263	1.072	20.2243

4.3. Discussion

The exploitation of nanotechnology has highly accelerated the diffusion of nanotechnology derived products in the consumer market which has sparked great concerns regarding its possible adverse effects on human health. Although the history of nanotechnology traces back to 1959, the first serious attention to potential adverse effects of nanotechnology was paid much later. In 2003, findings regarding the size effects of NPs on their distribution

and toxicity in animal studies were first presented which brought national and international attention to the issue [9]. Since then the importance of sound characterization of NPs was acknowledged by researchers as a necessary element in assessing NPs biological activity and toxicity.

TiO₂ and ZnO NPs used in the current study were procured commercially. Although the information and specifications on the NPs were provided from the manufacturer, a thorough characterization of these NPs was conducted in order to quantify all the necessary information relevant for toxicological studies. It was observed that these NPs were comparable to the ones which are used in sunscreens and other cosmetic products. The pristine size of TiO₂ and ZnO NPs measured were 22 ± 4 nm and 20 ± 2 nm respectively whereas their crystallite size were 16 nm and 17 nm respectively. Crystallite size refers to the size of a single crystal, whereas TEM gives the overall particle size. A particle may contain more than one crystallites, therefore, the crystallite size measured from XRD is lower than the particles size measured by TEM. As expected, the hydrodynamic size of NPs measured was larger than their pristine sizes. NPs have a higher tendency to form aggregates/agglomerates in the medium resulting in increased hydrodynamic size. This is due to the presence of ionic salts, amino acids, vitamins and glucose in the medium [10]. It was also observed that NPs behave differently in a different medium.

The high reactivity of NPs results in their agglomeration when dispersed in a solvent. There are various driving forces leading to aggregation/agglomeration of NPs. Some of the important factors are ionic strength, particle concentration and presence of protein and various ionic salts in the solvent. Ionic strength influences particles dispersion stability by altering the electrical double layer thickness. According to Derjaguin, Landau, Verwey and Overbeek (DLVO) theory, the stability of colloidal suspension is based on two forces: the electrostatic repulsion preventing aggregation of particles and the attractive van der Waals force which binds the particles together. In a weak electrolyte solution, the electrolyte compensating the charged surface of NPs extend far from the particle surface giving rise to long-range double layer interaction. Since Van der Waals force is relatively weak at long range, electrostatic repulsion dominates and the NPs suspension is stable. However, on

increasing the ionic strength, the electrical double layer gets compressed and the magnitude of electrostatic repulsion decreases, whereby van der Waals attraction dominates the stability of the suspension. Therefore, in the cell culture medium, the higher ionic strength of the medium leads to agglomeration of NPs [11]. Likewise, pH also affects the dispersion state by changing the zeta potential/surface charge of the particles [12]. Increase in pH of the surrounding environment causes an increase in agglomeration resulting in a higher hydrodynamic size of the particles [13]. Agglomeration rate increases with particles concentration. Adsorption of proteins onto the surface of the NPs also plays a decisive role in governing agglomeration. Protein adsorption acts as a surfactant and stabilized the NPs, suppression agglomeration [14]. Albumin, dominant protein in FBS has an affinity towards TiO_2 NPs. Therefore, in absence of albumin, TiO_2 NPs form large aggregates which tend to sediment rapidly. NPs tend to aggregate in most of the cell culture media, however, the effectiveness varies from medium to medium due to the difference in water chemistries which changes protein-NP interaction mechanisms. Phosphate ions also play an important role in NPs dispersion. Phosphate ions have higher affinity than the carboxyl groups of Bovine serum albumin (BSA) for Ca^{2+} site, hence, increasing the phosphate ions can prevent the adsorption of BSA on NPs surface [11]. The size of NPs aggregates/agglomerates were larger in the Epigro medium compared to DMEM. The reason for poor dispersion in the Epigro medium was due to the difference in serum content between the two medium. Unlike Epigro medium which was serum-free, DMEM was supplemented with 10% FBS. Whereas in media without serum, there was more aggregation/agglomeration of particles and hence the size measured was larger.

The solubility of NPs is another issue which is often overlooked. ZnO NPs are reported to partially dissolve in medium, therefore, the ZnO used in this study was measured for its solubility in both types on cell culture medium used. It was observed that the solubility of ZnO NPs largely depends on the medium, pH, and temperature [10]. Higher extent of dissolution was measured in DMEM than in Epigro medium. Both the medium has the same pH and were maintained at the same temperature. Therefore, the factor affecting the dissolution of ZnO NPs was the serum content of the medium. The NPs appeared to dissolve more in DMEM which contains 10% FBS. The protein corona on the particles

surface can affect the stability of NPs and affect their dissolution. The greater extent of ZnO dissolution in presence of serum was observed previously [15]. It must be noted that these measurements do not represent true saturation concentration of ZnO NPs as it was not known whether the solution had reached thermodynamic equilibrium with respect to the formation of aqueous Zn complexes within 24 hours. ZnO dispersion was incubated for 24 hours in order to replicate the actual condition used for toxicity studies.

NPs dose is another key factor affecting cellular responses. It is a parameter which needs to be handled carefully in order to correctly report a meaningful number especially in the context of in vitro models. It may, therefore, be more relevant if the dose is viewed as a two-faced parameter: the deposited amount of NPs (as only deposited NPs can interact with the cells) and the period of time for which they are interacting with the cells. It is also important to note that since only NPs deposited onto the cell layer (2D surface) are relevant, it is only logical that instead of absolute mass (no dimensions) or mass per volume i.e. concentration (3D), mass per surface area (2D) of cell layer or culture plate be used. This leads to the need for TWAD which considers the sedimentation profile of the NPs and can integrate the fraction deposited across the total incubation time. The ZnO solubility factor has also been considered in the calculation of TWAD factor. The TWAD obtained for NPs dissolved in the Epigro medium was higher compared to DMEM. This is due to the larger hydrodynamic size of NPs in Epigro medium which caused the NPs to deposit faster, thereby increasing the amount of NPs deposited at a certain time point. Therefore, although similar administered doses were used for both cell types, the actual dose received by keratinocytes was higher than fibroblasts.

4.4. Summary

In vitro toxicity studies are an essential component of risk assessment and therefore accurate characterization of NPs is an imperative prerequisite for understanding the biological response and underlying mechanism of NPs. There is a growing consensus on the necessity of precise characterization of NPs before using them for toxicity studies, in order to obtain reliable and reproducible toxicity results. In absence of such

characterization, the toxicity results will have limited value as the experimental conditions will have an unknown variability of NPs. The main factors influencing biological response from cells is NPs behavior. The behavior of a nanoparticle is dependent on their physical and chemical properties including size, shape, morphology, surface area, structure, crystallinity and so on. In this study, a sufficient and practical approach of NPs characterization has been implemented. In addition to NPs behavior, the dose is also a key parameter affecting the cellular response which has been handled carefully in order to correctly report a meaningful number in the context of in vitro models. To our knowledge, none of the in vitro studies reported in the literature, barring previous ones from our own group [16, 17], take into consideration dosimetry parameters like sedimentation kinetics that could directly skew data interpretation. Lastly, in cases where NPs are partially soluble, it is also pertinent that particulate effects are differentiated from ionic effects. In this current study, the specific effort has been given to characterize the dosimetry and solubility of the NPs in the culture system. The executive summary (key findings) are listed below:

- i. Size, shape, structure and specific surface area of TiO₂ and ZnO NPs used were comparable with the commercial ones used for the formulation of sunscreens and cosmetics.
- ii. NPs aggregation in serum-free cell culture medium (Epigro medium) was higher compared to medium containing 10% serum (DMEM medium).
- iii. TiO₂ NPs tend to aggregate more than ZnO NPs.
- iv. ZnO NPs were more soluble in medium containing serum.
- v. NPs dose received by the cells is based on the amount of NPs deposited onto the cells at a particular period of time.
- vi. NPs deposited faster in Epigro medium compared to DMEM medium, thereby increasing the dosage received by the cells at a particular time.
- vii. ZnO NPs deposited faster compared to TiO₂ NPs.

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Chapter 5*

Toxicological Influences of TiO₂ and ZnO NPs in Human Epidermal Keratinocytes

This chapter presents the findings on the interaction of TiO₂ and ZnO NPs with primary human keratinocytes, based on 2D cell culture models. The dose and time-dependent toxicity in keratinocytes, assessed by the measurement of cell viability and proliferation are presented in the first section of the chapter along with the toxicity contributed by Zn²⁺ ions dissolved from ZnO NPs in the biological medium. This is followed by NPs mediated oxidative stress which was assessed by measuring the increasing levels of ROS and MSO induced in the cells. Furthermore, the suppression of NPs mediated oxidative stress in the presence of NAC and the subsequent suppression of apoptosis in cells is presented. The fourth section presents the findings on NPs induced inflammation which is followed by NPs induced autophagy in keratinocytes. The interplay of autophagy and inflammation and the mechanism of NPs mediated toxicity in human epidermal keratinocytes is discussed.

5.1. Dose and time-dependent NPs toxicity

The response of human epidermal keratinocytes was evaluated after exposure to various administered doses and time points of TiO₂ and ZnO NPs. For evaluation of cytotoxic effects, keratinocytes were exposed to each NPs for 4 hours and 24 hours and measured for cellular metabolic activity (Alamar blue assay) and cellular proliferation (picogreen assay). Figure 5.1 shows the response of keratinocytes to various administered doses of TiO₂ and ZnO NPs at 4 hours of exposure.

Figure 5.1. Cytotoxic effects of TiO₂ and ZnO NPs to keratinocytes after 4 hours of exposure. AD- Administered Dose (μgml⁻¹), TWAD (mgcm⁻²hr), *p < 0.05 when compared to control (untreated cells) using one-way ANOVA.

Minimal effects on cell viability and proliferation were observed after 4 hours' exposure to TiO₂ NPs until a very high administered dose of 1000 μgml⁻¹ (TWAD 0.29 mgcm⁻²hr) at which 20% reduction in cell viability and 40% reduction in cell proliferation was

observed. On the other hand, ZnO NPs caused a much higher reduction in cell viability and proliferation; even at $25 \mu\text{gml}^{-1}$ (TWAD $0.0142 \text{ mgcm}^{-2}\text{hr}$) there was 64% reduction in metabolic activity and 70% reduction in cellular proliferation compared to the untreated cells. As incubation time was increased, both viability and proliferation of keratinocytes were reduced at higher administered doses as expected (figure 5.2).

Figure 5.2. Cytotoxic effects of TiO₂ and ZnO NPs to keratinocytes after 24 hours of exposure. AD-Administered Dose (μgml^{-1}), TWAD ($\text{mgcm}^{-2}\text{hr}$), * $p < 0.05$ when compared to control (untreated cells) using one-way ANOVA.

As the exposure time was increased, more pronounced toxic effects were observed. At TiO₂ administered dose of $100 \mu\text{gml}^{-1}$ for 24 hours (TWAD $0.57 \text{ mgcm}^{-2}\text{hr}$), cell viability was reduced by 20%, compared to the untreated cells, and at a higher administered dose of $1000 \mu\text{gml}^{-1}$ (TWAD $5.72 \text{ mgcm}^{-2}\text{hr}$), 62% reduction in cell viability was observed. Similarly, a significant reduction in cell proliferation was observed at the same dose with a maximum observed reduction of 60% at the highest administered dose of $1000 \mu\text{gml}^{-1}$ (TWAD 5.72

mgcm⁻²hr) measured. On the other hand, ZnO NPs after 24 hours' exposure caused cell viability to be reduced by 35% at 10 μgml^{-1} (TWAD 0.063 mgcm⁻²hr) and 90% at the highest tested administered dose of 100 μgml^{-1} (TWAD 0.628 mgcm⁻²hr). Likewise, there was a 93% reduction in cellular proliferation at higher administered dose of 100 μgml^{-1} (TWAD 0.628 mgcm⁻²hr) of ZnO NPs.

Figure 5.3. Phase contrast images of keratinocytes exposed to NPs at various administered doses of a) TiO₂ NPs, and b) ZnO NPs.

The cellular response was also observed through morphological changes in the cells. As shown in figure 5.3, the untreated cells showed healthy polygonal shapes and were well

attached to the growth surface. Not many changes were observed in the morphology of keratinocytes exposed to TiO₂ NPs for both 4 and 24 hours (figure 5.3a). However, drastic changes were observed in the morphological appearances of the keratinocytes exposed to ZnO NPs at both 4 and 24 hours (figure 4.6b); after 24 hours' exposure to 25 µgml⁻¹ (TWAD 0.157 mgcm⁻²hr) and above, the cells started to appear rounded and detached partially from the surface.

5.1.1. Effects of aqueous Zn²⁺

The dissolution of ZnO NPs in the biological medium has been shown in the previous chapter. Many studies have reported that the toxic cellular response by ZnO NPs is due to the aqueous Zn²⁺ ions in the complex formed by the dissolution of ZnO NPs. Therefore, to rule out the possibility that ZnO toxicity was due to the release of Zn²⁺ ions in the medium, the cellular response to a known concentration of Zn²⁺ ions in medium, based upon ICPOES analysis of ZnO solubility in the cell culture medium used was measured.

The contribution of aqueous Zn²⁺ generated by partial dissolution of ZnO, in the toxicity of keratinocytes was evaluated by exposing the cells to a known amount of aqueous Zn²⁺ in the culture medium. Based on the amount of aqueous Zn²⁺ measured by ICPOES, keratinocytes were exposed to ZnCl₂ solutions containing an equimolar concentration of Zn²⁺ ions compared to their counterpart ZnO NPs suspensions at the test concentrations. Cellular proliferation measured (figure 5.4) after exposing the cells to Zn²⁺ ions and ZnO NPs for 24 hours demonstrated that Zn²⁺ ions alone had minimal effects on cells up to a concentration of 3.337 µgml⁻¹ (the number of ions generated by a 10 µgml⁻¹ ZnO NPs suspension). In comparison, cellular proliferation was not significantly affected when the cells were exposed to an equimolar concentration of Zn²⁺ contributed by dissolution from 10 µgml⁻¹ of ZnO NPs, whereas, when the cells were treated to 10 µgml⁻¹ ZnO NPs (which contained both NPs and soluble Zn²⁺ ions), cell proliferation was significantly reduced. Zn²⁺ ions alone caused a significant toxic response in cells only at concentrations above 7.144 µgml⁻¹ (dissolved from ZnO 50 µgml⁻¹). This result confirmed that the released Zn²⁺ levels due to ZnO dissolution did not significantly contribute to a cellular response. Therefore, the observed effects reported herein were a direct result of cell-NP interactions.

Figure 5.4. Cell proliferation of keratinocytes exposed to various concentration of ZnO NPs and equivalent amount of aqueous Zn²⁺ ions dissolved from ZnO NPs the same concentration. *p < 0.05 compared to cells treated to ZnO NPs using one-way ANOVA.

5.2. NPs induced Oxidative stress

Even though keratinocytes could survive exposure to lower concentrations of NPs, their ability to maintain essential cellular functions with NPs exposure is still unclear. Metal oxide NPs are known to induce ROS, leading to oxidative stress by causing an imbalance in the redox state of the cells. Oxidative stress is often suggested as the possible mechanism of toxicity induced by metal oxide NPs [1]. Therefore, oxidative stress in keratinocytes exposed to TiO₂ and ZnO NPs was evaluated by measuring the ROS and MSO generated in the cells. Intracellular ROS generated in the cells exposed to NPs was examined by 2', 7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) while MitoSOX red mitochondrial superoxide indicator was used to detect the generation of MSO. The fluorescence reading demonstrated a significantly higher level of ROS and MSO signals in the cells exposed to the NPs.

Figure 5.5. ROS and MSO induced in keratinocytes exposed to TiO₂ and ZnO NPs for 4 hours. AD-Administered Dose (μgml^{-1}), TWAD ($\text{mgcm}^{-2}\text{hr}$), * $p < 0.05$ when compared to control (untreated cells) using one-way ANOVA.

Figure 5.5 shows the levels of ROS and MSO in keratinocytes exposed to TiO₂ and ZnO NPs for 4 hours. A significant increase in ROS was only observed at higher concentrations of TiO₂ NPs with an increase by 2 and 3.5 fold at an administered dose of $100 \mu\text{gml}^{-1}$ (TWAD $0.029 \text{ mgcm}^{-2}\text{hr}$) and $1000 \mu\text{gml}^{-1}$ (TWAD $0.29 \text{ mgcm}^{-2}\text{hr}$) respectively. Likewise, ROS was also increased after 4 hours' exposure to ZnO NPs, but the onset was at much lower doses: $25 \mu\text{gml}^{-1}$ (TWAD $0.0142 \text{ mgcm}^{-2}\text{hr}$) and $100 \mu\text{gml}^{-1}$ (TWAD $0.057 \text{ mgcm}^{-2}\text{hr}$) caused a significant increase in the ROS levels by 1.27 times and almost 2 times respectively.

The MSO levels in cells treated to TiO₂ NPs were lower than ROS levels at 4 hours' exposure. The significant increase in MSO levels was only observed at higher

concentrations of TiO₂ NPs: 100 µgml⁻¹ (TWAD 0.057 mgcm⁻²hr) and 1000 µgml⁻¹ (TWAD 0.57 mgcm⁻²hr) causing 1.3 times and 2.6 times increase compared to untreated controls respectively. In contrast, ZnO NPs exhibit more increase in MSO generation than ROS. However, the onset of the significant increase was again at much lower concentrations than that of the TiO₂ NPs counterpart: 25 µgml⁻¹ (TWAD 0.0412 mgcm⁻²hr) and 100 µgml⁻¹ (TWAD 0.057 mgcm⁻²hr) causing 2.2 and 2.6 times increase in MSO respectively. On increasing the exposure period to 24 hours, the ROS and MSO levels continued to increase strongly in a dose-dependent manner for both NPs as seen in figure 5.6.

Figure 5.6. ROS and MSO induced in human epidermal keratinocytes exposed to TiO₂ and ZnO NPs for 24 hours. AD-Administered Dose (µgml⁻¹), TWAD (mgcm⁻²hr), *p < 0.05 when compared to control (untreated cells) using one-way ANOVA.

As expected, again the onset of significant increase for both ROS and MOS levels was at much lower concentrations for ZnO NPs as compared to TiO₂ NPs. After 24 hours'

exposure, TiO₂ NPs at 100 µgml⁻¹ (TWAD 0.57 mgcm⁻²hr) and 1000 µgml⁻¹ (TWAD 5.72 mgcm⁻²hr) caused about 1.5 and 3 times increase in ROS levels respectively while ZnO NPs at 10 µgml⁻¹ (TWAD 0.063 mgcm⁻²hr) and 100 µgml⁻¹ (TWAD 0.628 mgcm⁻²hr) caused the ROS levels to increase by almost 1.5 and 4 times respectively. Interestingly, the MSO levels were higher than the ROS levels for both NPs exposure. TiO₂ NPs at 1000 µgml⁻¹ (TWAD 5.72 mgcm⁻²hr) caused an increase by almost 5 fold and ZnO at 100 µgml⁻¹ (TWAD 0.628 mgcm⁻²hr) caused a drastic increase by almost 7 times in the MSO level.

5.3. Oxidative stress and apoptosis suppression in presence of NAC

In order to determine whether oxidative stress contributed to toxicity by apoptosis, ROS generation was suppressed by using antioxidant N-acetyl cysteine (NAC). A significant reduction, typically around 20-30%, in the ROS levels was observed in NPs-treated keratinocytes at almost all administered doses tested, in the presence of NAC as an antioxidant after 24 hours' exposure (figure 5.7a). With the reduction in ROS, the percentage of cells which were viable or early apoptotic were increased (figure 5.7b). An increase from 71% to 84% at 1 µgml⁻¹ (TWAD 0.0057 mgcm⁻²hr) TiO₂ NPs in the presence of NAC was observed. Even at the highest TiO₂ administered dose of 500 µgml⁻¹ (TWAD 2.86 mgcm⁻²hr) tested, the percentage of viable cells increased from 41% to 64%. Consequently, late apoptotic cells or dead cells were also reduced in the presence of NAC.

Similar effects were observed for cells exposed to ZnO NPs (figure 5.8 a). Significant reduction in ROS levels was observed at all administered doses when treated with NAC. At ZnO 1 µgml⁻¹ to 25 µgml⁻¹ (TWAD 0.0063 mgcm⁻²hr to 0.157 mgcm⁻²hr), ROS level was dropped by a quarter in the presence of NAC. Likewise, an increase in the percentage of viable cells or early apoptotic cells was observed in apoptosis profile (figure 5.8 b). The most significant effect was observed at 25 µgml⁻¹ ZnO (TWAD 0.157 mgcm⁻²hr), where the percentage of viable cells increased from 15% to 50% when treated with NAC. Individually, the percentage of early, late apoptotic cells and dead cells were all reduced as viability was restored when cells were exposed to ZnO NPs in the presence of NAC compared to those exposed to ZnO alone.

Figure 5.7. a) ROS induced in keratinocytes treated with TiO_2 NPs with and without antioxidant NAC, and b) apoptosis induced by TiO_2 NPs with and without antioxidant NAC. AD-Administered dose (μgml^{-1}), TWAD ($\text{mgcm}^{-2}\text{hr}$), * $p < 0.05$ when compared to cells treated with TiO_2 without NAC using one sided Student's t-test.

Figure 5.8. a) ROS induced in keratinocytes treated with ZnO NPs with and without antioxidant NAC, and b) apoptosis induced by ZnO NPs with and without antioxidant NAC. AD- Administered dose (μgml^{-1}), TWAD ($\text{mgcm}^{-2}\text{hr}$), $*p < 0.05$ when compared to cells treated with TiO_2 without NAC using one sided Student's t-test.

5.4. NPs induced cytokines secretion

Numerous studies have reported that metal oxide NPs induced oxidative stress lead to

activation of pro-inflammatory responses [1]. In this study, it was observed that both TiO_2 and ZnO NPs resulted in changes in various cytokine levels secreted by the keratinocytes.

Figure 5.9. Levels of inflammatory cytokines secreted by keratinocytes at 24 hours' exposure to various administered doses of a) TiO_2 NPs, and b) ZnO NPs

At TiO_2 administered dose $500 \mu\text{gml}^{-1}$ the cytokine levels were increased when compared to untreated control as seen in figure 5.9a. The most significant increase was observed in IL-6 with almost 7 times increment. The other administered dose $10 \mu\text{gml}^{-1}$ and $100 \mu\text{gml}^{-1}$ did not show a much significant increase. Whereas, ZnO NPs caused more increase in

cytokines levels at all administered doses. IL-6 was increased by 11 times when treated with ZnO administered dose $10 \mu\text{gml}^{-1}$ as seen in figure 5.9b. Similarly, IL-2 and IL-8 showed 7 times increase compared to untreated cells. A dose-dependent increase in cytokine level was observed in IL-1 α , TNF- α , TNF- β , and sTNFRII.

5.5. NPs induced autophagy

As discussed earlier, TiO₂ and ZnO NPs induced oxidative stress in keratinocytes and increased the levels of secreted pro-inflammatory cytokines. It has also been established that NPs induced oxidative stress can lead to autophagy [2-4]. Hence, the effect of TiO₂ and ZnO NPs on induction of autophagy was evaluated.

Figure 5.10. a) Representative immunoblots showing LC3 II, p62 and actin, b) Graphs showing the fold change in LC3 II and p62 levels in keratinocytes exposed to TiO₂ and ZnO NPs. Differences are significant for * $p < 0.05$ against control.

Analysis of the autophagy markers p62 and LC3 II indicated an increase in the levels of both these proteins upon exposure to TiO₂ and ZnO NPs in a dose-dependent manner (figure 5.10a and 5.10b). Furthermore, to confirm the autophagy activity, the autophagic flux was studied in the presence of lysosomal inhibitors (ammonium chloride and leupeptin; NL). A significant increase in autophagic flux, similar to tamoxifen response, was observed upon treatment with TiO₂ (500 µgml⁻¹) and ZnO (50 pgml⁻¹ and 5 µgml⁻¹) (figure 5.11a and 5.11b).

Figure 5.11. a) Representative immunoblots showing LC3 II, p62, TRAF6 and actin; Graphs showing the fold change in b) LC3 II flux, c) p62 degradation, and d) TRAF6 degradation levels in keratinocytes exposed to TiO₂ and ZnO NPs with NL treated (+) and NL untreated (-). Differences are significant for *p<0.05, against (-).

Apart from the induction of autophagy, TiO₂ and ZnO NPs also induce an inflammatory response in keratinocytes as observed by the increased levels of proinflammatory cytokines secretion. Although significant autophagic degradation of p65 (Rel A) was observed at high dose exposure of TiO₂ (500 µgml⁻¹) NPs, upon exposure to ZnO NPs (figure 5.11a and 5.11c) no change in p65 levels was observed. TNF receptor-associated factor 6 (TRAF6), an adaptor protein in the Toll-like receptor (TLR) mediated inflammatory pathway also induces pro-inflammatory cytokines by an alternative pathway. The rate of autophagic turnover of TRAF6 under a low and high dose of both TiO₂ (100 fgml⁻¹ and 500 µgml⁻¹) and ZnO (50 pgml⁻¹ and 5 µgml⁻¹) NPs was studied. It was observed that TRAF6 was not significantly degraded by autophagy under basal (UT) or tamoxifen (TAM) induced an autophagic response (figure 5.11a and 5.11d). TRAF6 was also not degraded by autophagy under low administered dose of the NPs, however, under higher administered dose of the NPs, there was a significant increase in levels of TRAF6 degradation by autophagy (figure 5.11a and 5.11d).

Furthermore, an increase in levels of p65 (Rel A) upon treatment with a low administered dose of TiO₂ (100 fgml⁻¹) was observed, whereas, on treatment with a low dose of ZnO (50 pgml⁻¹) there was no significant change in p65 levels. Upon treatment with a higher dose of TiO₂ (500 µgml⁻¹), the p65 (Rel A) levels reduced to basal levels while increasing ZnO dose to 5 µgml⁻¹ showed no further change in p65 levels (figure 5.12a and 5.12b). p65 (Rel A) is activated by phosphorylation, and hence the effect of the TiO₂ and ZnO NPs on phosphorylation of p65 was also studied. Treatment of keratinocytes at a low dose of TiO₂ (100 fgml⁻¹) and ZnO (50 pgml⁻¹) showed no change in p65 phosphorylation (figure 5.12a and 5.12c). At a higher dose of TiO₂ (500 µgml⁻¹), there was a reduction in p65 phosphorylation, but increasing ZnO concentration to 5 µgml⁻¹ showed no change in phospho-p65 levels (figure 5.12a and 5.12c). Western blot analysis revealed increased levels of TRAF6 upon treatment with a low dose of both TiO₂ and ZnO NPs while increasing dose led to a drop in TRAF6 levels (figure 5.12a and 5.12d). Western blot analysis revealed increased levels of TRAF6 upon treatment with a low dose of both TiO₂ and ZnO NPs while increasing dose led to a drop in TRAF6 levels (figure 5.12a and 5.12d).

Figure 5.12. a) Representative immunoblots showing TRAF6, phosphorylation of p65 and p65 levels; Graphs showing the fold change in b) p65, c) p65 phosphorylation, and d) TRAF6 levels in keratinocytes exposed to TiO₂ and ZnO NPs. Differences are significant for * $p < 0.05$ against control.

5.6. Discussion

In this part of work, human epidermal keratinocytes exposed to TiO₂ and ZnO NPs at various administered dose and exposure time point were studied to identify the mechanism of NPs toxicity. The cytotoxic responses observed were consistent with most results reported in the literature. ZnO NPs were reported more cytotoxic to keratinocytes than TiO₂ NPs. With short-term exposure of 4 hours, TiO₂ NPs showed only mild effects to the cells, although significant at a highest administered dose, whereas on increasing the exposure period to 24 hours, more significant effects were observed without acute cell death. In

contrast, ZnO NPs caused acute cell death even at short-term exposure of 4 hours. This time and dose-dependent toxic behavior are in agreement with previously published results [2] [3]. Although the keratinocytes could survive a lower dose of NPs, their ability to maintain cellular functions are still unknown. It was observed that sublethal doses of TiO₂ and ZnO NPs could induce significant levels of ROS in keratinocytes. The ROS resulted in higher oxidative stress in cells which could swamp the cell redox defense system leading to disturbances in the normal functioning of the cells. Mitochondria are often a major target of NP-mediated ROS, causing damage to membrane phospholipids and consequent mitochondrial membrane depolarization [5], as observed in the current study with increased generation of MSO. Apoptosis is one of the major events implicated as the mechanism of cell death caused by oxidative stress and ROS triggered mitochondrial pathway. Both TiO₂ and ZnO NPs have been reported to cause oxidative stress-mediated apoptosis in human epidermal keratinocytes [6, 7]. By using NAC as an antioxidant, we confirmed that oxidative stress was an important mediator of TiO₂ and ZnO NPs-induced cell death. However, it was interesting to note that NAC regulated recovery was more significant in ZnO exposed than in TiO₂ exposed cells, suggesting mechanistic differences in cell interaction between the two NPs. At lower administered dose 10 µg/ml of ZnO NPs, there was only a 5% increase in the number of viable cells when treated with NAC. Likewise, at higher administered dose of 50 µg/ml, there was only a 1% increase in the number of viable cells in NAC treated groups. Interestingly, at administered dose 25 µg/ml, the NAC effect was observed to be beneficial with almost 35% increase in the viable cells. Therefore, it can be said that administered dose 25 µg/ml represents a transition of antioxidative strategy in response to the increasing dose of ZnO NPs.

Oxidative stress-induced cell damage has shown to activate pro-inflammatory cytokines [8]. Both TiO₂ and ZnO NPs are reported to elicit an inflammatory response through the secretion of a plethora of pro-inflammatory cytokine [9, 10]. However, another essential factor governing the effects of the NPs is dose. Hence, the cellular effects of these NPs on keratinocytes at low and high doses have been investigated. At high administered doses, both TiO₂ and ZnO NPs can be toxic to cells which may be contributed mainly by elevated levels of oxidative stress and inflammation [2] [11]. On the other hand, lower administered

doses, as low as 100 fgml⁻¹ for TiO₂ and 50 pgml⁻¹ for ZnO, resulted in autophagy activation after 24 hours' exposure. According to this study, autophagy appeared to be a cell survival response. The current study has further indicated the roles of TiO₂ and ZnO NPs induced autophagy on the inflammatory pathway. The NF-κB pathway has long been implicated as a prototype pro-inflammatory signaling pathway which is activated by cytokines like IL-1α and TNF-α [12]. The triggering of NF-κB-p65 (Rel A), a subunit of NF-κB transcription complex, in turn, leads to activation of pro-inflammatory genes [13-15]. Hence, the effect of TiO₂ and ZnO on the activation of p65 was investigated. At low administered dose (TiO₂ 100 fgml⁻¹) there was a significant increase in p65 levels, which reduced to basal levels when the cells were exposed to higher administered dose (TiO₂ 500 μgml⁻¹). The activation of p65 entails in phosphorylation and subsequent nuclear translocation. At higher administered dose (TiO₂ 500 μgml⁻¹), a significant reduction in phospho-p65 levels was observed. With the reduction of activated p65, nuclear translocation is reduced, which should result in reduced inflammation. It is also possible that the activation of autophagy at different administered doses of the NPs, which degrades various components of the inflammatory pathway, also prevents phosphorylation of p65 [16]. This corroborates with the significant reduction in phospho-p65 levels at a high administered dose of TiO₂ (500 μgml⁻¹) when autophagy is maximally activated.

Various studies have shown that NPs can be uptaken by cells through the endo-lysosomal pathway and be degraded by autophagy, although certain NPs can survive in the acidic lysosomal environment and impact the lysosomal pathway [16]. Our previous study has shown that TiO₂ can induce autophagy in keratinocytes [17]. Indeed, in the current study, it was confirmed that treatment with TiO₂ NPs resulted in activation of the autophagic pathway in a dose-dependent manner and is associated with induction of oxidative stress and mitochondrial depolarization. At high administered doses of TiO₂, there was the induction of autophagy in the keratinocytes which reduced phosphorylated p65 NF-κB (pNF-κB) levels, suggesting the autophagy induction to be a pro-survival mechanism. In addition to TiO₂, ZnO NPs have been also implicated to cause oxidative stress leading to ROS generation and DNA damage in keratinocytes [2]. As a result of increased ROS generation, there is activation of redox-sensitive transcription factor NF-κB as well as

secretion of the cytokine TNF- α [10]. However, no change in the activation of NF- κ B in response to different concentrations of ZnO NPs was observed. Similar to TiO₂, ZnO NPs also induced autophagy by increasing the levels of LC3 II, p62 and autophagic flux in a dose-dependent manner.

In this study, the autophagy was observed both as a direct effect of NP exposure as well as the cell's intrinsic response towards NPs induced oxidative stress and inflammation. At low administered doses (100 fg/ml for TiO₂ and 50 pg/ml) for ZnO, induction of autophagy was observed which is evidenced by increased levels and enhanced degradation of LC3 II and p62. Whereas, oxidative stress and inflammation were not observed at such lower administered doses. This suggests that the observed autophagy was a direct effect of NPs exposure. Additionally, the literature shows that both TiO₂ and ZnO NPs can be uptaken by the endosomes and accumulate in early endosomes, late endosomes and amphisomes [18]. Interestingly, amphisomes are cytoplasmic compartments that play a functional role in collecting autophagic substrates from endosomes and delivering them to the lysosomes [19, 20]. This corroborates with the increased LC3 II and p62 degradation profiles that were observed, suggesting that autophagy activation is a direct effect of NPs exposure. At higher administrative doses, TiO₂ and ZnO NPs resulted in elevated oxidative stress and inflammation in keratinocytes. It is reported that increased oxidative stress activates transcription factor Nrf2 which results in increased mRNA and protein levels of p62 [21]. p62 is a multidomain adaptor protein at the crossroads of autophagy and proteasomal pathways. Inhibition of proteasomal activity causes induction of p62 [21-24]. The increase in p62 could be the result of increased oxidative stress due to the high administered dosage of NPs, suggesting that autophagy could also be induced as an intrinsic response to oxidative stress. Additionally, increase levels of TRAF 6 was observed at lower dosages of TiO₂ and ZnO NPs. TRAF6 is an important player in the alternative inflammatory pathway, which in addition to activating inflammation, also activates autophagy in the beclin-mediated pathway [25]. All these evidence suggest that autophagy can be activated both as a direct effect of NPs exposure or in response to NPs induced oxidative stress and inflammation.

Accompanied by the increase in autophagy levels, there was also increased secretion of certain pro-inflammatory cytokines at high administered doses of the NPs. The most prominent increase was observed in IL-6 cytokine. Production of pleiotropic cytokine IL-6 is increased in human tissues in response to any damage. NPs are also known to upregulate various pro-inflammatory cytokines including IL-6 by activating nuclear factor-kappa B (NF- κ B) signaling, leading to cytotoxicity and cell death. It was observed that both TiO₂ and ZnO NPs resulted in changes in various cytokine levels secreted by the keratinocytes. The most prominent increase was observed in IL-6 production [26]. Among the various cytokines, IL-6 is a major mediator of the host response to an injury. The main function of IL-6 is to induce a host defense mechanism. While IL-6 is not usually induced by keratinocytes at normal conditions, it is induced in response to bacterial endotoxins, skin irritants, contact allergens and a broad range of dermatotoxic reactions [27]. As IL-6 is most often associated with a host defense mechanism, excessive production of IL-6 was observed when the keratinocytes were exposed to TiO₂ and ZnO NPs. IL-6 is highly dependent on IL-1 α [27]. It was observed that IL-1 α secretion was higher in keratinocytes treated to ZnO NPs compared to TiO₂. This might have resulted in higher levels of IL-6 secretion in keratinocytes exposed to ZnO NPs. Interestingly, at a very high administered dose of 25 μgml^{-1} for ZnO NPs, the secreted levels of several pro-inflammatory cytokines like IL-6 and IL-6sR were observed to be depleted. This can be due to various reasons, namely 1) high toxicity of the ZnO particles leading to cell death, which is the likely reason here as corroborated by cell viability and apoptosis results; 2) increased degradation of cytokines like TGF- β 1 [28] by increased autophagy activity; 3) reduced TRAF-6 levels due to its autophagic degradation, which may impair the production of certain cytokines like IL-6 and TNF [29]. Consequently, it can be confirmed that TiO₂ and ZnO NPs induce an inflammatory response in keratinocytes which may lead to apoptosis and cell death at higher doses.

At low administered doses of either TiO₂ or ZnO, a secondary inflammatory pathway was also activated, as observed by increased TRAF6 levels. TRAF6 is triggered by binding of the CD40 ligand to its receptor, recruiting TRAF6 proteins and downstream processing of p100 protein to p52, thus regulating transcriptional activation of inflammatory proteins

[30]. Although there is no direct evidence in keratinocytes, TNF- α and IL-1 β are known to increase TRAF6 expression in cultured astrocytes [31]. Interestingly, it was observed that over-activation of autophagy due to acute exposure to the NPs also led to the degradation of TRAF6, forming a feedback loop. Hence, at higher doses of TiO₂ or ZnO, TRAF6 levels are significantly reduced. This study demonstrates, for the first time, the degradation of TRAF6 by autophagy under the influence of TiO₂ or ZnO NPs.

Figure 5.13. Schematic diagram depicting the effects of low and high administered doses of TiO₂ and ZnO NPs on keratinocytes. At low administered doses of both NPs there is an upregulation of autophagy and TRAF6 levels. Both NPs induce inflammation by release of various cytokines at higher administered dosages. Autophagy is activated in a dose dependent manner under the influence of the NPs and plays a protective role against inflammation by (1) degradation of TRAF6 upon maximum activation. In addition to TRAF6 degradation, (2) autophagy also prevents p65 NF κ B activation in the TiO₂ treated condition.

It has been reported that high autophagy activity can prevent activation of NF- κ B downstream, preventing inflammatory response [32], and hence providing a protective mechanism not only to overcome the oxidative stress and DNA damage but also against inflammation. The current study provides evidence that the induction of autophagy by TiO₂ NPs inhibits both the classical p65 NF- κ B and TRAF6-mediated pathways whereas ZnO NPs only inhibits the TRAF6-mediated pathway to reduce the inflammatory response. This supports the observation that there was an overall lower amount of secreted cytokines in the keratinocytes exposed to TiO₂ NPs compared to the ZnO NPs. Therefore, assuming similar penetration of both NPs under physiological conditions, ZnO NPs may be more toxic compared to the TiO₂ NPs. Figure 5.13 shows the proposed model of TiO₂ and ZnO induced inflammation in relation to autophagy.

5.7. Summary

It is generally accepted that dermal exposure to NPs does not constitute significant health risks due to low levels of NPs penetration across the skin barrier. Despite this, current understanding of the physiological influence of such low levels of penetration is lacking and the jury is still out with regards to whether such influences may have an effect on skin health and beyond. This section of the study evaluates a range of physiological effects of TiO₂ and ZnO NPs exposure on human primary keratinocytes based on 2D cell culture. It was observed that at a higher dose, both TiO₂ and ZnO NPs possesses toxic effects to cells. NPs exposure at a lower dose, however, initiates autophagy induction in keratinocytes, which acts as a pro-survival response to NP-induced oxidative stress. Specifically, NPs induced autophagy prevents NF- κ B activation and promotes degradation of TRAF6, thereby contributing to cell survival. Nonetheless, with increasing levels of NPs exposure, oxidative stress induced by TiO₂ and ZnO NPs plays a central role in the onset of inflammation and eventual cell death by apoptosis. The physiological influence associated with dermal exposure and subsequent penetration of TiO₂ and ZnO NPs at the cellular level, therefore, hinges on the balance between autophagy and oxidative stress induction.

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Chapter 6

Toxicological Influences of TiO₂ and ZnO NPs in Primary Human Dermal Fibroblasts

This chapter presents the findings on the interaction of TiO₂ and ZnO NPs with primary dermal fibroblasts, based on 2D cell culture models. The dose and time-dependent toxicity in fibroblasts, assessed by the measurement of cell viability and proliferation are presented in the first section of the chapter along with the toxicity contributed by aqueous Zn²⁺ ions. This is followed by NPs mediated oxidative stress which was assessed by measuring the increasing levels of ROS and MSO induced in the cells. Furthermore, the suppression of NPs mediated oxidative stress in the presence of NAC and subsequent effects in apoptosis are presented. The fourth section presents the findings on NPs induced autophagy which is followed by the findings on the effects of NPs in growth factors secretion by fibroblasts. The growth factors secretion and its effects on keratinocytes growth and wound healing are discussed. Additionally, the different response of two cell types to NPs is discussed.

6.1. Dose and time-dependent toxicity of TiO₂ and ZnO NPs

The cellular response of human dermal fibroblasts to different administered dose of TiO₂ and ZnO NPs was evaluated by measurement of cellular metabolic activity through Alamar blue assay and cellular proliferation through picogreen assay. Various concentrations of TiO₂ and ZnO NPs were exposed to fibroblasts for 4 hours and 24 hours to measure cytotoxic responses. The cellular response of fibroblasts to various administered dose of TiO₂ and ZnO NPs at 4 hours' exposure is shown in figure 6.1.

Figure 6.1. Cytotoxic effects of TiO₂ and ZnO NPs to fibroblasts after 4 hours of exposure. AD- Administered dose (μgml^{-1}), TWAD ($\text{mgcm}^{-2}\text{hr}$), * $p < 0.05$ when compared to control (untreated cells) using one-way ANOVA.

At 4 hours' exposure, TiO₂ NPs caused minimal changes even at the highest administered dose of $1000 \mu\text{gml}^{-1}$ (TWAD $0.17 \text{ mgcm}^{-2}\text{hr}$) in which the cell viability and proliferation

were reduced by 5% and 15% respectively, but without any significant difference. In contrast to this, ZnO NPs caused significant toxicity from an administered dose of $10 \mu\text{gml}^{-1}$ (TWAD $0.0021 \text{ mgcm}^{-2}\text{hr}$) onwards. At ZnO $25 \mu\text{gml}^{-1}$ (TWAD $0.0054 \text{ mgcm}^{-2}\text{hr}$), the metabolic activity was reduced by 10% whereas 23% reduction was observed at $10 \mu\text{gml}^{-1}$ (TWAD $0.0021 \text{ mgcm}^{-2}\text{hr}$). At the highest administered dose, ZnO $100 \mu\text{gml}^{-1}$ (TWAD $0.21 \text{ mgcm}^{-2}\text{hr}$), metabolic activity was reduced by 20% whereas cellular proliferation was reduced by 35%. With the increase in the exposure period, the toxicity induced by NPs was increased as shown in figure 6.2.

Figure 6.2. Cytotoxic effects of TiO₂ and ZnO NPs to fibroblasts after 24 hours of exposure. AD- Administered dose (μgml^{-1}), TWAD ($\text{mgcm}^{-2}\text{hr}$), * $p < 0.05$ when compared to control (untreated cells) using one-way ANOVA.

At 24 hours of exposure, TiO₂ NPs still had minimal effects on fibroblasts. Reduction in metabolic activity was observed only at the highest administered dose of $1000 \mu\text{gml}^{-1}$

(TWAD $5.32 \text{ mgcm}^{-2}\text{hr}$) TiO_2 , in which metabolic activity was reduced by 20%, whereas a significant reduction in cellular proliferation was observed from TiO_2 administered dose $250 \text{ }\mu\text{gml}^{-1}$ (TWAD $1.33 \text{ mgcm}^{-2}\text{hr}$). At the highest administered dose of $1000 \text{ }\mu\text{gml}^{-1}$ (TWAD $5.32 \text{ mgcm}^{-2}\text{hr}$), almost a 50% reduction in cell proliferation was observed. On the other hand, ZnO NPs at 24 hours of exposure caused acute cell death.

Figure 6.3. Light microscopy images of fibroblasts treated with different concentrations of a) TiO_2 NPs, and b) ZnO NPs.

A significant reduction in both metabolic activity and cell proliferation was observed in ZnO $25 \mu\text{gml}^{-1}$ (TWAD $0.13 \text{ mgcm}^{-2}\text{hr}$) onwards. At the highest administered dose of $100 \mu\text{gml}^{-1}$ (TWAD $0.53 \text{ mgcm}^{-2}\text{hr}$), acute cell death was observed with almost 90% reduction in metabolic activity and proliferation. Likewise, at ZnO administered a dose of $25 \mu\text{gml}^{-1}$ (TWAD $0.13 \text{ mgcm}^{-2}\text{hr}$), a 30% reduction in metabolic activity was observed and a 40% reduction in proliferation was observed. The phase contrast images of fibroblasts showing morphological changes on exposure to TiO_2 and ZnO NPs are shown in figure 6.3. The signs of toxicity were manifested in the form of changes in cellular morphology as well. The untreated fibroblasts appear elongated and well attached to the growing surface. The morphology of the fibroblasts treated with TiO_2 NPs were similar to the untreated cells; not much morphological changes were observed even at the highest administered dose of TiO_2 . Additionally, cell proliferation from 4 hours to 24 hours was observed in cells treated with a higher administered dose of TiO_2 NPs as well. Unlike TiO_2 , ZnO induced toxic effects were observed in the phase contrast images of fibroblasts as seen in figure 6.3b. From the cellular morphology, it was clear that ZnO at a high administered dose of $25 \mu\text{gml}^{-1}$ and $100 \mu\text{gml}^{-1}$ caused acute cell death. These cells appeared rounded or shriveled compared with untreated controls. At 24 hours of exposure to higher concentrations, only cell debris was observed.

6.1.1. Effects of aqueous Zn^{2+} in cell viability

The main issue in toxicity screening of ZnO NPs is its solubility. The solubility of ZnO NPs in the DMEM has been shown in Chapter 4. The aqueous Zn^{2+} in the biological system may contribute to cellular toxicity as reported by previous studies. As such, it is unclear whether the acute cell death at higher concentration observed in fibroblasts is due to actual ZnO particles or simply due to aqueous Zn^{2+} in the medium. Therefore, in order to clarify this, known concentration of Zn^{2+} , based upon ICPOES analysis, was prepared by dissolving ZnCl_2 in culture medium and the cellular response to Zn^{2+} ions was measured. It was observed that Zn^{2+} ions alone did not have significant effects in cellular proliferation after 24 hours of exposure to all concentration tested (figure 6.4). At higher concentration of ZnO NPs: $50 \mu\text{gml}^{-1}$ and $100 \mu\text{gml}^{-1}$, acute cell death was observed, with an almost

complete reduction in cellular proliferation. In comparison, when the cells were exposed to an equimolar concentration of aqueous Zn^{2+} ions ($8.1 \mu\text{gml}^{-1}$ and $10.32 \mu\text{gml}^{-1}$), cell proliferation was reduced to only 70 to 80%. Likewise, when the cells were exposed to $10 \mu\text{gml}^{-1}$ ZnO NPs, a significant reduction in cellular proliferation was observed, whereas, when they were exposed to aqueous Zn^{2+} ions, dissolved from $10 \mu\text{gml}^{-1}$ of ZnO NPs, the cellular proliferation was reduced to 85%. The cellular effects observed in fibroblasts exposed to ZnO NPs was, therefore, a direct effect of NP-cell interaction. This result rules out the possibility that the toxicity observed by ZnO NPs was due to the release of Zn^{2+} ions in the biological system.

Figure 6.4. Cell proliferation of fibroblasts exposed to various concentration of ZnO NPs and an equivalent amount of aqueous Zn^{2+} ions dissolved from ZnO NPs at the same concentration. * $p < 0.05$ compared to cells treated to ZnO NPs using one-way ANOVA.

6.2. TiO_2 and ZnO NPs induced Oxidative stress by the generation of ROS and MSO

Numerous studies report that oxidative stress plays a central role in NPs mediated toxicity. Oxidative stress induced in fibroblasts exposed to TiO_2 and ZnO NPs for 4 hours and 24 hours was evaluated by measuring the levels of ROS and MSO generated in the cells. It was observed that even in 4 hours of exposure, NPs were able to generate significant

amounts of ROS and MSO in the cells. The fluoresce reading of ROS and MSO levels in the fibroblasts treated to TiO₂ and ZnO NPs for 4 hours is shown in figure 6.5.

Figure 6.5. ROS and MSO induced in fibroblasts exposed to TiO₂ and ZnO NPs for 4 hours. AD-Administered dose (μgml^{-1}), TWAD ($\text{mgcm}^{-2}\text{hr}$), * $p < 0.05$ when compared to control (untreated cells) using one-way ANOVA.

As shown in the figure, a significant increase in the ROS and MSO levels were observed at both TiO₂ and ZnO NPs exposure. TiO₂ administered dose 500 μgml^{-1} and 1000 μgml^{-1} (TWAD 0.11 $\text{mgcm}^{-2}\text{hr}$ and 0.211 $\text{mgcm}^{-2}\text{hr}$) caused 1.5 times increase in ROS levels when compared to untreated control. MSO levels generated by the cells were observed to be higher than ROS levels. TiO₂ administered dose of 250 μgml^{-1} (TWAD 0.054 $\text{mgcm}^{-2}\text{hr}$) caused 1.8 times increment in the MSO levels at 4 hours' exposure. Likewise, ROS and MSO levels in fibroblasts exposed to ZnO NPs were increased from ZnO administered dose of 10 μgml^{-1} . MSO levels were observed to be higher than the ROS levels at both TiO₂ and ZnO exposure. At 10 μgml^{-1} (TWAD 0.0021 $\text{mgcm}^{-2}\text{hr}$), a significant increase in

ROS and MSO levels by 1.2 times and 1.5 times respectively was observed. At a higher administered dose of $100 \mu\text{gml}^{-1}$ (TWAD $0.021 \text{ mgcm}^{-2}\text{hr}$), ROS was increased by 1.4 times whereas a drastic increase in MSO levels by 2.8 times was observed. The onset of ROS and MSO for ZnO NPs were at a much lower concentration as compared to TiO_2 NPs. Upon extending the exposure period to 24 hours, both ROS and MSO levels were increased strongly in a dose-dependent manner for both NPs exposure as seen in figure 6.6.

Figure 6.6. ROS and MSO induced in fibroblasts exposed to TiO_2 and ZnO NPs for 24 hours. AD- Administered dose (μgml^{-1}), TWAD ($\text{mgcm}^{-2}\text{hr}$), * $p < 0.05$ when compared to control (untreated cells) using one-way ANOVA.

As expected, with the increase in exposure time, the levels of oxidative stress were strongly increased. After 24 hours' exposure, TiO_2 $100 \mu\text{gml}^{-1}$ (TWAD $0.53 \text{ mgcm}^{-2}\text{hr}$) caused 2.2 times increment in ROS levels while the levels increased to 3.4 times at the highest administered dose of $1000 \mu\text{gml}^{-1}$ ($5.32 \text{ mgcm}^{-2}\text{hr}$). The MSO levels also showed a similar

pattern of increment, but the onset of a significant increase in ROS levels was observed at TiO_2 administered dose $50 \mu\text{gml}^{-1}$ (TWAD $0.27 \text{mgcm}^{-2}\text{hr}$) where a 1.5 times increase was observed. Similarly, at a highest administered dose of $1000 \mu\text{gml}^{-1}$ (TWAD $5.32 \text{mgcm}^{-2}\text{hr}$), 3.6 times increase was observed.

ZnO NPs, on the other hand, caused more increase in MSO and ROS levels at a higher administered dose. The significant onset of increase in oxidative stress levels was observed at ZnO administered dose $25 \mu\text{gml}^{-1}$ (TWAD $0.13 \text{mgcm}^{-2}\text{hr}$) where 2 times increase in ROS levels and 2.5 times increase in MSO levels was observed. At the highest administered dose of ZnO $100 \mu\text{gml}^{-1}$ (TWAD $0.53 \text{mgcm}^{-2}\text{hr}$), the ROS levels were increased by 3.3 times and much higher increase in MSO level was observed with almost 5.5 times increment.

6.3. N-acetyl cysteine (NAC) protected cells from undergoing apoptosis by suppressing ROS generation

It has been demonstrated that oxidative stress plays a critical role in apoptosis [1]. In order to determine whether the cell death was due to apoptosis induced by TiO_2 and ZnO NPs mediated oxidative stress, the ROS generation was suppressed by using antioxidant NAC and consequent oxidative stress and apoptosis was measured. NAC caused a reduction in ROS levels at almost all administered doses of TiO_2 NPs measured as shown in figure 6,7a. However, the significant reduction was observed at $250 \mu\text{gml}^{-1}$ (TWAD $1.28 \text{mgcm}^{-2}\text{hr}$) and $500 \mu\text{gml}^{-1}$ (TWAD $2.45 \text{mgcm}^{-2}\text{hr}$) with 15% and 41% reduction in ROS levels respectively. With the suppression in ROS, the percentage of viable cells or early apoptotic cells were increased as seen in figure 6.7b. The percentage of viable cells was increased at all administered doses of TiO_2 NPs. At TiO_2 $10 \mu\text{gml}^{-1}$ (TWAD $0.05 \text{mgcm}^{-2}\text{hr}$), 20% increase in viable cell population was observed in presence of NAC and at TiO_2 $100 \mu\text{gml}^{-1}$ (TWAD $0.5 \text{mgcm}^{-2}\text{hr}$), 15% increase in viable cells was observed. However, at higher concentration, not much significant increase was observed.

Figure 6.7. a) ROS induced in fibroblasts treated with TiO₂ NPs with and without antioxidant NAC, and b) apoptosis induced by TiO₂ NPs with and without antioxidant NAC. AD-Administered dose (μgml^{-1}), TWAD ($\text{mgcm}^{-2}\text{hr}$), * $p < 0.05$ when compared to cells treated with TiO₂ without NAC using one sided student's t-test.

Similar effects were observed when the cells were exposed to ZnO NPs in presence of NAC. Figure 6.8 shows effects of NAC in ROS and apoptosis in fibroblasts exposed to

ZnO NPs for 24 hours. As observed in figure 6.8a, ROS suppression in presence of NAC was observed in fibroblasts exposed to 10 μgml^{-1} of ZnO NPs and above. At ZnO administered dose 10 μgml^{-1} (TWAD 0.053 $\text{mgcm}^{-2}\text{hr}$), 15% reduction in ROS levels was observed and at administered dose 25 μgml^{-1} (TWAD 0.13 $\text{mgcm}^{-2}\text{hr}$), most significant reduction in ROS levels by almost 60% was observed. The percentage of early apoptotic, late apoptotic and dead cells were also reduced at all administered doses measured.

Figure 6.8. a) ROS induced in fibroblasts treated with ZnO NPs with and without antioxidant NAC, and b) apoptosis induced by ZnO NPs with and without antioxidant NAC. AD-Administered dose (μgml^{-1}), TWAD ($\text{mgcm}^{-2}\text{hr}$), * $p < 0.05$ when compared to cells treated with TiO_2 without NAC using one sided student's t-test.

As expected, suppression in ROS prevented cells from undergoing apoptosis as shown in figure 6.8b. The percentage of viable cells was increased at all administered dose of ZnO NPs measured. The most significant effect was observed at ZnO administered dose of 25 μgml^{-1} (TWAD 0.13 $\text{mgcm}^{-2}\text{hr}$) where the percentage of viable cells was increased by 20% when exposed to NPs in presence of NAC. Individually, the percentage of early, late apoptotic cells and dead cells were all reduced as viability was restored when cells were exposed to ZnO NPs in the presence of NAC compared to those exposed to ZnO alone.

6.4. NPs induced autophagy

Previously, autophagy induced in primary human keratinocytes exposed to NPs was discussed. The onset of autophagy in keratinocytes was observed at an administered dose as low as 100 fgml^{-1} of TiO_2 and 50 pgml^{-1} of ZnO NPs. Unlike keratinocytes, dermal fibroblasts exposed to TiO_2 and ZnO NPs did not show autophagy at 24 hours' exposure. In fibroblasts, the autophagy induction was measured by Monodansylcadaverine (MDC) staining. The MDC dye is a biochemical marker of autophagy which incorporates into multilaminar bodies through ion trapping mechanism [2]. Fibroblasts exposed to TiO_2 and ZnO NPs did not show MDC- positive staining as shown in figure 6.9.

Tamoxifen (4 μM) was used as a positive inducer of autophagy. An increase in autophagy level was observed in the fibroblasts exposed to Tamoxifen, however, on exposure to both TiO_2 and ZnO NPs, there was no significant increase in autophagy levels at all the administered doses measured. It was observed that at a higher administered dose of TiO_2 NPs 500 μgml^{-1} and ZnO 50 μgml^{-1} , there was a drop in autophagy levels. This might be due to cell death induced by NPs at higher administered doses.

Figure 6.9. MDC staining of autophagic vacuoles in fibroblasts exposed to various doses of a) TiO₂ NPs, and b) ZnO NPs.

6.5. NPs effects on inflammation and growth factors production

Various cytokines and growth factors are required to control the regulatory functions in the skin. Growth factor production by fibroblasts is a more relevant readout of fibroblast function. Hence, the growth factors produced by fibroblasts exposed to TiO₂ and ZnO NPs were measured using an array kit as shown in figure 6.10.

Figure 6.10. Growth factors secreted by fibroblasts exposed to a) TiO₂ NPs, and b) ZnO NPs.

It was observed that both TiO₂ and ZnO NPs resulted in changes in various growth factors secretion when compared to untreated controls. The profile of growth factors secreted by fibroblasts exposed to TiO₂ NPs is shown in figure 6.10a. It was observed that TGF-B secretion was reduced on the fibroblasts exposed to TiO₂ NPs. At TiO₂ 500 µgml⁻¹, TGF-B2 and TGF-B3 were reduced by almost 2 times compared to untreated control. On contrary, dose-dependent increase in secretion of most of the growth factors was observed. The most significant increase was observed in BFGF secretion with almost 5.7 times increase at TiO₂ 100 µgml⁻¹. It was noted that at a higher administered dose (500 µgml⁻¹) of TiO₂ NPs, the growth factors secretion was reduced. Likewise, ZnO NPs also resulted in increased secretion of most of the growth factors as shown in figure 6.10b. Similar to TiO₂ NPs, ZnO also resulted in a reduction of TGF-b2. At ZnO 25 µgml⁻¹, TGF-b2 levels were dropped by almost 7 times. For the rest of the growth factors, the dose-dependent increase was observed. The most significant increase was observed for BFGF and EGF secretion with 5 and 3 times increase respectively.

6.6. Discussion

In this section, human dermal fibroblasts exposed to TiO₂ and ZnO NPs at various administered doses and exposure time point were studied to identify the adverse effects of these NPs. TiO₂ and ZnO exposure indicated the range of toxicity in human dermal fibroblasts. Cytotoxicity results at 4 hours and 24 hours showed that ZnO caused greater toxicity compared to TiO₂, causing acute cell death at 24 hours. These results are in agreement with previous results that indicated mild adverse effects of TiO₂ and ZnO NPs at 4 hours exposure and substantial toxic impacts at 24 hours exposure in human dermal fibroblast [3]. Other studies also reported that ZnO NPs above 15 µgml⁻¹ significantly reduced cell viability and caused detachment of cells from the growth surface at 24 hours exposure whereas TiO₂ NPs did not show significant toxic effects on cell viability and morphology up to concentration 100 µgml⁻¹ measured [4, 5]. Although cell death was not observed at lower concentrations, the adverse effects of NPs in maintaining cellular function is still unclear. It was observed that sublethal concentrations of TiO₂ and ZnO NPs caused significant oxidative stress in fibroblasts. Oxidative stress is often reported to play

a central role in NPs mediated toxicity. Excessive oxidative stress can swamp the cell redox defense system resulting in damages to cell organelles such as cell membrane, mitochondria, and DNA which may lead to cell death by apoptosis. Mitochondria are also a major target of NPs-mediated ROS, causing damage to membrane phospholipids and consequent mitochondrial membrane depolarization [6]. A dose-dependent increase in MSO production was observed for both NPs exposure. Recent studies have reported that ROS and resulting oxidative stress play a critical role in apoptosis [1] which has also been demonstrated in this study. Apoptosis is one of the major events implicated as the mechanism of cell death caused by oxidative stress and ROS triggered mitochondrial pathway. It has been shown that both TiO₂ and ZnO NPs caused cell death via apoptosis with the onset of oxidative stress and mitochondrial superoxide. ZnO NPs induced oxidative stress-activated JNK signaling pathway leading to cell death via apoptosis of astrocytes has been reported previously [7]. When oxidative stress was suppressed with antioxidant NAC, recovery of cells from apoptotic to viable cells was observed which further confirmed that oxidative stress was an important mediator of TiO₂ and ZnO NPs-induced cell death.

Oxidative stress induced by NPs has been shown to activate the synthesis of inflammation mediators by causing disturbances in the normal metabolic activity of cells and tissue. It was observed that at a higher administered dose, both TiO₂ and ZnO NPs could be toxic to fibroblasts which may be contributed mainly by elevated levels of oxidative stress. With the increase in oxidative stress, cell death via apoptosis was also increased. In the previous section of this study, it was shown that autophagy was induced at a higher dose of TiO₂ and ZnO NPs, which played a protective role against NPs induced inflammation in human epidermal keratinocytes. However, in dermal fibroblasts exposed to TiO₂ and ZnO NPs, autophagy was not significantly induced at $p < 0.05$, as observed by MDC staining of autophagic vacuoles. It was observed that the fibroblasts were able to tolerate a lower administered dose of TiO₂ and ZnO NPs, as evident by the onset of oxidative stress only at a higher administered dose. Due to this reason, autophagy might not have been induced in the fibroblasts exposed to lower doses of NPs. However, it was surprising to observe that autophagy induction was not significant on the cells exposed to a higher dose of NPs

as well. It is well known that different cells react differently to similar stimuli. Although both keratinocytes and fibroblasts are primary skin cells, it was observed that they react differently to the same NPs.

It has been demonstrated that human epidermal keratinocytes depend on the presence of fibroblasts for efficient growth in skin tissue. Fibroblasts-derived growth factors are required by keratinocytes for their proliferation and migration [8]. Thus, the effects of TiO₂ and ZnO NPs in growth factors secretion by fibroblasts was measured. It was observed that NPs resulted in increased production of most of the growth factors, whereas some growth factors secretion was reduced. Transforming Growth Factor- β (TGF- β) is a multifunctional autocrine and paracrine regulators, which plays a crucial role in keratinocytes migration and proliferation [9]. TGF- β 2 is required for recruitment of fibroblasts and immune cells at the wounded area. It has been reported that in the wounds treated with antibodies against TGF- β 1 and TGF- β 2, there was a significant reduction in infiltration of immune cells at the wounded area [10]. The upsurge of TGF- β 3 has been reported to decrease scar formation [11]. Additionally, significantly lesser deposition of collagen I and III was reported [12]. In the fibroblasts exposed to TiO₂ and ZnO NPs, a significant reduction in TGF- β 2 and TGF- β 3 was observed with higher administered doses. Basic Fibroblast Growth Factor (bFGF/ FGF2) has a synergistic effect with (Epidermal Growth Factor) EGF on stimulating keratinocytes migration and promotes wound healing [13]. At TiO₂ 500 μgml^{-1} , BFGF dropped to the basal level. BFGF is clinically accepted and used for accelerated wound healing. It has been reported to improve dermal wound healing by recruitment of inflammatory cells at the wound site [14]. Lower levels of BFGF in fibroblasts might play a role in delayed wound healing of the skin explants exposed to TiO₂ NPs. Similarly, a significant drop in EGF level was observed when fibroblasts were exposed to TiO₂ 500 μgml^{-1} . EGF is involved in simulation, proliferation, and migration of keratinocytes and fibroblasts and facilitates dermal regeneration. They are required for mediating differentiation of keratinocytes for re-epithelization and dermal maturation [15]. It has been demonstrated that topical application of EGF resulted in enhanced wound healing [16]. The decreased levels of EGF in the fibroblasts exposed to higher administered dose of TiO₂ NPs could cause a delay in keratinocyte migration for wound closure. The

wound healing of skin explants in presence of TiO₂ and ZnO NPs is discussed in the next chapter.

In this study, we looked at the effects of NPs in both types of primary skin cells: human epidermal keratinocytes and human dermal fibroblasts. It was observed that these two types of cells, reacted differently to the NPs. The table 6.1 compares the cellular responses between the two cell types.

Table 6. 1. Comparison of cellular responses to NPs between two cell types.

Keratinocytes	Fibroblasts	Remark
<p>Toxic dose at 4 hours' exposure: TiO₂ 1000 µgml⁻¹ and ZnO 25 µgml⁻¹ (with acute cell death)</p> <p>Toxic dose at 24 hours exposure: TiO₂ 100 µgml⁻¹ and ZnO 10 µgml⁻¹</p>	<p>Toxic dose at 4 hours' exposure: TiO₂ no any significant effect and ZnO 25 µgml⁻¹</p> <p>Toxic dose at 24 hours exposure: TiO₂ 250 µgml⁻¹ and ZnO 25 µgml⁻¹</p>	<p>Keratinocytes were more sensitive to NPs compared to fibroblasts.</p> <p>Acute cell death was observed at 4 hours of exposure to ZnO 25 µgml⁻¹.</p> <p>ZnO was more toxic to both cell types compared to TiO₂ NPs.</p>
<p>Oxidative stress at 4 hours' exposure: TiO₂ 100 µgml⁻¹ and ZnO 10 µgml⁻¹</p> <p>Oxidative stress at 24 hours exposure: TiO₂ 100 µgml⁻¹ and ZnO 10 µgml⁻¹</p>	<p>Oxidative stress at 4 hours' exposure: TiO₂ 250 µgml⁻¹ and ZnO 10 µgml⁻¹</p> <p>Oxidative stress at 24 hours exposure: TiO₂</p>	<p>Both TiO₂ and ZnO NPs caused oxidative stress at subtoxic doses.</p>

	100 μgml^{-1} and ZnO 25 μgml^{-1}	
NAC suppressed ROS and protected the cells from apoptosis.	NAC suppressed ROS and protected the cells from apoptosis.	Oxidative stress plays a central role in cell death among both cells types. Oxidative stress triggers cell death via apoptosis in both cell types.
Autophagy induction by keratinocytes exposed to NPs more significant among keratinocytes. NPs also induced inflammation in keratinocytes.	Fibroblasts did not induce significant autophagy in response to NPs. NPs affect growth factors secretion which affects wound healing.	Mechanism of NPs induced toxicity is different among keratinocytes and fibroblasts.

While comparing the cytotoxic effects of NPs in keratinocytes and fibroblasts, it was observed that keratinocytes were more sensitive to NPs than fibroblasts. The onset of cytotoxic effects to keratinocytes was at lower administered dose compared to fibroblasts. This may be due to the difference in the cell type and also due to the difference in the biological medium used. It was observed that the tendency for NPs agglomeration/aggregation in DMEM was higher than Epigro medium, which increased their hydrodynamic weight. This affected the total amount of NPs transported to the cells at a certain time-point. As observed in Section 3.2, NPs settled faster in the Epigro medium compared to DMEM. Additionally, due to the higher viscosity of DMEM, the settlement of NPs took longer, reducing the fraction of NPs deposited over time. Although similar administered doses were used for both cell types, the actual amount of NPs deposited onto the cells at 4 hours or 24 hours (TWAD) was higher for keratinocytes compared to

fibroblasts. The higher amount of particles experienced by the keratinocytes might have resulted to cause more toxic effects compared to TiO₂ NPs. It was also observed that autophagy was triggered significantly in keratinocytes compared to fibroblasts. Even in the cells, treated with tamoxifen as a positive inducer of autophagy, a higher level of autophagy was exhibited by keratinocytes compared to fibroblasts. Fibroblasts, on the other hand, did not induce autophagy on exposure to both NPs.

6.7. Summary

In light of the wide application of TiO₂ and ZnO NPs in commercial products including sunscreen and cosmetics, a dose relevance study was performed in human dermal fibroblasts to explore the potential toxicity of the NPs across various doses. It was observed that at a higher administered dose, TiO₂ had minimal effects in fibroblasts whereas ZnO resulted in acute cell death. Similar to human epidermal keratinocytes, it was observed that oxidative stress played a central role in NPs mediated toxicity. Unlike keratinocytes, autophagy induction was not observed at both higher and lower administered doses, although oxidative stress was induced. However, autophagy induction in fibroblasts is insufficiently addressed which needs to be confirmed with further tests. Furthermore, it was observed that NPs also affected growth factors secretion by dermal fibroblasts. These growth factors might play a role in keratinocytes growth and wound healing which is discussed in the next chapter.

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Chapter 7

NPs Interaction with Viable Cells in Human Skin Explant and their Effects on Wound Healing

In this chapter, TiO_2 and ZnO NPs interaction with the epidermal keratinocytes was studied based on a 3D model system using human skin explants. The first section presents the findings on the interaction of TiO_2 NPs with skin explants and the second section presents the findings on the interaction of ZnO NPs with skin explants. Histological analysis of the cross-section of wounds performed with a combination of different staining techniques is presented. H&E staining was used to examine the morphology of the skin and specific keratin markers were used to study the wound healing process. Furthermore, NPs uptake and localization by the epidermal keratinocytes examined by the TEM has been presented. Furthermore, DNA damaging potential of the NPs was investigated using immunofluorescence technique.

7.1. Effects of TiO₂ NPs

In order to observe the effects of TiO₂ NPs in skin physiology, microscopic analysis of cross sections of the skin was performed. The skin sections were stained with H&E for observing the migration of keratinocytes for wound closure and any other physiological changes to skin structure. Immunoperoxidase staining was done to reveal the expression of specific keratin subtypes in the epidermis during wound healing.

7.1.1. Rate of wound healing

Methylene blue was used to stain the wounded area of the skin explants. Few representative images of the skin explants stained with methylene blue are shown in figure 7.1. The stained dark blue areas at the wound represent migrating keratinocytes and the unstained light areas represent the dermis or the unhealed area of the skin. The wound area in figure 7.1 (left) is fully stained at day 3 and day 7, representing full closure of the wound, whereas, in 7.1 (right) the light areas at the center represent that the wound healing is not complete. The rate of wound healing was calculated by measuring the healed and non-healed areas.

Figure 7.1. Wound area stained with Methylene blue at day 1, day 3 and day 7, (left side: healed wound, right side: non-healed wound).

In the figure, it can also be observed that the shape and size of the wounds are not similar. Therefore, calculating the wound healing on the basis of absolute area measurement becomes inaccurate when the wounds of different shape and sizes are compared. A relatively small amount of healing in a large wound will cause a bigger change in the healed area compared to a smaller wound. Calculating the wound healing rate by the measurement of the percentage change in the area could exaggerate the healing of smaller wounds. Therefore, in order to avoid this, Gilman's equation of linear wound healing was used to measure the rate of wound closure. According to this method, the linear healing of the wound is a function of the wound area and perimeter and is independent of the geometry and the size of the wound [1].

$$\text{Gilman's equation, } D = \Delta A/P$$

Where D = linear wound healing rate, ΔA = change in area and P = average of the initial and final perimeter of the wound.

Figure 7.2. a) Measurement of wound healing rate based on linear wound healing of the skin explants exposed to TiO₂ NPs, b. Evolution of the wound healed ratio for the width as a function of the wound healed ratio for the length.

On the basis of linear wound healing, it was observed that the rate of wound closure was reduced in the explants exposed to TiO₂ NPs as shown in figure 7.2a. However, no

significant difference was observed compared to untreated control. The figure shows the combined results of three independent experiments on different patient's skin with $n \geq 3$. The length and breadth of the healed wound were also measured. The evolution of wound healing ratio for width was plotted as a function of wound healing ratio for length as shown in figure 7.2b. The graph shows that healing process followed the $y=x$ trend for most of the wounds, such that the healing in the width and length directions correlated proportionally to each other. However, faster healing in the length direction was noticed on day 3 and day 7 for both untreated control and NPs treated wounds. It was not expected to observe faster healing in the length direction as the edges along the lengths of the wounds were located at the two apexes of the elliptical wounds where there should be more stress [37], which makes the healing slower. Unlike in vivo wounds, the wounds in cultured explants does not face any residual mechanical stress. This might have led to the observed faster healing at length direction as the stress induced by stretching and movements were not present. The graph also shows that for the samples that were not fully healed, the healing ratios were lower for the samples exposed to NPs.

7.1.2. Microscopic analysis of cross sections

7.1.2.1. Tissue morphology

The microscopic analysis of skin sections revealed that the migration of keratinocytes in most of the wounds started at day 1, progressed at day 3, and most of them were fully healed at day 7. Nevertheless, several wounds showed slower healing without full closure of wound even at day 7. The H&E sections of the cross-section of wounds at various time points are shown in figure 7.3.

As shown in figure 7.3, most of the wounds exposed to TiO_2 NPs showed similar wound closure compared to the control. At day 7, full closure of the wound was observed. On contrary, some of the explants did not show proper wound healing as shown in figure 7.4. At day 3, very little migration of keratinocytes was observed which did not seem to

progress on day 7. Instead, the migration tongue appeared rounded, as shown in figure 7.5, and did not progress significantly over time, showing the characteristic of a chronic wound.

Figure 7.3. H&E sections of skin explants exposed to TiO₂ NPs showing normal wound healing. Dashed lines indicate the original wound boundaries. Scale bar = 200 µm.

Figure 7.4. H&E sections of skin explants exposed to TiO₂ NPs showing abnormal wound healing. Dashed lines indicate the original wound boundaries. Scale bar = 200 µm.

Figure 7.5. Migrating tip at day 7 of skin explant treated with TiO₂ NPs at 500 µgml⁻¹.

In general, no significant modifications of wound closure with exposure to TiO₂ NPs were observed, based on H&E staining (figure 7.6). As abnormal wound closure was observed in some control samples as well, it could not be concluded if TiO₂ NPs played a role in wound closure. The percentage of fully healed samples exposed to TiO₂ NPs did not show any significant difference with exposure.

Figure 7.6. Percentage of fully healed samples at day 3 and day 7 of exposure to TiO₂ NPs.

7.1.2.2. Keratin expression

Keratin expression is specifically important during wound healing. It defines the differentiation and proliferation status of keratinocytes [2]. To observe the wound healing

process in the presence of TiO₂ NPs, specific keratin staining was performed in the skin sections. Figure 7.7 shows keratin 1 staining in the skin explants exposed to TiO₂ NPs at different time point and concentration.

Figure 7.7. Representative images of K1 expression in skin explants exposed to TiO₂ NPs at different time points. Scale bar = 100 µm.

Keratin 1 is expressed in the terminally differentiated keratinocytes. As the keratinocytes migrate to the upward layers, they are terminally differentiated, which could be observed in most of the samples. However, samples exposed to TiO₂ 100 µgml⁻¹ and 500 µgml⁻¹ for day 7, did not show positive staining for the cells at the suprabasal layers, when compared to the control, suggesting that the keratinocytes are still actively proliferating. It seems like in the non-healing wounds, although keratinocytes were not terminally differentiated, they failed to migrate and close the wound.

Keratin 1 dimerizes with Keratin 10 endogenously, so both of them are expressed by

terminally differentiated cells. Since the keratinocytes at the tip of the migrating tongue are actively proliferating, they do not express positive staining. Figure 7.8 shows keratin 10 staining in the skin explants.

Figure 7.8. Representative images of K10 expression in skin explants exposed to TiO_2 NPs at different time points. Scale bar = 100 μm .

As expected the suprabasal layers of keratinocytes show strong positive K10 staining and the staining was absent at the basal keratinocytes and the keratinocytes at the tip of the migrating tongue. However, in day 7 samples, the keratinocytes at the suprabasal layers did not show positive staining. Since this pattern of staining was expressed in control as well, it could not be concluded as the potential effects of TiO_2 NPs.

Keratin 5 is expressed by the actively proliferating keratinocytes; therefore, it is up-

regulated by the keratinocytes at the basal layer and tip of the migrating tongue. During wound healing, the proliferating keratinocytes migrate towards the wound for the closure of the wound bed. Therefore, downregulation of K5 could affect normal wound healing processes. Figure 7.9 shows K5 staining in skin explants.

Figure 7.9. Representative images of K5 expression in skin explants exposed to TiO₂ NPs at different time points. Scale bar = 100 µm.

Normal expression of K5 was observed in the control samples at all time points. However, in samples exposed to TiO₂ NPs for day 3 and day 7, very weak staining was observed in the wounds that did not heal. The down-regulation of K5 at the tip suggests that the keratinocytes are not proliferating, which could lead to a chronic wound. Keratin 14 dimerizes with Keratin 5 endogenously, so both of them are expressed by basal undifferentiated or proliferating keratinocytes. In figure 7.10, it was observed that K14 was

strongly expressed by the keratinocytes at the basal layers and it got weaker at the upper layers.

Figure 7.10. Representative images of K14 expression in skin explants exposed to TiO₂ NPs at different time points. Scale bar = 100 µm.

The expression of K14 appeared normal for most of the samples. In figure 7.10, the controls showed normal expression of K14 at both time points, showing stronger staining at the migrating tip of day 1 sample. Comparing this with day 3 and day 7 samples treated with the TiO₂ NPs, it could be observed that the staining was weaker at the migrating tip of the unhealed samples. This could have resulted in failure in wound healing even at day 7 for samples treated to NPs.

Keratin 16 is expressed by hyperproliferating keratinocytes. It can also be referred to as a

stress marker which is expressed at the stressed area of the wound. Expression of K16 is essential for keratinocytes proliferation and migration towards the wound bed. All samples expressed the normal pattern of K16 as shown in figure 7.11. For day 1 and day 7 samples, including the control, the staining was weaker compared to day 3 samples. The downregulation of K16 expression at day 7 could suggest that the keratinocytes at the wound area are no longer proliferating and therefore may result in failure of wound healing. However, this pattern was shown by control samples as well, therefore, it could not be concluded as the effect of NPs.

Figure 7.11. Representative images of K16 expression in skin explants exposed to TiO₂ NPs at different time points. Scale bar = 100 µm.

7.1.2.3. Collagen 4 expression

Collagen type 4 is the type of collagen which is mostly present at the dermal-epidermal junction of the skin and is responsible for the mechanical stability of the basement membrane. In the skin explants, the restoration of collagen was not observed until day 7 in all samples including control. Collagen 4 expressions in the wounds are shown in figure 7.12.

Figure 7.12. Representative images of collagen 4 expression in skin explants exposed to TiO₂ NPs at different time points. Scale bar = 100 µm.

7.1.3. TiO₂ uptake and localization

NPs uptake, localization and changes in ultrastructural structures in skin physiology with different TiO₂ concentration and time points was investigated through TEM. A large number of melanosomes were observed in the keratinocytes in the control samples as

shown in figure 7.13. TEM images of skin explants exposed to TiO_2 $100 \mu\text{gml}^{-1}$ at day 1 are shown in figure 7.14. It was observed that the uptake of TiO_2 NPs was dependent on time and concentration. More uptake was observed with higher concentration and longer exposure to NPs.

Figure 7.13. TEM micrographs of keratinocytes in skin explant without any NPs exposure (control)

Figure 7.14. TEM micrographs of keratinocytes in skin explant exposed to $100 \mu\text{gml}^{-1}$ TiO_2 NPs at day 1.

Figure 7.15. TEM micrographs of keratinocytes in skin explant exposed to 100 µgml⁻¹ TiO₂ NPs at day 7.

As observed in figure 7.14, on day 1, there was not much uptake of TiO₂ NPs by the cells. Clusters of TiO₂ NPs were observed outside the cells and at the ECM between the cells,

shown by the circle in figures A, B and C. A single nanoparticle was putatively observed inside the nucleus, figure C, denoted by an arrowhead. At day 7, higher uptake of TiO₂ NPs was observed as shown in figure 7.15. TiO₂ NPs uptake by the cells was observed to be through endocytic pits, as shown by the arrows in figures 7.15 A, B, and C. NPs were uptaken both individually and as agglomerates. Inside the cells, NPs were observed as clusters confined within endosomes, few NPs were seemed to be in the cytoplasm (figure C and D) without membrane-bound structure. The NPs containing vesicles migrated and localized towards the perinuclear regions (figure E and F). Some NPs could be observed suspended within the cytoplasm in the perinuclear regions. Notably, NPs were observed to be penetrating into the nucleus (figure G and H). Autophagosomes were also observed (figures I and J). On exposure of skin explants to a higher concentration of TiO₂ 500 µgml⁻¹, higher uptake on day 1 was observed compared to TiO₂ 100 µgml⁻¹ as shown in figure 7.16.

Figure 7.16. TEM micrographs of keratinocytes in skin explant exposed to 500 µgml⁻¹ TiO₂ NPs at day 1.

As shown in figure 7.16, NPs were observed to be perinuclear-localized (figure B, and C). Structural changes in the cell organelles were observed which seemed to be like autophagic structures (Figure E). The NPs were mostly localized in the vesicles containing melanosomes (figures B, C, and D). A single nanoparticle putatively inside the nucleus was also observed (figure E and F). A big cluster of TiO₂ NPs was found in the intercellular space between the cells beneath the granular keratinocyte layer (figure F) which was located far from the migrating keratinocytes. On increasing the exposure of TiO₂ 500 µgml⁻¹ 1 to day 3, further higher uptake was observed as shown in figure 7.17.

Figure 7.17. TEM micrographs of keratinocytes in skin explant exposed to 500 µgml⁻¹ TiO₂ NPs at day 3.

Figure 7.17. A and B shows the NPs present in the cells within the suprabasal layers of the epidermis which was located at the re-epithelized area of the wound. From the figures, it could be postulated that the NPs were localized together with melanosomes in the same lysosomal compartments. Almost all agglomerates of NPs present inside each keratinocyte were found within lysosomes containing melanosomes. Autophagy-like-structures were observed close to these lysosomes (figure F). TiO_2 individual particles were also clearly observed inside the nucleus (figure H and I, denoted by arrowheads). The uptake of TiO_2 NPs at day 7 was comparable to day 3 as shown in figure 7.18. As observed earlier, the TiO_2 NPs agglomerates were found together with the melanosomes. Autophagosomes were also observed (figure D).

Figure 7.18. TEM micrographs of keratinocytes in skin explant exposed to $500 \mu\text{gml}^{-1}$ TiO_2 NPs at day 3.

7.1.4. DNA damage

The TEM images showed that TiO₂ NPs were localized at the perinuclear region and were also able to penetrate into the nuclei of the keratinocytes. Immunofluorescence staining was performed to reveal if the NPs caused DNA damage to the keratinocytes. Phosphorylation of histone H2AX (γ H2AX) was used as a DNA damage marker. The presence of γ H2AX foci in the nucleus indicates the presence of double-strand breaks.

Figure 7.19. γ H2AX staining of skin explants exposed to TiO_2 NPs. Scale bar = 100 μm

γ H2AX immunofluorescence staining in the cross-section of the wounds exposed to various concentration and of TiO₂ NPs was done as shown in figure 7.19. For positive control of DNA damage, the skin explants were exposed to UV for 1 hour. From the figures, it can be observed that TiO₂ NPs did not induce DNA damage at both concentrations: 100 μgml^{-1} and 500 μgml^{-1} . The positive control showed an increase in γ H2AX positive cells whereas, in the explants exposed to TiO₂ NPs, the signal was not present. In the previous studies based on the 2D culture of human epidermal keratinocytes, it was reported that TiO₂ NPs above 50 μgml^{-1} induced DNA damage at 24 hours exposure [3] [4]. However, when the viable keratinocytes at the epidermal layers were exposed to the same concentration of TiO₂ NPs, DNA damage was not observed.

7.2. Effects of ZnO NPs

The skin explants were similarly exposed to ZnO NPs to observe their effects in skin physiology and wound healing. H&E staining was performed to observe physiological changes in skin structure and keratinocytes migration for wound closure. Immunoperoxidase staining was done to reveal the expression of specific keratin subtypes in the epidermis during wound healing. Additionally, immunofluorescence staining was done to reveal DNA damage induced by ZnO to the keratinocytes in the skin explant.

7.2.1. Rate of wound healing

Methylene blue staining of the wounds exposed to ZnO NPs at concentration 1 μgml^{-1} and 10 μgml^{-1} revealed that all the samples were fully healed at day 3 and day 7. Any significant changes with the untreated control were not observed.

7.2.2. Microscopic analysis of cross sections

7.2.2.1. Tissue morphology

H&E sections of the skin explants did not reveal any significant effects of ZnO NPs to skin explants at both concentrations. While normal wound healing was observed for most of the samples, some of the wounds showed delayed wound healing without full closure even at day 7. The H&E sections of the cross-section of the wounds are shown in figure 7.20.

Figure 7.20. H&E sections of skin explants exposed to ZnO NPs showing normal wound healing. Dashed lines indicate the original wound boundaries. Scale bar = 200 µm

As observed in the figure, wound healing started from day 1, progressed at day 3 and was fully healed at day 7. Conversely, for some of the wounds, abnormal or delayed wound healing was observed as shown in figure 7.21. Less migration of keratinocytes was observed on day 3 which did not progress up to day 7. At day 7, the tip of the wound edge appeared rounded, as shown in figure 7.22, and did not seem to be migrating, causing delayed wound healing. These wounds show the characteristics of the chronic wound which might not be healed as very little or no migration of keratinocytes were observed on day 7.

Figure 7.21. H&E sections of skin explants exposed to ZnO NPs showing abnormal wound healing. Scale bar = 200 μm

Figure 7.22. Migrating tip at day 7 of skin explant treated with ZnO NPs at 10 μgml^{-1} .

Figure 7.23. Percentage of skin fully healed skin explants exposed to ZnO NPs.

Similar to the skin explants exposed to TiO₂ NPs, no significant effects with exposure to ZnO NPs were observed. For some of the explants, both the control and ZnO exposed showed delayed wound healing ruling out the possibility of the effects of NPs. The ratio of non-healed wounds exposed to ZnO NPs did not show any significant difference with exposure as shown in figure 7.23.

7.2.1.2. Keratin expression

As discussed in the earlier section, specific keratin staining was performed in the cross sections of the skin to observe the wound healing process in presence of ZnO NPs. Figure 7.24 shows keratin 1 staining in the explants exposed to ZnO NPs at different time point and concentration. In normally healing the wound, K1 is expressed by the terminally differentiated keratinocytes.

Figure 7.24. Representative images of K1 expression in skin explants exposed to ZnO NPs at different time points. Scale bar = 100 μm .

As observed in most of the samples, the tip of the migrating tongue does not express K1, as they are actively proliferating and migrating for the closure of the wound. However, some abnormalities were observed as seen in skin explant exposed to ZnO $1 \mu\text{gml}^{-1}$ on day 3. The keratin staining was expressed by the keratinocytes at the tip of the migrating tongue, suggesting that they were terminally differentiated. The terminally differentiated keratinocytes are not able to proliferate and migrate for wound closure.

Keratin 10 is also expressed by terminally differentiated cells. Figure 7.25 shows keratin 10 staining in the skin explants. K10 staining was normally expressed in all the samples. The keratinocytes at the tip of the migrating tongue are undifferentiated, actively proliferating cells, hence they do not repress K10. Keratin 5 expression pattern is opposite of K1 and K10, as it is expressed by undifferentiated basal cells. The keratinocytes at the tip are undifferentiated proliferating cells, where K5 is upregulated. Figure 7.26 shows K5 staining of skin explants exposed to ZnO NPs.

Figure 7.25. Representative images of K10 expression in skin explants exposed to ZnO NPs at different time points. Scale bar = 100 μm

As expected, K5 was upregulated in the keratinocytes at the tip of the migrating tongue. However, in the sample exposed to ZnO $10 \mu\text{gml}^{-1}$ for day 3 and day 7, lighter staining was observed for the wounds that did not heal. The absence of K5 expression indicates that the keratinocytes in that region did not maintain their basal keratinocyte phenotype. This may have caused delayed or absence of wound healing for some of the wounds.

Similar to K5, K14 is also expressed by the basal undifferentiated or proliferating keratinocytes. Figure 7.27 shows K14 staining in the skin explants. In most of the samples, K14 was strongly expressed by the basal keratinocytes and the keratinocytes at the tip of the migrating tongue. However, abnormal expression of K14 was also observed in a few samples. The keratinocytes at the tip of the migrating tongue weakly expressed K14 in few wounds which had not healed. The failure of keratinocytes to differentiate could have resulted in delayed wound closure.

Figure 7.26. Representative images of K5 expression in skin explants exposed to ZnO NPs at different time points. Scale bar = $100 \mu\text{m}$

Figure 7.27. Representative images of K14 expression in skin explants exposed to ZnO NPs at different time points. Scale bar = 100 μm

Figure 7.28. Representative images of K16 expression in skin explants exposed to ZnO NPs at different time points. Scale bar = 100 μm

Keratin 16 is a stress marker expressed at the wound edge which is expressed by hyperproliferating cells. The expression of K16 is a hallmark of keratinocyte activation and differentiation. K16 staining in the cross sections of the wounds was normal for all wounds as shown in figure 7.28. K16 expression was significantly elevated in day 7 samples, compared to day 1 and day 3 samples. However, the staining patterns were similar between the control and those exposed to particles, therefore, it could not be concluded as NPs effect. Abnormalities in the expression of keratin, as observed in some explants, might be the reason for delayed wound healing, but strong evidence of NPs effect on the expression of these keratins was not observed.

7.2.2.3. Collagen 4 expression

Figure 7.29. Representative images of collagen 4 expression in skin explants exposed to ZnO NPs at different time points. Scale bar = 100 μm .

Collagen type 4 is the main collagen component of the basement membrane which underlines the epithelial cells. As discussed previously, collagen type 4 is important for maintaining the structure of the skin. In the skin explants, collagen 4 was not observed over the 7 days of culture, in all samples including control. Although the wounds were fully healed at day 7, collagen 4 did not show up at the basement membrane as shown in figure 7.29. From all the microscopic investigation of the skin explants, no significant effects of NPs in the skin physiology and wound healing process was observed.

7.2.3. ZnO uptake and localization

TEM was used to investigate the uptake, localization, and effects in the ultrastructure of keratinocytes at the epidermal layers of the skin exposed to ZnO NPs. Unlike TiO_2 , the uptake of ZnO was not as clear but more changes in the ultrastructure were observed.

Figure 7.30. ZnO NPs internalized by the keratinocytes in the skin explant.

As shown in figure 7.30, very few amounts of ZnO NPs were found inside the keratinocytes. At both ZnO concentrations: $1 \mu\text{gml}^{-1}$ and $10 \mu\text{gml}^{-1}$, the NPs were found inside the cells. The NPs were localized at the perinuclear regions, as seen figure A. A cluster of ZnO NPs was also observed at the junction between two cells, as seen in figure C. The particles were observed at the regions far from the migrating tip in the keratinocytes closer to the basement membrane (figure D). ZnO NPs were also found inside the nucleus of keratinocytes. The representative images are shown in figure 7.31.

Figure 7.31. ZnO NPs located inside the nucleus of keratinocytes.

As observed in the figure, ZnO NPs were observed inside the nucleus of the keratinocytes located at the migrating tip of the wound. The size measurement of the particles in figure A and B was 22 nm, which is the pristine size of ZnO NPs. However, the size of the ZnO observed in figure C and D was around 80 nm. Most likely it is due to the aggregation of NPs. The NPs inside the nucleus were also observed at day 7 of the skin explants exposed

to ZnO $10 \mu\text{gml}^{-1}$ as shown in figure 7.32.

Figure 7.32. ZnO NPs seen inside the nucleus of keratinocytes in skin explants exposed to $10 \mu\text{gml}^{-1}$ ZnO NPs at day 7.

Figure 7.32A shows a single particle close to the nucleus, in rest of the figures, particles can be seen inside the nucleus. The sizes of these particles found inside the nucleus were around 50 nm, which is bigger than the size of pristine NPs. It needs to be confirmed by elemental mapping whether the particles observed are indeed ZnO NPs. Along with the ZnO uptake by the keratinocytes, modification in the ultrastructure was also observed. Mostly autophagic compartments were observed in the keratinocytes closer to the migrating tips of the wounds exposed to ZnO NPs. The representative images are shown in figure 7.33.

Figure 7.33. Autophagic components observed in keratinocytes at the migrating tip of the wounds exposed to ZnO $10 \mu\text{gml}^{-1}$. Figures A, B and C are at day 3 exposure and figures D to F are at day 7 exposure.

7.2.4. DNA damage

The TEM images showed the presence of ZnO NPs inside the nucleus of keratinocytes. ZnO NPs have been reported to cause genotoxicity in keratinocytes. DNA damage was examined by γH2AX immunofluorescence staining in cross section of skin explants exposed to ZnO NPs at various concentration and time points. The skin explants were directly exposed to UV for 1 hour as a positive control for DNA damage. In the positive controls, a dramatic increase in γH2AX protein expression was observed in the nucleus of keratinocytes. In the skin explants exposed to ZnO NPs, γH2AX expression was observed positive in some keratinocytes. Mostly, the keratinocytes at the wound areas expressed positive staining. The representative fluorescence images of γH2AX staining in skin explants is shown in figure 7.34.

Figure 7.34. γ H2AX staining of skin explants exposed to ZnO NPs. Scale bar = 100 μm

The skin explants without exposure to ZnO NPs did not show positive staining of DNA damage in the nuclei of epidermal keratinocytes. In contrast, elevated expression of γ H2AX was observed in the nucleus of keratinocytes in the skin explants exposed to ZnO NPs. DNA damage was observed with both ZnO concentration and at all time points. At day 1 and day 3, some keratinocytes at the tip of the migrating tongue expressed DNA damage. The keratinocytes at the wound edge mostly expressed DNA damage as shown in skin explants exposed to $1 \mu\text{gml}^{-1}$ and $10 \mu\text{gml}^{-1}$ ZnO at day 3 and day 7.

7.3. Discussion

In the previous chapters, the understanding of the effects of TiO_2 and ZnO NPs in skin cells and the toxicity mechanism based on studies performed on 2D monolayer cell culture models were presented. The 2D cell cultures are the most common in vitro models for mechanistic studies on the toxicity of engineered NPs. The main advantages of 2D cell cultures are easier environmental control, cell observation, measurement and eventual manipulation compared to 3D cultures. However, there are many limitations associated with the use of 2D cell culture models. Although, they provide primary indications of cytotoxicity and cellular responses, they may not provide accurate results due to their inability to mimic the nature of the tissue. Hence, to obtain more adequate and detailed information on cell-NPs interaction, 3D skin explants have been used. 3D cell culture models mimic the in vivo microenvironment as well as cell-cell and cell-matrix interaction. They provide a very convenient model to study NPs penetration through the wound and their effects in wound healing. Thus, it is important to use a 3D model for toxicity assessment of these NPs to compare with the findings from studies performed on 2D cell culture models. Ex vivo human skin explants represent a model close to in vivo human skin, as it consists of melanocytes, Langerhans cells, keratinocytes and the whole structure of dermal fibroblasts. As such, this model allows studies that are closer to reality. Therefore, this section is focused on the toxicity and interaction of these NPs with cells in the 3D organization. Additionally, the direct interaction of NPs with the cells through an open wound and the resulting effects on wound healing were also studied.

From the microscopic examination of cross-sections of the tissue, it was observed that TiO₂ and ZnO NPs did not cause any significant effect in skin physiology and wound healing. Any macroscopic signs of skin irritation or aggravation of wounds were not observed in the skin explants after 7 days of exposure to TiO₂ and ZnO NPs. It has been reported that subchronic exposure of TiO₂ NPs on the dorsal skin of hairless rat for 56 consecutive days caused focal parakeratosis and spongiosis in the epidermis of skin [5]. Similarly, the formation of skin crusts with hyperkeratosis and papillomatosis were observed in rat skin in vivo after 90 days of exposures [6]. However, in this study, no morphological changes to the skin layers or the wounds were observed until 7 days of exposure. The wound healing process was normal for most of the wounds where the migration of keratinocytes was observed from day 1 and full closure of the wounds was observed on day 3 or day 7. However, in some of the wounds, keratinocytes failed to migrate and thus wound healing was obstructed. This phenomenon was observed for both untreated controls and the samples exposed to TiO₂ and ZnO NPs. The ratio of impaired wounds in skin explants obtained from each patient did not show any significant difference with NPs exposure. Therefore, it was not conclusive whether the NPs had any role to play in the wounds that did not heal up to day 7.

It is well known that wound healing depends on the majority of factors such as oxygenation, infection, age, sex, stress, obesity, alcoholism, smoking, nutrition and medical conditions [7]. Previous studies have reported that wound healing was affected by various factors, age being one of the significant factors reducing the wound healing efficiency [8-10]. In this study also, it was observed that most of the impaired wounds belonged to the skin explant excised from the oldest patient. The age of the patients donating the skin explants ranged between 41 to 64 years. Notably, in the sample obtained from the youngest patient aged 41 years, all wounds were observed to be fully healed at day 3, including the untreated controls and the wounds exposed to ZnO NPs. In order to investigate deeper into the process of wound healing, various keratin markers were used to stain the cross-section of the wounds at different time points. The expression of keratin markers in the skin explants exposed to NPs was comparable to the keratin expressed in the control samples. Some wounds showed abnormal expression of keratin, however, a similar pattern was observed for the untreated

controls as well, ruling out the possibility of potential effects of NPs. Table 7.1 summarizes the outcomes of various keratin staining.

Table 7. 1. The outcome of keratin staining in skin explants exposed to TiO₂ and ZnO NPs.

✓ Normal staining; ✕ Abnormal staining

Keratin 1							
TiO ₂	Day 1	Day 3	Day 7	ZnO	Day 1	Day 3	Day 7
Control	✓	✓	✓	Control	✓	✓	✓
100 µg/ml	✓	✓	✕	1 µg/ml	✓	✕	✓
500 µg/ml	✓	✓	✕	10 µg/ml	✓	✓	✓
Keratin 10							
TiO ₂	Day 1	Day 3	Day 7	ZnO	Day 1	Day 3	Day 7
Control	✓	✓	✕	Control	✓	✓	✓
100 µg/ml	✓	✓	✕	1 µg/ml	✓	✓	✓
500 µg/ml	✓	✓	✕	10 µg/ml	✓	✓	✓
Keratin 5							
TiO ₂	Day 1	Day 3	Day 7	ZnO	Day 1	Day 3	Day 7
Control	✓	✓	✓	Control	✓	✓	✓
100 µg/ml	✓	✕	✕	1 µg/ml	✓	✓	✓
500 µg/ml	✓	✕	✕	10 µg/ml	✓	✕	✕
Keratin 14							
TiO ₂	Day 1	Day 3	Day 7	ZnO	Day 1	Day 3	Day 7
Control	✓	✓	✓	Control	✓	✕	✕
100 µg/ml	✓	✕	✕	1 µg/ml	✓	✕	✕
500 µg/ml	✓	✕	✕	10 µg/ml	✓	✕	✕
Keratin 16							
TiO ₂	Day 1	Day 3	Day 7	ZnO	Day 1	Day 3	Day 7
Control	✕	✓	✕	Control	✕	✕	✓
100 µg/ml	✕	✓	✕	1 µg/ml	✕	✕	✓
500 µg/ml	✕	✓	✕	10 µg/ml	✕	✕	✓

Normal expression of keratin markers were observed in impaired wounds which had not healed until day 7. In the skin explants exposed to TiO₂ 100 µgml⁻¹ and 500 µgml⁻¹, the absence of K1 and K10 expression in the suprabasal layers of the skin was observed. However, this did not affect the wound healing as some samples with fully closed wounds also showed a similar staining pattern. Likewise, abnormalities in K5 and K10 expression were also observed, however, not all samples exposed to NPs were affected. Some of the

control samples also showed similar abnormal staining pattern. Thus, no general conclusion could be drawn on the effects of keratin expression during the process of wound healing on the skin exposed to TiO₂ and ZnO NPs.

The rate of wound healing was also measured by calculating the “linear wound healing rate”. Although it was observed that the rate of wound healing was reduced in the skin explants exposed to TiO₂ NPs, it was not statistically different. However, in the skin explants exposed to ZnO NPs, all the wounds were fully closed by day 3. The crosstalk of keratinocytes and fibroblasts plays an important role in wound healing. Growth factors and cytokines secreted by the fibroblasts supports keratinocytes migration and proliferation [11]. In the previous chapter, we investigated the effects of TiO₂ and ZnO NPs in growth factors secretion by dermal fibroblasts. It was observed that at TiO₂ 500 µgml⁻¹, the secretion of growth factors such as TGF-β2, TGF-β3, and EGF was highly reduced. EGF stimulates epithelization, fibroblasts proliferation and survival [12]. Inadequate levels of EGF have been reported to be observed in chronic wounds [13]. Likewise, in absence of TGF-β2 and TGF-β3, a significant delay in wound healing has been observed [14]. The reduced secretion of these growth factors by fibroblasts exposed to TiO₂ NPs might have resulted in delayed wound healing in skin explants exposed to TiO₂ NPs.

Furthermore, the interaction of TiO₂ and ZnO NPs with individual keratinocytes at the wound edge was investigated through TEM images of the skin explants. Dose and time-dependent uptake of TiO₂ NPs were observed in keratinocytes at the wound edge. Higher uptake of TiO₂ NPs was observed in skin explants exposed to TiO₂ 500 µgml⁻¹, day 7. The schematic figure representing NPs uptake and their effects to the keratinocytes is shown in figure 7.35.

Figure 7.35. Schematic representation of NPs uptake and DNA damage induced in keratinocytes in skin explant. The dashed line represents the initial cut area. The left side (Blue area) represents the effects of TiO_2 NPs and right side (Yellow area) represents the effects of ZnO NPs. Green color represents DNA damage induced in keratinocytes.

Surprisingly, TiO_2 NPs agglomerates were observed to be localized with the melanosomes in the cytoplasm of keratinocytes. Melanosomes are a distinct class of lysosome-related organelles derived from the endosomal compartment [15]. They are biosynthesized in melanocytes and transferred into keratinocytes after being fully synthesized. Clusters of melanosomes were observed in a membrane-bound structure in the cytosol of keratinocytes. Some of these cells containing melanosomes could be melanocytes as well, as it has been reported that melanocytes also take part in wound healing and migrate into the wounded area. Growth factors such as GEF and FGF also simulate melanocytes migration [16]. The membrane-bound structure containing melanosomes have been identified as secondary lysosomes [17]. It is well documented that in light skin, the melanosomes are distributed in keratinocytes as clusters of approximately 4 to 8 in lysosomes. Whereas, in dark skin type, melanosomes are larger and tend to be dispersed individually [18-20]. The skin explants used in this study were obtained from a Caucasian female with light skin tone, therefore, melanosomes were observed as clusters in lysosomes. From the TEM images, it was observed that TiO_2 NPs agglomerates were confined in the lysosomes containing melanosomes which were localized at the perinuclear regions. The accumulation of

melanosomes in the perinuclear region is critical for skin pigmentation and protection against UV radiation.

Notably, it was clear that TiO₂ NPs were internalized by the keratinocytes as agglomerates or individual particles via endocytic pits. These particles were then confined in lysosomal/endosomal structures. Agglomerated TiO₂ NPs in the lysosomes of keratinocytes has been reported before [21]. Lysosomes are known to attract each other. Therefore, it can be suspected that the lysosomes containing NPs fused with the lysosomes containing melanosomes. Hence, most of the TiO₂ agglomerates were confined in lysosomes together with melanosomes. This raises the question of whether TiO₂ NPs can affect normal functioning and life cycle of melanosomes. Several studies have been performed on melanosomes and their cycling in keratinocytes. The degradation of melanosomes in keratinocytes has been reported to occur when the keratinocytes move towards stratum spinosum and stratum granulosum. The number of melanosomes was reported higher in the keratinocytes at the basal layer and it decreased towards the stratum corneum. From this, melanosomes are assumed to undergo degradation along with keratinocytes differentiation [18]. Autophagy is also reported to degrade selected melanosomes in keratinocytes [15]. Since it was observed that TiO₂ NPs were able to induce autophagy in keratinocytes, its role in the degradation of melanosomes needs to be further investigated.

The clusters of TiO₂ agglomerates were also observed at the intracellular regions located far from the wound edge within 24 hours' exposure. It could be suspected that TiO₂ not only interacted with the keratinocytes at the wound edge where they were directly exposed to, but they were also able to migrate and reach the areas far from the migrating tip. Along with the NPs uptake, the presence of autophagic structures were also noticed in the keratinocytes, which further confirmed NPs ability to induce autophagy. In the skin explants exposed to ZnO NPs, not as many particles were observed in the keratinocytes. These may be due to partial dissolution of ZnO NPs inside the skin tissue. Additionally, the administered dose of ZnO NPs were much lower than TiO₂ NPs, resulting in a lesser amount of particles exposure. Although, ZnO uptake was lesser than TiO₂ NPs, the

structural changes in the keratinocytes exposed to ZnO NPs were more pronounced. The presence of autophagic structures in the skin explants exposed to ZnO NPs was observed to be higher compared to those exposed to TiO₂ NPs. This may be due to the effect of ZnO dissolution in an acidic environment. Many previous studies have reported the role of Zn²⁺ ions in lysosomal dysfunction and increased autophagosomes. In epithelial cells exposed to ZnO NPs, it was reported that Zn²⁺ ions specifically accumulate in the endosomes and lysosomes. More rapid dissolution of ZnO NPs in the acidic environment have been reported by previous studies where they also reported that ZnO NPs were dissolved in the lysosomes and caused lysosomal dysfunction [1]. Blockage of lysosomal degradation caused an increase in the amount of autophagosomes formation in the cytosol of undifferentiated T-cells [2]. Involvement of lysosomal dysfunction in the accumulation of autophagosomes in adipose tissue of obese mice has also been reported before [3].

Although not widely reported, it was found in this study that both TiO₂ and ZnO NPs were able to penetrate into the nuclei of the keratinocytes, albeit at low frequencies. Therefore, the possibility of DNA damage induced by NPs was further studied. It was observed that although TiO₂ NPs' ability to penetrate the nucleus of keratinocytes was higher compared to ZnO, ZnO NPs caused more DNA damage. DNA damage was observed in the keratinocytes at the tip of the migrating tongue and in the keratinocytes located at the wound edge. In our previous studies based on 2D monolayer culture, it has been shown that both TiO₂ and ZnO NPs were able to induce DNA damage in keratinocytes [22]. However, in the skin explants, it was observed that ZnO NPs have a higher potential in causing DNA damage. This may be again due to the effect of aqueous Zn²⁺ ions from the partial dissolution of ZnO NPs. Previous studies have reported that ZnO NPs penetrated inside the cells can enter into endocytic vesicles, which combines with vacuoles to form lysosomes [23]. At the acidic pH of lysosomes, ZnO NPs dissolve into Zn²⁺ ions, which are released in the cytoplasm. Both extracellular and intracellular Zn²⁺ has been reported to trigger DNA damage. Studies have shown that, even though ZnO NPs may not penetrate into the nucleus, Zn²⁺ ions can cause DNA damage in a dose-dependent manner [24]. However, in a study performed in human skin melanoma cell line, it was reported that DNA damage caused by aqueous Zn²⁺ ions were significantly lesser compared to similar

concentrations of ZnO NPs [25]. These findings suggest that intracellular Zn^{2+} has a higher potential to cause DNA damage.

Based on the 2D results, TiO_2 and ZnO NPs affected cell viability and proliferation, causing cell death at higher concentrations. However, when high doses of NPs were exposed to the wounds in skin explants, no such significant effects in cell proliferation or viability was observed. NPs had no significant effect on the normal functioning of keratinocytes and did not affect the wound healing process. This differential effects in 2D and 3D model indicated that the cellular response to TiO_2 and ZnO NPs were dependent on the culture systems. The toxic effects of TiO_2 and ZnO NPs might be overestimated from the nanotoxicity results based on 2D in vitro models. In 3D skin explants, it was observed that both TiO_2 and ZnO NPs could be internalized by the keratinocytes. Although these findings were observed in previous 2D studies, it was interesting to observe that these particles were able to penetrate the nucleus of keratinocytes even in 3D skin explants. However, only ZnO NPs were able to cause DNA damage in the keratinocytes. Genotoxic potential of both TiO_2 and ZnO NPs in keratinocytes have been reported in previous 2D studies. Yet it was interesting to note that in the 3D model, only ZnO NPs were able to induce genotoxicity. Additionally, through 3D studies, it was observed that the NPs uptaken by the keratinocytes were located close with the melanosomes in the same lysosomal compartment. Almost all TiO_2 clusters observed inside the keratinocytes were localized with the melanosomes. This typical behavior would not have been observed in keratinocytes cultured in vitro due to the lack of melanosomes in them. As such, 3D skin explant model plays a significant role in nanotoxicity studies.

There are certain disadvantages associated with 3D skin explant models which cannot be overlooked. Since the experiment is performed ex-vivo and the tissue is maintained in an artificial environment, the tissue tends to deteriorate with time. However, based on our histological evaluation, the epidermis of the skin explants cultured up to two weeks looked similar to day one and the dermo-epidermal junction was linked well with no changes observed on the dermis. Donor-to-donor variability is one of the major disadvantages of human skin explants. In this work, the skin explants were obtained from female patients

between ages 41 to 64, belonging to the same ethnic group. The age factor does affect skin explant. The slower rate of wound healing was observed in the skin explant obtained from the oldest individual. However, in this study, to overcome this issue, relative comparisons were made within each patient. The experiment was repeated on the skin explants obtained from various patients belonging to different age groups in order to obtain more accurate results. Furthermore, lack of systemic circulation is recognized as another limitation of the ex vivo skin model. As such, it lacks an influx of other immune cells and biochemicals such as neutrophils, natural killer cells and complement proteins from the circulatory system. However, ex vivo skin explants still provide a better model in studying nanotoxicity.

7.4. Summary

The current evidence shows that the penetration of metal oxide NPs through the intact human skin under normal condition is extremely unlikely due to the strong barrier function provided by stratum corneum. However, impairment of stratum corneum may lead to direct exposure of viable skin cells to the NPs and might also enhance penetration into the deeper skin layers. Therefore, in this part of the study, the interaction of NPs with viable cells through open wounds in full-thickness skin explants was investigated. It was observed that both TiO₂ and ZnO NPs do not affect the skin physiology or wound healing processes. Even though the rate of wound healing and the number of chronic wounds were not significantly influenced by the exposure to NPs, a potential impact on wounds was noticed. Although NPs did not affect keratinocytes viability at the wound edge, large uptake of NPs along with the structural changes in the keratinocytes was observed. ZnO NPs had higher toxic potential than TiO₂, which was evident by DNA damage observed in the keratinocytes exposed to ZnO NPs. However, these effects did not seem to affect wound healing of the skin explants in general. The valuable insights provided from this work is summarized as follows:

- i. Although NPs penetration through healthy human skin is at very low frequencies, they were able to penetrate and reach viable cells through an open wound.
- ii. In 2D studies, it was observed that NPs could affect cell proliferation and cause cell death. However, similar doses of NPs did not result in cell death or reduced cell

proliferation in skin explants. They also did not significantly affect the wound healing process.

- iii. Both TiO₂ and ZnO NPs were internalized by keratinocytes in skin explants.
- iv. Inside keratinocytes, almost all TiO₂ NPs were observed to be localized in the lysosomes containing melanosomes. (Their effects to melanosomes is not known yet.)
- v. Both NPs were able to penetrate into the nucleus of keratinocytes in skin explant.
- vi. ZnO NPs were able to cause DNA damage in the keratinocytes in skin explant.

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Chapter 8

Conclusion and Future Recommendations

This chapter draws together the major findings obtained from the study of the interaction between NPs and skin cells encompassed by this thesis. The results from each chapter discussed earlier are woven together to provide a final overview of the findings obtained from this research. The questions, during this study, that beg further consideration have been identified, and the direction for future studies has been provided to find the answers.

8.1. Conclusion

The widespread use of TiO₂ and ZnO NPs in various products, sunscreen, for example, begs serious investigation into its potential health hazard. Thus, in this thesis, the effects of TiO₂ and ZnO NPs on skin cells based on 2D monolayer cell culture and human skin explants with an open wound were investigated along with the wound healing process. The results obtained from the studies based on 2D monolayer cell culture model of human epidermal keratinocytes and human dermal keratinocytes indicated that both TiO₂ and ZnO NPs could cause cytotoxicity to the cells at certain doses. The cells were able to survive lower doses of NPs, however, at higher doses, NPs induced ROS and MSO elevated the levels of oxidative stress which lead to cell death. As postulated by most of the studies, oxidative stress proved to be the central mediator of toxicity leading to cell death. Induction of oxidative stress was observed at sub-lethal doses, but at higher doses, excessive oxidative stress lead to cell death via apoptosis. In human epidermal keratinocytes, induction of autophagy and inflammation was observed at 24 hours' exposure to both NPs. The onset of autophagy was observed at very low doses, which proved to act as a pro-survival mechanism and protected the cell death against NPs induced oxidative stress and inflammation. The results from this study indicated for the first time that NPs induced autophagy prevents NF-κB activation and promotes degradation of TRAF6, thereby contributing to cell survival. However, degradation of TRAF6 was not observed at higher doses of NPs, where eventual cell death by NPs induced oxidative stress and inflammation was observed. Therefore, it was concluded that the cellular response of NPs hinges on the balance between autophagy and oxidative stress induction. Surprisingly, in dermal fibroblasts exposed to TiO₂ and ZnO NPs, induction of autophagy was not observed. Based on in vitro studies in 2D cell culture models, both TiO₂ and ZnO NPs proved to be toxic to cells, however, the similar doses of NPs did not show any toxic effects to the viable cells residing in the skin explants. Likewise, NPs effects to growth factors and cytokines secretion by cells were observed on the 2D cell culture model, however, those effects did not seem to play a significant role in wound healing of skin explants. Both TiO₂ and ZnO NPs were observed to be internalized by the keratinocytes at the wound edge, and their penetration into the nucleus was also observed. This resulted in the formation of autophagy

structures in the cytoplasm of keratinocytes. ZnO NPs induced more autophagy structures compared to TiO₂ NPs and even caused DNA damages to the nucleus. In conclusion, with this study, it was confirmed that TiO₂ and ZnO NPs induced autophagy in epidermal keratinocytes. However, TiO₂ and ZnO NPs induced autophagy in human dermal fibroblasts was inconclusive. It was also confirmed that TiO₂ and ZnO NPs could be internalized by epidermal keratinocytes through an open wound but their effects in wound healing were not observed. Further studies with large sample size need to be done to confirm the NPs role in wound healing.

8.2. Future Recommendations

This thesis was focused on accessing the biological effects of TiO₂ and ZnO NPs' interaction with human skin cells and yielded some promising findings. However, many questions were also raised during the period of this research which was not initially postulated. Future studies need to be performed to answer those questions in order to provide a better picture of the impacts of NPs on human skin. Some of the recommendations for future studies are as follows:

8.2.1. Effects of TiO₂ and ZnO NPs in other skin cells

Besides keratinocytes, there are other cells types present in skin, such as melanocytes, Langerhans cells, Merkel cells and various immune cells in the dermis. On an event of penetration, all these skin cells might be subjected to NPs exposure. As it is known that different types of cells respond differently to NPs exposure, the interaction of NPs with different cell type available in skin must be studied. Primarily, single cell type studies are needed for mechanistic understanding, without confounding factors from other variabilities. They serve as screening tools as well to know what to look out for in more complex explant cultures.

Melanocytes are the cells located at the basal layer of the epidermis. Their main function is to synthesize melanin, a dark pigment primarily responsible to give color to the skin and

protect the skin from UV damage. Melanocytes synthesize melanosomes by the process of melanogenesis. During the synthesis of melanosomes, H_2O_2 and other cytotoxic molecules are secreted as intermediate products. Therefore, to protect themselves, they separate the synthesis of melanin in melanosomes and increase the levels of the anti-apoptotic protein [1]. Fully formed melanosomes are transferred to keratinocytes, where they form a melanin cap over the nucleus to protect it from UV damage. During wound healing, melanocytes also migrate into the wounded area. Growth factors such as GEF and FGF also simulate melanocytes migration.[1] It has been shown that TiO_2 and ZnO NPs can cause cell death via apoptosis pathway in keratinocytes and fibroblasts. Thus, the effects of NPs induced ROS in melanocytes needs to be investigated. Furthermore, from the TEM observation of the skin explants exposed to TiO_2 NPs, it was observed that high amounts of TiO_2 NPs internalized by the keratinocytes ended up inside the lysosomes containing melanosomes. As such, NPs might affect the normal functions of melanosomes. Additionally, the NPs could also affect the synthesis and transfer of melanosomes from melanocytes to keratinocytes. Melanoma, considered as the most dangerous type of skin cancer is developed from melanocytes. Therefore, it is important to investigate whether TiO_2/ZnO NPs are able to penetrate into the melanocytes or cause any cellular effects such as autophagy and/or DNA damage.

Langerhans cells are dendritic cells located on the stratum spinosum. They are also found in the papillary dermis, specifically around blood vessels. If the NPs are able to penetrate into the Langerhans cells, they might be transported into the blood vessels and lymph nodes and reach to various organs of the body. It has been reported that a small amount of Zinc form ZnO NPs in sunscreen was absorbed by healthy skin in a real-life environment and detected in blood and urine [2]. Therefore, NPs potential to penetrate into these cells or blood vessels in the dermis needs to be investigated. Additionally, immune cells such as macrophages, neutrophils, mast cells, dermal dendritic cells and lymphocytes residing in the dermis may also interact with the NPs. These cells play a role in immune response and protect the body from toxins. Therefore, to understand NPs interaction with the skin, it is essential to observe the effects of NPs in various types of skin cells.

8.2.2. Fate of NPs inside the skin tissue

It was observed that NPs agglomerates were internalized by the keratinocytes in the skin explants and they were also trapped in the intercellular spaces between the keratinocytes. It is not known how long these particles remain in the skin or how they are cleared out from the body. Agglomerates of TiO₂ NPs were also observed at the upper layers of the epidermis. It could be hypothesized that the NPs will eventually get rid of the NPs along with keratinocytes stratification. The NPs might as well reach the hair follicles or sebaceous glands and get excreted out along with the body fluids. If the NPs penetrate deeper into the dermis, they may reach the systemic circulation and translocate into other organs. There are many possibilities that could happen once NPs are able to penetrate into the skin. Human skin explants can be used to explore the pathways by which the NPs are cleared out from the skin.

8.2.3. NPs mechanism of toxicity in human dermal fibroblasts

In this study, it was observed that human dermal fibroblasts did not induce autophagy on NPs exposure. However, more experiments are needed in order to reveal the underlying mechanism. A different mechanism of NPs toxicity could exist which has not been yet explored. This needs to be explored further.

8.2.4. 3D human skin explants as realistic models for further exploration of nanotoxicity and wound healing

Human skin explant offers better opportunities to explore nanotoxicity. Due to the time constraint and limitation in a number of skin explants obtained from the hospital, enough data could not be generated to provide a concrete conclusion on the NPs effects in wound healing. Therefore, more experiments on skin explants are needed to obtain significantly representative results.

The skin explants can also be used to study the process of keratinocyte migration for wound closure. The mechanism of keratinocytes migration or extension of wound edge is a central question in wound healing which is still on debate. It is suggested that keratinocytes migrate in leap-frog or rolling mechanism where migrating keratinocytes at the suprabasal cells roll over the basal cells and lead the migrating tongue. The next suggestion is that keratinocyte migrate based on tractor-tread or sliding mechanism which postulates that whole keratinocyte layer moves forward as a block. It has not yet been clear which mechanism occurs during wound healing [3]. From the TEM images, a single cell with a large amount of TiO₂ NPs in the cytoplasm was observed at the suprabasal layer away from the migrating tongue. The basal cells at the tip of the migrating tongue are expected to internalize more particles. The cells at the suprabasal layers might have rolled over the basal cells at the tip of the migrating tongue which have internalized particles. As the suprabasal cells migrated over the basal cell, the basal cell got shifted over and pushed to the back. Therefore, a single cell with huge TiO₂ intake was observed at the suprabasal layer far from the migration tip. This observation supports the leap-frog theory of keratinocyte migration. Further studies need to be done with TiO₂ NPs as a marker to investigate the mechanism of keratinocytes migration.

In this study, it has been observed that even though NPs were toxic to keratinocytes, based on 2D cell culture model, they did not seem to be affected by the NPs when exposed in skin explants. The results obtained from 2D studies might not accurately reflect the actual phenomena occurring in the skin. Therefore, the studies which are commonly done in 2D cells such as oxidative stress, apoptosis, autophagy, inflammation induced by NPs should also be done in human skin explants in order to compare the toxic effects observed. It is reported that melanin can neutralize ROS [1]. Unlike the keratinocytes used for 2D cultures, high amounts of melanin were observed in the cytoplasm of keratinocytes in the skin explants. This melanin might play a role in balancing redox state of the cells by neutralizing the excessive ROS produced by NPs, thereby protecting the cells. Further studies need to be done to understand the effects of NPs on melanosomes and keratinocytes containing melanosomes.

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