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Toxicity of TiO_2 Nanoparticles

*Gohar I. Dar[1,2], Madiha Saeed[1,2], and Aiguo Wu[1]**

[1] *Cixi Institute of Biomedical Engineering, CAS Key Laboratory of Magnetic Materials and Devices, & Key Laboratory of Additive Manufacturing Materials of Zhejiang Province, Ningbo Institute of Materials Technology and Engineering, Chinese Academy of Sciences, 1219 Zhongguan West Road, Zhenhai District, Ningbo, 315201, P.R. China*
[2] *University of Chinese Academy of Sciences, No.19(A) Yuquan Road, Shijingshan District, Beijing, 100049, P.R. China*

CHAPTER MENU

2.1 Introduction

Nanotechnology and nanomedicine are the complementary disciplines having marvelous applications in many areas aimed at the improvement of human life. The development of nanotechnology has resulted in massive production of nanoparticle (NP)-based products that ultimately lead to the rapid development in their applications in diverse areas of life. However, NPs having various interfaces are capable to interact with the biological entities and impair their structure and functions. NPs are able to undergo cellular and molecular interactions in a biological environment. Therefore, the toxicity of NPs is a complicated issue [1–11]. The understanding of the underlying key mechanism of toxicity is of paramount importance in resolving this issue.

Titanium is considered as one of the most abundant and bioavailable element. It is the second most abundant metal as well as the ninth ultimate abundant ingredient in the Earth's surface [12]. Metal oxide-based NPs, especially titanium dioxide (TiO_2) NPs, have been extensively explored for their biomedical applications, including therapeutics and theranostics [5, 13, 14]. Owing to its low toxicity, biocompatibility, long-term photostability, strong oxidizing properties, and ease of

*Corresponding author: aiguo@nimte.ac.cn

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availability, titanium has been immensely utilized [15]. The worldwide production of TiO_2 accounts for 70% of the volume of pigments. The diameter of about 200–300 nm is typically used in various applications; however, small-sized NPs in the range of 10–50 nm are also used in sunscreens, catalysts, etc., since the refractive index is higher at the nanoscale. It is believed to be one of the most commonly used materials in various commercial products, including food, drugs, cosmetics, sunscreens, varnishes, coatings, and paints [16–19]. The estimated annual production of nano-TiO_2 only in the United States is 3800–7800 tons [19].

The photocatalytic killing efficiency of TiO_2 for cancer cells was reported by A. Fujishima et al. [20, 21]. TiO_2 is capable of generating different cytotoxic reactive oxygen species (ROS) (i.e. $O_2^{\bullet -}$, $OH^{\bullet}$, H_2O_2) [22–24]. Chen et al. studied a black TiO_2 (hydrogenated) with a band gap shift of TiO_2 near to1.5 eV [25]. The absorption increment in the near-infrared (NIR) region was verily enhanced, which attained the researcher's interest in black TiO_2 (b-TiO_2) nanomaterials (NMs) [26]. In 2015, pioneers Ren et al. inquired the application of b-TiO_2 in biomedical field as a potential photothermal therapy (PTT) agent [27], and then it was used for synergistic photodynamic therapy (PDT) and PTT [28, 29]. mSiO_2-coated B-TiO_2 core–shell nanocomposites and mesoporous TiO_{2-x} have been reported for NIR-triggered drug release [30, 31]. The potential of TiO_2 NPs to invoke the ROS under irradiation has been employed for PDT and sonodynamic therapy (SDT) in cancer cells [32–35]. The SDT and PDT are efficient and controllable mechanisms to make restrictions in tumor growth and may lead to future promising route. According to researches NP-based therapeutic routes are confident to conquer the confines of traditional treatment methods. However, there are two sides of a coin – the generated ROS might induce oxidative stress and severe toxicity [36, 37]. The exposure of ultraviolet (UV) light also induces severe damage to healthy tissues [38, 39].

Indeed, nanotechnology has a remarkable impact on society. However, there are numerous societal concerns about NP exposure that may derail its promising applications. NPs are entered in the human body by various pathways, including respiratory, digestive, intravenous injection, and dermal pathways. During these pathways, NPs can easily interact with biological systems. NPs may accumulate in the body for a long period or eliminate, depending upon their physiological properties. Moreover, the unexpected invasion of NPs in biological systems may disturb homeostasis, impair signaling pathways, and induce cell death, since biological systems work in coordination.

The major exposure routes of NPs are inhalation, oral, injection, and dermal. The continuous exposure of NPs, especially through inhalation, may cause potential health risks. The long-term exposure of NPs without necessary precautions may result in severe side effects and even mortalities [40]. However, careful studies of nanotoxicology while avoiding premature conclusions are prerequisite to ensure the safety of consumers and workers [41] (Figure 2.1).

NMs properties, size, surface chemistry, and behavior of biomolecules are of paramount importance for their safe biomedical applications. It is imperative to understand various exposure pathways of TiO_2 NPs, their biological effects, long-term behavior, fate, and mechanism of excretion. Therefore, material toxicity and long-term implications should be thoroughly explored [5]. Moreover, it is essential to understand NPs interactions with biological entities and to evaluate the impact of toxicity for their safe implementation [7].

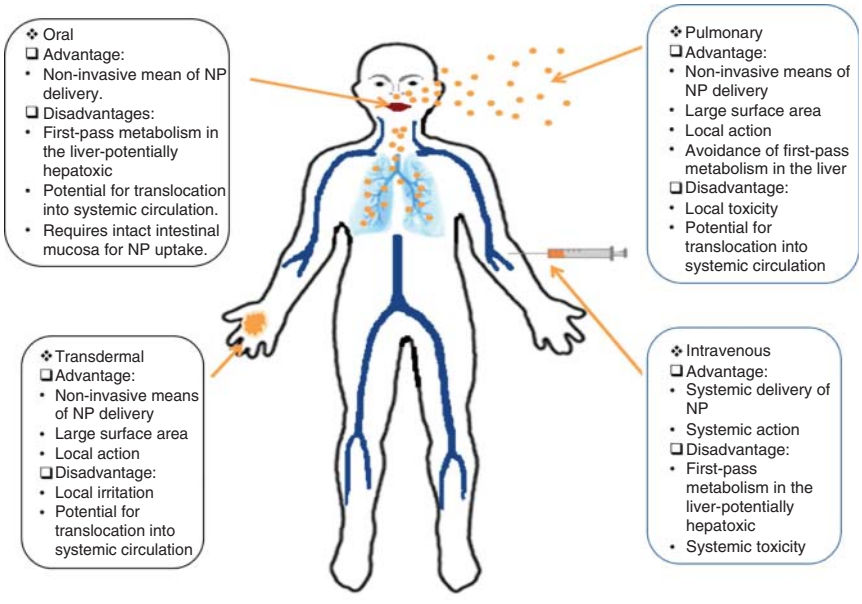

Figure 2.1 Description of various pathways of nanoparticle exposures throughout the body with advantages and disadvantages indications. Source: Reproduced with the permission of Mu et al. [230].

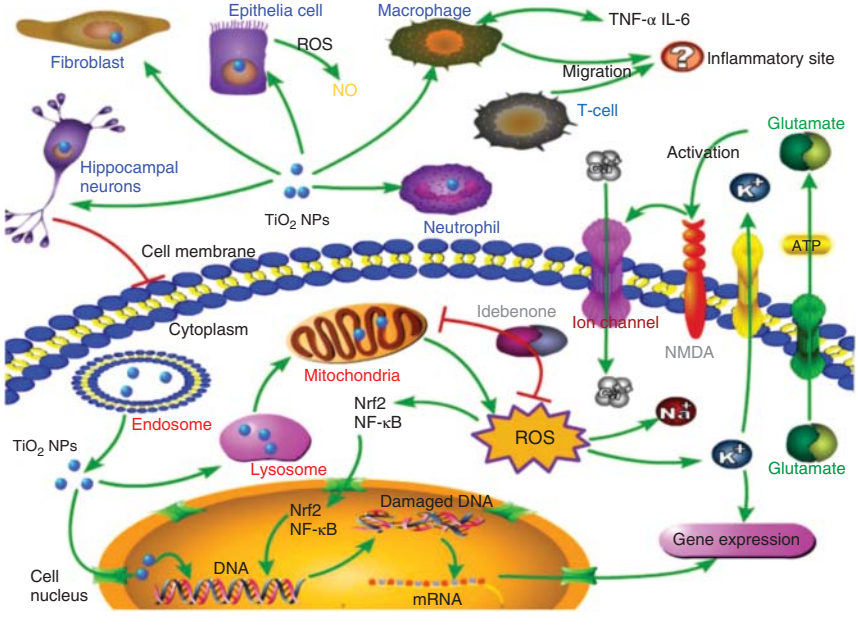

Figure 2.2 Graphical presentation of the toxicity effects in vertebrates and invertebrates through exposure of titanium dioxide nanoparticles. NMDA, *N*-methyl-D-aspartate. Source: Reproduced with the permission of Hu et al. [119].

Realizations of the immense potential of TiO_2 in various applications demand a thorough understanding of their interaction with cells and the ultimate mode of cell death as a product of this interaction. It is imperative to make judgments of the mechanisms of TiO_2-mediated toxicity. Herein, major modes of exposure, biodistribution, and clearance, as well as ultimate mechanisms of cell death under the exposure of TiO_2 NPs, are briefly discussed. Knowledge of the safe applications of TiO_2 NPs and their long-term fates is summarized. The potential toxicity of TiO_2 NPs and its implications are also discussed. The critical points that are highlighted must be considered for safe applications of TiO_2 (Figure 2.2).

2.2 Modes of Exposure, Biodistribution, Clearance, and Fate of TiO_2 NPs

NPs may adopt various routes to enter the human body, including inhalation, ingestion, intravenous injection, skin contact, etc. NPs are systematically distributed to distal organs and lymphatic system, where they interrupt to biological pathways before their clearance from the body. The activity and reactivity of NMs at nano–bio interface is determined by the rate of absorption, distribution, metabolism, and elimination (ADME). NMs are mainly distributed/accumulated in the liver, kidneys, spleen, central nervous system, bone marrow, lymph nodes, etc. [42]. Metal oxide-based NPs are found to be bio-reactive, which trigger various signaling pathways. Among other metal oxides, NPs of zinc oxide are comparatively more toxic, although TiO_2 NPs are also not bio inert [3].

2.2.1 Inhalation

Inhalation, in which lungs are mainly involved, is considered a promising systemic delivery route. NMs are inhaled and deposited in different pulmonary compartments of lungs, depending on their sizes. For instance, the smaller NMs are deeply penetrated into the lungs and may also translocate to other organs, including the brain. NMs are then cleared from different compartments, such as NMs in the gas-exchange region are cleared by alveolar macrophages (professional phagocytes or antigen-presenting cells) and subsequently drained into lymph nodes. Therefore, it is a slow clearance route, whereas NMs are rapidly cleared via the mucociliary escalator. Moreover, inhaled NMs may also translocate into pulmonary epithelial cells and bloodstream [42–44].

The physiochemical properties of NMs and their interactions with the biological interface can decide the magnitude of cellular and molecular responses. In rodents, both microparticles (μP) and nanoparticle adopt numerous transport routes within and out of the lungs, and μPs retain longer on the alveolar epithelium, while NPs can rapidly relocate into interstitial spaces. However, the other species may adopt different transport routes than the rodents [45]. The ultrafine TiO_2 NPs (20 nm) have been shown to stimulate the higher inflammatory response as compared with the comparatively larger particles (250 nm) in the lower respiratory tract of the lungs when exposed for 12 weeks. The higher

pulmonary effect is attributed to larger specific surface area, altered persistence, the increased retention, and interstitial access of ultrafine NPs. Therefore, the particle sizes and surfaces are reported to be more important than the mass of the retained particles [46].

Inhaled TiO_2 NPs (22 nm) appear in different compartments of lung tissue, capillaries, and on the luminal surface of the airways and alveoli. Subsequent translocation of NPs results in its accumulation in major organs of the body (the heart, liver, nervous system, etc.) [47]. The retention time of NPs in different compartments is also dependent on the exposure time and type of compartment in which NPs are trapped. Smaller-sized TiO_2 NPs are easily transported between different compartments/tissues and then translocated into the circulatory system [48]. The studies declare that deposited NPs in the alveoli of lungs can bypass the major clearance mechanisms within 24 hours of aerosol inhalation. The phagocytosed and free particles in alveoli and/or airways are cleared by the larynx [49]. After one hour of intratracheal instillation, about 4% of the delivered dose passes through the air–blood barrier and mainly retained in the carcass (4%). A small amount of TiO_2 NP translocates into the circulatory system, where they accumulate in almost all organs and tissues. From the absorbed/translocated TiO_2 NPs, about 5–20% passes through the gut epithelium and then undergoes to long-term clearance from the lungs via larynx. The persistent fraction of NPs in the lungs is mainly cleared by macrophage-mediated pathways [50].

The fate, as well as clearance of the inhaled NMs, is dependent on the lung compartment in which they are deposited in a size-dependent manner. Large-sized (1–10 μm) NMs are mainly penetrated in the conducting airways with ciliated epithelial cells, including trachea and bronchi, whereas small-sized (<100 nm) NMs are preferentially localized in alveoli. The clearance via mucociliary transport is considered as the fastest route, while the deposition into the bronchial pathways and lung periphery epithelium is a long-term and slow process. The deposited/accumulated NMs are then subsequently eliminated by translocation into the circulatory system and transportation to the draining lymph nodes and/or to the reticuloendothelial system (RES) [42, 49, 51, 52].

2.2.2 Oral Route

Oral route or ingestion is the most recommended path for drug administration as it extend the greatest degree of patient acquiescence [53]. Frequent consumptions of NMs impose toxicity in the gastrointestinal tract and various organs. NMs, including TiO_2, are capable to traverse the intestinal epithelial barrier and circulate into blood, resulting in accumulation in major organs over the long term [54]. Titanium is frequently ingested as an additive in various daily routine stuffs like in personal care, food, and in various consumer credentials. Food-grade TiO_2 (E171) is a major content of many products, such as sweets, candies, and chewing gums. Sunscreens and toothpaste are the main personal care products that contain up to 10% titanium by weight. Moreover, titanium is one of the major components in deodorants, shampoos, and shaving creams as well as in pharmaceutical products [53].

The biokinetic/biodistribution patterns significantly vary if the administration route is different. Most of the NPs are cleared by fecal route after oral administration of same TiO_2 NPs. It also results in its absorption (0.6%) in the intestinal membrane, a fraction of titanium (>0.001%) found in major organs after seven days. Therefore, NPs may subject to chronic exposure, due to its slow clearance, accumulation, and systemic circulation [55]. Size of particles play a main role in toxicity; therefore, TiO_2 NPs with size 25 and 80 nm are claimed to be more toxic than particles of 155 nm. NPs are absorbed by the gastrointestinal tract and transported to tissues and organs, where they gathered in the liver, spleen, kidneys, and lung tissues and may lead to nephrotoxicity and hepatic injury [56]. The extent of response and toxicity of NPs may also be affected by age. Young rats are found to be more susceptible to oral toxicity than adult rats. The liver and heart are more affected in young rats, while the liver and kidney are slightly affected in adult rats, suggesting that TiO_2 NPs may selectively target the liver even after oral exposure. NPs are mainly located in the mucosa of the stomach and small intestine, and the rate/extent of absorption from the gastrointestinal tract is found to be very low, Therefore, TiO_2 NPs may not be translocated into the circulatory system [57].

Inhaled or ingested NMs are excreted via mucociliary clearance pathways, in which they pass from the mouth to laryngopharynx, esophagus, which subsequently transports swallowed NMs into the stomach. NMs that are entrapped by hepatocytes are generally excreted to the intestines via bile and then may be translocated across the intestinal barrier or cleared from the body via feces [58]. Ingested NMs can be discharged as feces and urine via the gastrointestinal route. However, the fate of NMs is dependent on their physicochemical properties. Finally, NMs are released into the environment and may interact with living organisms [53]. It seems that the absorption of NPs across the gastrointestinal epithelium and systemic circulation greatly influence the fate of ingested NPs. The ingested NPs may interact with various biological pathways that cause potential toxicity.

2.2.3 Injection

The direct injection of NMs is considered as the rapid delivery route, such as intravenous, intratumor, and subcutaneous injections. TiO_2 (23.5 nm) can induce endothelial cell leakiness, which is even independent of apoptosis and oxidative stress and is attributed to intracellular signaling cascades. Moreover, injections of TiO_2 in mice results in leakiness of subcutaneous blood vessels [59]. Intracellular processing and uptake mechanism, including endocytosis initiate within four hours of NPs exposure/incubation and particles may release into the cytoplasm up to 48 hours [3]. Exposure of TiO_2 NPs may impair cell membrane, resulting in a disruption in cell adhesion, proliferation, and migration capabilities. It may also trigger signaling pathways, induce toxicity [9, 60] and immunogenicity [61], affect cytoskeleton structures [62, 63], and impair major organs, including the liver, kidney, lung, spleen, and brain [64], thus, impede its biomedical application.

Intravenously injected NMs are mainly cleared via the liver, kidneys, or RES. The distribution and fate of the NMs are dependent on the physicochemical properties. However, after subcutaneous and intratumor injection, the interstitial

lymphatic flow rate at the tumor site is also a contributing factor. Physicochemical properties, especially size, are one of the major parameters since small-sized NMs are absorbed into capillaries and rapidly cleared, whereas large-sized NMs are accumulated into major organs or drained into the lymphatic system [42]. Large-sized NPs are usually accumulated in the body for a long period and may clear by hepatic route. Results of a biokinetics study of relatively large-sized TiO_2 NPs (70 nm) showed that highest concentration of titanium is found in the liver (95.5%), 0.4% in the blood, and the detectable amount in almost all organs on day 1. NPs subsequently released via the liver, however, retained in tissues and organs till day 28 [65].

The negligible toxicity of black TiO_2 (b-TiO_2) NPs is reported in recent years, which suggest their biocompatibility nature and great potential in biomedical application [27, 29–31, 66–68]. No significant toxicity, inflammation, tissue damage, pulmonary fibrosis, or necrosis and no change in hepatic and kidney functions suggested nontoxicity nature of TiO_2 [27]. No significant changes in cell morphologies and no obvious cytotoxicity in the liver, epithelial, and brain capillary endothelial as well as in cancer cells intravenous injection suggest their biocompatibility [66]. b-TiO_2 are undertaken by the RES at 24 hours post intravenous injection [28, 66]. For b-TiO_2-based NPs, no obvious cytotoxicity and evident toxic effects against fibroblast and cancer cells are reported [29]. No inflammation, pulmonary fibrosis, and necrosis further suggested no liver and kidney dysfunction [30, 69]. Moreover, rapid excretion of polyethylene glycol (PEG)-coated TiO_2 via urine and feces after 48 hours of intravenous injection is indicative of great therapeutic biosafety [68]. *In vivo* long-term toxicity assessment of up to 90 days detected no obvious disorder and fluctuation in biochemistry parameters of the liver, kidney, brain, etc. Furthermore, the RES is suggested as a clearance mechanism [28].

The liver is mainly involved in the metabolism and clearance of NMs after intravenous administration; however, NMs are processed and eliminated in a size-dependent manner through three main mechanisms, such as a rapid renal clearance (if size <6 nm), a relatively slow hepatobiliary clearance (if size>6 nm), while persistent NPs are subsequently cleared via the mononuclear phagocyte system (MPS) that may take months to years [42, 58].

2.2.4 Dermal Route

The interactions between the skin and NMs, including cosmetic products and airborne NMs, are known as dermal interactions. Skin is consisting of different layers (such as stratum corneum, stratum spinosum, and stratum basale) that make the skin fairly impermeable and a protective barrier against external exposure [70]. TiO_2 NPs are a major component of commercially available sunscreens, owing to their unique properties, including UV scattering and reflecting capabilities. Although, it is generally believed that healthy and intact skin acts as an effective barrier that does not allow NPs to penetrate into stratum corneum. However, skin disrupting conditions (e.g. eczema) may be of serious toxicity concerns due to the increased NPs penetration [18, 71–75]. Therefore, special attention should be paid on safety evaluation before human use.

2.3 Cell Death Pathways Induced by TiO_2 NPs

2.3.1 Apoptosis

Cell death is mainly classified in at least three categories, such as apoptosis, necrosis, and autophagy. There are two main pathways extrinsic and intrinsic against molecular course that can lead to the apoptosis, such as the death receptor pathways and mitochondrial pathway. Various stimuli (e.g. heat, ROS, growth factors, etc.) can activate the intrinsic pathway, whereas the extrinsic apoptosis pathway may be connected to the intrinsic pathway through BH3-interacting domain death agonist (Bid). Upon destabilization of lysosomal membranes, the leakage of lysosomal hydrolases and cathepsins released into the cytosol activates mitochondrial apoptosis or non-apoptotic cell death pathways that allow cell death of apoptosis-resistant cells. Lysosomal membrane permeabilization (LMP) is a potentially lethal process in which the ectopic release of lysosomal proteases activates the additional hydrolases and caspases. The caspase pathway is indirectly activated by the proteolytic activation of Bid, which induce mitochondrial outer membrane permeabilization (MOMP). MOMP is a major checkpoint of both necrotic cell death and apoptotic that leads to caspase activation and release of cytochrome c. However, massive LMP may result in necrosis without caspase activation pathways [76–79]. Intrinsic apoptosis can be induced by a plethora of intracellular stress environments, such as DNA damage, oxidative stress, and many others. ROS act as the main stimulus to activate lysosomal destabilization. As a result, enhanced ROS generation precedes LMP and cell death. Several NMs are capable to stimulate signaling cascades by interacting with biological entities. It is believed that NMs can trigger extrinsic and intrinsic apoptotic pathways; however, NMs-induced intrinsic apoptotic pathways are mediated by increased ROS and oxidative stress. Redox properties of NMs promote the ROS generation, oxidative stress, and bio-related redox mechanisms [6, 60, 80–83].

Oxidative stress can be induced by the generation of ROS, which disturbs the various signaling pathways leading to the restrain of cell cycle, genotoxicity, and mitochondrial dysfunction in different cell lines. Therefore, oxidative stress and inflammatory responses are correlated with TiO_2 NP-induced toxicity [18, 54, 84]. TiO_2 NPs invokes the oxidative stress, DNA damage, and mitochondrial dysfunction are found to be mainly responsible for toxicity in inhalation models, but not frequently observed in intestinal models. However, cell exposure to TiO_2 NP may lead to an increase in intracellular ROS in intestinal epithelial cells. Furthermore, increased free calcium levels, structural disruption in cells, and changes in intracellular electrolytes are observed to be associated with the exposure of TiO_2 NPs [54]. The oxidative stress and pro-inflammatory responses are considered to be linked with size, surface, and concentration of internalized NPs [85, 86]. Exposure of TiO_2 NPs led to increased ROS production, reduced glutathione, induction of oxidative stress, and stimulation of inflammation-related genes. The increased ROS level triggers the activation of signaling pathways that induce caspase-dependent apoptotic death in the human bronchial epithelial cell line [87].

TiO_2 may induce toxicity by internalization of NPs and disturbing various cellular mechanisms. Physicochemical characteristics of NMs play a crucial role for their toxicity, including protein interactions, apoptosis, autophagy, genotoxicity, etc. [88]. TiO_2 NP can produce ROS in bronchial epithelial cells that undergo apoptotic cell death after being exposed with TiO_2 (15 nm) NP. NPs induce lipid peroxidation, destabilization of lysosomal membrane, which in turn release cathepsin B in order to activate caspase for apoptosis [89]. TiO_2 (24 nm) treated cells experience meaningful apoptosis, not dependent of the classical pathways (p53-Bax) [90]. TiO_2 (10–20 nm) induces oxidative stress, genotoxicity, lipid peroxidation, increment in hydrogen peroxide/nitric oxide production, and micronuclei formation in human bronchial epithelial cell line [91].

The decreased glutathione level and increased lipid peroxidation and ROS level, after exposure to TiO_2 NPs, may lead to cell death. ROS generation is the cause of initial mitochondria-mediated apoptosis and genotoxicity in the liver cells of human (HepG2 cells). Up-regulation of pro-apoptotic (Bax) and down-regulation of antiapoptotic (Bcl-2) is attributed to increased p53 expression as a result of TiO_2 exposure, which leads to cytochrome c release and ultimate formation of an apoptosome. Caspase-dependent pathways activation, such as caspase-9 and caspase-3, triggers the cascade of events and cell death [92]. Intragastric exposure of TiO_2 NPs for back to back (60 days) may result in their accumulation in the mouse hippocampus and ROS overproduction that leads to mitochondrial-mediated hippocampal apoptosis. TiO_2 NPs activate caspase-3 and caspase-9 and promote the Bax and cytochrome c while inhibiting Bcl-2 [93]. The expression of Bax, Bcl-2, cytochrome c, and p53 is altered by the exposure of TiO_2 NPs, suggesting the stimulation of intrinsic mitochondrial apoptosis pathway. TiO_2 NPs up-regulate the expression of caspase 9 without any significant change in the expression of caspase 8 and t-Bid in human bronchial epithelial cells [94]. TiO_2 NPs (<100 nm) stimulate ROS generation and oxidative stress in a concentration-dependent manner in mouse fibroblast cells [95].

TiO_2-induced pulmonary toxicity results in alveolar epithelial cell apoptosis. Activation of various signaling pathways, including cell cycle, complement cascades, and chemokines, as well as altered genes expression in an epithelial cell, was observed after being exposed with TiO_2 NPs (19–21 nm) [96]. In apoptosis-resistant transformed cells ($Bak^{-/-}Bax^{-/-}$), TiO_2 NPs (25 nm) induce lysosome-mediated cell death in a dose-dependent manner via apoptotic-independent (Bak/Bax independent) signaling pathway [97]. When exposed to UV irradiation, TiO_2 NPs (<100 nm) can induce caspase-dependent apoptotic cell death through ROS-mediated transcriptional upregulation of the death receptor and pre-apoptotic protein, like upregulation of the FAS (death receptor) and Bax activation (pre-apoptotic protein) in normal human cells [98].

The nature of the NMs and its interaction with cells/tissues/organ is crucial to activating a particular pathway; since the different molecular pathways are activated by the same type of NPs. NMs can trigger various cell death pathways, due to the extent of cellular insult, although it is difficult to declare a comprehensive mechanism for NMs-induced cell death due to conflicting data [84, 99].

2.3.2 Autophagy

Autophagy is critical factor to preserve the homeostasis and survival of cell under stressed circumstances, such as starvation, protein aggregate-induced stress, oxidative stress, and pathogen infection. It is a cytoprotective process to cope with stress that may lead to autophagic cell death in case of increased numbers of autophagosomes (in dying cells). Autophagy mechanism is defined as in which cytoplasmic materials, containing organelles and portions, are wrapped around a flat membrane cistern and form a double membrane vacuole (autophagosome) for degradation. The fusion of autophagosome with lysosomal and/or endosomal results in the formation of autolysosomes and amphisomes, respectively. Then the luminal contents of autophagosomes are digested and degraded by lysosomal hydrolases [100, 101].

It is generally believed that the increased ROS induces oxidative stress; however, it may also stimulate autophagy as a survival pathway, suggesting that ROS plays a vital role in regulating autophagic activity [100]. Autophagy is a fundamental cellular pathway that keeps cellular homeostasis. It is considered as a clearance/degradation mechanism for insoluble NPs, including TiO_2. In human primary keratinocytes, induction of autophagy is indicated as a pro-survival signal for the safe use of TiO_2 NPs. TiO_2-induced autophagy degrades toxic cytoplasmic content and maintains intracellular homeostasis [102, 103]. It is reported as a standard mechanism for rapid clearance of TiO_2 NPs (18 nm), which stimulate the cellular response in a dose-dependent manner without inducting ROS-mediated toxicity and oxidative stress (in human keratinocyte (HaCaT) cells) [104]. TiO_2 NPs (21 nm) can also induce autophagy in human brain-derived endothelial cells [105]. Rapamycin and its analogues activate the autophagy process by inhibiting its suppressor, mammalian target of rapamycin (mTOR). The AMP-activated kinase (AMPK) can sense altered intracellular AMP/adenosine triphosphate (ATP) ratio. Its activation stimulates autophagy while inhibiting mTOR. Therefore, regulation of autophagy is being carried out by various mTOR-dependent and mTOR-independent pathways [100]. After being exposed to TiO_2, autophagy acts as a protective and antioxidant system to relieve the toxic effects by triggering various signaling pathways, such as activation of AMPK and inhibition of mTOR. However, the solubility, surface reactivity, and chemical composition of NPs influence the cellular response and degrees of autophagy [103].

2.3.3 Crosstalk Between Apoptosis and Autophagy

The cell takes the "decision to die" after being exposed to lethal stimulus/inducer. Apoptosis is the default pathway that is initiated in reaction to most stimuli. However, the alternative pathway, autophagy activates as a cell death effector mechanism when apoptotic effectors are inhibited. Autophagy and apoptotic cell death are incompatible with each other when apoptosis inhibitors facilitate autophagy and apoptosis effectors inhibit autophagy [101].The well-regulated processes, i.e. apoptosis and autophagy, are crucial for maintaining homeostasis and development. The relation between the Beclin 1 (the autophagy protein) and

Bcl-2 (antiapoptotic protein) is a crucial point of convergence of the autophagic and apoptotic machinery [106]. Beclin 1 plays a major task in autophagy, also called "programmed cell survival." The "BH3-only members" of Bcl-2 family can promote apoptosis. In contrast to other BH3-only proteins, even an over-expressed Beclin 1 does not play a pro-apoptotic function. Caspase-mediated cleavage of Beclin 1 can stimulate crosstalk between apoptosis and autophagy. In apoptosis, the pro-autophagic activity of Beclin 1 can be inhibited by caspases. Caspase-3, 7, and 8 can cleave Beclin 1 in order to destroy its autophagic potential. Proapoptotic protein, Bax, induce apoptosis by promoting caspase-mediated cleavage of Beclin 1. However, Bcl-XL and non-cleavable Beclin 1 can restore autophagy. On the other hand, caspase-8 that acts as a death receptor–effector can also be cleaved by autophagy and evoked a feedback process. Therefore, apoptosis and autophagy might be activated by the same stimuli and share signaling pathways [107]. There are areas of crosstalk and interplay between apoptosis and autophagy, suggesting coordination between these pathways throughout homeostasis and development [100].

2.3.4 Necrosis

The massive LMP can cause necrosis [108] that are also reported as a mode of cell death after exposure of TiO_2 NPs; however, physiochemical properties, especially size, crystal structure, and exposure time might play the substantial job in order to decide the impact of toxicity and mode of cell death [84, 109, 110]. The overwhelming nanotoxicological studies make it difficult to distinguish between different cell deaths pathways [111]. It seems that lysosomes, dubbed "suicide bags," may conduct a crucial task in NMs-induced cellular reactions and may participate in apoptosis, autophagy, and in regulated necrosis.

2.4 Toxicity of TiO_2

Titanium dioxide is classified in three dominant crystallographic arrangements, i.e. brookite, rutile, and anatase, in which rutile have maximum stability and the last two structures are substantial in environment [112]. Every one of these forms has its own different attributions, environmental influences, and different applications. Besides this, in the company of light TiO_2 have adverse effect on microorganisms through output of ROS [113]. Anatase titanium NPs are employed in the production of some marketable products such as self-cleaning window coatings and sunscreens [114, 115]. Furthermore, commercial rutile titania and anatase exhibit the delivery of superoxide radical and release of H_2O_2 outside the cells of body and make an additional effect on mitochondria membrane that is called hyper-polarization [116]. For illustration, as TiO_2 are capable to take in large amount of UV radiation, the hydroxyl sort of species resigned in aqueous media may be referred to as the cause of essential harm to DNA, consequence of further environmental risks [117]. Hu et al. investigated the various metal oxide NPs *in vitro* cytotoxicity to the subjected organisms, and it was conformed that among

ZnO, Al_2O_3, SnO_2, CuO, Fe_2O_3, and TiO_2, the metal oxide titanium NPs exhibit lower results of toxicity with rising cation charge [118]. On the other hand, ZnO was referred as more extremely toxic among all other metal oxide NPs. Wang et al. investigated that titanium dioxide at nanoscale have great ability of oxidizing and production of free radicals [56]. Here we try to elaborate briefly about the toxicity of titanium NPs (TiO_2) with different aspects.

2.4.1 Cellular Uptake

The characteristics of NPs have great significance regarding toxicity, like their shape, solubility, aggregation, size, crystallinity, surface chemistry, charge, and surface area. These NP features can effectuate the subcellular localization, toxic effects, and cellular uptake [119]. Particularly, there are two ways of cellular uptake of NPs: one is active uptake and the other one is passive uptake, as active uptake is done by the process of endocytosis and the passive uptake is continued by free diffusion process. Geiser et al. investigated that, TiO_2 powders is employed in rats through inhalation, resultantly nano-sized NPs were not cleared but micron-sized particles were fascinatedly removed through alveolar macrophages. As phagocytes usually remove the particles larger than 500 nm [120] so the remaining smaller matter retained and caused adverse effect on tissues and make burden on other cells as well. Subsequently the inhalation liability and the free TiO_2 NPs were located in the cytoplasm of fibroblasts and endothelial cells [47]. Kocbek et al. investigated an *in vitro* study where the 25 nm TiO_2 NPs were uptaken by endocytosis in the human keratinocytes and also confirmed that the summation of NPs is in the amphisomes as well as in endosomes, supporting the endocytotic uptake mechanism [121]. Xia et al. examined the uptake of TiO_2 NPs that are fluorescence labeled and smaller in size <12 nm, and they are identified in the lung endothelial BEAS-2B cells, in the caveolar compartments, and in the late endosomal [122]. Further studies showed that the aggregated titanium NPs (TiO_2 NPs) that are less than 200 nm in size are qualified to access into the red blood cells, which have absence of phagocytic receptors, as long as other considerable size particles have capability to incline on surface of the cells [123].

2.4.2 Oxidative Stress Effectuates by TiO_2

In the case of TiO_2 NPs, the negative biological effects through oxidative stress has been chronic; by that proof it makes an increase in the production of oxidative output and ROS [87, 91, 124, 125]. Among various other adverse effects caused by the NPs, the oxidative stress is considered to be the main process for biological unfavorable effects. As oxidative stress is conveying by TiO_2 NPs in both cases with and without UV radiation. Uchino et al. reported that different sizes and crystalline morphologies have brought on the various measurements of hydroxyl radicals under UV irradiation, and these yield radicals associated to the cytotoxicity upon the ovary cells [126]. Dodd et al. further investigate that the TiO_2 NPs upon UV radiation produced the hydroxyl radicals, which are the dominant reason of the damaging results that respond to contribute carboxyl radicals [127].

In addition, studies showed that without photoactivation the TiO_2 nano-sized particles have also invoke the ROS and related biological unfavorable issues. Gurr et al. reported that the oxidative damage invoked in the human bronchial epithelial (BEAS-2B) cells is the result of combination of anatase and rutile TiO_2 NPs [128]. In another study, Petković et al. analogize the two different nano-sized pre-irradiated and non-irradiated anatase TiO_2 particles and their genotoxicity and cytotoxicity effects [128]. The result revealed that only TiO_2 particles has induced the oxidative damaging of DNA, and on the other hand non-irradiated NPs have only make slightly impact on DNA strand breakage, no aftereffect on cells survival. But after pre-irradiation both samples of NPs revealed same results of DNA breaks and oxidative damage as well as restrict the cell viability results. Kang et al. had confirmed the prompt of inflammation through the function of oxidative stress in TiO_2 NPs [129]. In summary, high concentrations of TiO_2 that induced large amount of oxidative stress can be commanding to cell damage-related reactions, although low level of oxidative stress may cause inflammation that might be energized through ROS activation of signaling tracks.

2.4.3 Genotoxicity

The views about the genotoxicity of TiO_2 NPs is argumentative and not so clear, early researches revealed that in standard assays there was no evidence of genotoxicity [130, 131]. But recently, researchers showed the results about genotoxicity of TiO_2 both in *in vitro* and *in vivo* studies. Some properties of NPs make some impact on genotoxicity of TiO_2 like those particles that are smaller in size showed more genotoxic effect than the larger particles in size instead of whatever their crystalline phase they have. Due to their smaller size, it's a bit easy for them to penetrate in nucleus and cytoplasm for accumulation inside [132]. Another effect agglomerations of TiO_2 NPs are being examined in various studies; whereas smaller sized almost 200 nm showed no invoke on genotoxicity, larger agglomerations showed some DNA damage results in various cell lines [126, 133, 134]. Due to photocatalytic characteristic of anatase TiO_2 NPs, they showed some more unfavorable effects than rutile TiO_2 particles [135]. In recent years, researchers use different test systems both *in vitro*, Ames test, DNA breaks, cell transformation, and micronucleus test, and *in vivo* studies on bone marrow cells of rat to examine the genotoxic effects of TiO_2. Gurr et al. examined different size of anatase TiO_2 NPs and rutile TiO_2 particles where anatase NPs having size about 20 nm showed some evidence of extension in micronuclei structure, while rutile and anatase of 200 nm size did not show any results [136]. In another study, high concentration of TiO_2 NPs is exposed to retinal pigment epithelial cells (RPE-19) and found that the particles were being observed in endoplasmic reticulum [137]. Jugan et al. reported in his work that both anatase and rutile TiO_2 NPs having spherical shape with the size of almost 12–140 nm showed the oxidative stress in A549 cells and invoke oxidative effect and single-strand interruption to DNA [136]. In this study it was also observed that TiO_2 NPs weaken the strength of cells in repairing the DNA through the inactivity of both sequences base excision repair (BER) and nucleotide excision repair (NER). Further studies revealed that TiO_2 NPs substantiate the genotoxicity and cytotoxicity effects in the human

amnion epithelial cells [138]. Other examinations revealed that increased in nitric oxide synthases (NOS) messenger RNA (mRNA) expression and extracellular ROS in NR8383 rat lung is being carried out due to TiO_2 NPs [139]. Bhattacharya et al. compared the effect of cytotoxicity and genotoxicity in the human bronchial epithelial (BEAS-2B) cells and human lung fibroblasts through employment of TiO_2 NPs and found that fibroblasts are more sensory [140]. In this examination, in the human lung fibroblasts, DNA adduct formation, and oxidative stress is being observed rather than DNA breakage by TiO_2 NPs. Trouiller et al. reported the micronuclei formation, signs of DNA double-strand interruption, and invoke of oxidative DNA damage in mice is being examined after oral exposure of TiO_2 by drinking water [141]. In this work authors observed also pro-inflammatory cytokines, and they claimed that for genotoxic effects the yield of inflammatory is accountable. Wang et al. used the comet assay, cytokinesis block micronucleus (CBMN) assay, human lymphoblastoid cells, and hypoxanthine-guanine phosphoribosyltransferase (*HPRT)* gene mutation assay for the finding of genotoxic effects caused by the TiO_2 NPs of 7–8 nm in size for different intervals up to 48 hours [142]. TiO_2 NPs invoke about fivefold increment (65 μg/ml) in tail moment and also increased 2.5-fold (130 μg/ml) in the binucleated cells. While Shi et al. observed in the examination on the BEAS 2B cells, they described that caspase 8/t-Bid pathway is not a cause of induced apoptosis through mitochondrial apoptosis [94]. These outcomes make the sense that effects of TiO_2 NPs are different for different cell lines. In another study conducted on human lymphocytes, it revealed that DNA breakage and invoke of MN configuration are also being carried out by the TiO_2 NPs [143]. The cells that were treated by TiO_2 NPs also showed the creation of ROS. Xue et al. observed the inhibitive characteristic of *N*-acetylcysteine (NAC) regarding the generation of ROS by exposing it to TiO_2 NPs. Falck et al. compared the coated and uncoated anatase and rutile TiO_2 NPs and showed that the uncoated NPs were more productive than SiO_2 coated TiO_2 NPs as regards the unsubstantial induction of micronuclei on BEAS 2B cells [144]. This work is further supported by Manoet al. by showing that rutile TiO_2 NPs revealed low activity in genotoxicity because of coating mechanism [145]. They examined the human acute monocytic leukemia (THP-1) cells and human pulmonary epithelial (NCI-H292) cells with PEG coated TiO_2 NPs and found that the induction stress and related cytotoxic effects were meaningfully decreased. Petkovic et al. studied the effects of genotoxicity with the employment of rutile TiO_2 NPs of <100 nm size (TiO_2-Ru) and anatase NPs of <25 nm size (TiO_2-An) in the human hepatoma HepG2 cells [146]. They revealed that the anatase TiO_2 NPs invoke the oxidized purines and DNA strand breaks. In another test conducted on Caco-2 cells, results revealed that anatase and rutile TiO_2 NPs make meaningful DNA damage and lactate dehydrogenase (LDH) leakage as compared with undiluted anatase TiO_2 exhibited by formamidopyrimidine [fapy]-DNA glycosylase (FPG)–comet assay [147]. Another factor per unit surface area also showed high cytotoxicity results of anatase and rutile TiO_2 NPs. For this purpose, researchers examined the WST-1 assay to reveal the significant mutual relation between cytotoxicity and anatase NPs surface area.

While some results in *in vivo* studies are contrasting in various cases, the investigators used the A549 cells against TiO_2 NPs of about 28 nm size with various

immersions up to 40 μg/ml for 24 h, and results showed no signals of ROS production [148]. In another study, Woodruff et al. revealed the results of uncoated TiO_2 NPs under the circumstances of Comet assays and Ames assays in the thymidine kinase heterozygote (TK6) cells, and no meaningful effects of genotoxicity was observed, and no DNA or oxidative DNA injury observed [149]. Linnainmaa et al. examined both TiO_2 NPs and fine particles (FPs) by employment of MN assays in the rat liver epithelial cells, and results revealed the negative response that there were no primary clastogenic potential [150]. Fisichella et al. showed that in their examination no adverse effects were detected on Caco-2 cells treated through TiO_2 NPs with core of rutile having concentration of 100 μg/ml for various interval up to 72 hours [151].

According to different results by different investigators, many contradictions are existing in the literature regarding to the genotoxicity effects by TiO_2 NPs. In some other studies, both *in vitro* and *in vivo*, various results showed positive response, and some of them reveals negative response, which were recorded by use of different cultured cells, various particle size distribution, and dispersion for illustration [152]. However, where there is some genotoxicity effect shown by the cells, most of them are closely connected with circulatory and respiratory system. Therefore, more research is required to justify the terms and conditions in which the genotoxicity effects of TiO_2 NPs will be examined [153, 154].

2.4.4 Reproductive and Developmental Toxicity

Whereas TiO_2 NPs may penetrate into placenta and fetal tissue through absorption, reproductive toxicity effects in human through exposure of TiO_2 NPs are not yet proven. On the other hand, when other species like abalone embryo and zebra fish are treated by TiO_2 NPs, and results revealed that it inhibit hatching, malformations, and induced impair reproduction [155, 156], although some contradiction is being observed in the case of zebra fish [157]. The research data is not sufficient to define the developmental toxicity effects in the mammals. Hougaard et al. investigated on mated C57BL/6BomTac mice through coated TiO_2 NPs by inhalation mechanism, and average changing is being recorded in the neurobehavioral site [158]. Yamashita et al. examined the pregnant mice intravenously with titanium and silica NPs having size of, respectively, 35 and 75 nm, and results showed some complex effects to pregnancy [159]. In the major organs, fetal brain, fetal liver, and in placenta, TiO_2 NPs were detected. In another work, Komatsu et al. worked on the testis leydig cells of mouse *in vitro* to check the cytotoxicity effects of carbon black and TiO_2 NPs, and results showed the TiO_2 NPs were more cytotoxic [160]. The leydig cells elevate the TiO_2 NPs and resultantly proliferation, and viability effects and gene expressions were observed. Limited research showed the results both *in vitro* and *in vivo*, and the reproductive toxicity was observed by TiO_2 NPs.

2.4.5 Carcinogenicity

There is no satisfactory understanding found about carcinogenicity mechanisms through metal induction. But in the cells, genetic and non-genetic influences

responded by TiO_2 NPs may exhibited the effects of carcinogenicity [161]. In the animal experiments, respiratory tract cancer was observed in subjected rats due to high absorption of TiO_2 NPs of about <100 nm in size with 10 mg/m^3 and TiO_2 FPs with 250 mg/m^3 with almost <2.5 μm size [162]. Heinrich et al. reported the carcinogenic effects caused by TiO_2 NPs of about 15–40 nm in size in rats and determined that NPs exhibited tumorigenic effects at immersion of nearly 10 mg/m^3 with interval of six months. Considering the equal concentrations of both TiO_2 NPs and TiO_2 FPs, the NPs showed more effects of carcinogenicity than FPs in rats. According to this distinction in results of carcinogenicity strength against TiO_2 NPs and TiO_2 FPs, risk evaluations and employment exposures to various diameters of both NPs and FPs should be mentioned [163]. Kuempel et al. determined the unfavorable effects of exposure employments of TiO_2 NPs to health through usage of lung dosimetry miniature [164]. In another study, hydrophilic TiO_2 NPs of about 25 nm size with various concentrations were employed on female rats through intratracheal instillation as interval of one week up to 30 weeks [165]. In order to examine the carcinogenicity and toxicological effects, Bernard et al. employed 5.0% of dietary coated TiO_2 NPs in fed diets of the rats for more than 100 weeks [166]. They didn't find any effects of either carcinogenicity or toxicity even for high dose (5.0%) of TiO_2 coated NPs.

In order to examine the skin cancer effects by TiO_2 NPs, Sagawa et al. conducted experiments on two-stage skin miniature by usage of silicon coated (35 nm) and non-coated TiO_2 NPs (20 nm) in the suspension of silicon oil and Pentalan 408 [167]. After the analysis, results showed that there were no effects of skin cancer monitored because of coated and non-coated NPs owing to no penetrations through skin. In another study, Newman et al. showed no skin carcinogenic effects were observed because TiO_2 were incapable of making an access to enter completely in dermal tissues [73]. Author suggested that further research is needed regarding to safety parameters to use TiO_2 NPs in skin burn conditions and in sunscreens. Pulmonary inquiries endure the carcinogenicity effects of TiO_2 particles through inhalation and intratracheal examinations. As long as dermal exposure revealed opposite results to pulmonary studies, TiO_2 NPs are non-carcinogenetic.

In epidemiological examinations, there were no evidence monitored about the correlation of raised in cancer risk of lungs against occupational exposure of TiO_2 NPs. Hence, more research didn't conduct investigation about the lung cancer risk and particle size of TiO_2 correlation, which raised the dispute about the evaluation of occupational carcinogenicity caused by TiO_2 NPs [141]. In other epidemiological studies without define particle size, no results were found in the support of TiO_2 NPs against lung cancer effects [168, 169]. As per focused studies of epidemiological, there were no relation being concluded of lung cancer symptoms/increased in cancer caused by TiO_2 NPs. Moreover, due to considerable agglomerations of NPs both *in vivo* and *in vitro*, there is a challenge to make safe evaluations of health hazards caused by fabricated NPs [170]. There is correlation existing between decrease in surface area that showed the agglomerations of TiO_2 NPs at an unvarying pH, and it was proven through experiment results [171]. Thus the unfavorable factor oxidative stress is caused by the influence of larger surface area against biological contexture [172]. Meanwhile, all TiO_2 NPs that

have marketable ability ensured their evaluation and managed properly their production and applications against different environment or conditions. At this particular point, risk assessments of TiO_2 NPs cannot be reliably defined due to less reliable data about dose reaction assay as well as on human exposure against TiO_2.

In many experiments conducted on animal models and on various cells, TiO_2 NPs and FPs showed the tumorigenesis, genotoxicity, and cytotoxicity effects [173]. As discussed earlier, mutations, DNA strand breakage, cell transformations, and chromosomal effectuations were included in various studies both *in vivo* and *in vitro*. The lack of balance in cellular redox was examined through oxidative stress in different types of cancer cells. The harmful effects of oxidative stress to cellular DNA can help to initialize the mutations. Further the mutations carried out in the DNA may lead to perform significant task in the growth of different cancer cells.

2.4.6 Immunotoxicity of TiO_2 NPs

The physiochemical characteristics of TiO_2 NPs allowed them to elevate immune cells like monocytes, leukocytes, macrophages, dendritic cells, and platelets. In the study of monoblastoid cell (U937), necrosis and apoptosis effects were invoked by the TiO_2 NPs in absorption comparable to that detected in plasma and blood [174]. Palomäki et al. investigated the silica-coated rutile TiO_2 and uncoated TiO_2 particles on the murine macrophages (RAW246.7) and murine dendritic cells (bm-DC). In their studies, results showed the enrich formulations of different proinflammatory cytokines invoked by TiO_2 NPs in corresponding cells [175]. Meanwhile, TiO_2 particles showed more toxicity in dendritic cells rather than macrophages. As TiO_2 NPs actuated the NLRP3 inflammasome, a sophisticated form of various proteins exist in the cytoplasm can dominant the IL 1β-secretion. There were separated illustration for long-term and short-term liability of neutrophils against anatase TiO_2 particles, as long as longer time viability exhibited the cytokine yielding and apoptosis inhabitation and short-term showed the altering of morphology that leads to move these cells. These assessments support that the TiO_2 NPs are neutrophil Agonist in *in vitro* studies. Moon et al. investigated the factors raised by TiO_2 in mice related to immune reaction, and results revealed the improved in expansion of subcutaneously breed B16F10 melanoma because of factors affecting immune such as macrophages, T-lymphocytes, killer cells, and B-cells [176].

2.4.7 Neurotoxicity of TiO_2 NPs

It was described in early studies that NPs passed through blood brain blockage and use olfactory track to reach in the nervous system [177–179]. In *in vitro* examination, brain microglia cell (BV2) showed the effects of oxidative stress against TiO_2 (Degussa P25) NPs, which further allied to inflammation, cell cycle, and apoptosis and in up and down inhibition of genes associated with energy metabolism [116, 124]. As long as TiO_2 (Degussa P25) showed contrast behavior, no toxicity effects were shown in N27 neurons as compared to BV2 cell, although TiO_2 Degussa P25 NPs exposure to complicated brain cell showed

neuron damaging in short period of time through ROS production. Liu et al. showed the apoptosis and oxidative stress effects against various dose rates by employment of TiO_2 NPs in the neuronal cell PC12 [180]. In another study, silicon coated rutile TiO_2 NPs were employed on mouse, and results showed these NPs have capability of developments of stem cells respecting to neurons [181]. These consequences revealed that TiO_2 NPs responded according to type of cell and intervene of oxidative stress. Scuri et al. conducted experiment on newborn rats through inhalation mechanism of TiO_2 NPs (12 mg/m^3), and results showed the up-regulations of lung neurotrophins articulation, leading to regulatory and sensitivity of neuronal cell growth that have association in asthma [182]. These outcomes were related to elaboration of moderate airway inflammation and airway hyperactivity.

2.4.8 Acute Toxicity of TiO_2

The studies about effects of TiO_2 NPs on humans is not explored widely. The value median lethal dose (LD_{50}) is actually used to determine the dosage outcome to death percentage (50%) of animals during experiments. Meanwhile, in 2002 Organization for Economic Co-operation and Development (OECD) officially ended this testing of acute toxicity rules (TG401), although there are other techniques and rules to examine the acute toxicity like dose–response method (TG425), up and down procedure (TG423), and fixed dose procedure (TG420).

In an inhalation examination, aerosols of TiO_2 NPs up to 50 mg/m^3 are employed on rats via inhalation mechanism for five days with interval of six hours a day [183]. Postmortem examination will be carried out either yet to 3 and 16 days after employment or as soon as after last liability. Lung inflammation is depending on dose to cause in the increment of neutrophil numbers and bronchoalveolar lavage fluid (BALF) cell, enzyme exertion, and overall contents of protein. The proper pathological measurements didn't exhibit the inhibition of systemic induction. In an examination conducted on mice to study inhalation through employment of TiO_2 NPs of size about 2–5 nm, results revealed the large number of alveolar macrophages and number of entire cells in the BALF [184]. Although mice regained its condition after three weeks of exposure, these results revealed the pulmonary inflammation caused by TiO_2 NPs. In another report against TiO_2 particles inhalation, microvascular unhealthy behavior in shoulder arteries were caused through TiO_2 NPs and FPs of size 21 nm and 1 μm [185]. TiO_2 NPs were more effective than FPs with respect to mass. The supplementary vascular condition consociated through particular matter (PM) liability of TiO_2 NPs of 100 nm in size and FPs 710 nm because of neurogenic actuation mechanisms [186]. Furthermore, after 24 hours of inhalation (TiO_2 NPs; 21 nm), low response of coronary arterioles against dilators were observed [187]. It is being observed that less lung burdens didn't make any special change in the BALF regulation against inflammation of lungs. These results are accepted and played a vital role between the cardiovascular complications and PM. Various cardiac complications like change in heart rate, blood clotting, and change in blood pressure can be aftereffect of PM exposure [188]. Liu et al. conducted intratracheal instillation experiment on rats by usage of body weight

(BW) 0.5–50 mg/kg dose of TiO_2 particles [189]. According to histopathological test concerned to lung tissues, the rats were effected by inflammatory lesions that was dependent on dose of TiO_2 NPs. Furthermore, TiO_2 NPs of size 21 nm and of 50 nm invoked less pulmonary toxicity effects than the TiO_2 particles of 5 nm in size. In another study, Kobayashi et al. examined the pulmonary reaction against different interval of 24 hours to 1 week through intra-tracheal instillation of TiO_2 NPs of 19 and 20 nm in size having concentration of 5 mg/ml. The more unfavorable pulmonary disorder effects were observed due to TiO_2 NPs had exposure of 24 hours than one-week exposure. Liu et al. studied the consequences on immune system due to intra-tracheal examination of TiO_2 NPs in the rats [190]. The results revealed the adverse effects on structure of cell and observed the disorder in the function of alveolar macrophages (AM) associated to immune system due to TiO_2 NPs exposure. These consequences were responsible for reduction in immune reaction against particular treatment of 5 and 200 nm TiO_2 NPs. The macrophage phagocytic capability was dependent on dose of TiO_2 NPs; capability was increased for low dose and decreased for high dose. Furthermore, the capability of chemotactic in macrophages were reduced after TiO_2 exposure as well as decreased the Fc receptors articulation on the surface of cell [191].

Warheit et al. investigated the dermal annoyance caused by anatase and rutile NPs (in H_2O) in rabbits and in mice (lymph node assay) for three days, and results showed no effects of skin irritations [152]. Another study results showed no prominent aftereffects of acute eye, vaginal mucous, and dermal irritation in mice caused by TiO_2 NPs exposure with interval of 1–48 hours [192]. In some other studies about toxicity of TiO_2 NPs (20 nm), after two weeks results showed provisory toxicity in Wistar skin of mice [193]. The reduction was observed in the balance of glutathione S-transferase (GST) and catalase activity. Moreover, increment was followed in the exertion of lipid peroxidation and in the lactate dehydrogenase, as well as increased in level of serum glutamic oxaloacetic transaminase creatinine, blood urea nitrogen, and in serum glutamic pyruvic transaminase were observed. Even so, no prominent effects were monitored through histopathological examinations at tissue status. Accordingly, consequences suggested that TiO_2 can induce the renal and hepatic toxicity through short-term dermal exposure in rats. Withal, in the operating skin, the hair follicles can be the spots from where the TiO_2 NPs were able to penetrate.

One-time oral activity of TiO_2 NPs of three different sizes 25, 80, and 155 nm in mice for two weeks were conducted to test the acute toxicity. After two-week exposure of TiO_2 NPs, results showed no significant acute toxicity. On the other hand, large coefficients of hepatic effects were observed in female mice through exposure of TiO_2 NPs of 80 and 25 nm in size. Post-exposure of particles revealed the hepatic renal harm effects through the alteration in pathology of the kidney and liver, as well as in serum biochemical measurements like blood urea nitrogen (BUN), alanine aminotransferase, LDH, and aspartate aminotransferase. Accordingly, systemic toxicity was not substantiating but biochemical alteration was revealed by various studies through oral liability. In nanomedicine, investigation can be done against the usage of TiO_2 NPs through the intraperitoneal examinations. In the study of intraperitoneal liability on

mice, anatase TiO_2 NPs of size 5 nm with high dose revealed severe effects like undo the level of lipid and blood sugar as well as involved in the injury of the kidney, myocardium, and liver [194]. Moreover, tracer functions of the kidney were fall down like BUN and uric acid; tracer functions of the liver like leucine, total protein, albumin levels, pseudo cholinesterase, alanine aminotransferase (ALT), and acid peptide were prominently increased because of TiO_2 NPs high dose. The glucose, lipoprotein cholesterol, and triglycerides content were raised prominently. Furthermore, the intraperitoneal injection of TiO_2 NPs in mice showed the value of LD_{50} was 150 mg/kg BW. TiO_2 NPs of 50 nm in size were injected intraperitoneally in mice with interval of one day up to two weeks and showed the acute behavior in the results like tremor, lethargy, and lack of appetite. The histopathological studies exhibited the lesions' effects due to penetration of certain number of TiO_2 NPs in the spleen. Small increase was also monitored in the aspartate aminotransferase (AST) and ALT levels as well as in the pulmonary vascular network, and thrombosis was detected. In another study by Ma et al., TiO_2 NPs of 5 nm in size showed the behavior of liver toxicity and injury of liver and inflammatory reactions also participating through NPs [195]. The assessment of enzyme-linked immunosorbent assay (ELISA) and real-time quantitative PCR (RT-PCR) through employment of TiO_2 NPs revealed that NPs caused the change in the protein articulation of various inflammatory tracks involving cross-reaction protein, interleukin (IL-4), IL-Iβ, IL-10, macrophage migration inhibitory factor (MMIF), and mRNA [196, 197], subsequently transferring TiO_2 NPs from abdominal cavity. NPs involved in the transformations of certain number of neurons to filamentous morphology remained shifted to inflammatory cells [198]. The intraperitoneally injection of TiO_2 NPs showed the systemic acute toxicity effects concerning biochemical and pathological sequel on major organs: the brain, heart, kidney, and liver.

Li et al. investigated the TiO_2 NPs effects on the erythrocytes, and results showed unpleasant symptoms like unusual hemagglutination, hemolysis, and sedimentation. These results were contrast to the TiO_2 FPs results of same treated cells. Another investigation was carried out through usage of TiO_2 NPs and FPs, outcomes of conducted experiment revealed that TiO_2 FPs exposure to erythrocytes showed the hemolysis 73 times more [199]. Although the plasma is the factor that can abrogate the hemolysis, accordingly *in vitro* state plasma can be helpful to stop hemolysis. In the experiment conducted on mouse macrophages (MH-S) cells, outcomes showed that anatase TiO_2 NPs of different sizes were less toxic [200]. Various studies *in vitro* exhibited the toxicity effects on circulatory cells through usage of TiO_2 NPs.

2.4.9 Sub-acute Toxicity of TiO_2

Rutile TiO_2 NPs of about 40 nm in size had coating of silicon dioxide (SiO_2) expose to lung tissues for two hours up to four weeks, and results of experiments showed the neutrophil attracted chemokine (CXCL 1), tumor necrosis factor (TNF)-α articulation, and pulmonary neutrophilia [201, 202]. Oberdorster et al. conducted the experiment to investigate the relationship between dose amount and TiO_2 particles (NPs and FPs) through intratracheal exposure to mice and rats [203]. The results showed the prominent effects of pulmonary

inflammatory factors that further involved in the increment of activity of LDH, acid α-glucosidase, and protein boost in BALF. The large surface area per mass of TiO_2 NPs was the suggested reason of the high toxicity. In another study Li et al. conducted an experiment on mice through intratracheal implant of TiO_2 NPs of size 3 nm with 12.2 mg/kg BW dose for four weeks (once a week) [204]. The results after four weeks of exposure showed adverse effects in the lungs and alter the alveolar-capillary wall. Furthermore, TiO_2 NPs were accessed to other pulmonary tissues like the kidney and liver through the blood circulation system and caused to injure the tissues up to significant level. The metabolomics assessment and standard methods were employed to investigate the oral toxicity effects against TiO_2 NPs in rats [205]. So by using main elements, 1H NMR and least squares assessments were held to examine the serum and urine. Furthermore, the chemical metabolic examination of urine revealed the increment of balance in citrate, histidine, citrulline, acetate, taurine, phenylacetylglycine (PAG), trimethylamine-*N*-oxide (TMAO), and α-ketoglutarate. Although in the balance of betaine, threonine, leucine, choline, 3-D-hydroxybutyrate, lactate, and pyruvate reduction were reported. The disturbances were showed in their investigations against amino acids, and energy balances may be the factors involved to create little adverse effects to the heart and liver through TiO_2 particles. Accordingly, to authors, to explore the NPs biochemical responses toward various instillations, the metabolomic assessment particularly based on nuclear magnetic resonance (NMR) can be more helpful and trustworthy.

2.4.10 Sub-chronic Toxicity of TiO_2 NPs

To compare pulmonary reactions against TiO_2 NPs, subchronic investigations were carried out on various species [206]. TiO_2 NPs with aerosol (0.5–10 mg/m^3) were employed on hamsters, female mice, and rats and results were observed against different intervals of time up to 13 weeks. After every interval, the selected lung activities, lymph nodes, and lung burdens were examined. Besides other activities lung burdens kept increasing with the control dose, and it showed maximum effects at the exposure closing in all under-examined species. At a point where the lung burdens balanced against TiO_2 NPs, rats showed high adverse inflammatory reactions than in mice. After this, improved fibroproliferative and epithelial alterations were reported. The disposal of TiO_2 NPs (10 mg/m^3) from the mice and rats' lungs were impaired, but in the case of hamsters, any dose concentration showed no significant effect during clearance.

In another investigation, Warheit et al. compared the intratracheal exposure to rats by employment of different TiO_2 NPs and FPs with diverse properties in term of size, crystalline morphology, and surface area [207]. Accordingly, the difference was large through two same size (100 and 25 nm) of particles in terms of surface area as 30-fold, but the reactions in lung inflammation had no significant diversity observed. So results suggested that surface area and size of particles are not the factors that make toxic effects on inflammatory responses. Wang et al. conducted an experiment to explore the effects of TiO_2 NPs of various sizes (25–155 nm) on female mice through intranasal way instillation on monoaminergic neurotransmitters with various interval up to 30 days [153]. To assay the effects of TiO_2 NPs in the area of murine brain, they engaged the

inductively coupled plasma mass spectrometry (ICP-MS). The reversed-phase high performance liquid chromatography (RP-HPLC) were used to make analysis about other neurotransmitters: 5-HT, dihydroxyphenylacetic acid (DOPAC), neurospora endonuclease (NE), homovanillic acid (HVA), domoic acid (DA), and 5-hydroxyindoleacetic acid (HIAA).

2.5 Alternative Perspective

There are reports demonstrating that TiO_2 NPs did not elicit significant biological responses and affect the cell-cycle progression [208], and they induce no significant cytotoxicity and inflammation [103, 197, 209, 210]. Under ambient conditions, it neither induces ROS generation nor oxidative stress in a lung-derived phagocytic cell line. Moreover, any toxic effects are neutralized by antioxidant defense mechanisms [103, 119, 210]. Its exposure does not affect cell morphology, cell proliferation, and survival [208, 211]. The study even reported no evidence of ROS-mediated oxidative stress in human intestinal cells (Caco-2); however, it suggested that the toxic potential is correlated with its surface area and the crystallinity [147]. No significant change in cell contractility and viability in human airway smooth muscle cells was observed after being exposed to TiO_2 (25 nm) [212]. It has also been reported as a biocompatible material for biomedical applications [15, 213–216].

The study reported no evidence of disruption in plasma membrane integrity, mitochondrial activity, and lysosomal function, thus suggesting negligible toxicity of the TiO_2 NPs [217]. Low concentration of TiO_2 NPs (<40 nm) neither show cell death nor disrupt epithelial integrity of intestinal cell lines [218], suggesting that NPs may be less toxic at low concentration [219]. No significant genotoxicity and oxidative stress are found after exposure of ultrafine and small-sized NPs (10 nm) [149, 152, 220].

ROS-induced oxidative stress is correlated with surface defects [221]. Therefore, surface passivation with silica or alumina may block ROS generation and ultimate oxidative stress. Moreover, protein corona also plays a crucial role in reducing toxicity [222]. Surface coating of TiO_2 NPs with biocompatible polymers, i.e. PEG, may minimize its toxicity [88, 145]. Though it is not considered a major problem [177], TiO_2 NPs of relatively small sizes are mainly eliminated via renal clearance [223, 224] and thus may induce minimum toxicity toward major organs. Therefore, the use of titanium in cosmetics, especially sunscreens, is also encouraged [73, 75].

Physicochemical properties, especially the size of TiO_2 NPs is found to be a critical factor in deciding the impact of toxicity [56, 85, 86, 220, 225–228], and minor changes in particle sizes may lead to significant alterations in inflammation and oxidative stress [85]. Micron-sized NPs might be less toxic than NPs [86, 220]. The studies point out that anatase TiO_2 NPs are more toxic than rutile TiO_2 NPs [18, 86], whereas TiO_2 possess minimum toxicity but may have the potential for long-term side effects, including epigenetic and mutagenic [229]. Indeed, there are conflicting data [18] about the toxicity of TiO_2 NPs that make it difficult to draw strong conclusions.

2.6 Conclusion

Nanotechnology and nanomedicine are the complementary disciplines having marvelous applications in many areas aimed at the improvement of human life; however, it is a double-edged sword. TiO_2 NPs are one of the most prevalent NMs. TiO_2 nano-products have a remarkable impact on society. However, there are numerous societal concerns about NPs exposure that may derail its promising applications. There are many challenges in recognizing and avoiding potential risks associated with TiO_2 NPs. Preventing exposure is the key that can be achieved with appropriate precautions and personal protection strategies.

TiO_2 NPs are capable to interact with biological systems as well as to cross various biological barriers. However, these interactions are heavily affected by the physiochemical properties of NPs and physiological systems. The physiological systems may have quite different strategies to counter toxicity and thus play a pivotal function in mitigating the adverse effects of NPs. Therefore, the biological activities and signaling pathways can be modulated by changing the physiochemical properties of NPs. The better understanding of underlying signaling pathways of cell death can be a straightforward approach to mitigate the unintended consequences.

The overwhelming majority of toxicological studies make it difficult to distinguish between the right and wrong. Notwithstanding, there is almost unanimous opinion among skeptics and proponents that the huge potential of TiO_2 NPs-based products requires immense attention to safety issues.

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