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REVIEW PAPER

Mechanism of oxidative stress involved in the toxicity of ZnO nanoparticles against eukaryotic cells

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ABSTRACT

ZnO NPs (zinc oxide nanoparticles) has generated significant scientific interest as a novel antibacterial and anticancer agent. Since oxidative stress is a critical determinant of ZnO NPs-induced damage, it is necessary to characterize their underlying mode of action. Different structural and physicochemical properties of ZnO NPs such as particle surface, size, shape, crystal structure, chemical position, and presence of metals can lead to changes in biological activities including ROS (reactive oxygen species) production. However, there are some inconsistencies in the literature on the relation between the physicochemical features of ZnO NPs and their plausible oxidative stress mechanism. Herein, the possible oxidative stress mechanism of ZnO NPs was reviewed. This is worthy of further detailed evaluations in order to improve our understanding of vital NPs characteristics governing their toxicity. Therefore, this study focuses on the different reported oxidative stress paradigms induced by ZnO NPs including ROS generated by NPs, oxidative stress due to the NPs-cell interaction, and role of the particle dissolution in the oxidative damage. Also, this study tries to characterize and understand the multiple pathways involved in oxidative stress induced by ZnO NPs. Knowledge about different cellular signaling cascades stimulated by ZnO NPs lead to the better interpretation of the toxic influences induced by the cellular and acellular parameters. Regarding the potential benefits of toxic effects of ZnO NPs, in-depth evaluation of their toxicity mechanism and various effects of these nanoparticles would facilitate their implementation for biomedical applications.

Keywords: *Cellular responses, Cytotoxicity, Oxidative stress, ROS generation, ZnO NPs*

INTRODUCTION

Over the past few decades, metal oxide NPs (nanoparticles), whose structures exhibit remarkably novel and improved physical, chemical, and biological properties attributed to their nano-scale size, have attracted much interest. Cytotoxicity effects of metal oxide nanomaterials such as ZnO NPs against different prokaryotic and eukaryotic cells have been observed through the several studies [1]. ZnO NPs are known to be one of the most important metal oxide nanomaterials due to their various medicinal use and biological applications. There are also several studies which prove the cytotoxic potential of ZnO NPs in mammalian cells [2-4]. An important aspect of the ZnO NPs application for their toxic effects is the requirement that they should not be cytotoxic to human cells. One of the critical features of ZnO NPs is their selective toxicity toward cancerous cells in comparison with normal human cells which shows potential benefits of these NPs as new antitumor drugs [5]. Thus, a high degree of cancer cell selectivity of ZnO NPs which have been indicated by recent studies elucidate their ability to surpass the indices of some currently used chemotherapeutic drugs in similar *ex vivo* studies [6,7]. ZnO NPs have been shown to exhibit preferential toxicity toward cancerous human myeloblastic leukemia cells (HL60) in comparison with normal peripheral blood mononuclear cells [5]. Ostrovsky et al. (2009) reported that the ZnO

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NPs exerted cytotoxic effect on several human glioma cell lines (A172, U87, LNZ308, LN18, and LN229) and no cytotoxic effect was observed on normal human astrocytes [8]. ZnO NPs have distinct influences on mammalian cell viability via killing cancer cells (HepG2, A549, and BEAS-2B) while posing no impact on normal cells like rat astrocytes and hepatocyte [9]. Therefore, several researches on the toxic influences of ZnO NPs have directed towards photodynamic therapy and nanomedicine as a new antitumor drug [1, 5, 10-12]. This review has focused on ZnO NPs with respect to their specific properties such as their relative ease of production, ability to alter their physiochemical characteristics, their inherent toxicity against cancerous and bacterial cells, and ability to functionalize them with chemotherapeutic drugs and cancer targeting molecules, which make them an appealing candidate for biomedical applications (13-17]. Extensive researches provided new approaches for a more comprehensive understanding of mechanism of NPs-induced toxicity. Induction of OS (Oxidative Stress) as a possible mechanism for cell damage by NPs is well evidenced. A wide range of nanomaterial species were shown to create ROS both *in vivo* and *in vitro* [18-20]. Although it is known that ZnO NPs are toxic to mammalian, the mechanism of toxicity has not understood yet. Several studies found that toxicity of nano-sized ZnO was induced by the generation of ROS [4, 6]. Table 1 shows the studies of the oxidative stress induced by ZnO NPs on different cell types.New insights into the complexity and roles of ROS in cytotoxicity have derived from extensive analyses of the possible influences of ROS on the cellular responses. Exposure to ZnO NPs leads to ROS generation and activation specific signal transduction pathways in target cells. Imbalance in the ROS production and antioxidant defense system of cells results in interferences with normal cellular processes which cause several different outcomes leading to cell death [21]. Biological systems can integrate multiple toxicity pathways into a limited number of pathogenic consequences [22, 23]. ZnO NP- induced ROS generation can cause a range of biological responses, depending on the relative level of ROS generation and the type of cellular pathways that are employed by OS [18, 22, 24]. Recent studies have indicated that the physicochemical properties of NPs (e.g., size, shape, particle surface area, crystal structure, solubility, chemical composition, method of the preparation, and dispersion factor] play decisive roles in determining their biological outcomes [18, 25, 26]. For example, NPs of smaller size can enter the mitochondria; consequently induce OS and cell death [22]. In this review, we summarized the literature from 1997 to 2014 discussed about oxidative stress as a potential mechanism of ZnO NPs toxicity. The focus of this review is on the elucidation of different proposed mechanisms of ROS production and consequent OS induced by ZnO NPs. Various mechanistic approaches were proposed to explain the tentative role of ZnO NPs in generating ROS. In spite of extensive researches about the toxicological influences of ZnO NPs, there is still no consensus on the exact mechanism of NP - induced toxicity. Since OS is an important determinant of NP-induced damage, it is necessary to define the different explanations given for the cellular ROS response resulting from NP. Considering different hypotheses about the exact mechanism of OS stimulation by ZnO NPs, we attempted to summarize the present state of knowledge on these mechanisms which makes this review valuable for determining the underlying mode of action of these NPs. The other purpose of this review was to characterize the ROS responses resulting from ZnO NPs. According to the close relationship between the physicochemical properties of ZnO NPs and their toxicity, a systemic toxicity screen with OS as a predictive model for ZnO NPs induced injury can be developed through the understanding of the multiple signaling cascades triggered by NP-induced ROS [27].

Mechanism of the toxicity of ZnO NPs

Among different proposed mechanisms, NPs-induced ROS generation and consequent OS is frequently observed and is the most discussed paradigm for NP toxicity [18, 43]. The oxidative stress mechanism of ZnO NPs could be attributed to the combination of more than one phenomenon including: generation of ROS on the surface of particles [34], dissolution and release of Zn^{2+} ions in the culture media [42], and physical interaction of ZnO NPs with the membrane wall leading to the deformation and rupture of membrane [42]. The ROS generation mechanism is different for each NPs, and the exact underlying molecular and cellular mechanism is not completely understood. Several potential mechanisms for this behavior were suggested which complicates the interpretation of the underlying mode of action for induction of OS. One potential mechanism may involve the generation of ROS by NPs themselves [5]. Here, we discuss different supposed mechanisms of ROS generation and consequent oxidative stress induced by ZnO NPs to have better understanding of their mechanism of toxicity.

Table 1. The literature reports on the toxicity of ZnO NPs induced by oxidative stress against eukaryotic cells

Cell type	Reference
Human bronchial epithelial cells (BEAS-2B)	(4, 22, 28, 29)
Human alveolar adenocarcinoma cells (A549)	(11)
Human epidermal cell (A431)	(21)
Mouse macrophage cell (RAW 264.7)	(22)
Primary mouse embryo fibroblasts (PMEF)	(30)
Human T lymphocytes	(6)
Zebrafish embryonic cell	(31)
Human colon cancer cells	(32)
Human myeloblastic leukemia cells (HL60)	(5)
Human liver cells (HepG2)	(33)
Human lung fibroblast cells (WI-38 133)	(34)
Human SHSY5Y neuronal cells	(35)
Rat alveolar epithelial cell monolayers (RAECMs)	(36)
Human lung epithelial cells (L-132)	(37)
Human normal skin cells	(38)
Human embryonic kidney cells (HEK293)	(39)
Human skin melanoma cells (A375)	(40)
RSC96 Schwann cells	(41)

Different mechanisms of ROS generation by ZnO NPs

There are several studies which attribute the toxicity of ZnO NPs to their capability of generating intracellular ROS. It means that OS is occurred by ZnO NPs as the production of ROS by these particles. From this point of view, the key factors involved in NP-induced OS include (i) light or UV activation of electron hole pairs leading to radical formation (ii) active electronic configurations and functional groups generation on the surface of NPs due to discontinuous crystal planes and defects and (iii) active redox cycling on the surface of NP owing to transition metal-based NP [18, 46]. Semiconductors have a void region known as band gap between the valence band and the conduction band. The movement of electrons from the valence band to the conduction band leaves holes or unoccupied states in the valence band [33]. In aqueous environments, the electrons and holes can react with oxygen and hydroxyl ions , respectively [18] to produce highly reactive free radical compounds including the superoxide anion radical (from electrons) and the hydroxyl radical (from holes) [10]. Moreover, shrinkage in nanoparticle size creates structural defects responsible for the presence of groups like increased interstitial zinc ions and oxygen vacancies (intrinsic defects in the crystal when an oxygen atom is removed from its place). Additionally, smaller NPs size is the reason for the disruption of well-structured electronic configurations, so as give rise to altered electronic properties on the particle surface [18, 25, 47]. Therefore, the crystal defects can lead to a large number of the electron donor or acceptor active sites which can migrate to the nanoparticle surface and contribute to the ROS generation. Thus, difference between NPs could explain their disparity in their cytotoxic effects. For instance, Si and Zn NPs with identical size and shape have diverse cytotoxicity responses owing to their composition and surface properties. ZnO has been shown to be more chemically active than SiO_2 and causes increased O_2^{-} formation resulting in OS [30]. Thus, chemical and surface characteristics of ZnO NPs, lead to spontaneous generation of ROS at their surface. Photoactivated ZnO NPs found to behave as an oxidizing agent with antibacterial and antitumor activity [1, 45]. Park et al. (2011) suggested that the toxicity of ZnO nanomaterials is related to ROS and the amount of ROS formation is dependent on their synthesis procedure as well as physicochemical characteristics [34]. Different explanations about various mechanisms of ROS generation by ZnO NPs are showed in Fig 1.

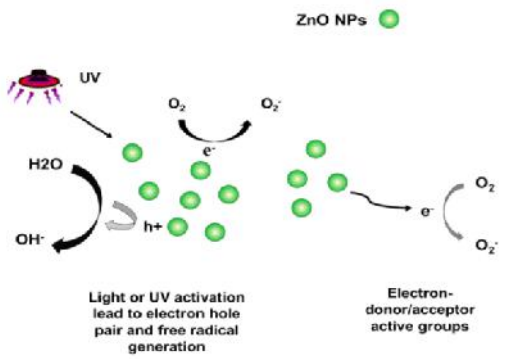

Fig. 1. Different mechanisms of ROS generation by ZnO NPs

From the other mechanistic point of view, we could discuss the sources of ROS based on the particle cell interactions [27]. It means that generation of free radicals occurs after interaction of NPs with cellular components such as mitochondria [33]. Boonstra and Post (2004) reported that disturbance of oxidative phosphorylation causes electron escape from the chain which can be accepted by molecular oxygen to form different ROS [52]. Asharani et al. demonstrated that disruption of mitochondrial respiration can enhance the levels of intracellular ROS [19].

The other relative new hypothesis for interpretation of ROS production as a consequence of ZnO contact with human cells highlights the existence of a different mechanism of cytotoxicity for ZnO NPs. In accordance with this new hypothesis some types of NPs have been proved to induce autophagy and autophagic cell death of human cells [57]. Yu et al. (2013) demonstrated that ZnO NPs induced ROS leads to cell death through authophagic vacuole accumulation and mitochondria dysfunction in normal skin cells [38]. Yu et al. (2009) showed that rare-earth oxide nanocrystals, including samarium (Sm)/europium (Eu) and gadolinium (Gd)/ terbium (Tb), induce serious autophagy in Hela cells [58]. Moreover, Chen et al. (2005) observed autophagic cell death in non-small cell lung cancer cells after nano-sized neodymium oxide treatment [59]. Another way of generating ROS is the activation of NADPH-oxidase enzyme which is a multisubunit enzyme and responsible for O^{2-} production in the membrane of phagocytic cells. These radicals can react with biological molecules (DNA, proteins, and lipids) leading to oxidative damage and apoptosis [33]. Another source of ROS generation in the cell is cytochrome P450 enzymes (P450s). The other suggested approach for oxidative damage is the metabolization of ZnO NPs by P450 cytochrome which can also be a source of ROS, as demonstrated in the case of other particles [60]. The other recommended process through which ZnO NPs show OS properties is via the production of nuclear factor-5ØßB (NF-5ØßB). Several metal oxide NPs such as Zn cause the NF-5ØßB activation [61-63]. The NF-5ØßB group of proteins activates genes responsible for defense mechanisms toward cellular stress and regulates miscellaneous functions such as inflammation, immune response, apoptosis, and cell proliferation. Once inside the nucleus, NF-5ØßB induces transcription of pro-inflammatory mediators resulting in inflammation and OS [61-63].

Correlation between the dissolution of ZnO NPs and oxidative stress

There are some reports which attribute the toxicity of ZnO NPs to particle dissolution and subsequent release of Zn^{2+} ions that lead to ROS mediated injury [20, 46, 48]. Although there are several publications which attribute ZnO NPs cytotoxicity to its dissolution, few of these studies have linked this to a mechanistic paradigm such as oxidant injury. However, the results of some studies showed the interrelationship between dissolution of ZnO NPs and the consequent oxidative stress. For example, data of an experiment showed that ZnO exhibiting major toxicity based on a mechanism of particle dissolution and Zn^{2+} release that engages ROS mediated injury [20]. Also, ZnO NPs produce intrinsically a small quantity of ROS and intracellular ROS mainly generates after ZnO NPs or the dissolved Zn^{2+} enters into the cells [41, 44]. The results of other experiment indicated that formation of ROS was the responsible mechanism of cellular toxicity and the release of Zn^{+} ions from the ZnO NPs, due to their instability in the acidic compartment of lysosomes, also increases the ROS generation [49]. Zhu et al. (2009) suggested that the combination of ZnO NPs and Zn^{2+} elicited embryonic toxicity by generating the ROS and/ or compromising the cellular OS response [31]. Fukui et al. (2012) concluded that ZnO NPs cytotoxicity came from the Zn^{+2} ions released from ZnO NPs in vitro and in vivo [50]. Furthermore, increases in its intracellular levels found to correlate with high levels of ROS and cell death [50]. In addition, these alternations in intracellular Zn^{2+} homeostasis exhibited powerful

stimulatory effects on multi-conductance cation channels in the inner membrane of mitochondria [41]. This can be attributed to the generation of intracellular ROS, which subsequently results in oxidative damage of organelles and cell apoptosis [41]. Findings of other experiment revealed that the release of transition metal cations by engineered NPs is responsible for the induction of OS [47]. Nel et al. (2009) reported that cytotoxicity of ZnO NPs may be directly related to ZnO dissolution through interactions at sequential nano-bio interfaces which can induce a series of harmful cellular outcomes such as ROS production [51]. Some studies showed that dissolution of ZnO NPs and excess release of Zn^{2+} leading to disruption of mitochondrial redox state. Experiments with isolated mitochondria have shown that Zn^{2+} interferes with cytochrome bc_1 and α-ketoglutarate dehydrogenase complex of the electron transport chain with subsequent inhibition of cellular respiration [53, 54]. Wiseman et al. (2006, 2007) revealed that excess free Zn^{2+} (from dissolved ZnO NPs) resulted in depletion of sulfhydryl groups in metallothionein-1 proteins and reduction of mitochondrial function [55, 56]. Impaired mitochondrial function due to increased intracellular Zn^{2+} causes elevated intracellular ROS, leading to apoptotic or necrotic cell death [55, 56].

Some experiments suggested that excess free Zn^{2+} arising from dissolved ZnO NPs disrupts zinc-protein interactions in mitochondria, leading to disruption of mitochondrial redox state and elevated intracellular levels of ROS, resulting in cell membrane injury and cell damage [36]. Several studies showed that ZnO induced ROS production at the mitochondrial level by the abnormal ultrastructural alterations in mitochondria, the increase in MitoSOX red ûuorescence, changes in membrane potential, and mitochondrial dysfunction [20, 56]. Furthermore, some studies were suggested that Zn^{2+} may contribute to ROS generation via activation of NADPH oxidase and nitric oxide synthase [53]. Xia et al. (2008) showed that Zn^{2+} may exert effects contributing to ROS generation, including activation of NADPH oxidase via protein kinase C as well as nitric oxide production [20].

Multiple cellular pathways involved in oxidative stress induced by ZnO NPs

Free radicals generated by ZnO NPs would interact with macromolecules including proteins, enzymes, membrane lipids, and even DNA that could be oxidized, modified, and destructured which finally leading to the damage and dysfunction of different cellular organelles. Therefore, depth evaluation of detailed various cellular responses to ZnO NPs-induced OS are valuable as a predictive model for their associated injury. An expanding field of researches has been done on the toxicity of ZnO NPs against different cell types which are explained in the following parts of this review.

DNA damage

One of the key possible modes thought to be responsible for the toxic effects exerted by nanomaterials via OS is DNA damage [22]. Hydroxyl radical ($^{\cdot}OH$) is one of the primary species responsible for DNA damaging. Certain NPs release transition metals ions from their surfaces which have the potential to convert cellular oxygen metabolic products such as H_2O_2 and superoxide anions to hydroxyl radicals [23]. ROS react with DNA causing damage to bases and DNA backbone [64]. There are different types of ROS-induced DNA damage, including single and double-stranded DNA breaks, base modifications (e.g. formation of 8-hydroxydeoxyguanosine adducts) and DNA cross-links, all of which if unrepaired possess the ability to initiate and promote carcinogenesis [65]. Toyokuni et al. (1998) reported that ROS are highly reactive molecules that can react with cellular macromolecules including DNA, lipids, and proteins and disturb the homeostasis of the intracellular milieu [66]. ZnO NPs reported to have a genotoxic potential in human epidermal cells even at low concentrations which may be mediated through lipid peroxidation and OS [21]. Malondialdehyde (MDA) is one of the main products of lipid peroxidation which is mutagenic and carcinogenic. It can react with DNA bases to form adducts to deoxyguanosine, deoxyadenosine and deoxycytidine [67, 68]. Yang et al. (2008) investigated the genotoxicity and oxidative effect of ZnO NPs on primary mouse embryo fibroblast cells. Their results showed that cytotoxicity and oxidative impairment of ZnO NPs were much greater than other non-metal NPs [30]. In agreement with this study, Zhao et al. (2013) demonstrated that ZnO NPs induces developmental toxicity, oxidative stress, and DNA damage on zebrafish embryos which is partly due to the dissolved Zn^{2+} [69]. Lin et al. (2009) demonstrated that ZnO NPs cause cytotoxicity and oxidative DNA damage in a dose- and time- dependent manner in human bronchoalveolar carcinoma-derived cells (A549) [28]. Moreover, NPs can

damage DNA via indirect mechanisms where they do not physically react with the DNA molecule, but with other cellular proteins such as cell division proteins [23]. The other plausible mechanism through which ZnO NPs-induced OS causes DNA damage is the activation of specific signaling pathways resulting in the release of pro-inflammatory cytokines.

Several studies revealed that exposure of endothelial cells to different NPs cause the inflammatory response which is nanoparticle composition and concentration dependent [70, 71]. In the study of Gojova et al. (2007) the production of ROS is an obvious candidate pathway for NPs induced inflammation, however more investigations are needed to exactly establish a connection between ROS generation and inflammatory responses [70]. Inflammatory factors found to induce DNA damage in the form of point mutations, chromosomal fragmentation, and DNA adducts formation. Also, these factors can prevent DNA repair and promote aberrant methylation patterns leading to changed gene expression profiles [72, 73]. Dufour and colleagues (2006) investigated the genotoxicity of ZnO NPs in Chinese hamster ovary (CHO) cells under the different irradiation conditions using the chromosome aberration test [74]. They observed that ZnO produced a concentration-related increase in chromosome aberrations in the dark. Chromosome aberrations induced by ZnO NPs showed to enhance in the presence of UV light.

ZnO NPs are genotoxic in somatic (keratinocytes, lymphocytes, hepatocytes) and germ cells [22, 33, 75]. Findings of Karlsson et al. (2008) showed oxidative DNA lesions in cultured human lung epithelial cells (A549) after exposure to ZnO NPs [76]. Exposure of human lung epithelial cells (L-132) to ZnO NPs found to cause DNA fragmentation illustrating apoptotic type of cell death [37]. Also, DNA damaging effects of ZnO NPs on human hepatocyte (L02) and human embryonic kidney (HEK293) cells were reported for which lipid peroxidation. It was suggested OS as the probable cause [37]. A significant increase in the Fpg sites was observed in the liver of people who treated with ZnO NPs indicating oxidative DNA damage [33]. The significant increases may create in tail length, percentage of DNA in tail, tail moment, and Olive tail moment after PMEF cells were treated with ZnO NPs [22]. Totally, it can be stated that some NPs owing to their small size are capable of reaching the nucleus and interact with DNA [77, 78]. While some other NPs like ZnO NPs may also exhibit an indirect effect on DNA through their ability to generate ROS [78]. DNA damage can disrupt normal cell functions which may lead to cancer or cell death. Regarding published documents, different explanations for the DNA damaging potential of ZnO NPs are reported (Fig. 2).

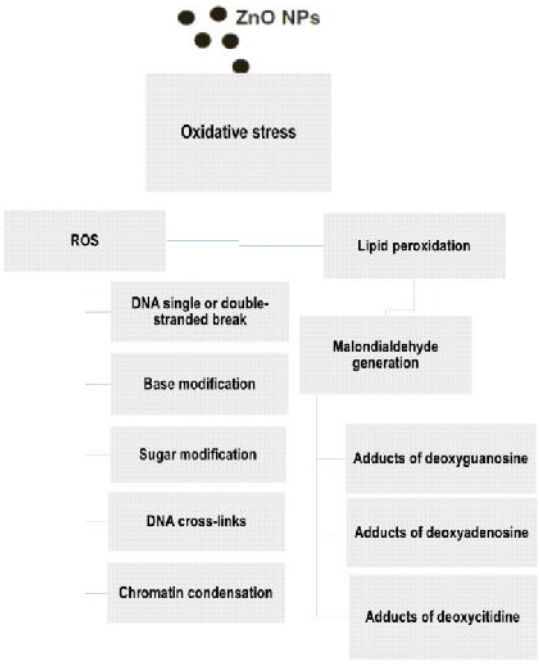

Fig. 2. Different mechanisms suggested for the DNA damaging potential of ZnO NPs

Gene expression

Although the previous studies showed that the NPs could induce OS and DNA damage, the studies about the genes related to oxidative damage were so inadequate. The documents could not supply the comprehensive information at the molecular level to indicate how the regulation of antioxidant enzyme activities and other negative effects is affected by NPs stress. Thus, in order to investigate the effects of ROS production in more detail, the essential genes related to antioxidant enzymes and oxidative-DNA repair system need to be evaluated. Assessment of oxidative damage caused by ZnO NPs in embryo-larval stages of zebrafish was determined by up regulation of Ucp2 and down regulation of Bcl-2, Gstp2, and Nqo1 gene expression [69]. Data of other research indicated that exposure of BEAS-2B cells to a sublethal concentration of ZnO NPs induces the expression of BNIP3 (BCL2/adenovirus E1B 19 kDa

interacting protein 3), PRDX3 (peroxiredoxin 3), PRNP (prion protein), and TRXND1 (thioredoxin reductase 1) genes which involved in OS and apoptosis [4]. Xia et al. (2008) [20] reported that ZnO NPs could induce increased NQO-1 and Nrf2 mRNA expression in RAW 264.7 and BEAS-2B cells [20]. In other study, ZnO NPs exposed L-132 cells showed upregulation of the metallothionein [MT) gene. MT is one of the essential biomarkers in metal-induced toxicity [37] which facilitates metal detoxification and protection from free radicals [79, 80].

Intracellular enzymes

Oxidative toxicity is defined as injurious effects due to cytotoxic properties of ROS which could activate/ inhibit complex arrays of antioxidant enzymes in the organism. Therefore, several intracellular enzymes were measured in different studies as biomarkers of OS to obtain more information [45, 81, 82]. Intracellular levels of SOD (superoxide dismutase) activity in primary mouse embryo fibroblasts (PMEF) cells exhibited a dose-dependent decrease after 24 h exposure to ZnO NPs [33]. ZnO NPs could induce OS in human epidermal cell line (A431) by decreasing catalase and SOD levels [22]. Zhao et al. (2013) reported the relationship between ZnO NPs-induced OS and antioxidant enzymes activities in embryo-larval zebrafish [69]. They showed that exposure to 100 mg/L of nano-ZnO cause the excessive production of ROS and a significant inhibition of SOD activity [69]. As levels of ROS produced by ZnO NPs was exceeded the capacity of cellular antioxidants, the antioxidant system could not eliminate them. Their results also confirmed that nano-ZnO had an inhibitory effect on catalase activity, suggesting that H_2O_2 generated by SOD was not removed completely by catalase directly and caused intracellular accumulation of ROS. Also, their results revealed that the GPx (gluthatione peroxidase) activity in ZnO NPs and corresponding soluble Zn^{2+} treated groups increased in comparison with control, indicating its enhanced capacity to scavenge H_2O_2 and lipid hydroperoxides [69]. Ahamed et al. (2011) showed that exposure of A549 cells with ZnO nanorods caused intracellular production of ROS leading to the induction of LPO (lipid peroxidation), SOD, and catalase, suggesting that OS might be the primary mechanism of ZnO nanorods toxicity in A549 cells [11]. Depletion in the SOD level was also found on 24-h exposure of human embryonic kidney cells to ZnO NPs [83, 84].

Hypercalcemia

Intracellular concentration of Ca^{2+} has major effects on cellular metabolism, signal transduction, and gene expression. Ca^{2+} concentration is tightly regulated while its increases in are associated with metabolic and energetic imbalance, cellular dysfunction, disease states which could be detrimental to the cell. Several studies demonstrated that NPs increase ROS and intracellular Ca^{2+} levels. Stone et al. (2000) found that ultrafine carbon black NPs induced opening of plasma membrane Ca^{2+} channels via a mechanism involving ROS [85]. Huang et al. (2010) demonstrated that ZnO NPs induce the intracellular ROS production and Ca^{2+} level in human bronchial epithelial cells (BEAS-2B) [4]. They suggested that ZnO may first interact with cytoplasm membrane due to the lipid peroxidation causing loss of cell membrane integrity leading to calcium influx through membrane channels [4]. ZnO NPs treatment was shown to induce a signiûcant and sustained Ca^{2+} concentration increase in RAW 264.7 and BEAS-2B cell lines [20]. ZnO NPs-induced calcium elevation may cause cytotoxicity by different mechanisms. Möller et al. (2005) reported that increase in intracellular calcium concentration activates calmodulin-dependent signaling pathways which are crucial for cytotoxicity and cytoskeletal dysfunctions which ultimately lead to cell death [86]. Intracellular calcium release is a major OS response that can lead to triggering the mitochondrial permeability transition pore (PTP) causes dissipation of the mitochondrial membrane potential and the possible release of proapoptotic factors and cell death [20]. Moreover, the cytotoxicity effects of ZnO NPs were reported to be associated with the release of Zn^{2+} from NPs and the subsequently increase of intracellular Ca^{2+} level which is another critical signal pathway closely related to cell apoptosis and necrosis [4, 41, 87]. Additionally, studies using insults other than NPs indicated that hypercalcemia and OS have mutual effect on each other. Findings demonstrated that the increase in intracellular Ca^{2+} concentration can activate enzymes (e.g., hydrolytic enzymes, proteases, dehydrogenases in the citric cycle) that generate ROS/RNS [88-90]. On the other hand, overproduction of ROS/RNS can oxidatively inactivate thiol-dependent Ca^{2+} pump, which in turn aggravates hypercalcaemia. Both ROS/ RNS and hypercalcaemia cause severe primary metabolic disorders lead to ATP depletion and eventually cell death (necrosis or apoptosis) [91-93].

Inflammation

In order to maintain the redox equilibrium at an intermediate level of OS, pro-inflammatory pathways are activated. Thus, the pro-oxidant effects of some NPs lead to the activation of signaling pathways and cytokine cascade that contribute to a wide range of cellular responses. Several metal oxide NPs such as Zn, Cd, Si, and Fe exert their toxic effects via ROS-dependent nuclear factor-5ØßB (NF-5ØßB) activation [61-63]. The NF-5ØßB family plays a key role in the regulation of inflammation and immune responses, cell proliferation, and apoptosis. During NPs mediated lung injury, activation of NF-5ØßB modulates pro-inflammatory cytokines production (TNF-5ØüÞ, IL-8, IL-2, and IL-6) by macrophages and lung epithelial cells. Ultrafine ZnO NPs reach the alveoli and cause pulmonary inflammation and symptomatic responses in the lung via an increase in pro-inflammatory cytokines TNF-α, IL-6, and IL-8 [94]. Data from the other study demonstrated that ROS production by ZnO NPs affects increased early growth response-1 (Egr-1) expression via extracellular signal-regulated kinase (ERK) activation leading to induction of transcriptional activations of TNF-α expression and generation of cellular inflammation [95]. Moreover, other several *in vitro* and *in vivo* pulmonary inhalation studies have been proved that ZnO NPs exposure induce the generation of various pro-inflammatory cytokines, such as IFN-γ, TNF-α, and IL-12 [6, 70]. Other work reported a significant decrease in cell viability, remarkable morphological changes, apoptosis induction via ROS production, and IL- 8 release, after treatment with ZnO NPs exposure suggesting that the OS was a key mechanism for their cell damage [3]. ZnO NPs at concentrations below those causing appreciable cell death has been found to induce the production of pro-inflammatory cytokines [6], suggesting that ZnO NPs could enhance tumor cell killing through the production of TNF-α when used at appropriate concentrations. Exposure of RAW 264.7 and BEAS-2B cells to ZnO NPs could effectively activate Jun kinase (JNK) cascade which occurred in parallel with increased TNF-α and IL-8 production, respectively [20].

Lipid peroxidation

One of the best known toxic effects of oxygen based free radicals is lipid peroxidation of unsaturated fatty acids present in membrane phospholipids. The MDA is one of the end-products of lipid peroxidation which is regarded as a marker of lipid peroxidation and related to the concentration of ROS generated. MDA content in the zebrafish exposed to high concentrations of nano-ZnO increased significantly compared to the control group (69]. The results of this study were in accordance with the conclusions from other researcher [96]. Findings of the other experiments revealed that ZnO NPs treatment at 50 and 100 μg ml^{-1} elevated the intracellular MDA concentration in a dose-dependent manner in the PMEF cell [30]. Formation of hyperperoxide as another marker of lipid oxidation by ROS was also indicated by the enhancement of LDH (lactate dehydrogenase) release after exposure of A431 cells to ZnO NPs [21].

An increase in lipid peroxides in HepG2 cells on exposure to ZnO NPs was also observed which represents another marker of OS. Lipid peroxidation can further give rise to more free radicals and damaged biomolecules [33]. Particle-dependent ROS formation and subsequent lipid peroxidation of cellular membrane were also proposed to explain ZnO NPs phytotoxicity to ryegrass [97] and yeast [98].

Mitochondrial dysfuction

Mitochondria are considered target organelles for cytotoxic injury. They have a critical role in maintaining cellular functions favoring aerobic ATP production. In addition to supplying cellular energy, they are involved in other processes, such as signal transduction, cellular differentiation, apoptosis, as well as the control of the cell proliferation and cell growth [3]. The mitochondrion is one of the major cell targets for NPs-induced OS and the intrinsic mitochondrial apoptotic pathway has an important role in metal oxide NPs-induced cell death. Different metal oxide NPs stimulate ROS-associated cell death via mitochondrial dysfunction [87, 99, 100]. Once NPs reach into the mitochondria, they can generate mitochondrial ROS via impaired electron transport chain and depolarization mitochondrial membrane [22].

NPs can catalyze the generation of superoxide anion ($O_2^{\cdot""}$) either by accelerating electron transfer to molecular oxygen or blocking the electron transport chain of mitochondria [51, 101]. Assessment of mitochondrial membrane potential ($\Delta\psi_m$) by Xia et al. (2008) showed that ZnO NPs could effectively increase the percentage of the cells with a lowered $\Delta\psi_m$ while CeO_2 and TiO_2 did not affect the $\Delta\psi_m$ [18]. Mitochondrial dysfunction and OS after exposure with ZnO NPS are investigated by different studies (Table 2).

Table 2. The literature survay on the mitochondrial dysfunction and OS induced by ZnO NPs

Cell type	Reference
Human colon carcinoma (Lovo) cells	(3)
Neuro-2A cells	(48)
Human hepatocellular carcinoma cells (HepG2)	(33)
Human embryonic lung fibroblasts (HELF) cells	(104)
Human epidermal (A431) cells	(21)
Human lung bronchial epithelial (BEAS-2B) cells	(4)
Human osteoblast cancer cell line	(1)
Normal skin cells	(38)
Rat alveolar epithelial cell	(36)

Apoptosis

Several studies have identified the oxidative stress as common pathway for ZnO NPs-induced apoptosis. Wang et al. (2014) showed that ZnO NPs trigger an increase of intracellular ROS in a dose-dependent manner followed by JNK phosphorylation, indicating that JNK signaling pathway was involved in apoptosis in response to ZnO NPs (102]. Also, a significant number of cells undergoing apoptosis were found in liver of treated mice compared to the control group suggesting that the oxidative stress might also lead to apoptosis during the in vivo exposure [33].

Sharma et al. (2012) showed that sub-acute oral exposure to ZnO nanoparticles in mice leads to an accumulation of nanoparticles in the liver causing oxidative stress mediated DNA damage and apoptosis [103]. Other study was designed to investigate the cytotoxicity, oxidative stress, and apoptosis caused by ZnO nanoparticles in human skin melanoma cells. Results demonstrate that ZnO NPs was responsible for induction of oxidative stress and apoptosis [40]. Data of the other experiment demonstrated that ZnO nanorod induced apoptosis in human alveolar adenocarcinoma (A549) cells through ROS and oxidative stress via p53, survivin, bax/bcl-2, and caspase pathways [11].

Different parameters influencing on oxidative stress mechanism

We focus in this review on the toxicity of ZnO NPs with respect to the oxidative stress paradigm. According to the results of several studies the principal proposed mechanisms for ZnO NPs-induced oxidative stress involve (a) the oxidative properties of the NP themselves, (b) particle dissolution and Zn^{2+} release, and (c) oxidant generation upon interaction of ZnO NPs with cellular components. It is evident that much remains unknown about the exact mechanism of ZnO NPs-associated with oxidative stress and in this field we have yet to reach a consensus on the underlying mode of the action. Finally, we hypothesize that for different conditions such as various environmental settings and physicochemical properties of ZnO NPs, different OS mechanisms are implemented. This result could be the reason of disagreement on the exact mechanism of oxidative stress induced by ZnO NPs. For example, much research indicated that different physicochemical properties including surface reactivity, particle size, surface charge, agglomeration state, crystal structure, shape, and dispersion factor influence on the ZnO NPs oxidative stress stimulation. One of the most important physicochemical characteristics of ZnO NPs is their solubility, one of the paradigms responsible for ZnO NPs-induced toxicity via oxidative stress. Therefore, it could be concluded that if environmental conditions of a nanomaterial (including surface free energy) favor particle dissolution in a suspending medium or biological environment the possibility of oxidative stress through the particle dissolution increases [105]. As an example, dissolution of Zinc NPs increased under acidic conditions as well as the presence of biological components such as amino acids and peptides [106]. These data verify the possibility that there is a correlation between exposure conditions of ZnO NPs and their underlying mode of action. Also, probing the various ZnO NPs properties such as size, shape, surface chemistry, roughness, and surface coatings allows the development of predictive relationships between structure and activity. For example, one of the

most important features of ZnO NPs which play a critical role in determining their mechanism of ROS generation is particle size. Nanoparticles of smaller size can enter the mitochondria of cells through various pathways, subsequently inducing oxidative stress and cell death via NP-cell interaction [22]. Differential oxidative stress mechanisms of ZnO NPs are also observed based on the differential cell type and consequently depending on the type of cellular pathways that are engaged by oxidative stress [18, 20, 107]. Results of an experiment showed that the uptake of labeled ZnO NPs in BEAS-2B cells occurs in caveolae. Instead, the any evidence of labeled particles in the lysosomal compartment was observed in RAW 264.7 cells, while toxic Zn^{2+} accumulation in this organelle was associated with organellar clumping and oxidative cell injury [20]. Considering the hierarchical OS model at the highest level of oxidative stress, a perturbation of inner membrane electron transfer and the open/closed status of the mitochondrial permeability transition pore (PTP) can stimulate cellular apoptosis and cytotoxicity. Hence, it seems that interaction between ZnO NPs and cellular organelles like mitochondria as one of the mechanisms of OS also depends on the strength of oxidative stress.

CONCLUSION

In this review, we focused on the various cellular pathways involved in the toxic effects of ZnO NPs with respect to the oxidative stress paradigm. Results of several researches demonstrated that ZnO NPs-mediated ROS responses orchestrate a series of pathological events such as genotoxicity and inflammation. Considering ROS generation as a major event during ZnO NPs-induced injury, it is essential to thoroughly characterized various cellular responses in order to predict the pathophysiological outcomes of ZnO NPs-induced toxicity. Besides, several studies reported that changes in structural and physicochemical properties of ZnO NPs can lead to changes in biological activities including ROS generation which is one of the most frequently reported NPs-associated toxicity. Therefore, different approaches for the manufacture of NP impact on their physicochemical characteristics as well as their toxicity. Thus, it is necessary to ensure the exact mechanism of ZnO NPs-induced OS for their maximum cytotoxic properties. Furthermore, extensive studies showed us that some other various parameters such as the type of cells treated with NPs, strength of OS, and environmental conditions are important factors determining the exact mechanism of oxidative stress at different experiments. Thus, a comprehensive characterization is essential for proper interpretation of toxicity data to differentiate between various mechanisms and prediction of the relative importance of particle-induced toxicity or dissolved Zn^{+2}þ effects in a given experimental setting. Hence, identifying the major responsible mechanisms of NPs-induced ROS and also underlying conditions determining the exact mode of the action facilitate design and manufacture of ZnO NPs with specifications according to our requirements. Moreover, it can be concluded that enough information about different cellular pathways triggered by ZnO NPs explain detailed correlation between the characteristics of NPs and induction of various cellular signal transduction. As a final result, it seems that comprehensive understanding of various influences induced by ZnO NPs at the genetic, molecular, and cellular levels provides new approaches in order to design NPs with properties for their improved efficacy as new antitumor or antibacterial drugs.

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