Interaction of nanoparticles with human skin

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2020

Goh, B. H. R. (2020). Interaction of nanoparticles with human skin. Master's thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/146115>

<https://doi.org/10.32657/10356/146115>

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**INTERACTION OF NANOPARTICLES WITH
HUMAN SKIN**

GOH HUAN RONG, BERNICE

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2020

INTERACTION OF NANOPARTICLES WITH HUMAN SKIN

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Master of Engineering

2020

Statement of Originality

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Abstract

One branch of nanotechnology is nanotoxicology, which studies the potential toxicity manifested from the nanomaterial exposure. Nanoparticles (NPs) have been incorporated as active ingredients in a diverse range of consumer products such as oral hygiene products and cosmetics, increasing the possible interaction between NPs and the human body. Due to their UV filtering ability, titanium dioxide (TiO₂) and zinc oxide (ZnO) NPs have been widely utilized in sunscreens and whitening creams. The widespread usage of these NPs has led to increase concerns on the possible penetration of these NPs into skin and potential adverse outcomes to human skin due to prolong exposure to nano-enabled consumer skin products. Current findings have reported the low amount of these NPs penetrating the intact skin barrier. However, penetration of these NPs through a compromised skin barrier remains unknown. In particular, the influence of NPs on wound healing has not been documented. In a wound scenario, application of nano-enabled skin products could lead to NPs interacting with the living skin cells and tissue. This interaction could induce potential toxic biological responses and consequently, leading to toxicity. Thus, this study aims to evaluate the interaction of TiO₂ and ZnO NPs with human epidermal keratinocytes, specifically in a wound setting and the biological outcomes resulted from this interaction. Most of the reported NPs interaction with keratinocytes studies were done on 2D monolayer cell cultures with only a handful done with animal models and *ex vivo* human skin explants. From the results gathered from cultured keratinocytes studies, TiO₂ and ZnO NPs at higher concentrations of 500 µg/ml and 25 µg/ml, respectively were observed to induce toxicity to cells. This toxicity manifested in numerous biological responses such as oxidative stress and DNA damage. These cellular responses were clearly dependent on the NPs dose. TiO₂ NPs increased autophagy in keratinocytes whereas ZnO NPs caused higher levels of oxidative stress and DNA damage in keratinocytes. These NP-induced biological responses such as oxidative stress and autophagy have brought upon an enhancement of keratinocytes migration as observed in the 2D scratch wounds. TiO₂ and ZnO NPs at concentrations of 10 µg/ml and 0.5 µg/ml, respectively increased the migration and proliferation of keratinocytes compared to untreated keratinocytes. The knowledge and positive controls established from these studies were utilized in 3D skin explant studies to

evaluate the biological outcomes that arise from TiO₂ and ZnO NP interaction with keratinocytes in a wound scenario. The positive controls of oxidative stress and DNA damage assays established from 2D studies were extrapolated to the 3D skin explant studies. Positive control of DNA damage assay was successfully implemented on the 3D skin explant whereas the positive control of oxidative stress assay has to be further validated. Overall, TiO₂ and ZnO NPs were observed to exhibit levels of cytotoxicity in high doses but at low doses, these NPs were able to aid in the process of wound healing in the context of keratinocytes migration and proliferation. This is applicable to the current consumer market as most of the consumer products use low doses of NPs.

Lay Summary

Sunscreens are one of the consumer products that have been widely used across the human population to provide themselves with a protection from the UV rays of the Sun. Over the years, it has been well reported that excessive exposure to the Sun would lead to an increase risk of skin cancer, which many had encouraged the use of sunscreens. Sunscreens have been categorized into two different types: organic and inorganic sunscreens. Organic sunscreens incorporated carbon-based chemicals in their ingredients whereas inorganic sunscreens utilized TiO₂ and ZnO NPs as mineral blockers to filter the harmful UV rays. NPs are materials that are small in size and invisible to human eye. Their small sizes have brought concerns to consumers about their incorporation into sunscreens as this would lead to an increase exposure of the skin to the NPs. The human skin is the largest organ and consists of different layers which have a barrier functionality to protect the human body from foreign particles. The chances of these NPs penetrating into the human skin have been documented to be low but possible penetration could occur when the skin barrier function is disrupted. Such scenario could be associated to skin diseases and skin injuries. Thereby, the focus of this study is to have a better understanding on the interaction of NPs with viable skin cells. In this study, NPs were observed to induce adverse effects on the skin cells, but these adverse effects would increase the rate of wound healing. However, the exposure to NPs was done on a monolayer cultured skin cells which does not reflect the actual exposure to NPs in the human skin. Therefore, human skin explants attained from patients that undergo abdominal surgeries were exposed to NPs. On the human skin biopsies, wounds were created with NPs administered to these wounds. Overall, this study has shown that TiO₂ and ZnO NPs would increase the wound healing rate through the cellular responses observed from the interaction between the NPs and skin cells which demonstrated the positive physiological impact of NPs on the skin cells.

Acknowledgements

First and foremost, I would like to convey my heartfelt gratitude and appreciation to my research supervisor, Associate Professor Ng Kee Woei, for his unwavering support and guidance. In addition, his patience and understanding have given me motivation to move forward. I am also thankful towards him for accepting me as his student.

A big thank you to my mentor, Dr. Magdiel Ingrid Setyawati, for her guidance throughout my Master journey, sharing with me all her knowledge. A thank you to my group members, who have played an important role in making this journey fruitful and enjoyable. At the same time, a thank you to other lab's group members for sharing their resources and knowledge.

Last but not least, a big thank you to my family and boyfriend for their encouragement and support. They have always believed in me and pushed me forward to achieve my goals.

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Abbreviations

NPs	Nanoparticles
TiO ₂	Titanium Dioxide
ZnO	Zinc Oxide
UV	Ultraviolet Light
SPF	Sun Protection Factor
HDF	Human Dermal Fibroblast
HaCaT	Human Epidermal Keratinocytes Cell Line
HEK	Human Epidermal Keratinocytes
DLS	Dynamic Light Scattering
TEM	Transmission Electron Microscopy
DMEM	Dulbecco's Modified Eagle Medium
PBS	Phosphate Buffer Solution
FBS	Fetal Bovine Serum
HRP	Horse Radish Peroxidase
DAB	3,3'-Diaminobenzidine
ROS	Reactive Oxygen Species
tBHP	tert-Butyl hydroperoxide
IRB	Institutional Review Board
PAS	Pre-Autophagosomal Structure
ATG	Autophagy Related
Au	Gold
4-HNE	4-hydroxynonenal
LC3-II	Microtubule-associated proteins 1A/1B light chain 3-II
γ -H ₂ AX	Phosphorylated histone H2AX

Chapter 1

Introduction

This chapter sets about an overview of nanotechnology which focus on nanotoxicology. Nanotechnology, in form of engineered nanoparticles (NPs), has been incorporated into a wide range of products. This has brought much attention to the safety and risks associated with the usage of NPs. To date, many metals and metal oxides NPs have been used in consumer products. Inorganic metal oxides NPs such as titanium dioxide (TiO_2) and zinc oxide (ZnO) were used in sunscreens and cosmetic products, yet potential risks to skin have not been fully understood. Thus, thorough investigation on their associated toxicity to human skin is warranted. In addition to the overview, the project's hypothesis and objective are documented, with the project's scope to be achieved.

1.1. Background

Nanotechnology has been demonstrated to improve the standard of lives, and has found its foothold in various fields, including electronic, household materials, cosmetics, and biomedicine. This is evident from numerous on-the-self cosmetics and dermatological consumer products that have penetrated the market.

As the number of commercial products containing engineered nanomaterials rises, the concern of the safety of these products increases. Nanomaterials' small sizes and associated properties may enhance their toxic potentials. The toxicity of any nanomaterials is dependent on the size, shape, concentration, surface properties and duration of exposure to nanomaterials. Engineered nanomaterials can enter the human body through inhalation, ingestion and dermal penetration, which the interaction of these nanomaterials with the human body could result in a toxic response to the human body.

Due to their unique properties, nanoparticles (NPs), which are defined as structures having size of 1 nm to 100 nm [1], have been incorporated into many products as a form of additives. One of these unique properties is the capability to absorb and scatter visible light. NPs like titanium dioxide (TiO_2) and zinc oxide (ZnO) are able to filter UV radiation, making these NPs to be a perfect material for UV filters in sunscreen products and rendering them transparent on the skin [2]. To date, there are 31 of sunscreen products that contain a combination of TiO_2 and ZnO NPs or either one of these NPs [3]. Considering the increased utilization of these NPs in various sunscreens and the fact that sunscreens are used in daily basis, it has become apparent that consumers' exposures to these NPs are real and the NPs accumulation in the skin could be expected. There is a legitimate concern whether these NPs would exert any detrimental effect in skin, and thus understanding the interaction between these NPs and skin system becomes pivotal.

To date the effects of these NPs exposures to the skin system studied in both *in vitro* and *in vivo* (animal) models. In the recent years, skin cells such as keratinocytes and fibroblasts were employed to study the effects of NPs interacting with the cells in *in vitro* setting.

Some of these interaction have led to cellular toxicities, such as DNA damage [4], autophagy [5] and inflammation [6] and the more concerning outcomes of carcinogenesis and mutagenesis [7]. Similarly, *in vivo* studies utilizing rat, mouse, and pig, showed similar cellular toxicities that observed in *in vitro* model.

Skin, the largest organ in the human body, is continuous and comprises of many layers with the mucous membranes lining the body's surface [8] acting as barrier for many foreign objects, such as allergens and microbes. In order for the NPs to interact with the skin cells, the NPs must first be able to penetrate the skin barrier. Thus, the topically applied NPs can only enter through transappendageal [9] and transcellular [10] route and upon entering the skin, most of these particles would remain in the upper layers of the stratum corneum [11]. However, studies suggested that disturbed skin barrier caused by long UV exposure or injury, could enhance the NPs penetration into the skin and their subsequent interaction with the different cell types present in the skin system [12]. Depending on the depth of wound, the NPs can penetrate deeper and interact with the dermis cells such as fibroblasts or the mast cells. Interaction between NPs and the respective skin cells could result in cellular toxicities which these toxicities would affect the rate of wound healing.

In a study completed by Zhou *et al.*, NPs such as ZnO would exhibit cytotoxicity that resulted in reduced cell viability. Reduced cell viability would then inhibit wound healing [13]. In a more recent study, cytotoxicity was observed in high concentrations of ZnO NPs treated fibroblasts which decreased the rate of fibroblasts proliferation and migration [14]. Nevertheless, ZnO NPs when incorporated into hydrogels were used as antimicrobial agents and tissue adhesives that facilitated the closure of wound in mice [15]. Additionally, Xu *et al.* reported that TiO₂ NPs treated Adipose Derived Stem Cells (ADSC) showed decreased cell migration speed and collagen contraction which are essential for closure of wound [16]. In contrast, TiO₂ NPs treated rats demonstrated increased aggregation of macrophages, fibroblasts, and collagen content in the wound, showing the improved wound healing effect due to TiO₂ NPs [17]. Overall, these studies highlighted that the wide variation in different test models resulted in difficulties assessing the effects of NPs interaction with underlying skin cells on wound healing.

In conclusion, this project aims to understand the possible associated outcome of NPs interaction with human epidermal keratinocytes and their effect on wound healing in the context of a scratch wound model and in a realistic exposures scenario using TiO_2 and ZnO NPs that are commonly found in personal care products.

1.2. Hypothesis

This thesis tests the hypothesis that NPs such as TiO_2 and ZnO can interact with human epidermal keratinocytes in a wound, and result in toxicological outcomes including autophagy, oxidative stress, and DNA damage induction, and affect the wound healing process.

1.3. Objective and Scope

The overall objective of this research is to generate a greater understanding in interaction between NPs and keratinocytes, which could implicate skin health and wound healing.

To achieve this objective, the scope of the project included:

1) Characterization of material properties of TiO_2 and ZnO NPs.

In this approach, TEM (Transmission Electron Microscope) and DLS (Dynamic Light Scattering) were employed to characterize TiO_2 and ZnO NPs physiochemical properties. TEM was used to measure the pristine sizes of TiO_2 and ZnO NPs. DLS was used to study the stability and behavior of TiO_2 and ZnO NPs when suspended in cell culture medium.

2) Exposure of TiO_2 and ZnO NPs to human epidermal keratinocytes (HEK) at physiologically relevant levels and understanding the effects of such exposure.

Various studies have reported the interaction of TiO_2 and ZnO NPs with different cell types in the human body. Some of these interactions have led to toxicological outcomes such as oxidative stress and mitochondrial damage

which is dependent on the dose of NPs. Therefore, NPs such as TiO₂ and ZnO relevant exposure on skin cells is required to understand the NPs interaction with the human skin when penetrated into human skin.

- 3) Correlation of the effects of these exposures to wound healing in a scratch wound model *in-vitro*.

TiO₂ and ZnO NPs were commonly incorporated into consumer products and this led to an increase exposure of NPs with the human skin. TiO₂ and ZnO NPs were observed to have an increased penetration through the compromised skin barrier [18] which could increase NPs interacting with the skin cells and affect the process of wound healing. Thus, it is essential to understand how these interactions would have an effect on the rate of wound healing.

- 4) Correlation of biological outcomes of TiO₂ and ZnO NPs interacting with viable skin cells through an exposure from *ex-vivo* open wound model in human skin explants.

The human skin is a complex model which has different layers and growth factors. The biochemical environment in the skin plays an important role in the interaction of TiO₂ and ZnO NPs with the viable skin cells. Therefore, it is crucial to understand how these NPs could affect the viable skin cells in the human skin.

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Chapter 2

Literature Review

This chapter provides a review on the current studies of nanotechnology and nanotoxicology. It starts with an introduction to nanotechnology and the common applications of nanotechnology. One of the prominent applications of nanotechnology is the incorporation of nanoparticles (NPs), such as TiO₂ and ZnO in personal care products. Properties and applications of TiO₂ and ZnO NPs were elaborated. After which, different routes of exposure to NPs were mentioned with a focus given to the dermal penetration of NPs. The anatomy of skin and the functions of different cells present in skin were explained in detail in this chapter. Following that, re-epithelization, a process of wound healing was mentioned in detail with how skin cells were greatly involved. Furthermore, the associated toxicity mechanisms of NPs interaction with 2D skin cell models were presented with recent findings reporting the adverse effects. Lastly, the chapter ends with current research on NPs influence on wound healing through studies done on 2D skin cell models.

2.1. Nanotechnology and Nanoparticles

Nanotechnology refers to the science and application of extremely small materials. The capability to manipulate materials into small dimensions, has enhanced the properties of materials, creating lighter weight, higher strength materials with other unique properties. Over the past two decades, nanotechnology has improved the lives of human and created an impact on many industry sectors, including personal care products, food, and medication.

The Greek term “nano”, meaning, “dwarf”, denotes one billionth meter, reflecting the object of minute proportions under the purview of this technology, the nanoparticles (NPs) [1]. Based on European Union (EU) in 2011 [2], “a material that contains 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 1 nm - 100 nm” is a nanomaterial. NPs can be categorized into two main groups: natural NPs and engineered NPs. Natural NPs are present in geological systems such as volcanic ash and ocean spray or biological systems such as virus and plants. However, engineered NPs are NPs that come from pure materials, which have controlled surface properties and exhibit specific size range from 1 nm to 100 nm. Engineered NPs can be classified into inorganic NPs, organic NPs and carbon-based NPs [3]. Examples of such NPs are metal-based NPs, chitosan NPs and carbon nanotubes respectively. NPs can be further classified into toxic or non-toxic, depending on their physical and chemical characteristics.

NPs are utilized in many different applications due to their unique ability to demonstrate enhanced properties on the nanoscale as compared to the bulk materials [4]. Taking advantages of the size-dependent properties of NPs, they are used in different fields, ranging from electronics to household wares but their predominance application is in the health and fitness industry (**Figure 2.1**) [5].

Figure 2. 1: Pervasive use of NPs in modern lifestyle products. Reproduced with permission from [5], Copyright 2020, John Wiley and Sons.

2.2. NPs in personal care products

NPs are incorporated in many consumer products to improve product quality and to achieve different properties such as antimicrobial and longer shelf life. As specifically to the cosmetics field, TiO_2 , ZnO and Ag NPs are the most commonly utilized NPs which are found in various products such as sunscreens and shampoos. From an online consumer database (Skin Deep Cosmetics Database) [6], TiO_2 NPs was found in 97% of personal care products, followed by ZnO NPs, 3% and last but not least Ag NPs, less than 1%. TiO_2 and ZnO NPs were used for their capability to absorb UV radiation [7] and hence, being incorporated in sunscreens and moisturizers to protect the skin from UV damage. Ag NPs have antimicrobial properties [8] and with that, they were used as preservatives in toothpastes and shampoos as well as an active ingredient in anti-acne creams.

In the cosmetics market, a wide variety of products were invented and produced each year. Having said that, consumers will always have “never to go without” skin care products

which include a sunscreen. Exposure to the Sun causes wrinkles, freckles, elastosis and skin cancers. Three main types of skin cancer; basal cell carcinoma, squamous cell carcinoma and melanoma [9]. Skin cancer develops in the cells of the epidermis and is ascribed to the UVA and UVB rays (290-400nm) that penetrate into the skin and causing permanent damage to the cells. With an increasing concern on skin cancer, many consumers are starting to use different forms of sun protection, be it a female or male. In a survey held by U.S. National Health in 2015, 75% of female adults and 67% of male adults use sun protection regularly. It was also mentioned in the survey that only 43.6% of female adults and 23.4% of male adults use sunscreens with Sun Protection Factor (SPF) 15 and above [10] while the others use shade or protective clothing that still allow some form of UV rays exposure.

Sunscreens can be categorized into organic and inorganic sunscreens. The organic sunscreens absorb UV radiation whereas the inorganic sunscreens reflect UV radiation, but both types of sunscreens perform the same function that prevents UV rays from reaching the cells in the epidermis [11]. Chemical organic UV filters are substances that can absorb UV radiation of a specific range of wavelength depending on the chemical structures of the substances [12]. Photostable filters, a type of organic UV filters dissipates absorbed UV radiation into heat and released into the surrounding. Followed by photounstable filters that are substances which absorb UV radiation and degrade or undergo chemical structure changes. Lastly, photoreactive filters are the least desired organic UV filters as the molecules of the substances jump to their excited state upon UV radiation absorption and interact with other substances. This can produce reactive oxygen species that may lead to undesired biological reactivity such as interaction with cells in the epidermis. Examples of commonly used organic UV filters are Octyl methoxycinnamate (OMC) and oxybenzone [13].

Physical inorganic UV filters are substances that can block the UV rays through reflection or scattering. The reflection and scattering properties of the UV filters depend on the refractive index, size of the substances, dispersion of the substances in the sunscreens and thickness of the sunscreens applied on the skin [14]. Examples of commonly used inorganic

UV filters are kaolin and metal oxides particles such as TiO_2 and ZnO [12]. Not only that, inorganic UV filters have to be accepted into cosmetic products. Inorganic UV filters tend to be opaque and whitish on the skin when applied, which this is not acceptable for cosmetic products and hence, the inorganic UV filters such as metal oxides particles have to be in the nanometers range. Being in the nanometers range may cause undesired interaction with cells in the epidermis that can lead to cytotoxicity.

2.2.1. Properties of TiO_2 NPs

Titanium does not exist in the metallic state but instead, it naturally oxidizes to form TiO_2 which is a compound that is widely utilized in different industries. This occurrence is due to the strong affinity between titanium and oxygen or other elements such as chlorine and hydrogen. TiO_2 takes form as a white, odorless, and noncombustible powder that comes in three types of structures, anatase, brookite and rutile. TiO_2 has a molecular weight of 79.9 g/mol, relative density of 4.26 g/cm³, boiling point and melting point at 2972°C and 1843°C respectively [15].

With an increasing interest on nanosized materials, TiO_2 NPs are synthesized since it is not naturally present. Over the years, different synthesis methods have been established and some of them include chemical vapor deposition (CVD), flame synthesis, and direct oxidation method. The reactivity of TiO_2 depends on the sizes of TiO_2 . The ROS production is mediated by the electron transfer process and thus, it is dependent on the band energy structure of the metal oxide. The band gap of the semiconductor is a function of the particle size [16], which smaller particles have smaller band gap, making it easier for electron transfer process to occur, and consequently increasing the ROS generation and the reactivity of TiO_2 NPs.

2.2.2. Applications of TiO_2 NPs

TiO_2 NPs are widely used across the world because it is considered an inert and safe material. It has been used in different applications such as paints, papers, pharmaceuticals,

food products, and cosmetics [15, 17]. Over the years, there is an increase in demand of TiO₂ NPs and that brings upon an increased TiO₂ NPs production volume. The global production in 2005 was estimated to be around 5000 metric tons and this has increased to around 10 000 metric tons in 2014 [18]. Most industries are switching from using bulk TiO₂ to TiO₂ NPs because of the fact that nano TiO₂ provides more advantages and application opportunities as compared to bulk TiO₂.

One of the common forms of nano TiO₂ are particles, which most of the commercial products incorporate TiO₂ NPs into their formulations. TiO₂ NPs were employed in different commercial products ranging from catalysts to foods and cosmetics. Food grade TiO₂ was added to foods to enhance the white color. On the other hand, TiO₂ NPs are able to reflect UV light and hence, they are added to food packaging materials to prevent photo degradation as well as prevent UV-induced spoilage and increase the shelf life of food [19].

Many food products such as chocolates and chewing gums subsume TiO₂ as one of the ingredients mainly because of its white pigment [20]. TiO₂ were also utilized to whiten skim milk [15]. Over the years, publications have reported the use of TiO₂, amounting approximately 0.1 - 6 µg per mg in tested food products. Further analysis showed that 15% of TiO₂ particles have sizes below 100 nm [21].

Apart from the food industry, the cosmetics industry added TiO₂ NPs into personal care products and cosmetics. In particular, TiO₂ NPs are heavily utilized in sunscreens because of the fact that they absorb UVB radiation (290 – 320 nm) and scatter UVA radiation (320 – 400 nm) which led to an increased sun protection factor (SPF) [22]. Moreover, TiO₂ NPs do not scatter visible light and hence, ensure the desired sunscreen transparency. In a study documented by Weir et al., the Ti amount in 13 different sunscreens were in a range of 14 – 90 µg per mg. Besides that, TiO₂ NPs are incorporated into deodorants, shampoos, face creams and toothpastes. The Ti concentration present in toothpastes varied from less than 0.1% to 0.5% product weight [23].

TiO₂ NPs are also employed in the pharmaceutical industry which they are utilized as a form of coating on medications such as low dose aspirin products. It was reported aspirin with coating contained 0.017 ± 0.005 µg of Ti per mg of product [23]. Another study had also reported the Ti content in drugs to be ranged from 0.0009 – 0.014 µg per mg of product [24].

2.2.3. Properties of ZnO NPs

Zinc does not exist as a pure element but has the natural tendency to oxidize into zinc oxide (ZnO) when it comes into contact with the oxygen in air. ZnO is an inorganic compound which has an appearance of an odorless white powder. It possesses a molecular weight of 81.4 g/mol, relative density of 5.61 g/cm³, boiling point of 2360°C and melting point of 1975°C [25]. ZnO can occur naturally or synthetically produced. On the contrary, ZnO NPs do not exist naturally but also synthesized to reach their nano structure.

To date, ZnO NPs can be produced through chemical precipitation method, solid state pyrolytic method, and biosynthesis method. Chemical precipitation method is widely used to produce ZnO NPs which two reagents are involved in the reaction. Solid state pyrolytic method is a low-cost synthesis method to produce ZnO NPs. Biosynthesis method is employed lately to produce ZnO NPs because it is environmentally friendly and biosafety.

2.2.4. Applications of ZnO NPs

ZnO NPs have been utilized in different applications ranging from biomedical fields to electronics industries and present in consumer products. One of its key applications is anticancer drug delivery to increase the formation of ROS from cancer cells and in turn, cause cancer cells injuries [26].

ZnO NPs possess electrochemical activity and high electron communication which make it favorable to be used in biosensor applications [27]. The popularity of ZnO NPs in biosensors was well demonstrated by Zheng et al. who showed excellent detection of trace

amounts of hydrogen peroxide in the body. The authors attributed this detection to ZnO NPs high surface area and magnificent dispersity, resulting in the enhancement of target molecules detection. Biosynthesized ZnO NPs used in this biosensor allowed it to be employed in the healthcare industries [28].

2.3. Human exposure: uptake route of NPs

The widespread applications of TiO₂ and ZnO NPs coupled with the possibility of their incidental release during manufacturing processes and waste handling of NP-containing products led to increased human exposure and potential health risks. Depending on the environment or NP-containing product used, NPs could enter human body through the inhalation, ingestion and dermal penetration.

2.3.1. Inhalation of NPs

Inhalation, one of the common routes for NPs to enter into human body. Workers working in the manufacturing industries have the highest chance of inhaling powdered NPs whereas consumers could inhale NPs from spray-based products. Upon entering through inhalation, NPs would reside in the lungs [29] and depending on the depth of penetration, NPs could reach different regions of the lungs. Based on a study done by Oberdorster et al., depending on the sizes of the NPs, the NPs could be found in various regions of the lungs such as the nasal and alveolar regions [30].

NPs that reside in the deeper regions of the lungs would be retained longer in the human body. This was further demonstrated by Oberdorster et al., who did a 12-week inhalation investigation on rats. From the results documented, TiO₂ NPs (20 nm) were retained in the rats for 501 days as compared to TiO₂ fine particles (250 nm) which remained in the body for 174 days [31].

2.3.2. Ingestion of NPs

NPs present in food products, water and medicine could enter through the human digestive system. The absorption of NPs occurs in the small intestine and colon which are the organs for the absorption of digested food substances. In a study conducted by Brun et al., TiO₂ NPs were present in the mouse gut after 6 hours since the exposure of 12.5 mg per kg body weight of TiO₂ NPs [32]. Similarly, mice fed with ZnO NPs (1600 Zn/kg food) supplemented food for 270 days was found to have significant ZnO accumulation in their small and large intestines [33].

Other than observing NPs in the small intestine and colon, NPs can also be present in the oral cavity. This was well documented by Tay et al., which used human oral buccal mucosa cells (TR146) in the study. Cellular uptake of TiO₂ NPs had occurred and localized in the TR146 epithelial cells [34]. Furthermore, an *ex vivo* study was done by Teubl et al. that supported the presence of NPs in the oral cavity. An observation of *ex vivo* explants of pigs contained TiO₂ NPs in the buccal mucosa was reported [35]. This suggests that NPs found in oral hygiene products could enter the human digestive system and retain in the organs.

2.3.3. Dermal Penetration of NPs

Skin lines the outer surface of the human body. Skin can be exposed to NPs through the surrounding environment or mainly topical applications of personal care products. It was estimated a topical application of sunscreen would introduce 12 – 55 mg of TiO₂ NPs per kg of body weight in a child whereas for an adult, it introduces 8 – 37 mg of TiO₂ NPs per kg of body weight. This observation could be due to skin of young children and infants not being fully matured and hence, less barrier functions to protect the skin [36]. Based on surface properties and sizes of the NPs, penetration through skin can occur in three different pathways: intracellular, intercellular and transappendageal that includes the hair follicles and sweat ducts [37]. Over the years, many research groups have studied the possible penetration of common NPs found in consumer products through the skin. Publications have shown *in vivo* studies supporting NPs penetrating into the skin. In a study conducted by Wu et al., TiO₂ NPs penetrated into the pig ear and located in deep layers of epidermis after 30 days of topical application [38]. Similarly, Osmond-McLeod et al. showed that

zinc concentration in the organs of hairless mice increases upon six topical applications of ZnO NP containing sunscreen [39].

It was further suggested to study the penetration of NPs on human subject as the thickness and skin permeability vary with different animal species and human. In a comparative study, Holmes et al., studied the possible penetration of ZnO and zinc ions through the human skin. No penetration of ZnO into the viable layers of epidermis was observed, except for the surface layers of stratum corneum whereas, penetration of zinc ions was observed. This could be attributed to the acidic environment on the surface of skin, which promotes the hydrolysis of topical applied ZnO into zinc ions [40]. Mavon et al. documented TiO₂ NPs penetration through *in vivo* and *in vitro* experiments following five hours of topical application of sunscreens. It was observed that TiO₂ NPs assembled in stratum corneum and hair follicles with no presence in the viable layers of epidermis [41]. In general, depth of penetration of NPs was reported to be affected by the surface characteristics or particles sizes. However, it was reported by Schulz et al. that regardless of surface coatings and particles sizes, the absorption of TiO₂ NPs through the human skin is minimal. The penetration of aluminium and silica coated TiO₂ NPs were mostly located in the surface of stratum corneum [42].

Nevertheless, the studies described previously were done on animal and healthy human skin models. It was reported in many publications that the penetration of NPs into intact skin is minimal, but studies reported on penetration of NPs into flexed or damaged skin is limited. Tinkle et al., reported that flexing of human skin allows the penetration of 0.5 and 1 μm particles as compared to unflexed human skin where no penetration was observed [43]. In contrast, Leite-Silva et al. documented no penetration of applied ZnO NPs into the viable layers of epidermis occurred even with the flexing and massage on human skin. ZnO NPs were mostly located in the upper layers of stratum corneum and skin grooves [44]. Interestingly, it was noted that penetration of NPs into damaged skin was minimal and has no difference as compared to the intact skin. Xie et al. reported no TiO₂ NPs (20 nm) penetration on both intact and slightly damaged rat skin [45]. In contrast, UVB-damaged pig skin showed TiO₂ and ZnO NPs penetrating upon topical application with sunscreen

formulation. Presence of TiO₂ NPs was found in deeper regions of stratum corneum and epidermis whereas ZnO NPs were accumulated in the surface layers of stratum corneum [46]. Overall, these studies have highlighted the wide variation in animal models, resulting in difficulties to assess the actual NPs penetration that is relevant to topical application of sunscreens.

On the other hand, studies have also reported repeated dermal applications of NPs could result in deeper penetration of NPs into the skin and distributed to different organs by blood vessels. Wu et al. reported that after 60 days of dermal exposure to topically applied TiO₂ NPs, it was present in deep layers of skin and organs such as spleen and brain of hairless mice [38]. However, Adachi et al. showed no evidence of NPs penetration into the skin of hairless rats after 56 days of dermal exposure to TiO₂ NP emulsions but, spongiosis (accumulation of fluid) and focal parakeratosis (nucleus retained in stratum corneum) were manifested in the skin [47] due to the contact between TiO₂ NPs and the skin.

2.4. Structure and Functions of Skin

The skin is the largest organ in the human body as it takes up 15% of total body weight [48] and overall body area of 2 m² [49]. The thickness of skin varies with gender, age and different body parts such as the palms and soles have higher thickness. The skin acts as a barrier that protects the human body from external threat and prevent penetration of foreign particles from the environment. Aside from protection, skin also regulates body temperature by perspiration and contains receptors that aid in the sensations such as touch and pain on skin. The skin comprises of three layers; the epidermis, the dermis and the subcutaneous tissue which is also known as the hypodermis (**Figure 2.2**).

Figure 2. 2: Schematic Diagram of Skin. Reproduced with permission from [50], Copyright 2020, LES LABORATOIRES SERVIER.

2.4.1. The Epidermis

Epidermis does not have blood vessels and hence, it is sustained by the dermis. Waste removal in the epidermis occurs via diffusion through the skin surface and dermal-epidermal junction. Epidermis consists 90% of keratinocytes where the cells are in a constant differentiation state. Keratinocytes are specialized cells that form a line of protection against the environment. Keratinocytes produce keratins that link them together. Thickness of the epidermis varies with different parts of the body where eyelids have the lowest thickness while palms and soles have the highest thickness [51].

Stratum basale (in the epidermis) located above the dermis composes of mainly keratinocytes and melanocytes. Keratinocytes attached to the basement membrane through hemidesmosomes. Keratinocytes would grow and divide from basement membrane, afterwards differentiate and move towards the superficial layers. On the other hand, melanocytes produce melanin that provides skin with a color. Melanin accumulates in an organelle called melanosomes. Melanosomes would be transported to the adjacent

keratinocytes for photoprotection that can lead to genetic damage. Merkel cells are also present in the stratum basale and are mechanoreceptors that necessary in sensation such as touch [52]. Sensitive areas of skin have greater amount of merkel cells.

Stratum spinosum (in the epidermis) is located above the stratum basale. It comprises mostly keratinocytes that grow and divide from the basement membrane and stratum basale. Langerhans cells belong to the skin immune system which originate from the bone marrow [53]. Langerhans cells detect and capture antigens present in the skin. Afterwards, Langerhans cells interact with lymphocytes to remove antigens from the skin.

Stratum granulosum (in the epidermis) is located above the stratum spinosum. In this layer, keratinocytes have an appearance of flattened shape and shrunken nuclei. In their cytoplasm, large keratohyalin granules can be observed. Keratohyalin granules stored profilaggrin and loricrin that were synthesized in this layer. Profilaggrin protein produces filaggrin that aggregates keratin filaments [54] while loricrin helps in the binding of keratin filaments.

Stratum corneum (in the epidermis), the outermost layer of skin. This layer consists terminally differentiated keratinocytes known as corneocytes. They have an appearance of flattened dead cells that have no nuclei and organelles. The lipid bilayers in between them consist of three main lipids: cholesterol, fatty acids and ceramides. Corneocytes are held together by corneodesmosomes. Corneocytes provide mechanical strength due to the keratin filaments present in the cells. The arrangement of corneocytes and extracellular lipids matrix provides a barrier that prevents water loss and penetration of microbes or toxins through the skin [55].

2.4.2. The Dermis

Dermis comprises of fibroblasts and blood vessels. This layer contains loosely attached collagen, elastin fibers and small blood vessels. The main function of this layer is to provide nutrients to the epidermis. In addition, it contains dense and irregular collagen with elastin

fibers. The strength and toughness of the skin were due to the collagen fibers which allow the skin to stay hydrated whereas elastin fibers provide elasticity to the skin. Fibroblasts, found throughout the dermis, produce extracellular matrix, proteoglycans collagen and elastic fibers. Proteoglycans provide compressional strength to the skin.

2.4.3. The Hypodermis

Hypodermis consists of adipose tissues and areolar connective tissues. This layer stores body fats and provides padding for the body. It acts as a thermal insulation and an energy reservoir. It is highly vascularized and hence, the perfect target site for subcutaneous injections.

2.5. Wound Healing

Wound healing is a biological process that occurs when the skin barrier is damaged. It starts from hemostasis which initiates upon wounding where blood vessels narrow, and the formation of a fibrin clot occurs. The constriction of blood vessels was to prevent and stop the bleeding. Platelets circulating in the nearby blood vessels would be recruited to the wound site to form a platelet plug. Thrombin converted fibrinogen to fibrin fibers which were used to stabilize the platelet plug [56]. Platelets plug and fibrin fibers would form a clot to prevent blood loss. Tissues surrounding the wound site and clot would release pro-inflammatory cytokines. After hemostasis, the inflammation stage takes place where inflammatory cells such as macrophages, neutrophils and lymphocytes stimulate inflammation.

At the wound site, neutrophils cleared the microbes and cellular debris present whereas macrophages released cytokines that would recruit and activate more lymphocytes. Macrophages were activated in the clearance of apoptotic cells which would stimulate skin cells to restore the damaged tissues [57]. Following inflammation of the wound, proliferation stage would occur whereby re-epithelization takes place. In this process, epithelial cells would proliferate and migrate to the wound site. Besides, re-epithelization,

angiogenesis, collagen and extracellular matrix (ECM) synthesis come about concurrently in the wound site. Fibroblasts in the dermis produced collagen, proteoglycans and glycosaminoglycans which are constituents of the ECM whereas endothelial cells contributed to angiogenesis by proliferation and migration [58]. Granulation tissue formation was observed in this stage. After which, remodeling of the newly formed tissues takes place. Keratinocytes migrate to the wound site to form a layer with newly stratified epidermis extending to the dermis. The granulation tissue composed of type III collagen was remodeled into scar tissue comprised of type I collagen by fibroblasts present in the granulation tissue. Type I collagen was aligned and cross-linked to reduce scar tissue thickness and provide strength to the newly formed tissue. The fibroblasts differentiated into myofibroblasts that expressed the smooth muscle actin [59]. Myofibroblasts facilitated in the wound closure by contraction of wound.

2.5.1. Re-epithelization

Re-epithelization is a process that restores the epidermis after an injury. This process is essential for wound healing and therefore, pivotal for the closure of wound. Keratinocytes, a major constituent in the epidermis are involved in re-epithelization. The epidermis is maintained by keratinocytes that proliferated in the basement membrane and differentiated as the cells moved to the superficial layers of skin. In the basement membrane, keratinocytes proliferated and expressed keratin intermediate filaments, K5 and K14. After proliferation, keratinocytes undergo differentiation as they moved towards the surface of skin while expressing keratin intermediate filaments, K1 and K10 in the stratum spinosum and stratum granulosum [60].

Upon skin injury, keratinocytes would be activated which the cells would migrate and proliferate to close the wound. Firstly, keratinocytes at the wound edge would lose adhesion between the cells and basement membrane. As keratinocytes are connected to one another by desmosomes and to basement membrane by hemidesmosomes, these connections have to be disassembled in order for migration to occur [61]. Several regulators were involved in the migration and proliferation of keratinocytes during re-

epithelization. PKC- α was activated in the process of migration to reduce the desmosomes' adhesive properties [62] and modulated the phosphorylation of $\beta 4$ subunits in $\alpha 6\beta 4$ integrin expressed by basal keratinocytes, resulting in increased disassembly of hemidesmosomes [63]. Migrating keratinocytes expressed keratin intermediate filaments, K6, K16 and K17, which these were reported to increase viscoelasticity of migrating keratinocytes [64]. With the migration of epithelial tongue, keratinocytes proliferated to ensure that the cells were sufficient to form a new stratified epidermis. Keratinocytes in the basement membrane have the ability to proliferate whereas keratinocytes in the other layers do not have this ability [65]. Keratinocytes proliferation is dependent on growth factors such as Epidermal Growth Factor (EGF) and Keratinocyte Growth Factor (KGF) availability, cells differentiation and cells attachment to a substrate. The ECM, integrins and growth factors worked together in regulating keratinocytes proliferation in re-epithelization. For instance, matrix metalloproteinase (MMP) is a component of ECM that worked with integrins to assist growth factors in keratinocytes proliferation [63]. Upon closure of wound by a layer of keratinocytes, the proliferation stopped.

2.6. NPs Induced Biological Responses

TiO₂ and ZnO NPs are incorporated into different types of personal care products including cleansing creams and sunscreens. These consumer products are topically applied on the skin which could potentially lead to the interaction between NPs and the skin. This has prompted concerns regarding the potential adverse effects associated. Thus, it is essential to understand how NPs and skin cells can interact as this would correlate to biological responses observed in the cells. Thereafter, these biological responses would account for the toxicity that could be observed in the cells or skin. This section provides the discussion on the latest research of NPs induced biological responses in the skin cells and human skin models.

2.6.1. Oxidative Stress

TiO₂ and ZnO NPs were documented to induce biological responses and these biological responses could be beneficial or deleterious to human. One of the paradigms for NPs induced biological responses is oxidative stress, a state where intracellular ROS production is higher than the antioxidant molecules in the cells. NPs can induce ROS production through two mechanisms: reactive functional groups on surface of NPs and redox reactions on the surface of transition metal NPs [66].

TiO₂ and ZnO are semiconductors and upon photoexcitation, they create an electron-hole pair which in turn generate ROS on the surface of NPs. When the electron-hole pair is in aqueous suspension, they react differently in water. Electrons in the conduction band would reduce oxygen to generate superoxide anions (O₂⁻) whereas holes in the valence band would remove an electron from hydroxyl ions, yielding hydroxyl radicals (•OH) in water [67]. Photocatalytic activity of NPs could trigger the production of ROS as that generates free radicals on the surface of NPs. The photocatalytic activity of ZnO NPs increases as the size decreases from 100 to 20 nm [68]. ROS generation by ZnO NPs would induce damage to cell membrane, mitochondria and DNA [69].

ROS are signaling molecules involved in cell signaling and keeping a homeostasis state. Some of the common ROS include hydrogen peroxide (H₂O₂), superoxide anions (O₂⁻) and hydroxyl radicals (•OH). At a cellular level, ROS are generated in trace amounts due to metabolism, mitochondrial respiration and inflammatory response [70]. To counteract the increased ROS in the cells, antioxidant defense is activated where antioxidant molecules such as glutathione (GSH) react with ROS or stimulate other antioxidants [71]. In a study documented by Saquib et al., catalase and glutathione, antioxidant enzymes that metabolized ROS, were observed to decrease significantly upon TiO₂ NPs exposure to WISH cells [72]. The authors attributed this to TiO₂ NPs binding to catalase, altering its structure and consequently causing the reduction of the antioxidant capacity[73]. **Table 2.1** summarizes the current studies of TiO₂ and ZnO NPs induced oxidative stress in different models.

Table 2. 1: Publications on TiO₂ and ZnO NPs induced oxidative stress in different models.

Nanomaterial (NM)	Particle Size (nm)	Concentration	Model	Observations	Reference
TiO ₂	21 nm (80% anatase and 20% rutile)	0.1, 1, 10, 25, 100 µg/cm ²	HaCaT	Dose dependent increase in superoxide but decrease in H ₂ O ₂ and nitric oxide levels	[74]
TiO ₂	10, 21, 25, 60 nm (anatase/rutile)	400 µg/cm ²	Hairless Mice	Increased malondialdehyde (MDA) level Decreased superoxide dismutase (SOD) activity in skin and liver tissues	[38]
ZnO	30 nm	20 to 0.008 µg/ml	Human epidermal cell line (A431)	Decreased glutathione level Reduced superoxide dismutase and catalase activities Increased lipid hydroperoxide formation	[75]
ZnO	< 50 nm, < 100 nm	5% and 10% dose (w/v)	Hairless Mice	Increased ROS generation Increased inflammation in skin	[76]

2.6.2. DNA Damage

NPs such as TiO₂ and ZnO are found to induce DNA damage through oxidative stress. One form of ROS that are produced is superoxide anions (O₂^{•-}). When this ROS comes upon transition metals (e.g. Titanium or Zinc), it would interact with the transition metal and produce hydroxyl radicals (•OH), another form of ROS. Hydroxyl radicals are able to cause DNA damage through interaction with the DNA [77]. The reaction of hydroxyl radicals formation is as follows:

DNA damage exists in the form of DNA adducts formation, DNA strands breakage, genes mutation and chromosomal fragmentation.

ZnO NPs were documented to induce DNA damage through the production of ROS and lipid peroxidation in human epidermal cell line (A431). Two reactions occurred, ROS reacted with DNA bases and backbones while malondialdehyde, a mutagen compound that formed through lipid peroxidation, reacted with DNA to form DNA adducts [75]. Comparably, TiO₂ NPs were observed to induce oxidative DNA damage in human epidermal cell line (A431). TiO₂ NPs treated cells were observed to induce the depletion in GSH level, enhanced ROS generation and lipid peroxidation that increased DNA damage and micronucleus formation [78].

Increased in DNA damage could also be caused by the disruption on the DNA damage repair pathways, including base excision repair (BER), nucleotide excision repair (NER) and DNA mismatch repair (MMR) [79]. BER recognizes, eliminates damaged bases and restores with the correct bases whereas NER removes damaged regions and replaces with the newly synthesized DNA regions. TiO₂ NPs were reported to immobilize the BER and NER pathways, and that would lead to the inhibition of the human lung carcinoma cells' ability to repair DNA damage [80]. Likewise, ZnO NPs were documented to suppress BER pathway in macrophages as two repair proteins, DNA polymerase β and Flap endonuclease 1 were observed with reduced expression [81]. Normally, cells with DNA damage are able

to recover from such damage but that has to depend on the severity of the damage. Skin fibroblasts (BJ cells) activated the p53 pathway upon DNA damage induced by ZnO NPs [82]. Furthermore, human dermal fibroblasts were able to activate ATM/Chk2 pathway as a response to DNA damage consequent to TiO₂ NPs exposure [83]. **Table 2.2** summarizes the current studies of TiO₂ and ZnO NPs induced DNA damage in different models.

Table 2. 2: Publications on TiO₂ and ZnO NPs induced DNA damage in different models.

Nanomaterial (NM)	Particle Size (nm)	Concentration	Model	Observations	Reference
TiO ₂	15 nm (anatase)	1, 3, 10, 30, 100 µg/ml	Primary human dermal fibroblasts	Increased phosphorylation of H2AX Decreased DNA synthesis and origin firing	[83]
TiO ₂	21 nm (70% anatase, 30% rutile)	0.5, 1, 5, 10, 50 µg/ml	HaCaT	Increased micronucleus formation Increased mitochondrial DNA damage	[84]
ZnO	30 nm	0.8 to 20 µg/ml	Primary human epidermal keratinocytes	Increased DNA damage	[85]
ZnO	32 nm	2.5 mg/cm ²	Hairless Mice	Increased 8-OHdG formation Increased DNA damage	[86]

2.6.3. Autophagy

Autophagy is a physiological process that ensures normal functioning of the body and maintaining the state of homeostasis in the cells. Autophagy is often referred to as a survival mechanism as this process involves the removal of intracellular pathogens, damaged cell organelles and proteins [87]. One of the prominent autophagy, macroautophagy delivers the cytoplasmic materials in a double membrane vesicle called autophagosome. The fusion of autophagosome with lysosome caused a formation of autolysosome that could degrade the unwanted cellular materials present in the cytoplasm.

Autophagy consists of five sequential steps: initiation of the phagophore, elongation of the phagophore, maturation of the phagophore which gives the autophagosome, formation of autolysosome and degradation of the components in autolysosome [88]. Phagophore is an isolation membrane and its initiation occurs in the pre-autophagosomal structure (PAS) where most of the autophagy related (ATG) proteins are located [89]. The elongation of the phagophore requires ATG5-ATG12 complex to interact with ATG16L1 to form a tertiary complex that associates with phagophore. Once the phagophore closes itself with some areas of cytoplasm, the autophagosome was formed. This tertiary complex would then dissociate from the autophagosome [90]. Autophagosome formation occurs at different areas in the cytoplasm. This formation involves microtubule-associated light chain 3 (LC3) and ATG proteins. During autophagosome formation, LC3-I proteins are transformed into LC3-II proteins that bound to the membrane of autophagosome [91]. The enzymes present in the autolysosome would degrade the enclosed cytoplasmic materials [92].

In normal circumstances, autophagy is activated during starvation and infection. Since autophagy is a process that degrades cellular components, irregular activation of autophagy could possess a risk to the cells. Thus, this is one of the biological responses required to study in order to understand the NPs mediated toxicity. **Table 2.3** summarizes the current studies of TiO₂ and ZnO NPs induced autophagy in different models.

Table 2. 3: Publications on TiO₂ and ZnO NPs induced autophagy in different models.

Nanomaterial (NM)	Particle Size (nm)	Concentration	Model	Observations	Reference
TiO ₂	22 nm	50, 100 fg/ml, 50 pg/ml, 50 ng/ml, 50 and 500 µg/ml	Primary human epidermal keratinocytes	Increased autophagic vacuoles Increased autophagosomes	[93]
TiO ₂	18 nm (anatase)	0.16 and 25.0 µg/ml	HaCaT	Increased LC3 puncta Increased p62 and NBR1 genes expression	[94]
ZnO	< 100 nm	0.5, 5, and 10 µg/ml	Human keratinocyte cell line (NCTC2544)	Formation of amphisomes NPs uptake in the amphisomes	[95]
ZnO	15.38 nm (width) and 82.34 nm (length)	10 µg/ml	Mouse skin epidermal cell line (JB6 Cl 41-5a)	Increased autophagic vacuoles Increased LC3, BECN1 and ATG5 proteins levels	[96]

2.7. Biological Responses in Wound Healing

TiO₂ and ZnO NPs were reported to induce biological responses such as DNA damage, oxidative stress, and autophagy in skin cells. These biological responses documented were ascribed to the interaction between the skin cells and NPs. This would lead to an effect on the cells functionality. This phenomenon could be clearly observed in wound healing where

cells proliferation and migration were required for the closure of wound. Depending on the biological responses, it could have a positive or negative impact on wound healing.

ROS would be produced within the cells or on the cells surface which these ROS could promote cells migration. In a study documented by K. Schröder *et al.*, ROS generated from NADPH Oxidase 1 mediated the basic fibroblast growth factor (bFGF), and that led to an enhancement of rat smooth muscle cells migration [97]. Cells migration required the involvement of cells proliferation which ROS could attribute to an increase in cells proliferation. Low concentrations of ROS stimulated the fibroblasts to proliferate as observed in the increase in cells number, cells morphology and thymidine incorporation [98]. Besides ROS production, autophagy was reported to promote cells migration. ROS activated JNK and p38 mitogen-activated protein kinases in keratinocytes which in turn, triggered the induction of autophagy in BNIP3 protein. This had contributed to an increase in the keratinocytes migration [99].

However, TiO₂ NPs were observed to inhibit cells migration, and this was attributed to the sizes of NPs. TiO₂ NPs with an average size of 300 nm at a concentration of 100 µg/ml demonstrated a decrease in NCI-H292 cells migration. This was ascribed to the increased uptake of NPs in the cells. Actin filaments of cells were required and involved during cells migration which NPs treatment collapsed the actin cytoskeleton, thereby decreasing cells motility [100]. Additionally, TiO₂ NPs were documented to disrupt the intracellular microtubules assembly and that had led to an increase in the cells traction. This had brought upon an enhancement of cells adhesion onto substrate and limiting cells migration [101].

2.8. Summary

NPs are one of the active ingredients that have been widely present in various consumer products such as sunscreens and wound dressing materials. Their high utilization in different products have brought to an increase in the interaction between the NPs and skin system. These interactions have led to a diverse toxicological outcome in the skin cells which aided in some of the cells physiological processes. One of the predominant cells

physiological processes is wound healing, which is a complicated process that involves different proteins communicating with one another to close the wound. The presence of wound in the skin allowed the NPs from consumer products to invade the skin and interact with the skin cells. This could affect the functionality of skin cells and in turn, influenced the closure of wound. Various studies have documented the influence of various NPs on cells migration and proliferation but that was dependent on the concentrations of NPs. Thus, a realistic dose of NPs relevant to consumer products on skin cells should be studied to correlate the realistic scenario of consumers using consumer products and their effects on wound healing.

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Chapter 3

Experimental Methodology

The materials and experimental methodology used in this study were described in this chapter. The methods used were categorized into ninth sections with the first section explained how NPs were characterized mainly using Transmission Electron Microscope (TEM) and Dynamic Light Scattering (DLS). The second section demonstrated how the NPs suspensions were prepared before using them as a treatment to cells. Third section stating how the cells were cultured. Fourth to sixth sections comprised of the different assays used for toxicity assessment of NPs exposure in the cells. Seventh section included how the scratch wound assay was conducted. Eighth and ninth sections stated how the skin explants were processed and the histological analysis performed, respectively.

3.1. Materials

3.1.1. Cells

Primary human epidermal keratinocytes (HEKs) of adult origin were purchased from American Type Culture Collection (ATCC PCS-200-011), USA.

3.1.2. Chemicals

Commercially available TiO₂ and ZnO NPs were utilized in this research study. P25 TiO₂-NPs were purchased from Evonik Corporation, Germany and ZnO-NPs were purchased from Melorium Technologies, USA. EpiGRO™ Human Epidermal Keratinocyte Complete Culture Media Kit (SCMK001) and Goat Serum were purchased from Merck Millipore, USA. 1X Phosphate Buffered Saline (PBS), Penicillin-Streptomycin and 0.25% Trypsin-EDTA were purchased from PAN Biotech, Germany.

DPX mounting medium, Tween 20, Triton X-100, Paraformaldehyde, Xylene, Bovine Serum Albumin (BSA), Chloroquine Diphosphate Salt, Propidium Iodide Solution, tert-Butyl hydroperoxide (tBHP) solution 70% in H₂O, L-Glutamine, Fetal Bovine Serum (FBS) and antibiotic-antimycotic were acquired from Sigma Aldrich, USA. Rapamycin was purchased from Enzo Life Sciences, Switzerland. Monodansylcadaverine was bought from Cayman Chemical, USA. ProLong™ Gold Antifade Reagent with DAPI, Hoechst 33342, Alexa Fluor™ 488 Phalloidin, CellROX™ Green Reagent, Haematoxylin and Eosin dyes were purchased from Life Technologies, USA. Target Retrieval Solution, Peroxidase Blocking Solution, Horse Radish Peroxidase (HRP) and 3,3'-Diaminobenzidine (DAB) were purchased from DAKO, USA.

3.1.3. Antibodies

Mouse anti-phosphorylated histone H2AX (γ -H₂AX) antibody and rabbit anti-4-hydroxynonenal (4-HNE) antibody were obtained from Abcam, USA. Rabbit anti-

microtubule-associated proteins 1A/1B light chain 3B (LC3-II) antibody was purchased from Novus Biologicals, USA. Alexa 488-chicken anti-mouse antibody, Alexa 594-chicken anti-rabbit antibody and Alexa 647-chicken anti-rabbit antibody were bought from Life Technologies, USA. Mouse anti-vinculin antibody and mouse anti- α -tubulin antibody were acquired from Sigma Aldrich, USA. Rabbit anti-acetyl- α -tubulin antibody and mouse anti-Ki-67 antibody were procured from Cell Signaling Technology, USA.

3.2. Methods

3.2.1. NPs Characterization

TiO₂ and ZnO NPs size and morphology were observed under a Transmission Electron Microscope (TEM), Carl Zeiss Libra 120 plus with an accelerating voltage of 120 kV. TiO₂ and ZnO NPs (20 μ g/ml) was dispersed in absolute ethanol. The suspension of each NPs with ethanol was sonicated using a probe sonicator (Qsonica Q125) for 2 minutes with an amplitude of 80%. After which, a drop of each suspension was placed onto a carbon-coated grid. NPs were left to dry fully at room temperature. Multiple TEM images were taken to determine the sizes of the NPs, while ImageJ software [1] was employed to quantify the sizes of the NPs. The NPs primary size data was determined by measuring at least 100 NPs, and the average measurement was represented as the primary size of the NPs.

Hydrodynamic sizes and zeta potentials of the NPs were measured using Dynamic Light Scattering (DLS), Malvern Zetasizer. NPs were dispersed in two different dispersants to investigate the NPs behaviour in different aqueous suspensions. 50 μ g/ml of TiO₂ and 200 μ g/ml of ZnO were dispersed individually in both ultrapure water and EpiGRO™. To investigate on the presence of protein corona formation on the NPs, an additional group for each NPs was prepared by firstly dispersing the NPs in EpiGRO™ for 20 minutes and followed by collecting the NPs through centrifugation and re-dispersing them in ultrapure water. The three suspensions of each NP were probe sonicated for 2 minutes with an amplitude of 80%. Immediately after sonication, the suspensions were analysed by DLS which the hydrodynamic sizes and zeta potentials were measured and recorded.

3.2.2. Preparation of NPs Suspensions

The stock suspensions of NPs were prepared individually at different concentrations. The stock suspension (5 mg/ml) of TiO₂ NPs was developed in ultrapure water followed by sonication with a probe sonicator for 5 minutes with an amplitude of 80%. After which, the stock suspension was diluted with the cells medium to reach the required concentrations (10 µg/ml, 100 µg/ml, and 500 µg/ml) that were used to treat the cells.

The stock suspension (500 µg/ml) of ZnO NPs was prepared in ultrapure water followed by sonication utilizing a probe sonicator for 5 minutes with an amplitude of 80%. After which, the stock suspension was diluted with the cells medium to reach the required concentrations (1 µg/ml, 10 µg/ml, and 25 µg/ml) that were used to treat the cells. The NPs suspensions were prepared instantly prior to treatment of cells, which has a time point of 24 hours.

3.2.3. Cell Culture

HEKs were cultured in EpiGRO™ Human Epidermal Keratinocyte Complete Culture Media Kit supplemented with 1% Penicillin-Streptomycin in 37°C and 5% CO₂, a standard cell culture condition. Cells were maintained to grow for three to four days with the medium being changed every two days until a confluency of 80% was reached. Once the cells were confluent, the cells were sub-cultured by trypsinizing them with 0.25% Trypsin-EDTA and centrifuging at 290g for 5 mins to obtain the cell pellets. After which, the cell pellets were re-suspended in medium. A hemocytometer was used to quantify the number of cells present.

The cells were seeded at a seeding density of 10,000 cells/cm² and incubated for 24 hours to allow complete attachment prior to any treatment.

3.2.4. Oxidative Stress Assays

Two assays were utilized to evaluate the oxidative stress level in the cells. One of the assays measured intracellular ROS level using CellROX whereas the other one measured the product of lipid peroxidation using immunofluorescence staining against 4-HNE. The treatment groups were positive control, treated with tBHP at a concentration of 200 μ M for 2 hours, TiO₂ NPs at concentrations of 10 μ g/ml, 100 μ g/ml and 500 μ g/ml with ZnO NPs at concentrations of 1 μ g/ml, 10 μ g/ml and 25 μ g/ml for 24 hours.

3.2.4.1. Intracellular ROS Level Measurement

CellROXTM Green Reagent was employed to measure the oxidative stress level in live cells. Briefly, the HEKs were seeded on 96-well Costar[®] clear bottom black well plates. The cells were administered with medium containing NPs at numerous concentrations and left to incubate for 24 hours. After the treatment time had ended, the cells were exposed to 5 μ M of CellROXTM Green Reagent and 10 μ g/ml of Hoechst 33342 for 30 minutes at 37°C. After which, the cells were washed rinsed with PBS and replaced with 200 μ l of fresh PBS for fluorescence reading, which was done by a microplate reader (Tecan Infinite M200, Tecan Inc., USA).

The intracellular ROS level was detected when the CellROXTM Green Reagent exhibited bright green fluorescence upon oxidation by ROS. CellROXTM Green Reagent has an excitation/emission wavelengths of 485/520 nm and Hoechst 33342 has an excitation/emission wavelengths of 350/461 nm which the fluorescence reading was used to measure the intensities of CellROXTM Green Reagent and Hoechst 33342. The fluorescence reading of wells containing PBS was used to correct the background.

3.2.4.2. Immunofluorescence Detection of Lipid Peroxidation

Reactive Oxygen Species (ROS) signals were visualized using the immunofluorescence staining. HEKs were seeded and grown on 15 mm cover slides in 12-well cell culture plates.

After an incubation which allows the complete attachment of cells, the cells were exposed to medium containing NPs at different concentrations for 24 hours. Thereafter, the medium containing NPs was removed, and the cells were washed with PBS.

Fixation of cells was done in 4% paraformaldehyde for 15 minutes, next, permeabilization was done in 0.2% Triton X-100 in PBS for 15 minutes followed by a blocking step in 2% BSA in PBS for 1 hour. After which, an overnight incubation at 4°C was done on the fixed cells with rabbit anti-4-HNE antibody (1:200). The primary antibody was diluted in 0.1% Triton X-100 and 0.2% BSA in PBS before adding to the cells. Fixation, permeabilization and blocking steps were done in ice to prevent protein degradation process.

After an overnight incubating with primary antibody, the cells were washed thrice with PBS. A secondary antibody incubation was implemented for 1 hour in room temperature with Alexa 594-chicken anti-rabbit antibody (1:400). Cells were stained with Hoechst Dye (1:2000) for nucleus staining and Alexa Fluor™ 488 Phalloidin (1:200) for actin filaments staining, which were mixed with the secondary antibody. The secondary antibody, Hoechst Dye and Phalloidin were diluted in 0.1% Triton X-100 and 0.2% BSA in PBS before adding to the cells. After the staining of cells was completed, the cover slides were mounted with ProLong™ Gold Antifade Reagent containing DAPI. The images were taken using an Olympus Fluorescence Microscope. Keratinocytes with more than 5 dots were counted as 4-HNE positively stained cells. Percentage of the positively stained cells over the total population of cells (n=150 cells) of each treatment group was determined, and the value was normalized against untreated control group.

3.2.5. DNA Damage Detection

Another toxicity associated to NPs exposure is DNA damage, which this could be due to oxidative stress. Metal based NPs produce reactive oxygen species (ROS) upon oxidation by free radicals. ROS would interact with the DNA and caused DNA damage. Immunofluorescence staining against γ -H₂AX was used to detect DNA double strand breaks. The treatment groups were positive control, exposed to UV for 15 minutes, TiO₂

NPs at concentrations of 10 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, and 500 $\mu\text{g/ml}$ and ZnO NPs at concentrations of 1 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$, and 25 $\mu\text{g/ml}$ for 24 hours. DNA damage signals were observed using the immunofluorescence staining. HEKs were seeded on 15 mm cover slides in 12-well cell culture plates. After an overnight incubation for complete cells attachment, the cells were treated with medium containing NPs at different concentrations for 24 hours. Thereafter, the medium containing NPs was removed, cells were rinsed gently with PBS followed by proceeding to immunofluorescence staining as narrated in section 3.2.4.2. The primary antibody employed was mouse anti- $\gamma\text{-H}_2\text{AX}$ antibody (1:200) and the secondary antibody was Alexa 488-chicken anti-mouse antibody (1:400). Keratinocytes nuclei with more than 5 dots were counted as $\gamma\text{H}_2\text{AX}$ positively stained cells [2]. Percentage of the positively stained cells over the total population of cells ($n=150$ cells) of each treatment group was determined, and the value was normalized against untreated control group.

3.2.6. Autophagy Assays

Autophagy is a physiological process that ensures normal functioning of the body. This process involves the removal of intracellular pathogens, damaged cell organelles and proteins. The influence of NPs exposure on autophagy has not been studied fully and hence, it was required to observe the effect of NPs on autophagy. Immunofluorescence staining against LC3B was used to detect autophagosome. The treatment groups were positive control, treated with Rapamycin at a concentration of 200 nM for 24 hours, TiO_2 NPs at concentrations of 10 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$ and ZnO NPs at concentrations of 1 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$, and 25 $\mu\text{g/ml}$ for 24 hours. At the last 4 hours of treatment, 50 μM of chloroquine diphosphate was added to each treatment group to block the fusion of lysosome to autophagosome.

3.2.6.1. Monodansylcadaverine (MDC) Staining

Monodansylcadaverine is a fluorescent dye to detect autophagy. MDC surrounds autophagic vacuoles where the dye would interact with the cell membranes through ion

trapping and other mechanisms. HEKs were seeded in 96-well Costar® clear bottom black well plates and left to incubate for the cells to attach. The cells were then treated with medium containing NPs at different concentrations and left to incubate for 24 hours. After the treatment had completed, the cells were treated with 10 µg/ml of Monodansylcadaverine and 10 µg/ml of Hoechst 33342 for 15 minutes at 37°C. After which, the cells were washed twice with PBS and replaced with 200 µl of fresh PBS for fluorescence reading, which was done by a microplate reader (Tecan Infinite M200, Tecan Inc., USA).

MDC has an excitation/emission wavelengths of 335/512 nm and Hoechst 33342 has an excitation/emission wavelengths of 350/461 nm which the fluorescence reading was used to quantify the intensities of MDC and Hoechst 33342. The fluorescence reading of wells containing PBS was used to correct the background.

3.2.6.2. Immunofluorescence Detection of Autophagosome

Autophagy signals were imaged using the immunofluorescence staining technique. HEKs were seeded on 15 mm cover slides in 12-well cell culture plates. After an incubation of 24 hours for cells attachment, the cells were administered with medium containing NPs at various concentrations for 24 hours. Thereafter, the medium containing NPs was removed, cells were rinsed gently with PBS followed by proceeding to immunofluorescence staining as narrated in section 3.2.4.2. The primary antibody utilized was rabbit anti-LC3B antibody (1:200) and the secondary antibody was Alexa 594-chicken anti-rabbit antibody (1:400). Keratinocytes with more than 5 dots were counted as LC3-II positively stained cells. Percentage of the positively stained cells over the total population of cells (n=150 cells) of each treatment group was determined, and the value was normalized against untreated control group.

3.2.7. Scratch Wound Assay

Human epidermal keratinocytes played an important role in the re-epithelization process where keratinocytes migration facilitated the closure of wound. An *in vitro* scratch wound assay was implemented to demonstrate the migration of keratinocytes. HEKs were seeded in 48-well cell culture plates with a seeding density of 125 000 cells/cm² and incubated for 24 hours to allow complete cells attachment. Once the formation of a confluent monolayer has completed, the cells would be treated with medium containing NPs at different concentrations. The treatment groups were positive control, treated with Rapamycin at a concentration of 200 nM for 24 hours, TiO₂ NPs at various concentrations of 10 µg/ml, 50 µg/ml, and 100 µg/ml and ZnO NPs at concentrations of 0.1 µg/ml, 0.5 µg/ml, and 1 µg/ml for 24 hours. After the exposure time had ended, the cells would be scratch-wounded with a clear pipette tip and left to incubate for 24 hours to allow the migration of keratinocytes. The keratinocytes migration was monitored at 0 hour and 24 hours using an Olympus Microscope. The relative wound closure was determined as follows:

$$\text{Wound Closure} = \frac{\text{Wound Area at } t = 0 - \text{Wound Area at } t = 24}{\text{Wound Area at } t = 0} \times 100\%$$

3.2.8. Human Skin Explants and Wound Generation

Human skin explants were attained from the abdomen of anonymous female donors that undergo plastic surgeries. The collection of human skin explants was done in a local hospital with ethical approval by the NTU Institutional Review Board (IRB-2017-06019). Upon collection from the hospital, the skin explant was rinsed first with PBS, followed by 70% ethanol, and last, PBS containing 2% antibiotic-antimycotic. After which, the dermis and subcutaneous fat were detached from the skin, leaving behind the epidermis. On the viable epidermis, 8mm of biopsy punches were made. On each biopsy punch, a wound was generated by making a small cut. Once the wound was created, the biopsies were treated with tBHP solution or UV by adding 10 µl of tBHP solution in medium or exposed to UV, respectively. The negative control groups were treated with PBS in medium. Skin biopsies were placed on Falcon[®] deep well plates and inserts and maintained for different timepoints: 1 day, 3 days and 7 days in 37°C and 5% CO₂. They were kept in an air-liquid interface

where the bottom part was exposed to DMEM supplemented with 4 mM L-Glutamine, 1% antibiotic-antimycotic and 10% FBS and the wound area was exposed to air. After the respective treatments were completed, the skin biopsies were fixed in PBS containing 4% paraformaldehyde.

3.2.9. Histology Analysis

Once the biopsies were fixed for 24 hours, the biopsies were processed for histology analysis. Skin biopsies were dehydrated in ascending order of ethanol: 70%, 80%, 90% and two changes of 100%, first 100% ethanol dehydration for an hour and incubated overnight in second 100% ethanol dehydration. After which, skin biopsies were transferred to two different bottles of xylenes, first xylene incubation for 7 hours and incubated overnight with second xylene. Afterwards, skin biopsies were permeated with two changes of paraffin wax, first paraffin wax for 8 hours and left overnight in second paraffin wax. Finally, they were embedded into cassette blocks and proceeded with sectioning using microtome. Each biopsy was section into 5 μm thin sections and attached onto glass slides. These sections were then kept overnight in 37°C to ensure complete attachment of tissue sections to glass slides.

3.2.9.1. Haematoxylin and Eosin (H&E) Staining

Before staining of tissue sections, dewaxing of sections was done with three different bottles of xylene, followed by rehydration in descending order of ethanol: 100%, 90%, 80% and 70% at a time interval of 3 minutes each. After that, they were soaked in tap water for 3 minutes. Once the tissue sections have been rehydrated, they were stained with haematoxylin dye for 8 minutes, and 30 seconds of washing in running water. Next, the tissue sections were placed in acid alcohol containing 70% ethanol, 29% deionized water and 1% hydrochloric acid (37%) for 15 seconds, with 30 seconds of washing in running water right after. After which, they were transferred to Scott's tap water (20 g/l of magnesium sulfate and 2 g/l of sodium bicarbonate) for 5 minutes, accompanied by 30 seconds of washing in running water. Lastly, they were counterstained with eosin dye for

2 minutes, with an additional 30 seconds of washing in running tap water. Upon the completion of staining, tissue sections were dehydrated ascending order of ethanol: 70%, 80%, 90%, 100% and xylene followed by mounting of tissue sections with DPX mounting medium. They were left to dry overnight before microscopy analysis.

3.2.9.2. Immunoperoxidase Staining

Before staining of tissue sections, the sections were heated up to 60°C for 1 hour, dewaxed with xylene, rehydrated in a series of descending ethanol followed by tap water for 3 minutes. Next, the sections were heated up to 120°C in an oven with an antigen retrieval solution. Afterwards, the sections were dipped in two changes of PBST containing 0.5% Tween 20. Sections were quenched in endogenous peroxidase blocking solution for 30 minutes. The sections were then placed in running water and rinsed with PBST for 5 minutes, respectively. After which, the sections were blocked with 10% goat serum in PBS for 1 hour. The sections undergo an overnight incubation at 4°C with primary antibody. The primary antibody was diluted in 10% goat serum in PBS before adding to the sections. On the following day, sections were placed in running tap water for 10 minutes. After which, they were placed in PBST for 5 minutes. The sections were then treated with Secondary antibody Envision+/Horse Radish Peroxidase (HRP) and incubated for 1 hour. The sections were washed in running water followed by PBST. DAB reagent was used as the colouring agent to indicate the signals produced by the antibodies. 100 µl of DAB reagent was added to each section and the colour development was observed. By placing the sections in tap water, the colour development had stopped. Afterwards, they were washed in running tap water for 5 minutes. The sections were then counterstained with haematoxylin, dehydrated in ascending order of ethanol and xylene followed by mounted with DPX mounting medium.

3.2.9.3. Immunofluorescence Staining

Before staining of tissue sections, the sections were heated up to 60°C for 1 hour, dewaxed with xylene, rehydrated in a series of descending ethanol followed by tap water for 3

minutes. Next, the sections were heated up to 120°C in an oven with an antigen retrieval solution. The sections were then dipped in two changes of PBST. After which, the sections were placed in PBS containing 10% goat serum for 1 hour. The sections placed under an overnight incubation at 4°C with mouse anti- γ H₂AX antibody (1:200). The primary antibody was diluted in PBS containing 10% goat serum before adding to the skin sections. On the following day, sections were kept in running tap water for 10 minutes and rinsed in PBST for 5 minutes. The sections were then probed with Alexa 488-chicken anti-mouse antibody (1:250) and Hoechst Dye (1:2000) for nucleus staining for 1 hour. Afterwards, the sections were washed in PBS before mounting with ProLongTM Gold Antifade Reagent. They were left to dry overnight before images were taken using an Olympus fluorescence microscope.

3.3. Statistical Analysis

Results reported for all experiments in this study were expressed as mean $\pm$ standard deviation of at least three replicates ($n = 3$). Statistical significance was analyzed using two tailed Student's t-test, p value of < 0.05 was considered statistically significant.

References

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- [2] L. Hernández, M. Terradas, M. Martín, L. Tusell, and A. Genescà, "Highly sensitive automated method for DNA damage assessment: gamma-H2AX foci counting and cell cycle sorting," (in eng), *International journal of molecular sciences*, vol. 14, no. 8, pp. 15810-15826, 2013.

Chapter 4

Results and Discussion

In this chapter, TiO₂ and ZnO NPs used in this study were characterized. Important characteristics of NPs such as size, shape, and hydrodynamic properties were displayed in the first part of this chapter. Second part talks about the biological responses observed in human epidermal keratinocytes upon exposure to TiO₂ and ZnO NPs. Three biological responses were discussed: oxidative stress, DNA damage and autophagy. After which, the influences of these NPs on migration and proliferation of HEK were studied.

4.1. NPs Characterization

4.1.1. Physiochemical Properties of NPs

NPs interaction with cells could elicit biological responses that in turn lead to adverse effects. It has been demonstrated that physiochemical properties of NPs such as surface area, size and shape play an important role in the reactivity of NPs and modulate this nano-bio interaction [1]. For example, plate-like and needle-like nanosized hydroxyapatite was reported to induce higher amounts of cell death as compared to spherical and rod-like nanosized hydroxyapatite [2]. Therefore, it is essential to understand the physical properties of TiO₂ and ZnO NPs prior to exposing HEK and human skin explants to these NPs.

Commercialized spherical TiO₂ NPs (Aeroxide P25) from Evonik Corporation and spherical shaped ZnO NPs from Melorium Technologies were used in this study. Primary sizes of the TiO₂ and ZnO NPs obtained from TEM micrographs (**Figure 4.1**) were 24.55 ± 6 nm and 21.09 ± 5 nm respectively which further proved that the NPs used were in the nano size range. Crystalline composition of TiO₂ NPs was reported with 73-85% anatase, 14-17% rutile and 2-13% amorphous [3] whereas ZnO NPs was documented to have a crystalline zincite structure [4]. The surface areas of TiO₂ and ZnO NPs were reported to be 50 ± 15 m²/g [5] and 47 ± 1 m²/g, [4] respectively.

Figure 4. 1: Representative Transmission Electron Microscope (TEM) micrographs of (A) TiO₂ NPs and (B) ZnO NPs. Scale bar = 100 nm.

4.1.2. Hydrodynamic Properties of NPs

Hydrodynamic characterization was performed on the TiO₂ and ZnO NPs using dynamic light scattering (DLS) to understand their behavior when suspended in the cell culture medium. The results demonstrated that TiO₂ NPs suspended in ultrapure water aggregate with an average diameter of 169 nm (**Table 4.1**). Nonetheless, when the TiO₂ NPs were suspended in HEK cell culture medium, EpiGRO, an observation of formation of larger aggregates with an average diameter of 244.8 nm was made (**Table 4.1**). Similar increase in hydrodynamic diameter was observed in the study documented by Ji *et al.*, where TiO₂ NPs suspended in deionized water showed an average diameter of approximately 200 nm as compared to TiO₂ NPs suspended in various cell culture mediums and showed an increase in average diameter of 800 to 1000 nm [6].

The increased in hydrodynamic diameter was also observed in the case of ZnO NPs in which agglomerate size was detected to increase from average diameter of 367.6 nm in ultrapure water suspension to 598.2 nm in cell culture medium suspension (**Table 4.1**). The same phenomenon was observed in a study presented by Hsiao and Huang where ZnO NPs

dispersed in deionized water demonstrated an average diameter of 279 nm as compared to ZnO NPs dispersed in cell culture medium showed an increase in average diameter of 936 nm [7]. This increased in hydrodynamic diameter of the NPs could be attributed to the biomolecules such as proteins present in the cell culture medium. These proteins would get adsorb onto the NPs surface and formed a protein corona, increasing the overall NPs diameter [8]. In a study conducted by G. U. Nienhaus *et al.*, among the different types of proteins tested, the formation of protein corona led to an increase in NPs size which was measured using fluorescence correlation spectroscopy (FCS) [9]. The thickness of protein corona was dependent of the protein orientation adhered to the NPs. The protein corona formed around the NPs would affect the cellular interaction and in turn caused potential adverse effects.

In the cell culture medium, the presence of large number of biomolecules could interfere with the hydrodynamic size measurement of the NPs. In a study conducted by R. Yusoff *et al.*, TiO₂ NPs when suspended in BSA containing medium, formed a translucent layer of material about 5nm on the surface of NPs. This was observed under TEM and was attributed to protein corona formation [10]. Therefore, an additional step, centrifuging of NPs suspended in cell culture medium was included to remove the unbound proteins that could interfere with the NPs' reading with DLS. TiO₂ and ZnO NPs were at first suspended in cell culture medium and afterwards, centrifuged to collect the NPs with protein corona before resuspending the NPs into ultrapure water. TiO₂ NPs was observed with an average diameter of 189.7 nm whereas ZnO NPs was showed with an average diameter of 415.5 nm (**Table 4.1**). These hydrodynamic diameters were in between the diameter of NPs suspended in ultrapure water and cell culture medium, suggesting the removal of loosely bounded proteins NPs surface while those strongly bounded proteins were retained.

An observation of protein adsorption onto the NPs surface was made due to the change in ζ -potential reading where the NPs adopted the protein charge. TiO₂ NPs suspended in ultrapure water were observed with a positive charge of +9.24 mV whereas TiO₂ NPs dispersed in cell culture medium were observed with a negative charge of -10.3 mV (**Table 4.1**). This observation was similar with the results reported by Ji *et al.*, where TiO₂ NPs

dispersed in ultrapure water showed a positive charge of +30 mV as compared to a negative charge of -7.4 to -14 mV when TiO₂ NPs were suspended in various cell culture mediums [6]. The same trend was observed for ZnO NPs, where ZnO NPs demonstrated a positive charge of +13.8 mV in ultrapure water as compared to a negative charge of -11.8 mV in cell culture medium. Similarly, ZnO NPs were reported with a positive charge of +25.9 mV in ultrapure water while registering a negative charge of -2.4 mV in cell culture medium [7]. Both TiO₂ and ZnO NPs carried a negative charge of -24.8 mV and -16.1 mV respectively after centrifuging and resuspending in ultrapure water. This negative charge was attributed to the protein corona that was formed on the surface of NPs after removing the free proteins and salts in the medium. Upon comparison between the NPs suspended in ultrapure water and resuspension in ultrapure water, TiO₂ and ZnO NPs suspended in ultrapure water gave a zeta potential of +9.24 mV and +13.8 mV, respectively. After resuspension in ultrapure water, these NPs gave a zeta potential of -24.8 mV and -16.1 mV, respectively. The increased in negative charges suggested adsorption of proteins onto the surface of NPs. This adsorption has led to a change from positive surface charged NPs to a negative charged protein layer surrounding the NPs. With this adsorbed protein layer on the NPs, electrostatic repulsion force between the NPs has increased, and that prevented agglomeration of NPs, forming a stable NP colloidal.

Table 4. 1: Summary of hydrodynamic properties of TiO₂ and ZnO NPs.

Dispersant	TiO ₂ NPs		ZnO NPs	
	Hydrodynamic Size (nm)	ζ-potential (mV)	Hydrodynamic Size (nm)	ζ-potential (mV)
Ultrapure Water	169 ± 16.31	9.24 ± 2	367.6 ± 26.21	13.8 ± 0.611
Complete EpiGRO™ Cell Culture Medium	244.8 ± 3.983	-10.3 ± 0.641	598.2 ± 28.48	-11.8 ± 0.404
Resuspension in Ultrapure Water	189.7 ± 2.146	-24.8 ± 0.361	415.5 ± 55.57	-16.1 ± 0.321

4.2. NPs Induced Biological Responses

4.2.1. NPs Induced Oxidative Stress

One of the common mechanisms of NPs toxicity are oxidative stress that arises from the production of ROS and free radicals. Metal oxide-based NPs induced ROS in the cells, which led to a state of imbalance between the ROS and antioxidant molecules which resulted in oxidative stress [11]. Wright *et al.* and Sharma *et al.* reported that TiO₂ and ZnO NPs were capable of inducing oxidative stress in human keratinocyte cell line [12], [13], resulting in subsequent cell death. Thus, the oxidative status of TiO₂ and ZnO NPs treated primary human epidermal keratinocytes were investigated by quantifying the production of ROS in the cells. This was done by measuring the intracellular ROS in the treated keratinocytes upon 12 hours exposure to TiO₂ and ZnO NPs. CellROX green reagent was utilized as the dye shows weak fluorescent signal at non-oxidized state and would only exhibit bright green photostable fluorescence that binds to DNA of the cells upon oxidation by ROS. Therefore, the oxidative stress in keratinocytes could be examined through the measured fluorescence signal intensity.

As shown in **Figure 4.2A**, the generation of intracellular ROS on TiO₂ and ZnO NPs treated keratinocytes denoted an increased in the intracellular ROS production by 1.21-fold to 1.55-fold following exposure to TiO₂ and ZnO NPs for 12 hours. An approximately of 1.37-fold to 1.46-fold increase in generation of ROS were observed in ZnO NPs at 0.1 µg/ml to 1 µg/ml treated HEK. Introduction of TiO₂ NPs at 10 µg/ml to 100 µg/ml to HEK induced an increase of 1.25-fold to 1.32-fold in ROS generation. TiO₂ NPs at 500 µg/ml induced an increase of 1.43-fold while ZnO NPs at 10 µg/ml and 25 µg/ml caused the ROS levels to increase by 1.49-fold and 1.55-fold, respectively. This result is in good agreement with the previous studies documented by Gautam *et al.*, where TiO₂ NPs at 100 µg/ml and 500 µg/ml induced an increase of 1.5-fold and 2-fold respectively while ZnO NPs at 10 µg/ml and 25 µg/ml caused the ROS levels to increase by 1.5-fold and 2.5-fold respectively in primary HEK [14].

In addition to measuring the direct intracellular ROS production, cells oxidative status could also be determined by the byproducts of the oxidation process occurring in the cell. HEK exposed to TiO₂ and ZnO NPs for an extended time of 24 hours were observed to undergo an extensive lipid peroxidation process in which the free radical species oxidize the cell membrane lipids. This process leads to the formation of oxidation products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). 4-HNE reacts rapidly with thiols and amino groups which makes it a cytotoxic product that could exert a genotoxic effect in humans [15]. 4-HNE is considered as one of the major producers of oxidative stress and a dominant lipid peroxidation product, thereby commonly used as bioactive marker for lipid peroxidation [16]. Keratinocytes treated with NPs were exposed to 4-HNE antibody and immunofluorescence (IF) staining was utilized to facilitate visual observation and quantification of 4-HNE to probe the effects of TiO₂ and ZnO NPs on lipid peroxidation.

For the positive control, HEK were treated with tBHP (200 μ M) for 4 hours. tBHP is a well-known inducer for oxidative stress in HEK and has been widely used as a positive control in oxidative stress-related studies [17]. tBHP-treated HEK (**Figure 4.2**) showed the presence of punctate signals. These signals have indicated the formation of 4-HNE in HEK following exposure to tBHP. Indeed, as depicted in **Figure 4.2**, lipid peroxidation was depicted in the HEK treated with ZnO and TiO₂ NPs. 4-HNE IF (**Figure 4.2B**) displayed the presence of punctate signals following NPs treatment for 24 hours and these signals were absent in the case of untreated HEK. Quantitative analysis of the IF images indicated that NPs exposure led to a dose dependent increase of 4-HNE punctate signals (**Figure 4.2C**). This is in good agreement with the intracellular ROS measurement (**Figure 4.2A**) as increase in intracellular ROS generation was perceived following the administration of NPs onto HEK. An approximately 5.5-fold and 6.5-fold increase in formation of 4-HNE were observed in ZnO NPs at 10 μ g/ml and 25 μ g/ml treated HEK, respectively. Introduction of TiO₂ NPs at 10 μ g/ml and 100 μ g/ml to HEK induced an increase of 3.34-fold and 4.08-fold, respectively. This indicated that ZnO NPs caused a higher level of oxidative stress in HEK as compared to TiO₂ NPs. The increased formation of 4-HNE suggested that NPs treatments increased the production of ROS which in turn, increased

the oxidation of cell membrane lipids. Overall, both intracellular ROS production and 4-HNE formation strongly suggest that both ZnO NPs and TiO₂ NPs treatments induced oxidative stress in HEK.

Figure 4. 2: NPs treatments induced oxidative stress in HEK.

(A) Intracellular ROS generated in TiO₂ and ZnO NPs exposed HEK for 12 hours. HEK treated with tBHP (200 μ M, 1 hour) served as positive control. Data represents means $\pm$ SD (n=3), Two tailed Student's t-test, * $p < 0.05$, compared with untreated control. (B) Oxidative stress in HEK upon 24 h exposure of ZnO and TiO₂ NPs was ascertained through immunofluorescence staining of lipid peroxidation marker, 4-HNE (red puncta). Cell nuclei were visualized in blue while the actin filament was visualized in green. Scale bar = 100 μ m. (C) Quantitative analysis of the IF images, which showed an increase in the number of 4-HNE positively stained cells following ZnO and TiO₂ NPs treatment. Data represent means $\pm$ SD (n=150), Two tailed Student's t-test, * $p < 0.05$, compared with untreated control, # $p < 0.05$, compared with the paired group.

4.2.2. NPs Induced DNA Damage

DNA damage, another form of NPs toxicity eventualized due to oxidative stress. Numerous studies have demonstrated that induction of ROS following NPs treatments could lead to oxidative DNA damage in *in vitro* and *in vivo* models [18]. Metal oxide NPs, for example ZnO NPs could dissolved in the extracellular environment or be internalized by the cells. Upon internalization, ZnO NPs dissolve into Zn²⁺ ions in the acidic lysosomal environment. Once Zn²⁺ ions entered the cells, it induced ROS. ROS would attack the DNA, resulting in DNA damage in the form of double strand breaks in the cells and activated the γ -H₂AX pathway [19]. γ -H₂AX is used as a biomarker for DNA damage to monitor double strand breaks of DNA. Induction of γ -H₂AX occurred initially upon the onset of DNA damage and the signals could be detected within minutes [20]. This has led to the increasing use of γ -H₂AX in various studies to assess DNA damage.

For the positive control, HEK were exposed to UV for 15 minutes. UV has been well-documented to induce DNA damage in cells and skin [21] which has the potential to cause skin cancer. UV-exposed HEK (**Figure 4.3**) showed the presence of punctate signals in the nuclei of cells. These signals have indicated the double strand breaks in HEK following exposure to UV. Undoubtedly, as illustrated in **Figure 4.3**, DNA damage was depicted in the HEK treated with ZnO and TiO₂ NPs. γ -H₂AX IF (**Figure 4.3A**) exhibited the presence of punctate signals following NPs treatment for 24 hours and these signals were absent in

the case of untreated HEK. Quantitative analysis of the IF images indicated that NPs exposure led to a dose dependent increase of double strand breaks (**Figure 4.3B**). This result was supported by a previous study conducted by Sharma *et al.* where ZnO NPs induced a dose dependent increase of DNA damage in HEK. A tail length of 26.37 to 70.46 μm was observed for HEK treated with 4 to 10 $\mu\text{g/ml}$ ZnO NPs [22]. An approximately 4.8-fold and 7.04-fold increase in DNA double strand breaks were observed in ZnO NPs at 10 $\mu\text{g/ml}$ and 25 $\mu\text{g/ml}$ treated HEK, respectively. This indicated that ZnO NPs were internalized by HEK and elicited a genotoxic response by the dissolution of ZnO NPs into Zn^{2+} ions in HEK. Introduction of TiO_2 NPs at 100 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$ to HEK induced an increase of 4.03-fold and 4.97-fold, respectively. This suggested that TiO_2 NPs were uptake by the HEK and triggered DNA damage within the cells. Overall, TiO_2 and ZnO NPs treatments induced DNA damage in the form of double strand breaks in HEK.

Figure 4. 3: NPs treatments induced DNA damage in HEK.

(A) DNA damage in HEK upon 24 h exposure of ZnO and TiO₂ NPs was ascertained through immunofluorescence staining of DNA damage marker, γ -H₂AX (green puncta) in the cell nuclei (blue). Scale bar = 100 μ m. (B) Quantitative analysis of the IF images, which showed an increase in the number of γ -H₂AX positively stained cells following ZnO and TiO₂ NPs treatment. Data represent means $\pm$ SD (n=150), Two tailed Student's t-test, * $p < 0.05$, compared with untreated control, # $p < 0.05$, compared with the paired group.

4.2.3. NPs Induced Autophagy

Autophagy is one of the mechanisms associated to nanotoxicity as NPs has been demonstrated to damage the lysosomes. Lysosomes would engulf the NPs and caused destabilization of lysosomal membrane [23]. NPs have been reported to be able to both induce and block the autophagy process. Engineered NPs induced oxidative stress has been reported to induce activation of autophagy pathway to remove the intracellular oxidative

byproducts [24]. In contrast, Au NPs were reported to block autophagic flux [25]. During autophagy, LC3-I proteins is covalently conjugated to phosphatidylethanolamine (PE) found on cell membranes to form LC3-II [26], which subsequently binds to autophagosome. Considering the pivotal role LC3-II protein in the autophagy, it was chosen an autophagic biomarker in this study.

For the positive control, HEK were treated with rapamycin (200 nM) for 24 hours. Rapamycin has been widely utilized as a positive control for autophagy in various studies and known to induce autophagy [21, 27]. Chloroquine was added to all treatment groups in the last 4 hours of treatment. It was added to inhibit the fusion of autophagosome with lysosome thereby, preventing the degradation of LC3-II. Rapamycin-treated HEK (**Figure 4.4**) showed the presence of punctate signals in the cells. These signals have indicated the formation of autophagosome in HEK following exposure to rapamycin. As shown in **Figure 4.4**, autophagy was detected in the HEK treated with ZnO and TiO₂ NPs. LC3-II IF (**Figure 4.4A**) demonstrated the presence of punctate signals following NPs treatment for 24 hours and that were absent in the case of untreated HEK. Quantitative analysis of the IF images indicated that NPs exposure led to an increase of formation of autophagosomes (**Figure 4.4B**). Similarly, a study conducted by Zhao *et al.* showed TiO₂ NPs induced an increase of autophagy in HEK. Onset of autophagy was observed in HEK exposed to 100 fg/ml TiO₂ NPs [28]. An approximately 2.21-fold and 2.38-fold increase in formation of autophagosomes were observed in TiO₂ NPs at 10 µg/ml and 100 µg/ml treated HEK, respectively. Introduction of ZnO NPs at 10 µg/ml and 25 µg/ml to HEK induced an increase of 1.98-fold and 2.07-fold, respectively. These suggested that HEK had internalized TiO₂ and ZnO NPs, inducing a stress response within the cells and hence, activated autophagy as a survival mechanism.

Besides detecting the presence of autophagosomes, the measurement of autophagy level in NPs exposed keratinocytes was also conducted. This was done by measuring the autophagy vacuoles present in the treated keratinocytes upon 24 hours exposure to TiO₂ and ZnO NPs. Monodansylcadaverine was utilized as the dye is a fluorescent compound that would embed into autophagy vacuoles through interaction with the membrane lipids, providing a

direct detection of autophagic vacuoles. Thus, the autophagy level in keratinocytes could be determined through the measured fluorescence signal intensity.

As shown in **Figure 4.4C**, the autophagy level on TiO₂ and ZnO NPs treated keratinocytes denoted an increased in the production of autophagic vacuoles by 1.09-fold to 1.22-fold following exposure to TiO₂ and ZnO NPs for 24 hours. An approximately of 1.16-fold to 1.22-fold increase in formation of autophagy vacuoles observed in ZnO NPs at 0.1 µg/ml to 1 µg/ml treated HEK. Administration of TiO₂ NPs at 10 µg/ml to 100 µg/ml to HEK caused an increase of 1.11-fold to 1.17-fold in autophagic vacuoles formation. ZnO NPs at 0.5 µg/ml resulted an increase in the autophagy level by 1.22-fold. This result is in good agreement with the previous studies documented by Zhao *et al.*, where TiO₂ NPs at 10 µg/ml induced an increase in the formation of autophagic vacuoles and LC3-II proteins that bound to autophagosome membranes [28]. Overall, TiO₂ and ZnO NPs treatments triggered autophagy in HEK.

Figure 4. 4: NPs treatments induced autophagy in HEK.

(A) Autophagy induction in HEK following 24h exposure of ZnO and TiO₂ NPs was ascertained through immunofluorescence staining of autophagy marker, LC3-II (red puncta) in the cells. Cell nuclei were visualized in blue while the actin filament was visualized in green. Scale bar = 100 μ m. (B) Quantitative analysis of the IF images, which showed an increase in the number of LC3-II positively stained cells following ZnO and TiO₂ NPs treatment. Data represent means $\pm$ SD (n=150), Two tailed Student's t-test, * $p < 0.05$, compared with untreated control. (C) Autophagy induction monitored through monodansylcadaverine (MDC) which stains autophagic vacuoles produced following HEK exposure to TiO₂ and ZnO NPs for 24 hours. HEK treated with Rapamycin (200 nM, 24 hours) served as positive control. Data represents means $\pm$ SD (n=3), Two tailed Student's t-test, * $p < 0.05$, compared with untreated control.

4.3. NPs Influence on Wound Healing

4.3.1. NPs Increased Keratinocytes Migration

TiO₂ and ZnO NPs were manifested to induce biological responses such as oxidative stress, DNA damage and autophagy in HEK. These biological responses were due to the interaction between the TiO₂ and ZnO NPs and HEK. TiO₂ and ZnO NPs at different concentrations were seen to induce biological responses on a toxicity level where cell death could be effected with high doses of NPs such as TiO₂ NPs at 500 μ g/ml and ZnO NPs at 25 μ g/ml. This had led to a detrimental effect on cell functionality. Nonetheless, ROS and autophagy are multifaceted physiological regulators and have been demonstrated to be involved in many skin responses, including wound healing [29]. It is therefore interesting to understand the interplay of NPs induced autophagy with wound healing events.

Wound healing is a complex process which involves keratinocyte migration and proliferation to close the wound. Therefore, the effect of the NPs on HEK migration was studied utilizing the in vitro scratch wound model. As shown in **Figure 4.5** TiO₂ and ZnO NP treated HEK exhibited an increased in migration through increased in scratch wound closure. TiO₂ NPs exposed wounds displayed an increased closure rate by 1.11 to 1.36-fold whereas ZnO NPs treated wounds demonstrated an increased closure rate by 1.84 to

2.47-fold. This increase in HEK migration could be attributed to the biological responses observed in **Figure 4.2B and 4.4A**. NPs such as TiO₂ and ZnO entered HEK through endocytosis and upon entering the cells, ROS could be generated from the interaction between TiO₂ and ZnO NPs and cells organelles or dissolution of ZnO NPs. The generation of ROS led to the formation of oxidative by-products which triggered the autophagy pathway to remove the damaged cell organelles and proteins for cell survival [24]. This was dependent on the doses of ROS generated as low doses of ROS could initiate cell survival processes whereas high doses of ROS could trigger cell death [30]. This was concomitant with the increase in autophagy shown in **Figure 4.4C**. The increased generation of intracellular ROS and formation of autophagic vacuoles suggested that low doses of TiO₂ and ZnO NPs treatments were capable of promoting cell migration through the induction of biological responses that were essential for cells survival and development. The positive control used in this study was rapamycin, a known inducer of autophagy. In a study conducted by Zhang *et al.*, autophagy was documented to promote epidermal keratinocytes migration through the accumulation of ROS [31]. Overall, low doses of both TiO₂ and ZnO NPs treatments stimulated HEK migration.

Figure 4. 5: NPs induce increase in HEK migration.

(A) Confluent monolayer of HEK was scratch wounded with a clear pipette tip. White solid lines demarcated the wound boundaries of the monolayer and the migration of keratinocytes were recorded at 0h and 24h post scratching. Scale bar = 100 μ m. (B) Quantitative analysis of the phase contrast images, which shows an increased in the wound closure following ZnO and TiO₂ NPs treatment. Data represent means $\pm$ SD (n=3), Two tailed Student's t-test, * $p < 0.05$, compared with untreated control.

It was hypothesized that autophagy was the driving force for increased migration observed in HEKs scratch wound assay. Since rapamycin is a known inducer for autophagy event in cells, it was used in this study to test the hypothesis. Rapamycin's role in inducing autophagy mediated cell migration and wound healing has been reported by J. Zhang *et al.* (2019) where autophagy contributed to keratinocyte migration [31]. In addition, P. Liang *et al.* (2018) documented that autophagy promoted angiogenesis through increased proliferation and migration of cells [32]. With the enhancement of angiogenesis, the rate of wound healing would increase. To fully understand the mechanism behind the increased

migration of HEKs upon NPs treatments, inhibitor study utilizing chloroquine which inhibits autophagy and N-Acetyl-L-cystine, which acts as anti-oxidant were utilized.

As shown in **Figure 4.6**, upon the addition of chloroquine into TiO₂ and ZnO NPs treated groups, the rate of wound closure decreased significantly. TiO₂ NPs added with chloroquine exposed wounds displayed a decreased closure rate by 1.24-fold whereas ZnO NPs added with chloroquine treated wounds demonstrated a decreased closure rate by 1.62-fold. Comparing the untreated control and control added with chloroquine treated groups, the difference in rate of wound closure was minimal.

Figure 4. 6: NPs added CQ induced a decrease in HEK migration.

Quantitative analysis of the fluorescence images, which shows a decreased in the wound closure following ZnO and TiO₂ NPs treatment. Data represent means $\pm$ SD (n=3), One tailed Student's t-test, * p < 0.05, compared with untreated control, # p < 0.05.

In **Figure 4.7**, NAC was added into TiO₂ and ZnO NPs treated groups to observe the difference in rate of wound closure by blocking the oxidative stress pathway. As depicted in the graph, the rate of wound closure decreased significantly upon the addition of NAC, denoting the effects of oxidative stress in HEKs migration. NAC, an antioxidant reduced the excess generation of ROS in HEKs which was caused by the addition of TiO₂ and ZnO NPs. With the reduction of ROS generation, HEKs migration decreased through decreased in scratch wound closure. TiO₂ NPs added NAC exposed wounds displayed a decreased closure rate by 3.03-fold whereas ZnO NPs with NAC treated wounds demonstrated a decreased closure rate by 3.62-fold. Overall, through this inhibitor study, oxidative stress was the contributing factor to HEKs migration. NPs induced oxidative stress led to an increased rate of wound closure in NPs treated groups.

Figure 4. 7: NPs added NAC induced a decrease in HEK migration.

Quantitative analysis of the fluorescence images, which shows a decreased in the wound closure following ZnO and TiO₂ NPs treatment. Data represent means $\pm$ SD (n=3), One tailed Student's t-test, * p < 0.05, compared with untreated control, # p < 0.05.

Focal adhesions (FA) are the cells' contact points with the substrate on which they attach, move and grow. Cell adhesions are required for the regular cell processes such as migration, proliferation and differentiation [33]. Uptake of NPs by cells has been demonstrated to decrease cell adhesions that lead to sparsely connected actin filaments [34]. Vinculin is used as a biomarker for adhesion as it is one of the major focal adhesion complex proteins that bind the actin filaments of cells to the substrate. It has been known that cells migrate by modulating the number and the size of these FA complexes [35]. It is hypothesized that the NPs induce the destabilization of the FA proteins on HEK, decreasing the cells to adherence to the substrate that in turn help to increase their cellular migration. Therefore, focal adhesion vinculin was examined to ascertain the degree of cell migration.

Immunofluorescence staining (**Figure 4.8**) showed the presence of vinculin clustering (green signals) at the cell periphery. These signals indicated the focal adhesions in HEK under physiological condition where cell adhesions to the substrate are required for the cells to perform the necessary cell functions such as migration and proliferation [36]. It was noted that the untreated HEK displayed the hallmark of mature FA complex that were enlarged and elongated. In comparison, immunofluorescence staining of TiO₂ and ZnO NP treated HEK showed a reduction in focal adhesions expression as evidenced by the decreased in intensity and number of vinculins present in the HEK (**Figure 4.8**). This is in good agreement with the wound closure measurement (**Figure 4.5B**) which shows increased wound closure following the introduction of NPs treatment onto HEK. This indicated that TiO₂ and ZnO NPs resulted in an enhancement of HEK migration through the decreased in focal adhesions expression. However, this was in contrast to the study reported by Tay *et al.*, where TiO₂ NPs were documented to increase the focal adhesions of epithelial cells. This could be attributed to the high doses of TiO₂ NPs used in the study [37]. Overall, the decreased in focal adhesions expression supported the increased migration observed in TiO₂ and ZnO NP treated HEK.

Figure 4. 8: NPs treatments decreased HEK focal adhesions.

(A) Focal adhesion expression in HEK upon 24 h treatment of ZnO NPs and TiO₂ NPs were done with immunofluorescence staining whereby vinculin was visualized in green and cell nuclei were visualized in blue. Scale bar = 10 μ m. (B) Quantitative analysis of the fluorescence images shows a decrease in HEK focal adhesion following ZnO and TiO₂ NPs treatment. Data represent means $\pm$ SD (n=30), Two tailed Student's t-test, * $p < 0.05$, compared with untreated control.

In addition to visualizing the focal adhesions of HEK, tubulin could be studied when it comes to understanding the migration of HEK. Microtubules consist of tubulins that are the major constituents in the cytoskeleton which provide the cells their shape and structure. During the migration of cells, the microtubules would rearrange asymmetrically by cells adhesions and contractions to move the cells forward [38]. Microtubules have two different ends, the plus end (β -tubulin) and the minus end (α -tubulin). Autophagy was reported to promote acetylation of α -tubulin which could aid in the migration of cells [39]. α -tubulin is used as the biomarker for microtubules whereas acetylated α -tubulin is used as the biomarker for remodeling of microtubules in cells migration. Increase in the acetylation of tubulin was attributed to increase cell motility. Similarly, the presence of acetylated tubulin was noted on the migrating wound-edge fibroblasts that are orientated towards the cell's leading edge [40]. Thus, the remodeling of microtubules in HEK could be examined through the anti-acetylated α -tubulin staining.

Through IF staining (**Figure 4.9**), increased acetylated α -tubulin expression was noted in positive control rapamycin treated HEK as compared to control group. These signals have demonstrated the remodeling of microtubules in HEK under the migration of HEK. Similarly, increase of microtubules remodeling was observed in TiO₂ and ZnO NPs treated HEK as evidenced by the increase of acetylated α -tubulin intensity (**Figure 4.9**) on the NPs treated HEK. This is in line with the wound closure measurement (**Figure 4.5B**) which showed as increased in wound closure following the administration of NPs treatments onto HEK. This suggests that TiO₂ and ZnO NPs treatment resulted in an increased of HEK migration through the increased in microtubules remodeling. This is in line with the study documented by Lin *et al.*, where ZnO NPs from 0.1 μ g/ml to 10 μ g/ml were proved to significantly remodeled the microtubules structure of epithelial cells [41]. Overall, the

increased in microtubules remodeling induced by the NPs contributed to the increased migration in HEK.

Figure 4. 9: NPs treatments increase HEK microtubules remodeling.

ZnO NPs and TiO₂ NPs treated HEK visualization were done with immunofluorescence whereby acetylated α -tubulin was visualized in green , α -tubulin was visualized in red and cell nuclei were visualized in blue. Scale bar = 100 μ m.

4.3.2. NPs Increased Keratinocytes Proliferation

Wound healing requires the involvement of cell proliferation and migration where cells at the wound edges would have to migrate while cells behind the wound edges would have to proliferate to close the wound [42]. Uptake of NPs could affect the proliferation of cells by exerting detrimental effects on cell cycle progression [43]. TiO₂ NPs were reported to impact cell proliferation negatively in a dose and size dependent manner [44]. Ki-67 is a

nuclear protein that is associated with cell proliferation and hence, is used as a biomarker to detect proliferating keratinocytes.

As depicted in **Figure 4.10**, immunofluorescence staining showed the presence of Ki-67 in the nuclei of HEK. These signals indicated the proliferation taking place in the in vitro wound healing model. In fact, as displayed in **Figure 4.10**, increased proliferation was observed in HEK treated with TiO₂ and ZnO NPs as compared to control. Ki-67 IF (**Figure 4.10**) evinced the increased occurrence of Ki-67 following NPs treatment for 24 hours. This is in line with the wound closure measurement (**Figure 4.5B**) as increased in wound closure was observed following the introduction of NPs treatments onto HEK. This suggests that TiO₂ and ZnO NPs led to an increased HEK proliferation which could contribute to the increase of wound healing rate. This is in good accordance with the study done by Botelho *et al.*, where TiO₂ NPs at low doses from 20 µg/ml to 150 µg/ml were shown to increase the proliferation of human gastric epithelial cells [45]. In summary, NPs induced proliferation accorded the HEK with increased wound healing capacity.

A

Figure 4. 10: NPs induced increase in HEK proliferation.

(A) HEK proliferation following 24h exposure of ZnO NPs and TiO₂ NPs were done with immunofluorescence. Proliferation marker, Ki-67 was visualized in red, actin filaments were visualized in green and cell nuclei were visualized in blue. Scale bar = 100 µm. (B) Quantitative analysis of the fluorescence images, which shows an increase in the HEK proliferation following ZnO and TiO₂ NPs treatment. Data represent means $\pm$ SD (n=3), Two tailed Student's t-test, * $p < 0.05$, compared with untreated control.

4.4. Summary

Overall, TiO₂ and ZnO NPs were able to interact with skin cells, HEK and resulted in a series of biological responses including oxidative stress, DNA damage and autophagy. The biological responses induced were dependent on the dose of NPs administered to the HEK. At high dosage of NPs, HEK exhibited cytotoxicity which was able to lead to cells death. However, at low dosage of NPs, these biological responses were able to impact the HEK positively. This was attributed to a dose-response relationship where low doses of NPs

provide stimulation and high doses of NPs demonstrate inhibition [46],[47]. Under low doses of TiO₂ and ZnO NPs, the migration and proliferation of HEK were enhanced which suggested that re-epithelization could be achieved at an increased rate. These NPs have demonstrated the possible increase rate of wound healing in a 2D study.

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Chapter 5

Conclusions and Recommendations

To date, TiO₂ and ZnO NPs penetrating through a disrupted skin barrier has not been widely studied. Most of the studies on TiO₂ and ZnO NPs interacting with human skin cells were done in in vitro and only a handful in in vivo. Thus, more studies should be done on in vivo or ex vivo models to evaluate the potential toxic responses that could arise from NPs cellular interaction and by using these models, representation of NPs in human skin could be done accurately. In the current in vitro studies, most of the wound healing studies were done using high doses of NPs and acute exposure. Thus, it would be interesting to study the effects of NPs on wound healing through low dosage and chronic exposure. Besides studying the effects of NPs on keratinocytes migration and proliferation, fibroblasts could be used as the next skin cell model since fibroblasts are also involved in wound healing. Lastly, the chapter ends off with the positive controls tested on human skin explants. The positive controls were established from the NPs treated keratinocytes and extrapolated to human skin explants.

5.1. Conclusions

This dissertation aims to study the biological response associated to NPs interaction with the skin cells including their effects on induction of oxidative stress, DNA damage, autophagy and wound healing process. With the increase usage of NPs based consumer products, more consumers were exposed to NPs through inhalation, ingestion and dermal penetration. As compared to inhalation and ingestion, dermal penetration of NPs was not studied fully. This brought to the concern of using NPs based skin care products such as sunscreens. Sunscreens incorporated TiO_2 and ZnO NPs to block harmful UV rays from the Sun which led to expose the skin cells to NPs. In this thesis, TiO_2 and ZnO NPs were used to treat the human epidermal keratinocytes for 24 hours to study the induced biological responses and their effects on wound closure. This is different from the nano-bio interaction studies where the effects of high doses treatment of NPs on keratinocytes cytotoxicity and functionality were reported. This study serves to highlight the importance of low doses treatment on keratinocytes which is more relevant to the real-world scenario and its influence on the functions of keratinocytes. This provides a better understanding of nanosafety and differentiates from the traditional cytotoxicity studies.

A conclusion of TiO_2 and ZnO NPs inducing biological responses such as oxidative stress, DNA damage and autophagy was made through the observation of this study. At high doses of these NPs, the biological responses could cause cells death which indicated the dose-dependent toxicity of NPs. However, low dose exposure of these NPs resulted in no toxicity being observed. Interestingly, low dose NPs exposure lead to increase in HEK migration and proliferation. This was hypothesized to be facilitated by the reactive oxygen species and autophagy modulation in the HEK. Low doses of NPs could initiate low doses of reactive oxygen species that could activate cell survival processes such as autophagy to facilitate cellular signaling including wound healing as shown in the increased migration and proliferation. This had brought the possibility of NPs increasing the rate of wound healing.

5.2. Recommendations

5.2.1. Low Dose and Chronic Exposure of NPs

Most of the NPs associated toxicology studies were done on high doses and acute exposure while only a handful was done on low doses or chronic exposure. In a study reported by Falagan-Lotsch *et al.*, Au NPs were able to induce an adaption in human dermal fibroblasts (HDF) after a chronic exposure. 0.1 nM Au NPs were treated on HDF at the same concentrations but for different exposure periods. For the acute exposure, HDF were exposed to Au NPs for 24 hours, after which, the NPs were removed, and the cells were left to grow for 20 weeks. For chronic exposure, HDF were exposed to Au NPs constantly for 20 weeks. HDF treated with acute exposure showed an increased oxidative stress and inflammation genes expression as compared to chronic exposure. These genes were present in the HDF after NPs were removed for 20 weeks [1]. In another study documented by Wu *et al.*, long term exposure to ZnO NPs at a non-toxic dose of 0.5 µg/ml on human keratinocytes (HaCaT) were able to induce an adaptation in HaCaT. HaCaT were able to better cope with the oxidative stress introduced by these NPs interaction with the cells [2]. This has brought the possibility of chronic exposure to NPs at a low dose could affect the migration and proliferation profile of human epidermal keratinocytes (HEK), resulting in the alteration in the wound healing process.

5.2.2. NPs Effects on Fibroblasts Functionality

HEK and HDF are the major components in the human skin where HEK were located in the epidermis while HDF were located in the dermis. In the process of wound healing, HEK and HDF have to work with one another to produce growth factors that would stimulate the closure of wound. From this study, an observation of TiO₂ and ZnO NPs inducing biological responses upon interaction with HEK was made. These biological responses were able to impact the HEK positively in terms of migration and proliferation. The same effects would have to be examined in the NPs treated HDF. It would be interesting to study whether TiO₂ and ZnO NPs were able to stimulate similar biological responses upon

interaction with HDF after which, increase the migration and proliferation of HDF. In addition, studying the migration and proliferation, HDF is involved in the production of collagen and ECM, which are pivotal in wound healing process. In a study produced by Han *et al.*, Nitric Oxide NPs were able to accelerate the collagen deposition which demonstrated the ability to increase the rate of wound healing [3]. Another study done by Casco *et al.*, illustrated that Iron Oxide NPs were able to increase the production of ECM through an increased production of collagen IV [4]. Therefore, it would be important to understand how TiO₂ and ZnO NPs could affect the functions of fibroblasts.

5.2.3. NPs Effects on Human Skin Explants

The interaction between TiO₂ and ZnO NPs with HEK have been studied in this study where the interaction has manifested in a series of biological responses such as oxidative stress, DNA damage, autophagy, and wound healing. These biological responses exhibited different cellular responses such as cells death and increased cells migration. However, this study was done on confluent monolayer of cells which does not represent the human skin accurately. The human skin consists of different cell types and growth factors that help the skin to perform its functions. Therefore, it is necessary to study the effects of NPs interaction with the compromised human skin where penetration of NPs could occur. A scenario of compromised skin barrier would be a wound on the human skin. This increased the penetration of NPs into the human skin, giving rise to a direct contact of NPs with the skin cells. This could possibly affect the rate of wound healing.

A preliminary study was conducted on human skin explants where it depicts the human skin accurately. The human skin explants were obtained from the abdomen where the dermis and subcutaneous fats were removed, leaving behind the viable epidermis. In the epidermis, majority of the cells present are keratinocytes. Therefore, treating the 2D cultured HEK with TiO₂ and ZnO NPs would help in understanding what are the biological responses observed after NPs cellular interaction and accurate signals to look out for in 3D human skin explants treated with TiO₂ and ZnO NPs.

5.2.3.1. Migrating Tongue of Wound

Figure 5. 1: Hematoxylin & Eosin Staining of Human Skin Explant.

Keratinocytes in migrating tongue (red arrows) and wound edge (red lines) visualization were done with immunohistochemistry whereby the top purple layer is the epidermis and bottom pink layer is the dermis. Scale bar = 100 μm .

A wound would be generated on 8 mm of viable epidermis biopsies and thereafter, the TiO_2 and ZnO NPs suspensions would be dropped onto the wound. This allows TiO_2 and ZnO NPs to interact with the underlying keratinocytes and observed the biological responses after interaction. The microscopic analysis of skin sections was used to observe the biological responses induced in viable epidermis after exposure to TiO_2 and ZnO NPs for 1 day, 3 days and 7 days.

As illustrated in **Figure 5.1**, the migrating tongue (as shown in red arrows) of the wound could be observed from the microscope. Once the NPs suspensions were dropped onto the wound, the keratinocytes in the migrating tongue and wound edge would be exposed to NPs and thus, induced various biological responses. Therefore, different immunostaining techniques would be required to observe the different biological responses.

5.2.3.2. Oxidative Stress in Human Skin Explants

As mentioned in **Section 4.2.1**, exposure to NPs led to oxidative stress. The reactive functional groups on the surface of NPs would generate free radicals and ROS upon cellular interaction. These free radicals would then attack the cell membrane lipids, forming 4-HNE as the end product. From the results observed in **Section 4.2.1**, tBHP could be used as a positive control for oxidative stress. Thus, increased concentration of tBHP with an extended period of treatment was used to produce the positive control for 4-HNE staining in human skin explants. Human skin explants were treated with tBHP at a concentration of 600 μM for 4 hours to serve as positive control.

The skin biopsy treated tBHP was sectioned into numerous skin sections that could be used for different types of stainings. Prior to 4-HNE staining, the skin sections were stained with hematoxylin and eosin dyes to observe the skin morphology. As represented in **Figure 5.2**, the keratinocytes-containing melanin were present near the basement membrane with a handful of them away from the basement membrane. Melanin is a pigment produced by melanocytes found in the basement membrane. Melanin is transported in melanosomes to the nearby keratinocytes where melanin would aggregate near the surface of keratinocytes. This prevents the genetic information in nucleus of keratinocytes from damaging by the ultraviolet light from the Sun [5].

Figure 5. 2: Hematoxylin & Eosin Staining of Skin Section treated tBHP.

Epidermal cells-containing melanosomes in migrating tongue (red arrows) and wound edge (red lines) visualization was done with hematoxylin and eosin staining whereby cell nuclei were visualized in blue and cytoplasm were visualized in pink. Scale bar = 100 μm .

After skin morphology observation, skin sections were stained with 4-HNE antibody to detect the presence of 4-HNE in the skin. As illustrated in **Figure 5.3**, keratinocytes in the migrating tongue and wound edge were observed to have a brown staining after treated with tBHP. The skin section without tBHP served as a control was detected with no brown staining throughout except for the keratinocytes-containing melanin near the basement membrane. This has to be further validated with another staining color to ensure that the brown staining observed was 4-HNE and not melanin produced by the melanocytes.

Figure 5. 3: Positive control of 4-HNE Staining.

Keratinocytes in migrating tongue (red arrow) visualization were done with immunoperoxidase whereby 4-HNE was visualized in brown dots (colored boxes) in the cell and cell nuclei were visualized in blue. Scale bar = 10 μm.

5.2.3.3. DNA Damage in Human Skin Explants

As documented in **Section 4.2.2**, DNA damage can occur upon exposure to NPs. NPs would be taken up by the cells and interact with DNA. Interacting with DNA could lead to inhibition of DNA replication, DNA base modification and DNA single or double strands break [6]. From the results obtained in **Section 4.2.2**, UV could be used as a positive control for DNA damage. Therefore, increased exposure time to UV was used to produce the positive control for γ -H₂AX staining in human skin explants. An exposure of an hour was done on human skin explants with UV to serve as positive control.

As represented in **Figure 5.4**, the keratinocytes in the migrating tongue and wound edge were observed to have green punctate signals in the nucleus after exposed to UV. The skin

biopsy without exposure to UV served as a control produced tissue section without punctate signals.

Figure 5. 4: Positive control of γ -H2AX Staining.

Keratinocytes in migrating tongue and wound edge visualization were done with immunofluorescence whereby γ -H₂AX was visualized in green punctate signals (white boxes) in the cell nuclei and cell nuclei were visualized in blue. Scale bar = 100 μ m.

Nevertheless, TiO₂ NPs increased the rate of wound healing in rats through increased aggregation of fibroblasts and collagen deposition [7]. This had proved the potential effects of NPs on *in vivo* skin model which accentuated the importance of studying TiO₂ and ZnO NPs interaction with the human skin.

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