Antibacterial Action of Zinc Oxide Nanoparticles against *Staphylococcus aureus* in Broiler Breast Fillet

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A B S T R A C T

Zinc oxide can be called a multifunctional material thanks to its unique physical and chemical properties. Practical application of zinc oxide nanoparticles (ZnO NPs) suspension with different concentrations (5, 8, and 10 mM) were investigated against *Staphylococcus aureus* artificially inoculated into broiler breast fillets. The results indicated that ZnO suspensions (5, 8 and 10 mM) had a significant inhibitory effect on the growth of *Staphylococcus aureus* during 12 days of refrigerator storage at 4ºC. Accurately, ZnO NPs 10 mM showed the highest reduction percentage (25.35%) to *Staphylococcus aureus* from 8.6 to 6.42 log cfu/g, compared to other concentrations (5 and 8 mM). Thus, that the antibacterial activity of ZnO was concentration dependent.

Key words: ZnO nanoparticles - broiler breast fillets -Antibacterial Activity-*Staphylococcus aureus*.

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1. INTRODUCTION

Poultry meat has become a mass consumer product throughout the world. Poultry is a food that has been highly appreciated by man since time immemorial. It is an important, low cost source of animal protein, rich in nutrients, phosphorous, other minerals and B-complex vitamins (FAO, 2010). Food borne illness in human beings due to bacterial pathogens and their toxins are well known all over the world (Hazairwala et al., 2002). Food borne illness leads to a substantial economic and quality of life by a way of acute morbidity and chronic sequels (Duff et al., 2003). In this respect, nanotechnology represents the vanguard of the wave of innovations that is perhaps going to shake the agro-food sector in the years ahead. The advent of nanotechnology which involves the manufacture and use of materials with size of up to about 100 nm in one or more dimensions has brought great opportunities for the development of materials with new properties for use as antimicrobial agents (Roco, 1999). These novel and enhanced material properties are the results of the increase in relative surface area that occurs as particle size decreases down to the nano-scale. Nanosize materials are also more biologically active compared to the same material in the macro or micro scale (Ren et al., 2009). Nanosized inorganic compounds display strong antibacterial activity at low concentrations and unique chemical and physical properties (Rai et al., 2009). A key advantage of inorganic nanoparticles in their stability under extreme conditions, such as high temperatures and pressures. (Sawai 2003). Also some inorganic nanoparticles are considered non-toxic because they contain minerals essential to the human body (Roselli et al., 2003). Most antibacterial inorganic materials are metallic nanoparticles and metal oxide nanoparticles such as silver, copper, titanium oxide, and zinc oxide (ZnO) (Bradley et al., 2011). In general, Zinc oxide (ZnO) is one of the five zinc compounds that are currently listed as GRAS by the FDA (FDA, 2011). ZnO powder has been used for decades as an active ingredient for dermatological applications in creams, lotions, and ointments because of its antibacterial properties (Sawai, 2003). Futher, ZnO NPs as nontoxic and biocompatible, have been utilized in many biological applications in daily life such as drug carriers, cosmetics and medical devices. However, these NPs have not been applied in fresh meat. The present work was carried out to evaluate the efficacy of ZnO NPs (20nm) as antimicrobial agents on the survival of *Staphylococcus aureus*

artificially inoculated into broiler breast fillet (in vivo).

2. MATERIALS AND METHODS

2.1. Preparation of Bacterial strain:

The tested *Staphylococcus aureus* was biochemically identified by Animal Health Research Institute, Egypt The bacterial strain was maintained in tryptic soy agar (Merck, Germany). Four to five isolated colonies of the tested strain were picked up and inoculated in tubes of sterile peptone water 0.1% (Merck, Germany) (5 ml in each) then incubated at 37°C/24 hrs (Saeed and Tariq, 2005). From this culture, dilutions up to 10^{10} were plated on Baired Parker agar (Merck, Germany) to determine the cell concentration. The cell count was adjusted to10^7 cfu/ml (7 log cfu/ml) for *Staph. aureus* (Kantachote and Charernjiratrakul, 2008) (the infective dose of enterotoxin may be achieved when the population of *Staph. aureus* reaches a level of > 10^5 cfu/g) (Stewart et al., 2003) with tube dilution methods. The number of cfu/ml was considered as initial inoculum load to be inoculated into broiler breast fillet samples.

2.2. Preparation of zinc oxide nanoparticles:

ZnO nanoparticles with (diameter, 20 nm; purity, 99.98%) were purchased from NanoTech Egypt for Photo-Elecronics according to NT-ZONP brand with certificate of analysis. To obtain a homogenous solution of nanoparticles at different concentrations, including concentrations (5Mm, 8mM and 10 mM),150 ml distilled water were added to each concentration of nanoparticles in glass containers and sonicated for 30 min. for uniform dispersion and formed a colloidal suspension. Then, the resulting homogenous suspensions were autoclaved for 30 minutes to be sterilized (Mottaki et al., 2014).

2.3. Broiler breast fillet samples:

A total of 1200 grams of random samples of fresh raw broiler fillets were purchased from different shops and supermarkets at El-Gharbia province. The samples will keep in separate plastic bags and transferred directly to the laboratory in an insulated ice box under complete aseptic conditions without undue delay. In the laboratory, all samples were divided into 4 equal groups, (each group was 100 grams) washed with sterile distilled water and stored at 4ºC until use.

2.4. Artificial inoculation of broiler breast fillet samples with staphylococcus aureus:

Broiler breast fillet samples were dipped in 150 ml of sterile pepton water 0.1% (Merk, Germany) containing *Staph. aureus* at approximately 7 log cfu/ml for 15 min. at room temperature (25ºC). After dipping, the fillet samples were kept at room temperature for 20 min. to allow attachment and absorption of bacteria (Dubal et al., 2004). Then, *Staphylococcus aureus* in the samples was enumerated to get the initial load before dipping in ZnO nanoparticles suspensions.

2.5. Application of zinc oxide nanoparticles:

Broiler breast fillet samples with known load of the tested organism *(staph.aureus)* were dipped in sterile distilled water (control), 5 mM ZnO NP suspension, 8 mM ZnO NP suspension and 10 mM ZnO NP suspension at room temperature (25ºc) for 15 min. All groups were properly packed in polyethylene bags, labeled and stored at 4ºC. Sensory analysis (overall acceptability) and *Staph.aureus* count analysis were conducted on days 0, 3, 6, 9 during storage, using the serial dilutions and spread plate technique (FDA, 2001). The above experiment was performed in triplicate.

2.6. Sensory examination:

Overall acceptability of all samples was carried out using a 10 - point standardized numerical scale, where 10 corresponded to 'components characteristic of the highest quality'. The panel consisted of 10 members of the staff who were familiar with meat characteristics was conducted during storage according to Kanatt et al., (2010).

2.7. Bacteriological analysis:

Staphylococcus aureus count was applied using standard methods (FDA, 2001). Thereafter, black shiny colonies with narrow white margins surrounded by a clear halo zone extending into the opaque Baired parker medium were counted and expressed as colony forming units (log cfu/g). The mean of log cfu/g calculated from two replications of each sampling point were expressed as bacterial survival counts.

2.8. Statistical analysis:

All the obtained data were statistically analyzed by one-way analysis of variance (ANOVA) using SPSS package (SPSS 19.0, Chicago, IL, USA). Significant ($P < 0.05$) differences between treatments were determined using Duncan's post hoc test. Data were expressed as means ± standard deviation (SD).

3. RESULTS

3.1. Sensory examination:

The results obtained in table (1) showed that the mean values of overall acceptability in case of using ZnO NPs (5mM) were 6.33 ± 0.5, 8.00 ±1.00, 7.00 ±1.00, 4.67± 0.58(log cfu/g) at zero day, 3rd day, 6th day and 9th day of the refrigerated storage period at 4 ºC, respectively. While in case of using ZnO NPs (10 mM) the mean values were 6.67 ± 0.58, 7.67 ± 0.58, 6.67 ± 0.58, 7.00 ±1.00, 6.67± 0.58 (log cfu/g) at zero day, 3rd day, 6th day, 9th day and 12th day of the storage period at 4 ºC respectively. In case of using ZnO NPs (8mM) the mean values were 8.33 ± 0.58, 8.33 ± 0.58, 7.00 ± 1.00, 7.67 ± 0.58, 7.00 ±0.00 (log cfu/g) at zero day, 3rd day, 6th day, 9th day and 12th day of the refrigerated storage period at 4 ºC, respectively. Comparing with the mean value of overall acceptability in the control samples which was 8.00 ± 1.00(log cfu/g) at zero day then the samples showed extreme discoloration and off-odor on the third day of storage indicating complete spoilage.

3.2. Bacteriological examination:

Table (2) revealed *Staph.aureus* counts (log cfu/g) artificially inoculated into raw broiler breast fillet samples during refrigerated storage with different concentrations of ZnO NPs (20nm). The present data exhibited the potential of ZnO NPs as food preservative against *Staph.aureus* in broiler breast fillet samples. The initial counts of *Staph.aureus* in broiler breast fillet samples after inoculation were 8.60± 1.07 log cfu/g. At zero day counts of *Staph.aureus* in broiler breast fillet samples were 8.38 ± 1.24; 8.16 ±1.04 and 8.01± 0.23 log cfu/g, after treatment with ZnO NPs 5mM, 8mM, 10 mM ZnO NPs respectively, Comparing with *Staph. aureus* count in the control ($P < 0.05$) which was 8.52 ± 1.20 log cfu/g. By 3rd day of refrigerated storage period such count of *Staph.aureus* slightly decreased to 7.75± 0.17; 7.75± 0.74 and 7.49 ± 0.76 log cfu/g after treatment with ZnO NPs 5mM, 8mM, 10 mM ZnO NPs, respectively, as well as they were acceptable from aesthetic points without off odor or discoloration compared with the control +ve. Samples which showed extreme discoloration and off-odor on the 3rd day of refrigerated storage indicating complete spoilage. While, the 6th day of refrigerated storage of treated samples with ZnO NPs 5mM, 8mM, 10 mM ZnO NPs, the mean counts of *Staph.aureus* were 7.52 ±0.65; 7.35±0.68 and 7.25±0.62, respectively, as well as they were still acceptable without off odor or discoloration. By 9th day of refrigerated storage, count of *Staph.aureus* decreased to 7.32 ± 0.77; 6.65 ±0.60 and 6.42 ± 0.54 log cfu/g, after treatment with ZnO NPs 5mM, 8mM, 10 mM ZnO NPs, respectively. As illustrated in table (3) ZnO NPs 5mM reduced the growth of *Staph.aureus* 2.56%; 9.88%; 12.56% and 14.88% after zero day, 3rd day,6th day and 9th day of refrigerated storage, respectively. At the concentration of ZnO NPs 8 mM reduced the growth of *Staph.aureus* 5.12%; 11.98% ; 14.53 and 22.67% after zero day, 3rd day,6th day and 9th day of refrigerated storage, respectively. Moreover ZnO NPs 10 mM reduced the growth of *Staph.aureus* 6.86%; 12.91% ; 15.70% and 25.35% after zero day, 3rd day,6th day and 9th day of refrigerated storage, respectively.

Table (1): Effect of ZnO NP (20 nm) on overall acceptability of raw broiler breast fillet samples during refrigeration storage (n=30)

Groups	Zero day	3rd day	6th day	9th day	12th day
Control	8.00 ± 1.00^{ab}	Spoiled	Spoiled	Spoiled	Spoiled
5 mM ZnO NP (20 nm)	6.33 ± 0.5^{b}	8.00 ± 1.00^{a}	7.00 ± 1.00^{a}	4.67 ±0.58^{b}	Spoiled
8 mM ZnO NP (20 nm)	8.33 ± 0.58^{a}	8.33 ± 0.58^{a}	7.00 ± 1.00^{a}	7.67 ± 0.58^{a}	7.00 ±0.00
10 mM ZnO NP (20 nm)	6.67 ± 0.58^{ab}	7.67 ± 0.58^{ab}	6.67 ±0.58^{a}	7.00 ± 1.00^{a}	6.67 ±0.58

The values represent Mean ± SD of three experiments. Means within a column followed by different letters are significantly different ($P < 0.05$). Score System for Sensory Evaluation (Kanatt *et al.,* 2010):

9: Excellent	6: Good	3: Poor
8: Very very good	5: Medium	2: Very poor
7: Very good	4: Fair	1: Very very poor

Table (2): Effect of different concentrations of ZnO NP (20 nm) on *Staphylococcus aureus* count (log cfu/g) artificially inoculated into raw broiler breast fillet samples during refrigeration storage (n=30)

Groups	Zero day	3rd day	6th day	9th day
Control	8.52 ± 1.20[a]	----	----	----
5 mM ZnO (20 nm)	8.38 ± 1.24 [a]	7.75 ± 0.17 [a]	7.52 ± 0.65 [a]	7.32 ± 0.77 [a]
8 mM ZnO (20 nm)	8.16 ± 1.04 [a]	7.57 ± 0.74 [a]	7.35 ± 0.68 [a]	6.65 ± 0.60 [a]
10 mM ZnO (20 nm)	8.01 ± 0.23 [a]	7.49 ± 0.76 [a]	7.25 ± 0.62 [a]	6.42 ± 0.54 [a]

(----): Organoleptically spoiled samples. Initial load of *Staph. aureus* = 8.60 ± 1.07 log cfu/g. The values represent Mean ± SD of three experiments. Means within a column followed by same letters are non-significant ($P < 0.05$).

Table (3): Reduction % of *Staphylococcus aureus* count (log cfu/g) artificially inoculated into raw broiler breast fillet samples treated with different concentrations of ZnO NP (20 nm)

Groups	Zero day		3rd day		6th day		9th day	
	log	%	log	%	log	%	log	%
Control	0.08	0.93	----		----		---	
5 mM ZnO (20 nm)	0.22	2.56	0.85	9.88	1.08	12.56	1.28	14.88
8 mM ZnO (20 nm)	0.44	5.12	1.03	11.98	1.25	14.53	1.95	22.67
10 mM ZnO (20 nm)	0.59	6.86	1.11	12.91	1.35	15.70	2.18	25.35

4. DISCUSSION

Depending on the concentration, ZnO NPs could significantly reduce *Staph.aureus* count in broiler breast fillet samples. Our result is similar to those reported by (Etman, 2015; El-Fiky, 2014) who studied the effect of ZnO NPs on *L. monocytogenes* in ready to eat meat products and fresh minced meat, respectively. Finally, (Mirhosseini and Arjmand, 2014) proved the activity of ZnO NPs with acetic acid on *Staph. aureus* in mutton meat.

The exact antibacterial mechanism of ZnO nanoparticles is still unknown. However, many antibacterial mechanisms of ZnO nanoparticles such as the formation of reactive oxygen species (ROS), the interaction of nanoparticles with bacteria, subsequently damaging the bacterial cell, and the release of Zn^2+ have been proposed (Manna, 2012). Many studies have indicated that the formation of ROS is the main antibacterial mechanism of ZnO nanoparticles (Zhang et al., 2008; Espitia et al., 2012). The antibacterial effect of ZnO can also be the result of mechanical damage to the cell membrane caused by the abrasive surface of nanoparticles, since ZnO NPs have been considered to be abrasive due to surface defects such as edges and corners (Stoimenov et al., 2002). The orientation of ZnO which affects the biocidal activity of ZnO because of its various randomly oriented spatial configurations, which exhibits higher antibacterial action compared with those of regularly arranged structures (Ramani et al., 2014).

Particle size and concentration of ZnO-NPs play important roles in the antibacterial activity. ZnO-NPs antibacterial activity directly correlates with their concentration as reported by several studies, the activity is size dependent, however, this dependency is also influenced by concentration of NPs (Sirelkhatim et al., 2015). Larger surface area and higher concentration are accountable for ZnO NPs antibacterial activity (Peng et al., 2011). ZnO NPs of smaller sizes can easily penetrate into bacterial membranes due to their large interfacial area, thus enhancing their antibacterial efficiency. Studies investigated on the impact of particle size on the antibacterial activity, and authors found that controlling ZnO NPs size was crucial to achieve best bactericidal response, and ZnO NPs with smaller size (higher specific surface areas) showed highest antibacterial activity (Zhang et al., 2007). The dissolution of ZnO NPs into Zn^2+ was reported as size dependent. Moreover, the generation of H_2O_2, affected by size and concentration of NPs. The smaller particle gives larger surface area so, greater antibacterial activity. (Padmavathy and Vijayaraghavan, 2008). So, inhibition of bacterial growth is completely

related to the concentrations of ZnO NPs and the initial number of bacterial cells. As a matter of fact, antibacterial activity of ZnO NPs is a dose-dependent issue.

5. CONCLOSION

The noble properties and attractive characteristics of ZnO NPs confer significant toxicity to organisms which have made ZnO NPs successful candidate among other metal oxides. The antibacterial activity of ZnO nanoparticles is size and concentration dependent. It is still not clear whether ZnO NPs are safe for human health. Results to date show that ZnO NPs are safe up to a certain level, but may become toxic at higher concentrations. In future, more research should be focused on the safety evaluation and antimicrobial activity of ZnO NPs in vivo need to be undertaken.

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