Zinc oxide nanoparticles significant role in poultry and novel toxicological mechanisms

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Abstract

Zinc oxide nanoparticles (ZnO NPs) have involved a lot of consideration owing to their distinctive features. ZnO NPs can be described as particularly synthesized mineral salts via nanotechnology, varying in size from 1 to 100 nm, while zinc oxide (ZnO), it is an inorganic substrate of zinc (Zn). Zn is a critical trace element necessary for various biological and physiological processes in the body. Studies have revealed ZnO NPs' efficient immuno-modulatory, growth-promoting, and antimicrobial properties in poultry birds. They offer increased bioavailability as compared to their traditional sources, producing better results in terms of productivity and welfare and consequently reducing ecological harm in the poultry sector. However, they have also been reported for their toxicological effects, which are size, shape, concentration, and exposure route dependent. The investigations done so far have yielded inconsistent results, therefore, a lot of additional studies and research are required to clarify the harmful consequences of ZnO NPs and to bring them to a logical end. This review gives an overview of the possible role of ZnO Nano particles in the poultry industry, primarily as dietary supplements that effect on the growth, health, and performance of the birds. In addition to the anti-bacterial mechanisms of ZnO NPs and their promising role as anti-fungal, and anti-colloidal agent, this paper also covers the toxicological mechanisms of ZnO NPs and their consequent toxicological hazards to vital organs and the reproductive system of poultry birds.

1. Introduction

'Zinc' is the second most abundant and crucial nutritional trace element found in the body (De Grande et al., 2020; Wan and Zhang 2022). Zn is requisite for several biological processes, like growth, metabolism, reproduction, wound healing (Liu et al., 2011), and bone and feather development (Shao et al., 2014; Kwiecień et al., 2017). More than 200 enzymes involved in a variety of physiological functions, such as immunology, antioxidant capacity, and other epigenetic activities, require Zn as a cofactor (Ao and Pierce 2013). These enzymes further regulate the synthesis and breakdown of lipids, nucleic acids, proteins, and carbohydrates and include almost all recognized categories of enzymes (Classen et al., 2011). Zn acts as a structural element in many different proteins and enzymes, such as superoxide dismutase (SOD), which is a crucial part of the antioxidant defense system (Bao and Choct 2009). However, the body lacks any unique system for storing Zn; therefore, consistent dietary usage of Zn is required in order to maintain its homeostasis in the body and to enable Zn to sustain its multiple functions (Bonaventura et al., 2015). The poultry industry contributes significantly to the growth of the economies of many nations, especially emerging nations, and serves as a decent, inexpensive, and accessible source of animal protein (Abo-Al-Ela et al., 2021; Ganapathy et al., 2021; Tahir et al., 2021; Onunkwo et al., 2021). The primary goal of the poultry industry is to improve the performance and health of the chicken by providing safer feed (Khan et al., 2012; Mahmood et al., 2022; Malik et al., 2022). Presently, both organic (amino acids, Zn chelates of glycine, lysine, or methionine, and their hydrate and sulfate analogues) and inorganic (Zn oxide [ZnO], Zn acetate, and Zn sulfate [$ZnSo_4$]) Zn substrates are used as nutritional feed additives (Naz *et al.*, 2016; Imran *et al.*, 2020; Ali et al., 2022). However, ZnO or $ZnSo_4$ are primarily used for Zn supplementation in

poultry (De Grande et al., 2020). As per the guidelines of the National Research Council (NRC), the per-day Zn requirement for poultry is 40 ppm (NRC, 1994). However, on commercial levels, feed manufacturers supplement their feed with an additional 100–120 ppm of Zn to speed up the growth of their chicks (Feng et al., 2010). But this increase drives up the price of producing feed, and in the long run, Zn excretion in feces may cause environmental pollution. It also reduces vitamins and unbalances other microelements (Sagar et al., 2018; Akhavan-Salamat and Ghasemi 2019; Reda et al., 2021) like cadmium, iron, and copper by reducing their digestibility (Suttle 2010). Although these issues can be partially resolved by boosting Zn's bioavailability (Sagar et al., 2018; Akhavan-Salamat and Ghasemi 2019; Reda et al., 2021; Samy et al., 2022).

Nanotechnology has been a current trend in several fields, and there has been widespread adoption of it (Alkhtib et al., 2020). NPs refer to particles having a size of less than 100 nm in three dimensions (Titma et al., 2016; Bakr et al., 2020). The nanoforms, or NPs, of essential minerals, have lately been investigated for their effects on the growth, health condition, feed efficiency, and performance of animals, including poultry (Patra and Lalhriatpuii 2020; Tammam et al., 2020). NPs have the potential to distinctively deliver vital trace minerals in animal feed, along with boosting mineral bioavailability and digestibility (Alkhtib et al., 2020; Youssef et al. 2020). This increased availability could be due to the particle's minor size and increased surface area to volume ratio (Rajendran 2013; Li et al., 2016; El-Dawy et al., 2023). These NPs manifest distinctive physical, biological, and chemical properties in comparison to their large-sized particles (Patra and Lalhriatpuii 2020). They are likely to alter the biological impacts produced by them as compared to their bulk materials (Patra and Lalhriatpuii 2020), and the amount entailed is also less (Sri Sindhura et al., 2014). NPs can be successfully manufactured utilizing any physical, chemical, or biological process (Abd El-Hack *et al*., 2021; Altaf and Umair, 2022).

ZnO NPs have attracted more attention recently, primarily on account of their small particle size and increased surface reactivity (Mohd Yusof et al., 2019; El-Bahr *et al*., 2020). On an annual basis, nano-ZnO is the third most manufactured metal nanoparticle worldwide, after nano-silicate and nano-titanium (Piccinno et al., 2012). The Food and Drug Administration of the US has listed ZnO as "Generally Recognized as Safe" (GRAS) (FDA-2015) because of its non-toxic properties (Pulit-Prociak et al., 2016), so the usage of its nano-form as a feed additive is well justified. The feed's source of Zn affects the Zn absorption rate (De Grande et al., 2020). Numerous investigations seeking to find out more about the impacts of Zn NP addition on poultry's growth rate, intestinal architecture, and immunological response utilized ZnO NPs because of their good biocompatibility, affordable price, and minimal toxicity (Jiang et al., 2018). ZnO NPs are tremendously promising for biological applications, especially as antimicrobial agents (Sirelkhatim et al., 2015; Jamdagni et al., 2018). The antibacterial characteristics of ZnO NPs are far better than those of traditional ZnO (Padmavathy and Vijayaraghavan 2008). ZnO NPs outperform traditional Zn sources as they have a progressive effect on both the antioxidant defense system, performance of chicken (Mohammed and Safwat 2013; Mohammadi et al., 2015; Ali et al., 2017; El-Katcha et al., 2017; Eskandani et al., 2021). Numerous studies have documented the effectiveness of ZnO NPs in preventing the development of an extensive range of disease causing agents (Jayaseelan et al., 2012; Moghaddam *et al*., 2017; Saravanan et al., 2018), which could potentially offer an alternative to

conventional antibiotics. However, in contrast to this, some studies have reached out to test the toxic properties of ZnO Nanoparticles in various biological systems, including mammalian cells (Wang et al., 2010), bacteria (Sinha et al., 2011), and the reproductive system (Liu et al., 2016; Liu et al., 2017). As a result, it is imperative that further research be conducted to assess the potential efficacy and toxicity of ZnO NPs so that their usage as a supplement in poultry can be made effective and safe.

2. Potential Role Of Zno Nps As A Dietary Supplement

Zn has a limited bioavailability in the animal body, so dietary consumption must be consistent. In general, the various forms of Zn sources utilized in animal feed are both organic Zn like Zn acetate, Zn methionine, and Zn propionate, and inorganic Zn such as $ZnSO_4$ and ZnO (Schlegel et al., 2013; Khoobbakht et al., 2018). Although organic Zn has better bioavailability than inorganic Zn, its use in the diets of animals is limited as it is overpriced (Zhao et al., 2014; Khoobbakht et al., 2018). Secondly, the low utilization rate of traditional inorganic Zn as a feed additive for animals is the major problem. As a result, producers and animal nutritionists utilize more dietary Zn than is normally advised to maximize animal performance (Bratz et al., 2013). This excessive feed addition of Zn eventually leads to an increased Zn level in the excreta, which has negative environmental repercussions (Rajput et al., 2018) In addition, the body's ability to maintain vitamins and the stability of other nutrients may be impacted by excessive dietary Zn supplementation (Zhao et al., 2014). In such a scenario, the development of nanotechnology linked to the nanoscale has increased the effectiveness and bioavailability of trace elements in the diet of animals (Gopi *et al*., 2017). Current studies have concentrated heavily on ZnO NPs' impact on animal productivity and their promising use as a nutritional supplement and a substitute for traditional Zn (Wang et al., 2017; Zhao *et al*., 2017; Abedini *et al*., 2017; Khajeh *et al*., 2018). Owing to their compact size, ZnO NPs, when added to feed, improve and raise the rate of Zn assimilation in the gastrointestinal tract (GIT), which in turn enhances Zn uptake by the animal body and ultimately increases its bioavailability (Gangadoo et al., 2016; Tsai et al., 2016). These NPs are competent enough to pass through the GIT and distribute themselves further into the bloodstream and intended organs (Mohd Yusof et al., 2019). The secretion of Zn in feces is also reduced because of the increased bioavailability of ZnO NPs, thereby palliating environmental pollution (Mohd Yusof et al., 2019).

3. Effect On Growth Performance.

The animal's growth performance has reportedly been improved by the incorporation of ZnO NPs in the feed. When broiler chickens were fed diets of ZnO Nanoparticless at absorptions of 20 and 60 mg/kg, they gained more body weight and had improved food alteration ratios (FCR) associated to when traditional Zn was used (Zhao et al., 2014). At an optimal dosage of 20 mg/kg, biologically active ZnO NPs enhanced the antioxidant capacity and growth performance of broilers (Xueting et al., 2018). Likely, adding ZnO NPs at absorptions of 60 and 90 mg/kg to the feed considerably increased weight of body, dressing weight, and carcass weight of broiler chickens (Khah et al., 2015). Forty-two days of supplementation with ZnO NPs at a level of 40 mg/kg have been observed to improve the creation

chickens (Lina *et al*., 2009). Similarly, the ZnO NPs in the dietary addition to poultry cause an increase in the redness value and intramuscular fat in the breast muscles, along with an increased percentage of eviscerated yield. It also raises pH in thigh muscles along with increased levels of average daily gain (ADG), DM, and ADF. However, drip loss is observed in the thigh and breast muscles, while shear force is decreased only in thigh muscles (Liu et al., 2011). According to an investigation by Ahmadi et al., (2013), introducing ZnO NPs up to a threshold of 120 ppm caused a decrease in the concentration of breast cholesterol in chickens. The putative function of Zn in lipid metabolism could provide an explanation for this. Incorporating ZnO NPs into poultry diets brought about a substantial reduction in triacylglycerol (TAG) and serum total cholesterol (TC). This outcome could be attributed to Zn's role in improving fat metabolism or lowering dietary lipid absorption (Ibrahim et al., 2017). Although ZnO NPs at higher levels, like 100 mg/kg, suppress developmental performance, demonstrating the impacts of ZnO NPs as feed additives is dependent on their degree of concentration and must be given at the right scale (Zhao et al., 2014). So, the nutritive addition of ZnO NPs can enhance the growth performance of birds along with improving the quality of poultry meat, but specifying an optimum dose is necessary.

4. Effects On Egg Quality And Quantity.

The poultry industry places a significant amount of attention on the eggshell because it preserves the safety of the inside ingredients and provides an oval shape to the cell. It also works as a shield against harmful microbes, as it has been demonstrated that shell flaws increase the danger of bacterial spoilage of eggs. Broken or cracked eggshells result in significant financial loss and decrease the safety and quality of egg products (Samiullah et al., 2014). Zn is a constituent of the enzyme carbonic anhydrase, which is crucial for eggshell development and for enhancing its tensile strength (Mabe et al., 2003). In particular, Zn influences the isthmus epithelium’s structure, which contributes to the formation of the eggshell membrane (Nys *et al*., 2014; Rodriguez-Navarro *et al*., 2015). Older layer hens frequently have weak eggshells, which causes eggs to break easily. Travel et al., (2011) projected that the incidence of fractured and damaged eggs during the latter production phase could reach as much as 12–20% due to age-related deterioration in eggshell strength. The eggshell strength and thickness were raised by the diet supplemented with Zn methionine and ZnO NPs in comparison to normal ZnO (Abedini *et al*., 2018). Additionally, the diets with ZnO NPs demonstrated increased egg production. This might be justified by the significant part that Zn plays in the formation and release of the reproductive hormone. This reproductive hormone helps to increase the bioavailability and absorption efficacy of ZnO NPs for egg production. Likely, the layer hens treated with organic Zn and ZnO NPs had thicker eggshells, which was likely due to the increased bioavailability and retention of Zn in the body (Tsai et al., 2016). Laying hen performance was enhanced by dietary supplements of ZnO NPs or zinc methionine, with 80 mg per kg being optimal (Li et al., 2019). Therefore, adding ZnO NPs to layer hen feed could improve egg quality and address the issue of weak eggshells in older layers. So, in this context, ZnO NPs are found to be more efficient than organic Zn and could be replaced in the field.

5. Effects On Intestinal Morphology (In Combination With Probiotics)

Intestinal morphology is crucial for improved nutritional absorption because larger villi help the intestine absorb nutrients more effectively. Nutritional supplementation of ZnO NPs alone has stronger benefits for improving intestinal morphology. However, the probiotic supplementation with ZnO NPs dramatically improves intestinal morphology, enhancing the body's ability to absorb other nutrients and improving overall health (Yusof *et al.*, 2019). It has been hypothesized that probiotics can lower intestinal pH altering the gut microflora and causing an increase in short-chain fatty acids (Jiang et al., 2015), which in turn increases the solubility and absorbability of minerals (Byrne et al., 2015). Several studies in recent years have combined probiotics with nano-minerals like selenium nanoparticles (Saleh, 2014) and ZnO NPs. Studies by Khajeh *et al.*, (2018) observed a synergistic effect when ZnO nanoparticles and probiotics were consumed together leading to enhanced villus width and height in broiler chickens. In comparison to traditional ZnO and ZnO nanoparticless (25 mg/kg), the results exhibited that broiler chicken fed ZnO NPs (50 mg/kg) plus probiotics improved the villi height to crypt depth ratio. A summary of the role of ZnO NPs used as a feed, Additives in poultry has been shown in Fig. 2.

6. Effects On Enzymes

Zn, being an essential mineral in the body, functions as a catalyst or as a cofactor in several enzymes, such as superoxide dismutase (SOD). SOD is a key player in the anti-oxidant defense system, protects cells from oxidative stress, and is crucial for the cleansing of superoxide free radicals (Prasad 2014). Fathi et al., (2016) gave ZnO NPs to broiler chickens at an amount of 20 mg/kg, and the activity of copper-zinc superoxide dismutase (Cu-Zn-SOD) was significantly affected. Cu-Zn-SOD is a metalloenzyme and a member of the ubiquitous SOD family (Perera et al., 2016). Although at higher concentrations, the activity of Cu-Zn-SOD was not appreciably impacted. Layer hens fed 80 mg/kg of ZnO NPs and organic Zn had higher levels of SOD in their liver and pancreatic tissues than those given conventional Zn (Abedini *et al.*, 2018). According to Ahmadi et al., (2014), supplementing broiler chicken feed with 60–90 ppm of ZnO NPs greatly boosted the activity of SOD in comparison to the control and those birds that were given a 30 ppm level. They discovered that higher Zn levels have an inhibitory effect, as the lowest activity of the SOD enzyme was observed at 120 ppm. There is a common consensus that ZnO NPs and organic Zn have high bioavailability, which increases Zn retention, decreases its excretion, and boosts the SOD activity. Additionally, catalase is a known antioxidant enzyme that works to shield cells from ROS-induced oxidative damage (Jiang et al., 2009). Catalase activity decline is associated with a rise in oxidative stress (Duzguner and Kaya 2007). In the Zhao et al., (2014) study, broilers given ZnO NPs at a level of 20 mg/kg had significantly greater serum catalase activity, indicating that NanoParticles supplementation induced antioxidant activity. The enzymatic activity of catalase in the liver tissue samples is gradually inhibited by the increasing supplementation of ZnO NPs up to 100 mg/kg. The (mRNA) expression of cancer necrosis factor alpha (TNF-α), interleukin 10 (IL-10), and interleukin 1β (IL- 1β) was enhanced by ZnO NPs supplementation (Wang et al., 2018). Therefore, increased bioavailability of Zn in the body has a positive regulatory effect on certain enzymes.

7. Effects On Immunity

Supplementing birds with ZnO NPs show a positive impact on their immunological status as Zn improves the functions of the immune system. Zn is necessary for thymulin to produce thymocytes and peripheral T-cells via thymus secretion (Bonaventura et al., 2015). Hence, the high bioavailability of Zn increases thymulin activity, which in turn encourages immunological responses in the body of the animal (Mohd Yusof et al., 2019). Additionally, ZnO NPs have been shown to preserve the stability of lysozyme (Chakrabort *et al*., 2010). In layer hens, the addition of ZnO NPs at a concentration of 80 mg/kg in feed raised sheep red blood cell (SRBC) antibody (Ab) titers in comparison to traditional Zn. Additionally, this ZnO NPs-containing diet showed a stronger cellular immunological retort to antibody titers in contradiction of Newcastle sickness (Abedini *et al*., 2018). In a comparison of the nutritional effects of inorganic Zn (traditional Zn), ZnO NPs, and organic Zn (Zn-methionine) on broilers, it was identified that the latter two had higher antibody titers against SRBC than the former one (Sahoo et al., 2014). So, all of these studies point to the fact that ZnO NPs have a beneficial effect on Zn bioavailability, which in turn affects Zn's ability to be absorbed by the body and affects the immune response.

Table 1
Potential role of ZnO NPs as dietary supplement in poultry

ZnO NPs	Effects	Dose/conc of ZnO NPs	Model Bird	Salient findings	References
	On body health and overall productivity	20–60 mg/kg	Broiler chickens	↑ Body weight Better FCR	Zhao et al., 2014
		20 mg/kg (Biologically active)	Broilers	Enhanced antioxidant activity and growth performance	Xueting et al., 2018
		60 and 90 mg/kg	Broilers	↑ Live body weight ↑ Dressing weight ↑ Carcass weight	Zhao et al., 2014
		40 mg for 42 days	Broilers	Enhanced production and dressing performance	Lina *et al*., 2009
		-	Poultry	↑ Redness value and intramuscular fat in breast muscles ↑ Eviscerated yield % ↑ pH in thigh muscles along with ↑ ADG, DM and ADFI	Liu et al., 2011
		-	Poultry	Drip loss in thigh and breast muscles ↓ Shear force in thigh muscles	Liu et al., 2011
		-	Poultry	↓ TAG ↓ TC	Ibrahim et al., 2017
		120 ppm	Broiler	↓ Breast cholesterol concentration	Ahmadi et al., 2014
	On Egg Quality and Quantity	80 mg/kg Along with Zn-methionine	Layer Hen	Thicker egg shells ↑ Egg production	Abdeni *et al*., 2018; Li et al., 2019
	On Intestine morphology	50 mg/kg plus probiotics	Broiler chickens	↑ Villi height to crypt depth ratio ↑ Solubility and absorbility of minerals	Khajeh *et al*., 2018 Bryne *et al*., 2015

↑= increase ↓= decrease

On Enzymes	20 mg/kg	Broiler chicken	Cu-Zn-SOD activity affected	Fathi et al., 2016
	80 mg/kg	Layer hen	↑ SOD level in liver and pancreas as compared to conventional Zn	Abdeni *et al*., 2018
	60–90 mm	Broiler chicken	Boosted SOD in comparison to control and those given 30 ppm	Ahmadi et al., 2014
	120 ppm	Broiler chicken	Lowest activity of SOD indicating ↑ Zn level have inhibitory effect	Ahmadi et al., 2014
	20 mg/kg	Broiler chicken	↑ Serum catalase activity	Zhao et al., 2014
On Immunity	80 mg/kg	Layer hens	↑ SRBC Ab titer	Shaoo *et al*., 2014

↑= increase ↓= decrease

8. Significant Role Of Zno Nps As An Antibacterial Agent

ZnO NPs demonstrate bactericidal action in contradiction of both gram-negative/positive bacteria (Arabi et al., 2012). Furthermore, they efficiently combat spores that can withstand high pressure and temperature (Rosi and Mirkin 2005). The stability, concentration, shape, and size influence the bactericidal potential of ZnO NPs. For instance, Stevia leaves were used in the green-mediated production of rectangular-shaped ZnO NPs, which showed a strong bactericidal action against *Staphylococcus aureus* and *Escherichia coli*. with a minimum inhibitory concentration (MIC) value of 2.0 µg/mL, the outcomes demonstrated that these rectangular-shaped ZnO NPs at lower concentrations had a greater antibacterial action (Khatami et al., 2018). Similarly, the antibacterial action of zinc oxide nanoparticles in the form of nanorods generated by a cell-free supernatant of *Bacillus megaterium* was examined against a strain of *Helicobacter pylori* that was multidrug-resistant too. According to the TEM examination, cells treated at a dose of 17 g/mL for 60 minutes with ZnO NPs exhibited damage to the cell membrane, causing the cellular content to leak out and ultimately leading to cell death. Comparatively, the cell that had not been exposed to ZnO NPs had an intact cell membrane and was complete. The authors also hypothesized that these nanorods behaved as a needle that harmed the cells by piercing the bacterial wall (Saravanan et al., 2018). ZnO NPs can offer a new avenue as anti-bacterial agents in poultry, but due to recommended *in-vivo* evaluation methods, they are still underutilized.

9. Antibacterial Mechanisms.

The antibacterial mechanisms incorporate means and processes by which numerous bacteriological cell actions, including their breakdown, active transport, and enzyme action, are inhibited, leading to their death. Various scientists have suggested certain probable bactericidal mechanisms, but the precise antibacterial mechanism of ZnO NPs is still unknown (Mohd Yusof et al., 2019). Among the several proposed antibacterial mechanisms adopted by ZnO NPs, one is via the development of ROS reactive oxygen species, abbreviated as ROS (Happy Agarwal *et al.*, 2018). It develops when subjected to UV and mostly consists of reactive species, e.g., superoxide anion (O_2^-), hydroxyl ions (OH^-), and hydrogen peroxide (H_2O_2). These responsive species are produced on the NP's surface, where they react with the hydroxyl groups and take up water to generate hydroxyl radicals and H^+. They eventually create O_2^- with the existence of oxygen (Król A et al., 2017). In the presence of H^+ and electrons, this O_2^- then further reacts with H^+ to make HO_2 and produce H_2O_2 (Sirelkhatim et al., 2015). Ultimately, this H_2O_2 permeates the bacterial cell membrane and damages its DNA, proteins, and lipids, causing injury and ultimately cell death (Xia et al., 2008). With the exception of H_2O_2, OH^- and O_2^- can only be found on the exterior of bacterial cell membranes because of their negative charge (Zhang *et al.*, 2007). Another theory for the antibacterial action of ZnO NanoParticles is over their "electrostatic binding" to the bacterial cell membrane. ZnO NPs can adhere to negatively charged bacterial cells more easily due to their positive zeta potential, which results in their entry into the cells (Seil and Webster 2012). This interaction could harm the bacterial cell's integrity and alter the plasma membrane structure, allowing internal contents to seep out and ultimately leading to cell death (Jayaseelan et al., 2012).

Another potential mechanism for ZnO NPs' antibacterial action is that these small NPs have easier penetration into the cells and greater surface reactivity, so they end up releasing the Zn^{2+}. Later on, the poisonousness of Zn^{2+} on the biomolecules of the bacteriological cell induces their death. One of the primary hypotheses in antibacterial processes is that Zn^{2+} is released from ZnO NPs (Soren et al., 2018). The size and morphology also affect the release of Zn^{2+}. For example, the amount of Zn^{2+} released from small spherical-shaped NPs is greater than the amount released from rod structures because of the reduced surface area, which brings about equilibrium solubility (Chang et al., 2012). Another hypothesis states that when bacteria are subjected to ZnO NPs, their permeability significantly increases, impairing normal transport across the plasma membrane (Auffan et al., 2009), and ultimately causing cell death. Similarly, another proposition states that ZnO NPs may permeate and damage bacterial cells by reacting with components that comprise sulfur and phosphorus, like deoxyribonucleic acid (DNA) (Arabi et al., 2012). Apart from all these proposed mechanisms, ZnO NPs' antibacterial activity is determined by their surface area and concentration, while particle shape and crystalline structure have a minimal impact (Arabi et al., 2012). Some other investigators also stated that antibacterial activity is inversely correlated with size, i.e., the smaller the ZnO particle, the more enhanced its antibacterial activity is (Shrivastava *et al.*, 2007). Thus, it may be inferred that ZnO NPs buildup in bacterial cells impedes their regular metabolic functions, ultimately resulting in cell death.

10. Zno Nps Utilization As An Anti-fungal Agent.

In warm, humid climates, fungi generate secondary metabolites known as mycotoxins. They are damaging to the productivity and well-being of poultry (Mgbeahuruike et al., 2018). Mycotoxins, produced by fungi like the *Aspergillus*, *Penicillium*, and *Fusarium* species, are frequent contaminants in animal feed (Vila-Donat et al., 2018). In the poultry feed sector, depending on the locale, the main worry among mycotoxins is aflatoxins. The most deadly and frequent toxin in food among aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), and aflatoxin B2 (AFB2) is aflatoxin B1 (AFB1) (Yang et al., 2012). They have been found to contaminate animal feed in a high percentage (Kosicki et al., 2016) with detrimental consequences seen on the health and performance of the animals. Countless investigations have been conducted to investigate the antifungal potential of ZnO NPs. *Aeromonas hydrophila's* produced ZnO NPs demonstrated a maximal inhibitory zone of antifungal activity against *Aspergillus flavus* (19mm ± 1.0 mm) (Jayaseelan et al., 2012). Moreover, an antifungal investigation of biologically mediated synthesis of ZnO NPs conducted by Jamdagni et al., (2018) against *Fusarium oxysporum*, *Botrytis cinerea*, *Alternaria alternata*, *Penicillium expansum*, and *Aspergillus niger* indicated ZnO NPs were efficacious in contradiction of all the tested fungi, and in particular, *Aspergillus niger* was identified as being delicate to ZnO NPs with 16 µg/ml as the lowest MIC value. Therefore, keeping in mind this perspective, ZnO NPs may potentially replace traditional fungicides as an antifungal agent and possibly stop the emergence of fungicide resistance.

11. Activities Of Zno Nps As An Anti-coccidial Agent.

In the rearing of livestock and poultry, coccidiosis is one of the most prevalent enteric diseases. This disease is brought on by the Eimeria-genus enteric protozoa, which infects the intestinal mucosa, leading to bloody diarrhea, decreased weight gain, and increased death rates (Wajiha and Afridi 2018). There has been scientific data about ZnO NPs that shows they possess anticoccidial properties (Dkhil et al., 2015). Milani et al., (2017) noticed that weaned piglets, when provided ZnO NPs at dosages of 15, 30, and 60 mg/kg, had their gut microbiota stabilized. According to an in vivo investigation into the anticoccidial activity of ZnO NPs, *Eimeria papillata*-infected mice produced 29.7 × 103 ± 1500 oocysts/g of feces, while treated mice had reduced excretion of 12.5 × 103 ± 1000 oocysts/g of feces. ZnO NPs are effective against pathogenic microbes, and numerous investigations have shown that their supplementation impacts the gut microbiome of domesticated animals (Feng et al., 2017; Yausheva et al., 2018). However, due to a lack of in vivo assessment methodologies, their use as an anti-microbial cause in animal farming is still neglected.

Similarly, an intestinal microbiota-based study by Yausheva et al., (2018) on broiler chicken supplemented with ZnO NPs demonstrated increased biological activity of the cecal microbiota. The researchers proposed that ZnO NPs be considered a promising bactericidal treatment for broilers. Feng et al., (2017) fed ZnO NPs to hens for nine weeks, and they displayed a reduction in the abundance of bacteria in the ileum, especially *Lactobacillus*; yet, this effect was dependent on dose. *Lactobacillus* is the most common bacterial genus in animal guts (Jiao et al., 2016). It is crucial in modulating the level of certain pathogenic bacteria, (Feng et al., 2010) so its decrease in the GIT is of special relevance (Jiao et al., 2016). Yausheva et al., (2018) discovered the amount of *Lactobacillus* in the cecum of broiler

chickens decreased with the dietary addition of ZnO NPs, whereas the level of other pathogenic microorganisms did not change. This suggests that ZnO NPs supplementation can also control pathogenic microbes in an animal's digestive system. However, this contrasts with a study by Xia et al., (2017), which found that giving piglets ZnO NPs at a dosage of 600 mg/kg increased the abundance and diversity of the microbiota in their ileum and colon. Researchers also pointed out that *Firmicutes*, *Lactobacillaceae*, and *Lactobacillus* were becoming more numerous in the colon. This development may be advantageous and aid in maintaining a more balanced micro-ecosystem of the gut.

Due to their inherent features, ZnO NPs are the perfect antimicrobial agent (i.e., anti-bacterial, anti-fungal, and anti-coccidial) and a replacement for traditional antibiotics. Their unique mechanisms differ from those of conventional antibiotics, inhibiting the emergence of multidrug-resistant bacteria. Additionally, multiple in vitro investigations into ZnO NPs' antibacterial activities have demonstrated their remarkable capacities to suppress the development of a variety of bacterial species at potentially reduced levels. However, the biggest concern associated with their use as antimicrobial agents is their detrimental effects on useful gut microbes. Therefore, a lot more research is required to clarify their impacts on the poultry gut environment and ecosystem.

12. Toxicological Effects Of Zno Nps.

Apart from ZnO NPs’ promising use as a feed supplement, they are also inclined to have unhealthy impacts on the animals. Their toxicological threat is still debatable, as a few studies have noted their therapeutic advantages while others have highlighted their toxicity to living organisms (Bondarenko et al., 2013; Ma et al., 2013; Cho et al., 2013; Talebi et al., 2013; Jo et al., 2013; Lopes et al., 2014).

12.1 Toxicity mechanisms.

There has been a tremendous amount of work published in the last ten years that aims to reveal the underlying processes of ZnO NPs induced toxicity, but the complete scope of these mechanisms is still uncertain and pretty much unknown (Chong et al., 2021). There are several hypothesized ideas regarding their toxicity, such as that they are capable of readily entering cells, attaching to membranes, or releasing Zn^{2+}, which in turn causes oxidative stress that is mediated by DNA damage and peroxidation of lipids and eventually results in apoptosis (Swain et al., 2016; Król et al., 2017; Rajput et al., 2018). The most established underlying mechanism for ZnO NPs’ toxicity among the various theories put forth is ROS generation (free radicals) and the subsequent oxidative stress (Chong et al., 2021), induction of inflammation, and ultimately cell death (Xia et al., 2008). According to Saliani et al., (2016), a combination of ensuing events leads to this oxidative stress: (1) ROS formed on the element's surface; (2) disintegration and liberation of Zn^{2+} ions; (3) ZnO NPs’ physical interface with the biomolecules.

ROS are oxygenated radicals produced by mitochondria as a result of metabolic ramifications. They operate as signaling molecules in numerous crucial physiological processes like immune response, signal transduction, and gene transcription (Handa *et al.*, 2015; Liu et al., 2018). At high levels, ROS are

the main offenders responsible for the development of oxidative stress in the cells, tissues, and organs. Glutathione (GSH) levels were measured as oxidative stress biomarkers in animal studies, together with the expression and activities of glucatalase (CAT), SOD, and glutathione peroxidase (GPx) which are the main antioxidant enzymes (Pinho et al., 2020). SOD speeds up superoxide's dismutation to oxygen and less reactive peroxide (H_2O_2), which can then be nullified by GPx and CAT (Hammond and Hess 1985; Siddique et al., 2013; Jeeva *et al*., 2015). These anti-oxidant enzymes modulate the amount of ROS in the cells and also prevent the more production of other ROS and hydroxyl radicals. The physiologic unbalance between ROS and antioxidants results in oxidative stress, and crucial biological molecules like DNA or RNA, lipids, and proteins are also damaged, which invariably causes cellular damage and ultimately cell death (Ighodaro and Akinloye 2018; Nandi et al., 2019). Likewise, rats given oral exposure to ZnO NPs for seven days straight showed depletion in SOD and CAT (Attia et al., 2018). Several experiments utilizing animal models demonstrated that ZnO NanoParticles could disturb the redox equilibrium, generating oxidative stress and a series of ROS-mediated damage (Zhao et al., 2013; Mansouri et al., 2015; Aijie et al., 2017; Xiaoli et al., 2017; Attia et al., 2018; Khorsandi et al., 2018; Shahzad et al., 2019; Liu et al., 2020; Mir et al., 2020; Abdel-Halim et al., 2020).

An oxidation process in which unsaturated fatty acids or phospholipids in the cellular membrane are targeted by ROS is known as lipid peroxidation. It changes the fluidity and porosity of the membrane, along with impeding the functionality of membrane-linked enzymes (Saxena 2014; Chang et al., 2014; Sukhanova et al., 2018). It has been shown that lipid peroxidation produces conjugated diene hydroperoxides and unstable molecules, which then break down into different aldehydes and may act as biomarkers in assessing oxidative stress (Fuchs *et al*., 2013). The most mutagenic lipid peroxidation final product is malondialdehyde (MAD). MAD might generate adducts by retaliating with nucleosides that could cause mutations, cell cycle arrest, strand breakage, and ultimately apoptosis. This could lead to the onset of inflammation and related diseases (Ayala et al., 2014; Ma et al., 2014). Figure 3 sketches it out.

Earlier research has also demonstrated a rise in MDA levels in animals treated with ZnO NPs (Mansouri et al., 2015; Aijie et al., 2017; Xiaoli et al., 2017; Wang et al., 2017; Khorsandi et al., 2018; Attia et al., 2018; Liu et al., 2020; Kong et al., 2020; Abdel-Halim et al., 2020). The increased production of ROS brought on by exposure to ZnO NPs may result in DNA oxidative damage. The deoxyribose backbone and all bases are known to be particularly reactive with the HO• radicals, which can break DNA strands, oxidize nucleotides, and produce DNA adducts, all of which may eventually result in mutagenicity and carcinogenesis. (Cadet et al., 2003). Increased levels of the phosphorylated histone H2AX (γH2AX) have been seen in animals given ZnO NPs, implying breaks in the DNA strand (Pati et al., 2016; Liu et al., 2017; Zhai et al., 2018). These findings corroborated research from other groups that showed ZnO NPs had the ability to cause DNA damage. For oxidative DNA, 8-OHdG has long been employed as a biomarker (Islam et al., 2019). This 8-OHdG synthesis has been linked to the emergence of cancer and degenerative illnesses owing to its capacity to cause GC-TA mispairing mutations (Qing et al., 2019). Aside from this, mice treated with ZnO NPs showed significant micronuclei production in their blood marrow cells and peripheral blood (Pati et al., 2016). These DNA injuries may set off apoptosis by activating the p53

pathway, which would then result in tissue damage (Ma and Yang 2016; Abass *et al.*, 2017; Yousef et al., 2019).

Along with oxidizing biomolecules, ZnO NPs can also cause harmful impacts on organs through the inference of inflammation caused by oxidative stress. As a matter of fact, the oxidative stress and inflammation brought on by ZnO NPs work collaboratively to highlight the gradual harm to the tissues and organs (Chong et al., 2021). In his review, Biswas., (2016) clearly outlined the relationship between oxidative stress and inflammation and also illustrated their interdependence. According to Biswas, oxidative stress imparts the establishment of inflammation, and inflammation may produce ROS, which would exacerbate oxidative stress. Therefore, while investigating the negative effects of ZnO NPs in diverse organs, both inflammation and oxidative stress have frequently been detected concurrently (Attia et al., 2018; Liu et al., 2020). In animal model organisms, ZnO NPs caused inflammation in several organs via attracting inflammatory cells and activating pro-inflammatory mediators and cytokines in local tissue cells through mitogen-activated protein kinase (MAPK) and NF-κB signal transduction pathways (Saptarshi et al., 2015; Tang et al., 2016; Liu et al., 2017; Almansour et al., 2017; Qiao et al., 2018). Particular consideration should be paid to this idea that the kind and quantity of native immune cells vary in tissues depending on the tissue type, which also influences how much of these cytokines and chemokines are produced. Consequently, the immunomodulatory alterations that cause tissue injuries may differ among various tissue types (Najafi et al., 2018).

12.2 Factors responsible for toxicity:

Studies have indicated the consequences of toxicity depend on size (Hanley et al., 2008; Guo et al., 2008), dose/concentration (Deng et al., 2009), surface composition, and morphology (Pujalte *et al.*, 2011). Wang et al., (2016) conducted an in vivo experiment whose findings indicated that higher dosages of ZnO NPs, i.e., 5000 mg/kg, were hazardous to mice. It induced a decrease in body weight along with a relative increase in the weight of the brain, pancreas, and lungs. In addition, Zn buildup was seen in the bones, kidney, liver, and pancreas. However, long-term supplementation with ZnO NanoParticles at 50 and 500 mg/kg manifested little toxicity, indicating that large amounts of ZnO NPs can be detrimental (Wang et al., 2006; Najafzadeh et al., 2013; Wang et al., 2016; Tang et al., 2016). Moreover, the shapes and sizes of NPs affect their toxicity, so smaller NPs in the range of 3–6 nm are easier to excrete from the kidneys than larger NPs of around 30 nm, which stay in the liver (Burns et al., 2009). Additionally, bigger NPs have a tendency to remain in the kidney for a prolonged duration because glomerular filtration has a slower excretion mechanism, and this prolonged retention might result in organ damage (Kumar et al., 2010). Additionally, irrespective of their specific surface area, the distinct shapes of nanoparticles also influence their toxic effects. Wahab et al., (2016). On cancer and normal cells, nanorods showed increased cytotoxicity and inhibitory effects, respectively, because they have a wide and useful surface area. This may possibly cause cells to experience increased oxidative stress. Additionally, all of the previously mentioned investigations employed chemically generated ZnO NPs, which could be a potential source of the ZnO NPs' inherent toxicity due to the circumstances of the chemical reactions in the typical method.

So, the synthesis of ZnO NPs via microbes must be taken into account owing to their biocompatibility and controllable NP size and shape.

12.3 Toxicological effects of ZnO NPs on vital organs of the body.

The target organs of ZnO NPs on oral exposure are the heart, liver, pancreas, spleen, and bones (Wang et al., 2008). Mice were given 300 mg/kg of ZnO NPs orally for fourteen days in a row. This resulted in raising ALP and ALT serum values as well as pathological liver lesions (Sharma et al., 2012). According to the histopathological analysis, Wistar rats' heart and liver tissue had focal hemorrhages and necrosis after being exposed to oral ZnO NPs at a high dose of 400 mg/kg because of oxidative stress (Saman et al., 2013). Likely oral ZnO NP deliverables at a concentration of 20 mg/kg body weight in lambs produced toxic consequences, including elevated blood urea nitrogen (BUN) and creatinine levels, which specify renal impairment (Najafzadeh et al., 2013). According to Lee et al., (2016), ultra-fine sized particles or ZnO NPs interfered with the metabolism of lipids in lung tissues, but a study by Zhang et al., (2018) demonstrated that ZnO NPs disturbed the lipid metabolism in the livers, which in turn disrupted blood lipid levels and altered the plasma metabolome, all of which were ultimately linked to hepatic steatosis.

Wang et al., (2017) used a serum biochemical assay to assess the toxicity of supplementing weaned piglets with lower doses of ZnO NPs. Weaned pigs fed 1200 mg/kg ZnO NPs showed no changes in their serum activities of LDH, GPT, or GOT, demonstrating that supplementing ZnO NPs up to a certain dose does not cause toxicity. Yan et al., (2012) used metabolomics in their nanotoxicological studies to demonstrate nephrotoxicity in rats following an oral dose of 50 nm ZnO NPs for fourteen days in a row. Moreover, Lee et al., (2016) recognized respiratory toxicology following inhalation of ZnO NPs. These two studies looked at metabolomic changes in the bronchoalveolar lavage fluid (BALF), lungs, and kidneys after ZnO NPs administration. Similarly, Zhang et al., (2018), employing a metabolomics-based strategy, found that the amounts of metabolites implicated in anti-oxidative processes, energy, and lipid metabolism in the livers of hens varied as a result of ZnO NPs. Additionally, the dose-dependent effects were more pronounced during the short exposure period of 4 weeks than they were throughout the extended exposure period of 24 weeks. These findings were consistent with past studies showing that ZnO NPs disrupted the TCA cycle, which led to the use of alternate energy sources.

12.4 Toxicological effects on the reproductive system.

Although some studies and research are available on the harmful impacts of ZnO NPs on the reproductive system (Jo et al., 2013; Talebi et al., 2013; Zhao et al., 2015; Zhao et al., 2016) yet their impacts or toxicity-associated pathways in the female reproductive system are poorly understood. The molecular intuitions of ZnO NPs on the female reproductive systems are still unspecified. As a matter of fact, neither particular pathways regulated by NPs nor changes in protein expression following exposure to ZnO NPs have been documented. Hong et al., (2014a,b) observed a substantial decrease in fetal weights and a rise in abnormalities following the administration of 400 mg/kg/day ZnO NPs. Yet the Zn concentration in fetal tissue did not differ significantly between the experimental and control groups.

Brun et al., (2014) discovered that Zn^{2+} was the only factor associated with the impact of ZnO NPs. But according to Poynton et al., (2011) and Chen et al., (2014) ZnO NPs' exposure impact was connected to both Zn^{2+} and NPs. Zhao et al., (2015) investigated the impact and the molecular perspectives (a precise mechanism) of ZnO NPs on the granulosa cells of female reproductive systems by using chicken ovarian granulosa cells (GCs) as a model. This research found that the treated GCs had intact NPs, which prevented granulosa cell development. This work also demonstrated that Zn^{2+} and whole NPs have differing effects on gene and protein expression engaged in particular pathways. NPs and $Zn^{2+}$are therefore dissimilar and cause different effects.

The ovarian development of pubescent hens was hampered by ZnO NPs (Liu et al., 2016). Likely, Liu et al., (2017) found that oocyte stage exposure to ZnO NPs inhibits embryo development. The -H2AX and NF-B pathways may greatly influence this inhibition. The influence of ZnO NPs treatments caused intact NPs to be found in ovarian tissue (in-vivo) and in cultured cells (in-vitro). This is significant since the in-vitro culture data and in-vivo embryonic data were quite similar. The deficits brought on by treatment with ZnO NPs persisted over cell generations, and their embryo toxicity engaged both NF-kappaB and gamma-H2AX pathways. A study demonstrated that copper oxide and silicon dioxide NPs are crucial for ZnO NPs' toxicity. Both intact NPs and Zn^{2+} contribute to the harmful effects of ZnO NPs. This study and others indicate that ZnO NPs are harmful to the female reproductive system, specifically the ovaries (oocytes), and consequently to the embryo (embryo toxic). So ZnO NPs produce negative effects on the female reproductive system via controlling particular signaling pathways. This brings up the potential health risks that ZnO NPs could have on the human female reproductive system, as they are part of numerous daily edible and household products. However, there is still much to learn about ZnO NPs and how they selectively influence genes and proteins associated with triggers toxicity in the reproductive system.

12.5 Overcoming toxicity.

In light of cumulative scientific findings on the toxicity of ZnO NPs, numerous techniques have been used to create safer NPs without altering their distinctive physicochemical characteristics. The surface-bound chemical modification is a common method utilized for altering the surfaces of NPs, and it plays an important role in their biological interactions too (Luo et al., 2014). So, by using this technique, NPs are coated with particular substances like polyethylene glycol (PEG) (Luo et al., 2014), silica (Chia and Leong 2016) (Ramasamy et al., 2014), chitosan (Onnainty et al., 2016), and organosilanes (Yung et al., 2017). Chia *et al.*, (2016) used fine layers of silica for the surface alteration of ZnO NPs. It was successful in lessening their cytotoxic effects on epithelial cells as it limited the dissociation of ZnO NPs to Zn^{2+}. Although higher concentrations of silica coating on ZnO NPs still caused cytotoxicity in mammalian gut cells, indicating this silica coating is ultimately not benign (Chia and Leong 2016). Among the coating materials, PEG is frequently employed to modify the surface of NPs due to its biodegradability and biocompatibility (Król et al., 2017). According to numerous studies, PEG coating significantly reduces the NPs' toxicity by controlling the ROS production and the release of Zn^{2+} (Martinez et al., 2011). The greater than before the energy of cells treated with PEG-coated ZnO NPs suggested that PEG surface

modification interferes with the pathways that cause cytotoxicity, whereas untreated ZnO NPs caused cytotoxicity in the tested cell line. So, the surface modification approach has the potential to be an excellent method for reducing the hazard risks associated with NPs. However, this method needs to be re-evaluated when taking into account the manufacturing costs associated with large-scale production

13. Conclusions, Outlook And Prospects.

ZnO NPs have potential characteristics that make them competent enough for use in the poultry sector. They exhibit great potential to be used as a feed supplement because of their small size, higher bioavailability, and strong absorption rate as compared to traditional inorganic Zn sources, along with cutting down on the amount needed. Their usage improves the body's rate of Zn utilization and minimizes environmental impact by lowering undigested Zn in excreta. They also look promising therapeutic agents owing to their bactericidal, fungicidal, and anti-coccidial actions on a broad range of poultry microbes. In the near future, ZnO NPs might take the place of traditional antibiotics and anti-fungal drugs, which are responsible for the emergence of multidrug resistant microorganisms. However, they are capable of producing toxicity in birds, which generally depends on the size, shape, and NPs concentration along with the route of exposure. Their *in-vivo* toxicity investigations using metabolomics-based approaches are rare. So, the use of ZnO NPs in poultry may provide a new source of significant improvements, particularly in terms of production and health, but their toxicity is still questioned, so more research is required to determine it precisely.

Declarations

Competing interests.

All authors have declared that there is no further conflict of interest and financial matter involved in the work's publication.

Authors' contributions.

This manuscript not submitted elsewhere for publication as well as the author's contribution mentioned below,

Arjmand Fatima[a]*:* Manuscript writing and experimental data investigations and analysis

Tean Zaheer[b]*:* Experimental data analysis and have been involved in drafting the manuscript or revising it critically for important intellectual content.

Kaushik Pal[c,*]*:* Plan of Research direction and supervision for the execution of experimental analysis and draft moderation with submission.

Rao Zahid Abbas[b]***:* Mentorship guidance and supervision, as well as resources of common research facilities of experimental investigations through central facilities.

Tayyaba Akhtar[d]*:* Substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data.

Sultan Ali[a]*:* Accountable for all aspects of the work in ensuring that experiment related to the accuracy or integrity of any part of the work is appropriately investigated and resolved.

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Availability of data and materials.

All data are available with us and will submit once a reasonable request needed.

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Figures

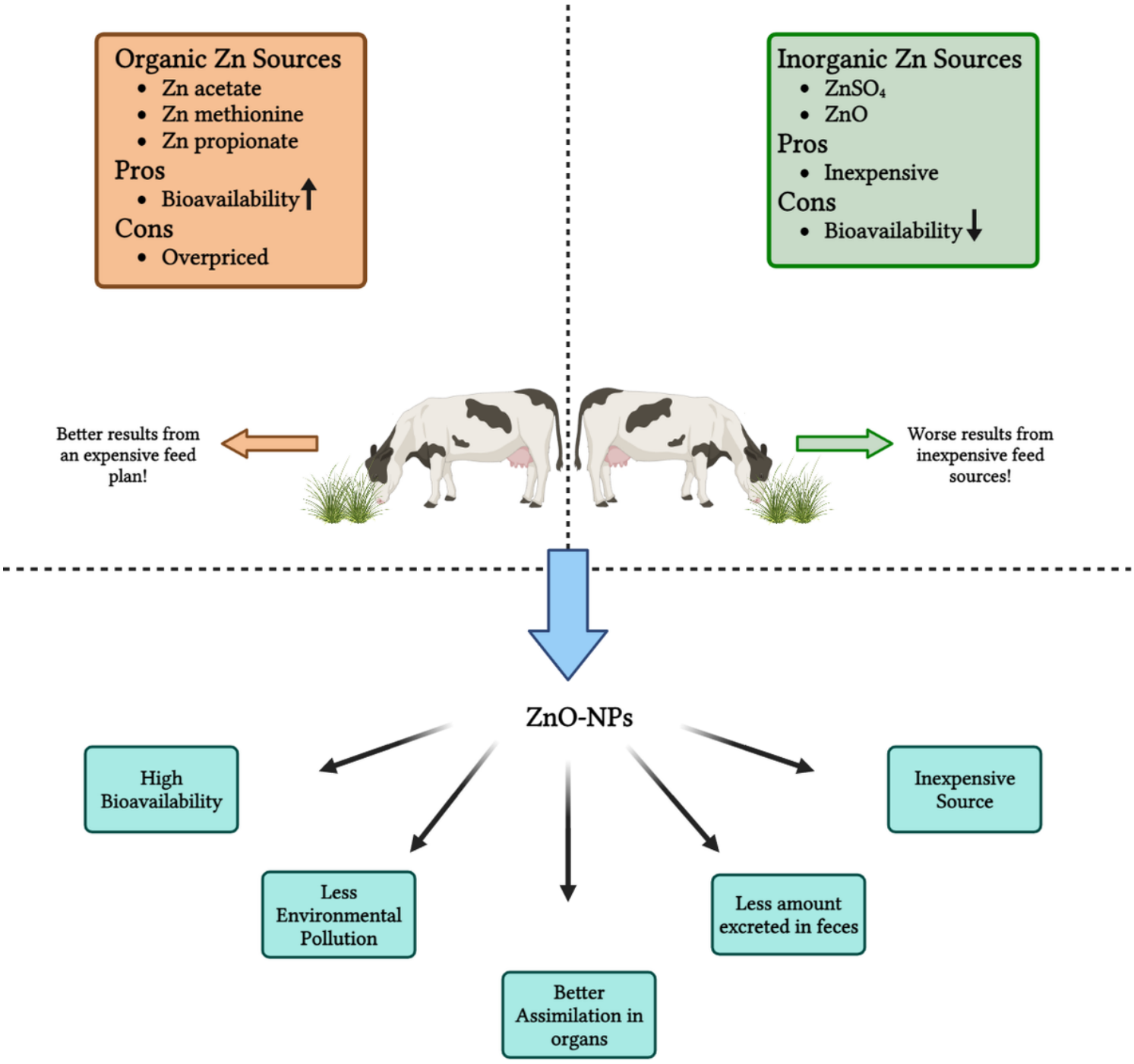

Figure 1

Both organic and inorganic sources of Zinc in feed of animals tend to have their own plus and minus points that rendered the researchers to keep looking for an alternate way to induce Zinc that turned out to be ZnO-NPs. These nanoparticles have better bioavailability of Zn in body, better assimilation in various organs, less excretion through fecal matter which results in less environmental population and all that too from an inexpensive source.

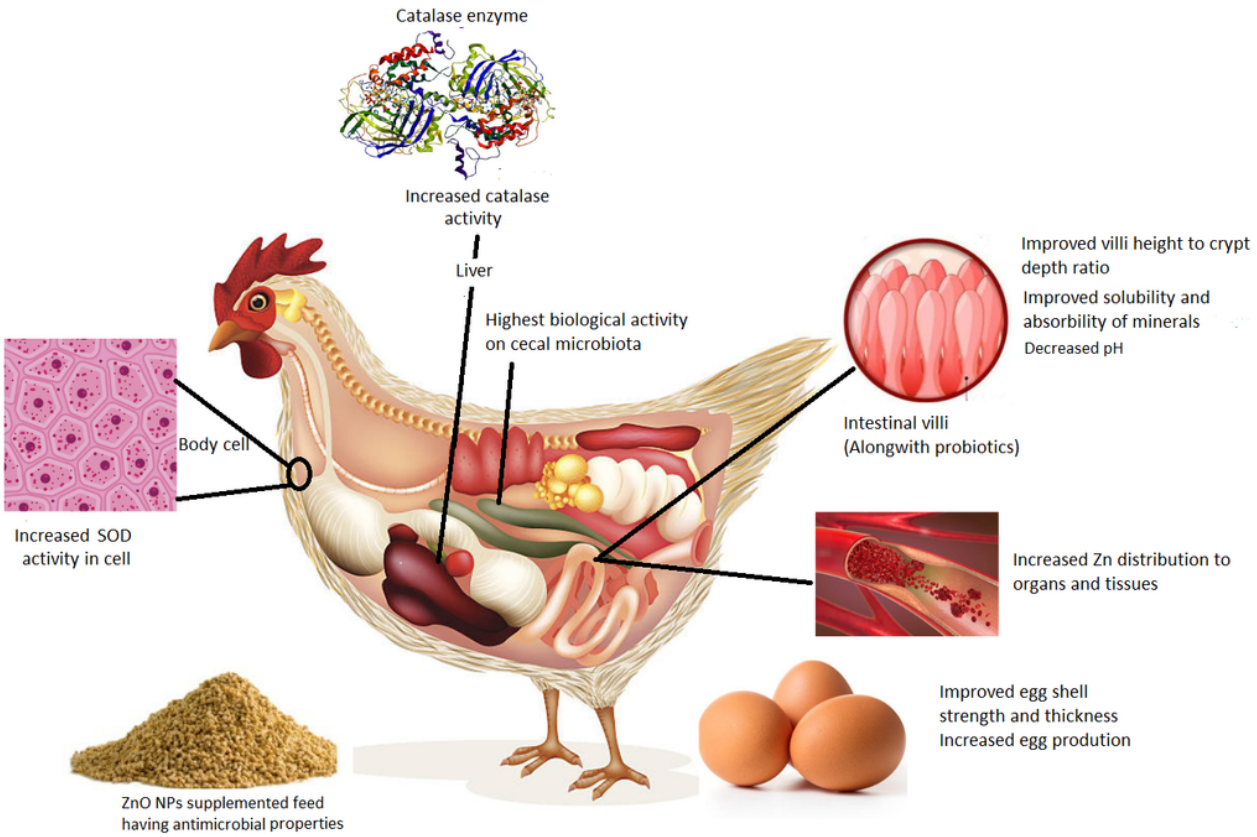

Figure 2

Potential Role of ZnO NPs as Feed Additive

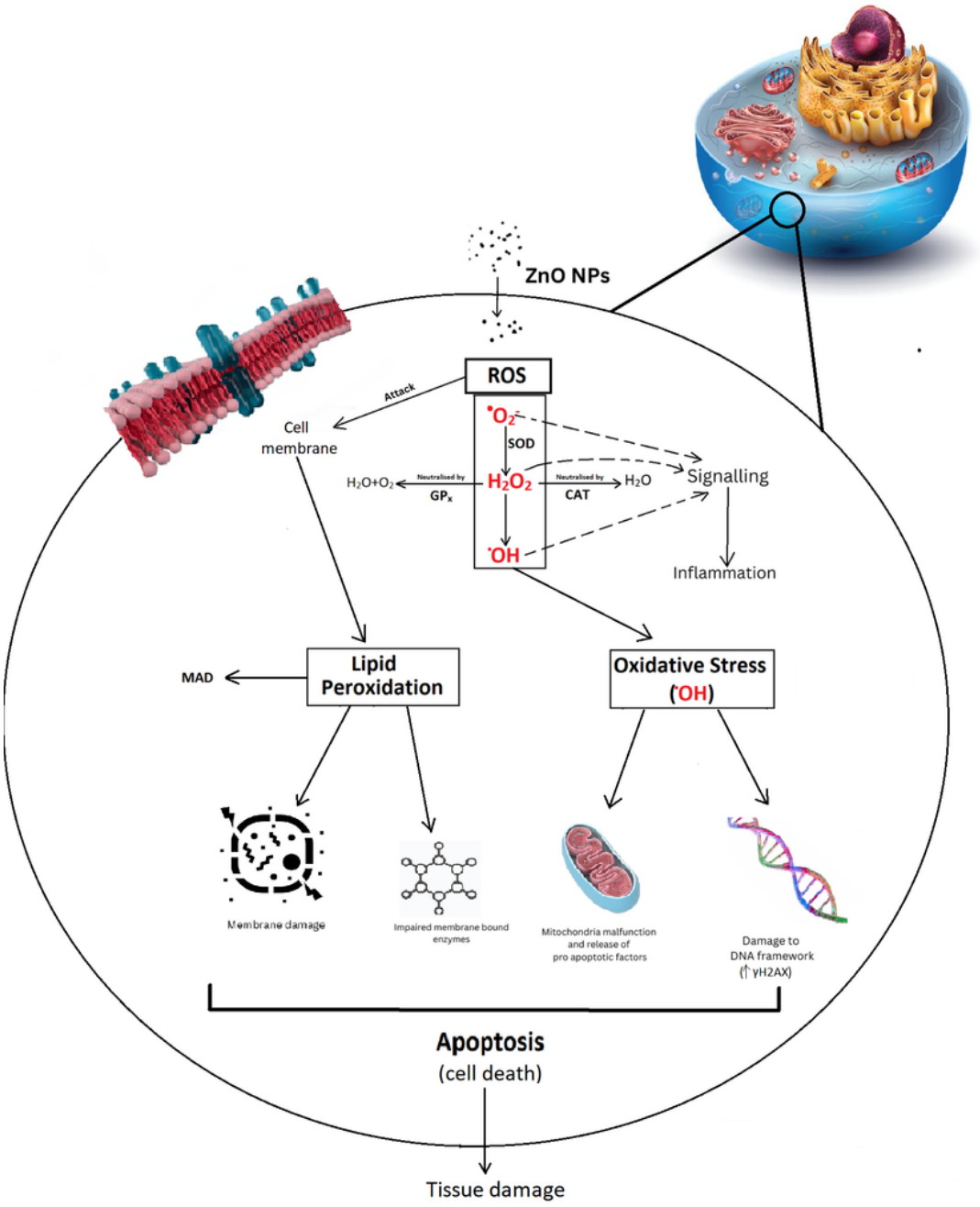

Figure 3

Toxicity mechanism via ROS mediated by Lipid Peroxidation and Oxidative stress

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