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Chapter

Biological Role of *Withania somnifera* against Promiscuity of Zinc Oxide Nano Particles and Its Interaction with Macrophages

Jitendra Kumar, Chander Datt, Surya Kant Verma and Kavita Rani

Abstract

In agriculture and food industry, nanotechnology can be utilized to improve crop yield, food quality, shelf life, safety, cost and nutritional benefits. Zinc is a trace element and its deficiency causes health problems in human beings and animals. The use of zinc oxide nanoparticles (ZnO NPs) is growing exponentially in food industry, biomedicine and nanofertilizer segment. A remarkable presence of nanomaterials in ecosystem and consumer products can cause adverse effects. Hence, it is an important challenge for the use of nanoparticles in agriculture as fertilizer to enhance plant yield on one hand and their interaction with the cells of the innate immune system in animals on the other hand. So, public concern about their potential toxicity is increasing. ZnO NPs interact with cells and produce harmful effects in a dose dependent manner. The reactive oxygen species generation might be a reason for the toxicity of ZnO NPs. The toxicity is caused due to dissolved Zn^{++} ions by absorption which causes adverse effect on phagocytosis and oxidative stress by free radical while *Withania somnifera* induced the phagocytosis activity by antioxidant mechanism thus having protective effects. It is emphasized that further research is needed on the use of nanoparticles in agriculture, animal husbandry, and human health sector so that their safer levels for use could be ascertained.

Keywords: agriculture, immunotoxicity, macrophages, nanofertilizer, nanoparticles, *Withania somnifera*, zinc oxide

1. Introduction

Nanotechnology is an emerging technology which can lead to a new revolution in many fields of science [1]. Nanoparticles (NPs) are gaining importance recently due to their exciting applications in different fields like biomedical, pharmaceutical, agriculture, etc. The properties of the materials change as their size approaches the nanoscale, and nanoparticles have a very high surface area to volume ratio and high energy. Application of nanoparticle in the agriculture and food sectors is relatively new as compared to their use in health sector.

In India, more than 60% of the population survive on agriculture, but unfortunately this sector is facing various global challenges. Therefore, nanotechnology has a dominant position in transforming agriculture and food industry. Nanotechnology has a great ability to transform conventional agricultural practices and boost yield and growth of corps. Zinc oxide NPs (ZnO NPs) are used as fertilizer which support their growth and improves production [2].

Zinc oxide NPs may be used as a source of Zn in supplements and functional foods [3]. ZnO NPs also act as antimicrobial agents against harmful bacteria. The antimicrobial activity of ZnO NPs has been partly attributed to their ability to penetrate into microbial cells and animal cells and generate reactive oxygen species (ROS) that damage cellular components thereby leading to cytotoxicity [4]. A single oral dose of ZnO NPs caused hepatic cell injury, kidney toxicity, and lung damage [5]. The studies in frogs showed that ZnO NPs exhibited more toxicity than a dissolved form of Zn which was attributed to their greater ability to induce oxidative damage in cells [6]. The administration of ZnO NPs increased all liver enzymes [7]. In this chapter, we attempted to explore the potential use of nanoparticles in agriculture, biological, and pharmacological significance of *Withania somnifera* against the promiscuity of zinc oxide ZnO NPs and their interaction with macrophages.

2. Use of zinc oxide nanoparticles in agriculture and animal husbandry

2.1 ZnO NPs and their potential use in agriculture

Once nanomaterials (NMs) are released to the environment, they accumulate in ecosystems and pose threats to living organisms; therefore, it is important to understand the behavior of NMs in soil and to assess the risks for arable soil ecosystems [8]. About 260,000–309,000 metric tons (MT) of NMs were produced globally in 2010 [9], and worldwide consumption of NMs is likely to grow from 225,060 to 585,000 MT during 2014–2019 [10]. The third most commonly used metal-containing NMs are ZnO NPs with an estimated global annual production between 550 and 33,400 tons [11]. The concentration of ZnO NPs in the environment was found to be 3.1–31 μg kg^{-1} and 76–760 μg L^{-1} in soil and water, respectively [12]. ZnO NPs can strongly attach to soil particles. They exhibit low mobility at various ionic strengths [13] and show higher sorption compared to ionic zinc, and possible uptake mechanism has been illustrated in **Figure 1.**

2.2 Effects of ZnO nanoparticles on animal health

Unplanned use of ZnO NPs as nanofertilizer in agriculture leads to their entry in the food chain, and ultimately nanofertilizers enter in the body directly or indirectly, and their interaction with immune cells may have deleterious effects. The effects of ZnO NPs on the immune system are not completely understood. Some researchers postulated that increased cytosolic Zn^{2+} and the generation of ROS play important roles [16]. In innate immune, cells recognize ZnO NPs via toll-like receptors (TLR) which bind to corresponding antigens ZnO NPs and activate signal transduction pathway and inflammatory response. The ZnO NPs induce apoptosis and necrosis in macrophages in relation to their important role in the clearance of entered particulates and the regulation of immune responses during inflammation.

Withania somnifera (L.) Dual (Solanaceae) Indian ginseng or Indian winter cherry is a medicinal plant. Different parts of the plant have been used in Ayurvedic medicine formulations. Withaferin A is a steroidal lactone found in the leaves and

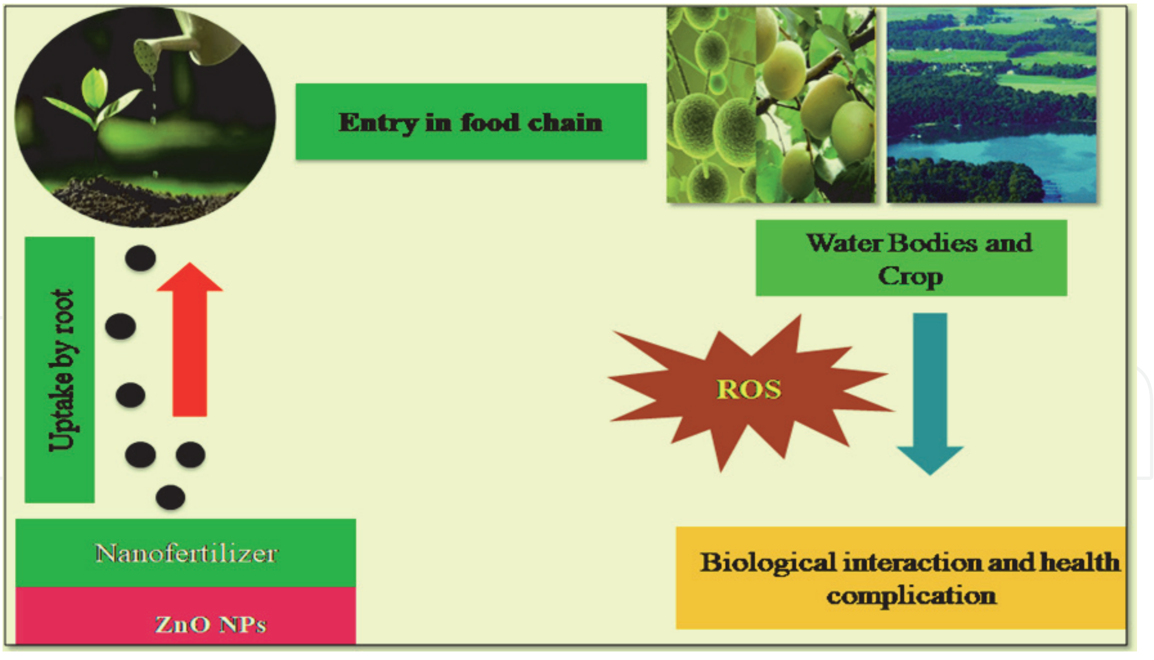

Figure 1.
Plant uptake, transport and environmental transformation mechanism of nanofertilizer (ZnO NPs) into ecosystem and entry in food chain [14, 15]. Designed by first author using Google as tool.

roots *of W. somnifera*. The biological properties of crude root extracts have been largely reported and only a few are related to the pure compound (withaferin A) as immunomodulatory function.

3. Immunological health importance of zinc as microelement

Zinc is crucial for normal development and function of cells mediating innate immunity, neutrophils, and natural killer cells (NKs). Phagocytosis, intracellular killing, and cytokine production are affected by Zn deficiency. Zinc is a micronutrient required by organisms and plays a vital role in maintaining immune and macrophage function. There is a progressive decline in immune response with the advance in aging due to the deficiency of Zn [17]. There is impairment of monocytes, reduced cytotoxicity in NK cells, and reduced phagocytosis in neutrophils [18]. Zinc is also a major intracellular regulator of lymphocyte apoptosis [19]. Impaired immune function in elderly subjects due to Zn deficiency has been shown to be reversed by an adequate Zn supplementation [18]. The beneficial effects of lower doses of Zn (≤ 50 mg/d) on immune function have been reported while very high doses of Zn (≥ 150 mg/d) may impair cellular immunity [20].

4. Mechanism of innate immunity in animals

The defense system is the bedrock of living systems and innate immunity is an integral part of health. It is the first line of the defense mechanism of the body from lower organism to mammals. Any alteration in innate immunity leads to disease conditions; however, adaptive immunity plays a great role in defense mechanism. Innate immunity has two arms, i.e., the afferent and efferent. The afferent arm is lipopolysaccharide (LPS) or endotoxin [21]. As to the effector arm of innate immunity, Hunter (1774) first recognized leukocytes at the site of inflammation. The cellular theory of immunity was given by Metchnikoff, 1884 [22] and must be recognized in the functional analysis of innate immune cells. Massart and Bordet had showed that injured cells secrete chemicals that attract phagocytes in 1917. The myeloid cells in invertebrates' are precursors of the innate immunity. Macrophages are professional immune cells that engulf and destroy foreign particles. Myeloid cells include mononuclear phagocytes and polymorph nuclear phagocytes. Macrophages are mononuclear phagocytes derived from blood monocytes. Macrophages are distributed in all parts of the body of the host and also present within the parenchyma of the heart, lungs, liver, brain, and peritoneal cavity. Pathogens invading the host body through any route are killed by macrophages. Macrophages have the potential ability of supervisory of innate immunity. Reactive oxygen intermediates are produced in phagosomes of neutrophils and macrophages. Superoxide radicals (O_2^-) are generated by the p91 subunit of cytochrome form (O_2) [23]. Superoxide (H2O2) is produced from O_2^- anions where superoxide dismutase is the catalytic enzyme.

$$2O_2 + \text{NADPH (oxidase)} \rightarrow 2O_2\bullet^- + \text{NADP} + H^+ \quad (1)$$

$$2H^+ + 2O_2\bullet^- = H_2O_2 + O_2 \quad (2)$$

Hypochlorous acid (HO Cl), a reactive halide, is produced from H_2O_2 by the action of myeloperoxidase. These radicals not only kill microbes directly but also generate other metabolites for this purpose, and singlet oxygen can be generated by O Cl^-, the former being strangely reactive with C: C double bands. Hydroxyl radicals can be produced where HO Cl react with superoxide.

$$Cl^- + H_2O_2 + H^+ = \text{HO Cl} + H_2O \quad (3)$$

$$O_2\bullet^- + \text{HO Cl} = O_2\bullet^- + OH\bullet + Cl^- \quad (4)$$

Therefore, hydroxyl radical (OH•) could be produced were agent using upper oxide as substrate, reactive nitrogen species (NO•) can be produced.

$$O_2\bullet^- + H_2O_2 = OH^\bullet + OH + O_2 \quad (5)$$

$$O_2\bullet^- + NO\bullet = ONOO \quad (6)$$

Complement, lactoferrin, lysozyme, and antimicrobial peptides are the humoral component of innate immunity. Lysozyme present in the saliva and tear inhibits the cell wall synthesis in bacteria. Complement is an enzymatic proton which plays an important role in innate immunity. Antimicrobial peptides and C-reactive proteins (CRP) are also having defense ability by disrupting plasma membrane.

5. Macrophages and their roles in animal health

The macrophages are mononuclear phagocytes and are committed progenitor cells in the bone marrow [24]. There are mainly two types of phagocytic cells, namely, macrophages and dendritic cells (DCs), which have similar cell surface receptors but different functional activities which are short-lived, and their life span depends on the nature of immune response [25]. All types of macrophages are differentiated from circulating monocyte and DCs by their expression of Fc, F4/80, and CD11b receptors. Macrophages are the main inducers of the adaptive T cell responses. Macrophages are skilled in scavenging dead cells, cellular debris, phagocytosis, and remodeling after tissue injury [26]. Their names and phenotypes vary based on their anatomical location. Physiological characters and significant roles of macrophages are listed in **Table 1** [27].

Sr.No.	Characteristic	Macrophages
1.	Precursor cells and origin of macrophages	Progenitor myeloid cell of bone marrow
2.	Maturation Site of macrophages	All tissues of body
3.	Phenotypic difference	CD68+,F4/80+ (mouse) or EMR1+ (human), CD107b+,(Mac-3+)
4.	Mature macrophages cells in circulation	No (or very less)
5.	Mature cells recruited into tissues from circulation	No
6.	Proliferative ability of mature cells	May vary by subpopulation (M_2 macrophages can proliferate)
7.	Mature cells normally present	Connective tissues
8.	Life span	7 to 30 days
9.	Detection of pathogens and initiation of inflammation	Have ability
10.	Inflammation	Increase
11.	Antigen presentation	Main cell for this work
12.	Detoxification of animal venom	Have ability

Table 1.
Physiological characters and significant roles of animal macrophages.

There are three main subtypes of macrophages: one is classically activated M_1 macrophages which play an important role in host defense and antitumor immunity, while another one is M_2 macrophages, the suppressor and regulator of wound healing. Third type is M_3 which are phagocytic cells that continuously express different types of receptors that facilitate removal of necrotic tissue, aged red blood cells, and toxin molecules from the circulation. Macrophages maintain tissue homeostasis, while macrophages and DCs act as sentinel cells for the immune response [26]. Neutrophils are recruited and inflammation is promoted by mediators as indicated by macrophages [28]. Last main classes of macrophages are known as regulatory macrophages and are similar to suppressive M_2 macrophages [29]. Regulatory macrophages are induced by toll-like receptor (TLR) agonist in the presence of prostaglandin, apoptotic cells, and immunoglobulin G (IgG) immune complexes and defined the release of the immunosuppressive cytokines IL-10 and TGF-1 [29]. Regulatory macrophages are poor antigen-presenting cells (APCs) and have the inclination to induce T_{H2} and regulatory T cell responses that can further suppress antitumor and chronic inflammatory responses.

5.1 Significance of macrophages in innate immunity

The macrophages are important cells of immune system that function in innate and adaptive immunity and can play major role in the protective and pathogenic activity. Different types of pattern recognition receptors including biosensor like C-type lectin receptors, helicase RIG like receptors, NOD-like receptors and TLRs are expressed in macrophages.

The invading pathogens, foreign substances (ZNFs, silica, and stone dust particle), microbes and dead and dying cells are recognized by these receptors as a danger signal [30]. Adaptors induced signaling causes myeloid differentiation and regulation of inflammatory vesicle formation activity. This cascade further triggers antimicrobial activity of M_1 macrophages by stimulating the production of cytokines TNF and IL-1 [31]. Apart from innate immunity, macrophages also play an important role in wound healing [32].

5.2 Promiscuity and interaction of zinc oxide nanoparticles with macrophages

The ZnO NPs have been engineered, synthesized, and commonly used in products including sunscreens, cosmetic products, food and medical materials. These play a significant role in the biomedical area for disease diagnosis and therapy [33].

ZnO NPs are also widely used in the food industry and as nanofertilizer in agriculture. The wide application of ZnO NPs increased the chance of human and animal's exposure [34]. They can be absorbed into the body and redistributed into various organs after environmental exposure [34]. Hence, the safety assessment of nanoparticle is mandatory. The ZnO NPs can influence the immune system and affect the process of diseases and the emergency responses of immunity governed by macrophages [35]. ZnO NPs are foreign particles, and the macrophages play an important role in the recognition, processing, and removal of ZnO NPs [36]. The ZnO NPs interact with soluble proteins to form a halo corona that affects NP activity. The composition of protein coronas varies according to the size of NPs [37]. Protein coronas of NP surface have two layers including hard corona nearer to the NP surface and soft corona composed of reversibly adsorbed materials and large-size NPs phagocytized by macrophages through nanoparticle protein complexes corona. ZnO NPs deflate phagocytosis of macrophages and show cytotoxic and bactericidal activities by enhancing oxidative stress which may disrupt bacterial outer cell membrane and causes cell apoptosis. The ZnO NPs also inhibit nitric oxide (NO) production through the NF-Kβ signaling. NO reduces ZnONPs toxicity in rice seedlings by regulating oxidative damage and antioxidant defense systems [38].

5.3 Mechanism of ZnO NPs induced toxic effects on cells of immune system

The effects of ZnO NPs on the immune system depend on their physicochemical properties [39]. A nanotoxicological effect of NPs depends on the size, size distribution, surface area, electrostatic charge, and solubility [40]. The ZnO NPs are more water-soluble which result in more dissolution of toxic ions and ROS production [41]. ZnO NPs undergo endocytosis into the macrophages cells, dissolve into bioavailable zinc ion, and increase oxidative stress through ROS which cause immunotoxicity. The important factor for the immunotoxicity of ZnO NPs is ROS. Intracellular ROS production has at least some contribution in cell death induced by ZnO NPs [42, 43].

6. Effect on epithelial barriers of innate immunity

The important components of the innate immune system are epithelial barriers, phagocytic cells (dendritic cells, polymorphonuclear leukocytes, monocytes/macrophages), phagocytic leukocytes, basophils, mast cells, eosinophils, natural killer cells, circulating plasma proteins. TLR is the main signaling in the innate immunity which induce expression of inflammatory gene. Metal oxide nanoparticles trigger the TLR signaling pathway.

7. The innate immune system and role of TLRS signaling pathway

The innate immune system relies on the recognition of pathogen-associated microbial particles (PAMPs) through a limited number of germ line-encoded pattern recognition receptors belonging to the family of TLRs [44]. The activation of TLR signaling induces cytokines production and phagocytosis of macrophages along with catalytic activity of NK cells. More importantly, TLR signaling activation can also enhance antigen presentation via upregulating the expression of major histocompatibility complex (MHC) and co-stimulatory molecules (CD80 and CD86) on dendritic cells leading to adaptive immunity activations. Nanoparticles enhanced TLR signaling pathways which act as adjuvants [45]. The TLR antagonists or inhibitors that reduced the inflammatory response would have beneficial therapeutic effects in autoimmune diseases and sepsis [46].

7.1 Effect of NPs on TLR signaling of innate immunity health

The TLR is a type I transmembrane receptors which contain an N-terminal domain (leucine-rich repeat) and a C-terminal toward cytoplasm. When macrophage receptors recognized PAMP, TLRs recruit a TIR domain such as MyD88 and TRIF and initiate signaling events called downstream signaling by the secretion of different inflammatory molecules (chemokines, inflammatory cytokines, and IFNs I) [47]. The TLR signaling is responsible for the transcription of inflammatory and immune responses genes [48]. ZnO nanoparticles induced MyD88-dependent proinflammatory cytokines via a TLR signal pathway [49]. The TLRs may have important roles in NP uptake and for their cellular response which is directly proportional to the size of NPs.

7.2 Effects of NPs on phagocytic cells

Macrophages and dendritic cells have phagocytic activity; hence they readily uptake nanoparticles. Therefore, magnetic nanoparticles and nanoparticles-based PET agents were usually used for the visualization of macrophages in human diseases (cancer, atherosclerosis, myocardial infarction, aortic aneurysm, and diabetes).

8. Indian ayurvedic medicinal plant: *Withania somnifera*

*Withania somnifera (*Ashwagandha), an Indian Ayurvedic medicinal plant, is a green shrub and belongs to the Solanaceae family. For over 3000 years, Indians cultivated and applied its whole plant extract or separate constituents in Ayurvedic and indigenous medicine [50]. It was shown to have anti-inflammatory, antitumor, anti-stress, antioxidant, immuno-modulatory, hematopoietic, and rejuvenating properties thus benefiting the endocrine, cardiopulmonary, and central nervous systems [51]. It inhibits immunologically induced inflammation and a variety of pharmacological effects in *Babl/c mice* [52]. Various mechanisms have been proposed to explain the antitumor activity of Ashwagandha including potent anti-inflammatory, anti-angiogenic, anti-metastatic, pro-apoptotic, and radio-sensitizing properties [53]. An extract of *W. somnifera* showed immunological activity in *Balb/c mice*. Treatment with different doses of *W. somnifera* root extract (20 mg/dose/animal; i.p.) enhanced the total WBC counts. *W. somnifera* extract along with sheep red blood cell antigen (SRBC) increased antibody titer in circulation and plaque-forming cell numbers (PFC) in the spleen and reduced the delayed-type hypersensitivity reaction in mice model. Withania extract improved in phagocytic activity of macrophages when compared to untreated mice. The immunomodulatory effects of *W. somnifera* against ZnO NP-mediated toxicity in *Balb/c* mice study showed a dose-dependent reduction in phagocytosis, an increase in the levels of NO production along with upregulation of *TLR6, and arginase* gene. However, the adverse effect of ZnO NP on macrophages was reduced by *W. somnifera* extract and withaferin A with decreased *TLR6* overexpression and improved phagocytic activities [14].

8.1 Pharmacological and medicinal activities of withaferin a

Withaferin A is the key withanolide prototype which has been shown to have anti-inflammatory [54], antitumor [55], anti-angiogenesis [56], radio-sensitizing activity, and chemopreventive [57] and immunosuppressive [58] properties. Withaferin A is highly reactive because of the ketone containing unsaturated

A ring, the epoxide in B ring, and unsaturated lactone ring. In another study, withaferin A inhibited NF-κB at a very low concentration by targeting the ubiquitin-mediated proteasome pathway in endothelial cells. In vitro experiments demonstrated that withaferin A interfered with TNF-induced NF-κB activation at the level or upstream of IKKβ [57]. Withaferin A inhibited the expression of iNOS in the lipopolysaccharide (LPS)-stimulated murine macrophage cell line [59]. Withaferin-A inhibited, LPS-induced COX-2 expression and PGE2 production in BV_2 murine microglial cells [60]. Both pre and post-treatment of astrocytes with Withaferin-A attenuated LPS-induced production of tumor necrosis factor-α and the expression of COX-2 with expression of induced nitric oxide synthase by blocking the NF-κB activity [61]. Treatment with withaferin A increased SOD, catalase, and glutathione peroxidase activity in rat brain frontal cortex and striatal concentrations [62].

9. Conclusions

Nanoparticles are gaining importance recently due to their exciting applications in different fields like agriculture, human health, and livestock sector. Increased application of ZnO NPs is clearly indicating the adverse effect on immunity. It is, therefore, necessary to explore safety level of ZnO NPs and their role in humans and animals. The future work must be placed in the context of current risk assessments which must be associated with ZnO NPs toxicity and safety level and their uses. Further research work is emphasized for elucidating the nature of ZnO nanoparticles and their fate in living and non living matrices which can serve as to safeguard the ecosystem functioning. The ecosystem strengthening should be in term of agriculture and livestock particularly concerns production, food resource, immune and health status of animals. The role of *W. somnifera* as an antidote to immune complication induced by ZnO NPs exposure needs further research.

Author contributions

The authors' responsibilities were as follows: Dr. Jitendra Kumar, Dr. Chander Datt, Suryakant Verma and Kavita Rani conceived and designed the chapter.

Conflict of interest

The authors declare no competing interest.

Notes/thanks/other declarations

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