Effects of Zinc Oxide Nanoparticles on Growth, Carcass Characteristics and Intestinal Tight Junction Protein Gene Expression in 35-day old Broiler Chickens

V. Mozhiarasi
Tamil Nadu Veterinary and Animal Sciences University
R. Karunakaran (drrkarunakaranphd@yahoo.com)
Tamil Nadu Veterinary and Animal Sciences University
L. Radhakrishnan
Tamil Nadu Veterinary and Animal Sciences University
P. Raja
Tamil Nadu Veterinary and Animal Sciences University
N. Pazhanivel
Tamil Nadu Veterinary and Animal Sciences University

Research Article

Keywords: Broiler chickens, Carcass characteristics, Growth, Intestinal health and Zinc oxide nano particles

Posted Date: November 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2243628/v1

Abstract

In the present study, the effect of zinc oxide nanoparticles (ZnONPs) at different concentrations (20, 40 and 60 mg/kg) in broiler chicken diet on growth, carcass characteristics and intestinal health of broiler chicks were evaluated. The ZnONPs synthesized by physical method was characterized for its nanoparticle properties and evaluated for its *in vitro* toxicity using different cell lines. *In vivo* feeding trials was conducted with 150-day-old broiler chicks randomly assigned to five dietary treatments in three replicates for the period of 35 days with ZnONPs. The results indicated that ZnONPs supplemented group showed significantly higher ($p < 0.05$) body weight gain and the lower feed conversion ratio (FCR) compared to control. The carcass characteristics like meat pH and dressing percentage revealed the significance differences in the ZnONPs supplemented group. The intestinal histomorphology revealed significantly higher ($p < 0.05$) crypt depth in ileum and villi length to crypt depth ratio in duodenum of ZnONPs supplemented group. The zinc elemental concentration in the serum found to be significantly higher and the mRNA expression of intestinal tight junction protein genes like mucin-2 and claudin-3 found to be significantly upregulated in the ZnONPs supplemented group. It could be concluded that supplementation of ZnONPs in broiler diet could improve production performance, intestinal health status and can be used as an effective feed additive in broilers.

Introduction

Poultry production in India has emerged as one of the fastest growing sectors among various livestock-based sectors with growth rates of 8.51 and 7.52% in egg and broiler production, respectively (BAHS, 2019). Nutritional interventions have contributed to high growth of broiler production during recent decades in India and there is huge demand for broiler meat and vast scope for broiler industry. Zinc is an important nutrient for animals, which can act as antioxidant, glandular development, poultry performance, protein and carbohydrate metabolism and cofactor in more than 300 metalloenzymes (Hussien et al., 2021). It promotes growth and act as antibacterial agent, modulates the immunity and reproduction of the animals (Asheer et al., 2018). The uniqueness of zinc is that, it is the second most abundant trace element in the animal body but at the same time it cannot be stored, thus demanding the regular dietary intake. In the last few decades, the interest of researchers to nano forms of minerals has been increased. This might be due to their higher reactivity, huge surface to volume, stability, bioactivity, bioavailability, controlled particle size and site-specific targeting (Youssef et al., 2019). Because of their small size in nature (< 100 nm), the nanomaterials have unique physical properties that can influence their uptake, distribution, and behaviour in the body (Schmidt et al., 2009). An enhanced bioavailability of a mineral source could reduce the amount of a mineral that is added to a diet to meet mineral nutritional requirements, which would reduce the amount of mineral excreted by birds (Cheng et al., 1998). Nanotechnology facilitates the development of nano vehicles for nutrients (including trace minerals), allowing efficient delivery to improve digestion and absorption for better nutrient metabolism and physiology. Recently, nanoforms of essential minerals have been explored to improve the growth performance, feed utilisation and health status of animals (Patra & Lalhriatpuii, 2020). Nano minerals are interesting alternatives for inorganic and organic minerals for animals that can substantially enhance the bioavailability and reduce pollution (Abdelnour et al., 2021) and may be used at lower doses in livestock feed to provide better results than the conventional Zinc sources (Swain et al., 2016). It has been indicated in several studies that nano-zinc is an emerging alternative feed supplement for poultry as it surpasses the conventional zinc sources, even at lower doses, in terms of growth performance, meat quality, improved serum concentration of zinc and intestinal barrier function of broiler chickens (Hafez et al., 2017; Khah et al., 2015; Hatab et al., 2022 and Zhang et al., 2022). This study, therefore, aimed to investigate the effect of zinc oxide nanoparticles (ZnONPs), in comparison to inorganic and organic zinc, on growth performances, carcass characteristics, serum biochemical profile and intestinal health status of broiler chickens.

Materials And Methods

Synthesis of zinc oxide nano particles:

Synthesis of zinc oxide nanoparticles was carried out using Planetary Ball mill (Retsch – PM 100). To synthesize zinc oxide nano particles different combinations of time duration (7 or 8 or 9 hours), quantity of inorganic zinc oxide (5 or 10 g) and number of balls each having 5 mm diameter (45 or 50 or 55) were used for optimization. The ball mill was operated at 250 RPM speed. Finally, ball milling conditions to synthesize the zinc oxide nanoparticle was optimized with following conditions: Approximately 5 g of food grade zinc oxide was added in the 50 ml capacity zirconium jar with 50 balls each having 5 mm diameter operated for the total running time of 9 hours with 30 minutes interval.

Characterization of zinc oxide nanoparticles

Dynamic light scattering (DLS) and zeta potential analysis were carried out to assess the average size and stability of synthesized nanoparticles (particle size analyzer -Horiba SZ-100). For further confirmation, the particle size and shape were investigated using high resolution transmission electron microscope (Jeol/JEM 2100) operating at 200 kV voltage. The crystalline nature and the average crystallite size of synthesized zinc nanoparticles were determined by X-Ray Diffraction technique (Bruker D8 Advance) with 2θ ranging 0 – 80^{o} (λ=1.5406 A°). The average crystallite size of ZnONPs were calculated using Debye-Scherrer's equation: $D=K\lambda/\beta Cos\theta$, where 0.89 is Scherrer's constant (K), λ is the wavelength of X-rays, θ is the Bragg diffraction angle, and β is the full width at half-maximum (FWHM) of the diffraction peak. The characteristic functional group of the synthesized zinc oxide nanoparticles was analyzed by Fourier Transform Infra-Red (FTIR) spectroscopy (Thermo Nicolet iS5o) in the region of 4000 cm^{-1} to 100cm^{-1} The presence of zinc oxide nanoparticles was confirmed by UV–Vis spectroscopy (Perkin Elmer Lambda 365) in the wavelength region of 200 nm to 1000 nm. The elemental concentration (in percentage) of zinc in synthesized nano particle was estimated using Inductively Coupled Plasma Mass Spectrometry (Thermo Fisher iCAP RQ ICP-MS).

In vitro cytotoxicity Assay:

The cytotoxicity of zinc oxide nanoparticles was assessed by MTT assay using BHK-21, Vero and Primary Chick liver culture cell lines. The cell lines were maintained in 10 % fetal bovine serum and Dulbecco's modified eagle medium (DMEM) at 37°C with 5 % CO_2 incubator. Cells (10^8 cells/ml) were seeded into 96-well plates and after 24 hours of incubation, the media was discarded. The zinc oxide nanoparticle at different concentrations (6.25, 12.5, 25, 50 and 100 µg /ml) were dispersed in DMEM and added to each well in triplicate and incubated for 72 h. After 72 hours, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide) dye (5 mg / ml) was added to all the wells and incubated at 37 °C for four hours. After the incubation period, the media containing MTT dye was removed from all the wells and about 100 µl of DMSO was added and the absorbance was measured at 570 nm using ELISA reader (Agilent HTX multi-mode reader).

Bird Management and Experimental Design:

A total of 150-day old broiler chicks (Cobb 400) were procured and randomly allocated in a completely randomized design for 5 treatments, each of which had 3 replicates of 10 broiler chicks for 35 days. The dietary treatments consisted of a maize- soyabean meal based diet supplemented with inorganic zinc at 100 % of the requirement as per Bureau of Indian Standards, 2007 was taken as control (T_1), basal diet supplemented with organic zinc at 60 mg/kg (T_2), basal diet supplemented with nano form of zinc at 60 mg/kg 75 % of the requirement level (T_3), basal diet supplemented with nano form of zinc at 40 mg/kg 50% of the requirement level (T_4), basal diet supplemented with nano form of zinc at 20mg/kg 25 % of the requirement level (T_5). Broiler chicks had *ad libitum* access to feed and water, and the light program was 23 hours of light per day. The details of experimental design and feed ingredient compositions are furnished in the Table 1 and Table 2, respectively. The experimental rations were formulated as per BIS, 2007 specifications. Based on the nutrient requirement and anticipated growth rate, the experiment was divided into three phases namely, pre-starter phase (0-7 days), starter phase (8-21 days) and finisher phase (22-35 days).

Growth Performances:

Body weights of individual birds were measured on day 1, 7, 14, 21, 28 and 35. The weight gain of individual birds was calculated from weekly body weight data. The feed intake of each replicate was recorded by subtracting the quantity of residual feed (g) from feed offered (g). The FCR was calculated by dividing the respective feed intake of the chicks by weight gain during the respective period.

Carcass characteristics:

At the end of experimental period (35 days of age), six birds (two birds from each replicate) from each treatment group were selected for the carcass characteristic study and were kept off feed for a period of 12 hours prior to slaughter but given *ad libitum* access to water. The pre slaughter live weights of the birds were recorded. Feathers were then plucked and the birds were eviscerated and weighed to determine the dressing percentage. Further, the pH, Water Holding Capacity and Shear force value of breast muscle meat sample were analyzed.

Serum Biochemistry:

Blood samples were collected from six birds from each group on day 35 and centrifuged at 3000 rpm for 5 minutes to separate serum. The serum was analysed for Glucose, Total protein, albumin, Cholesterol, Alanine transaminase (ALT), Uric acid (UA), calcium and phosphorus using A15 Biosystem random access analyzer.

Concentration of zinc in the serum:

The serum sample was diluted with distilled water (10-fold dilutions) and the concentration of zinc in the serum was analyzed using Atomic Absorptive Spectrophotometer (ZEEnit 700P).

Intestinal Morphometry:

The intestinal morphometry was studied by collecting the intestinal samples from six birds per group from the duodenum, jejunum and ileum, fixed in 10 % of neutral buffered formalin, dehydrated manually, embedded in paraffin wax, cut to 3 µm thick, stained with haematoxylin and eosin. Histological indices measured using an image analyser software (Magvision) included villus length (from the top of villi to the junction of the villus and crypt), crypt depth (defined as the depth of the invagination between adjacent villi) and villus height to crypt depth (V / C) ratio.

Intestinal Tight Junction protein gene expression analyis:

The ileum tissue was collected from different treatment groups and treated with 750 µl of Trizol Reagent (Sigma), mixed thoroughly by vigorous pipetting and incubated at room temperature for 5 minutes. About 200 µl chloroform was added, mixed well, incubated at room temperature for 5 minutes and centrifuged at 12000 rpm for 15 minutes at 4ºC. The upper aqueous layer was collected and equal volume of ice-cold isopropanol was added and incubated at -20 ºC for 20 minutes. Centrifugation was carried out at 12000 rpm for 10 minutes at 4ºC and the supernatant was discarded. The pellet was washed with 1 ml of 70 % of ice-cold ethanol and centrifuged at 10000 rpm for 10 minutes at 4ºC. The supernatant was discarded and pellet was air dried. The pellet was resuspended in 20 µl of NFW, quantified and stored at -80 ºC until further use. About 500 ng of RNA was used for synthesis of cDNA using iScript (TaKaRa) as per the manufacturer's instructions. The gene expression analysis of intestinal tight junction protein genes (Zona occludens-1 (ZO-1), Mucin-2 & Claudin-3) were carried out by specific primers (Table 3) with the following PCR conditions: an initial denaturation

step at 95ºC for 3 min, followed by 40 cycles at 95ºC for 30 sec, the annealing and extension temperature at 60ºC for 30 sec, and a final extension step of 72ºC for 35 sec. The melt curve analysis was carried out to assess the specificity of the amplified products.

Statistical analysis:

All the data of this study was grouped and subjected to statistical analysis by one-way ANOVA using SPSS, version 20.0 statistical package. Pair wise differences in means were computed using Duncan test. The obtained results were expressed as means and standard deviations.

Results

Characterization of zinc oxide nano particles:

The Dynamic Light Scattering (DLS) results revealed the average particle size of synthesized zinc oxide nanoparticles was 4.4 ± 1.45 nm (Fig 1) and the average zeta potential value obtained was - 35.4 mV (Fig 2). Inductively Coupled plasma mass spectrometry analysis revealed that nano zinc contained 74.78 % elemental zinc. X ray diffraction pattern showed 2θ values at 31.907˚, 34.554˚, 36.393˚, 47.672˚, 56.738˚, 62.985˚, 66.517˚, 68.104˚, 69.192˚, 72.713˚, and 77.054˚ (Fig 3) which could be indexed as the zinc oxide wurtzite structure (JCPDS Data Card No: 36-1451). The average particle size of the sample was found to be 5.078 nm which is derived from the FWHM of more intense peak corresponding to 101 planes located at 36.393° using Scherrer's formula (Talam et al. 2012) which is comparable with the particle size obtained in DLS technique. Using HR-TEM morphology and particle size of the synthesized zinc oxide nanoparticles were assessed. It was found that most of the ZnONPs are in nano meter scale and are hexagonal in shape with the average particle size of 41.2 nm (Fig. 4).

The FTIR spectrum of synthesized zinc oxide nano particle showed the peaks at 3428.96 cm^{-1}, 2921.43 cm^{-1}, 2849.8 cm^{-1}, 1630.02 cm^{-1}, 1022.17 cm^{-1}, 989.43 cm^{-1}, 872.77 cm^{-1} and 448.42 cm^{-1} (Fig. 5). In our study, UV-visible absorption spectroscopy is used to examine the optical properties of nano particles. UV-Vis absorbance spectrum of zinc oxide nanoparticle shown in Figure 6a. ZnO nano particle exhibited the absorption peak between 350 – 360 nm and diffuse reflectance spectra revealed characteristic absorption edge near 380 nm with high reflectance of 50 % in the visible region (Fig. 6b)

***In - vitro* cytotoxicity assay:**

The cytotoxicity effects of zinc oxide nano particles in different cell lines (Vero and BHK-21) and primary chick liver culture were assessed by MTT. The results of ZnO nanoparticles on Vero, BHK-21 and primary chick liver culture cells are presented in Fig. 7. In primary liver culture cells, the maximum cell viability was observed at the lowest concentration of zinc oxide nano particles (6.25 µg/ml). The present study suggested that zinc oxide nanoparticles were exhibited no cytotoxicity upto the concentration of 100 µg/ml in both cell lines and primary culture.

Growth performances:

Effects of dietary zinc oxide nanoparticles (ZnONPs) supplementation on feed intake, weight gain, feed conversion ratios (FCR) in broiler chickens from day 1 to 35 were presented in Table 4. In overall trial period, 60 mg/kg of zinc oxide nanoparticles supplemented group revealed significantly higher ($p<0.05$) body weight gain compared to control and no significant difference ($p>0.05$) with 20 mg/kg of zinc oxide nanoparticles supplemented birds. Feed Conversion Ratio was significantly lower ($p<0.05$) in the birds supplemented with 60 and 20 mg/kg of zinc oxide nanoparticles when compared to control and other treatment groups.

Carcass characteristics:

The results indicated that there was no significant difference ($p>0.05$) among the treatment group in shear force value and water holding capacity as shown in table 5. Whereas, 60 mg/kg of zinc oxide nanoparticles supplemented birds had significantly lower ($p<0.05$) meat pH. The results indicated that ZnONPs fed groups had significantly higher ($p<0.05$) dressing percentage compared with other treatment groups.

Serum Biochemistry:

There was no significant difference ($p>0.05$) in the glucose, total protein, albumin, cholesterol, ALT, uric acid, calcium and phosphorus values between the treatment groups (Table 6).

Concentration of zinc in the serum:

Effects of zinc oxide nanoparticles on serum zinc concentration was given in Table 7. In serum, the zinc concentration revealed highly significant difference ($p<0.01$) between zinc oxide nano particles supplemented group and control.

Intestinal Morphometry:

Among treatment groups, there was no significant difference($p>0.05$) in ileum villi length and jejunum Villi length and crypt depth ratio (Table 8). The villi length and crypt depth in duodenum and jejunum and villi length to crypt depth ratio in ileum was significantly ($p<0.05$) higher in birds supplemented with of 20 mg/kg ZnONPs compared to control (Fig. 8, Fig. 9 and Fig.10). Further, in our study crypt depth in ileum and villi length to crypt depth ratio in duodenum there was a significant difference ($p<0.05$) between zinc oxide nano particles supplemented group and other groups. Supplementation of zinc oxide nanoparticles showed significant increase ($p<0.05$) in crypt depth in ileum and villi length to crypt depth ratio in duodenum values when compared

to other groups. Based on the data presented in table 8, the highest villi length, crypt depth and villi length to crypt depth ratio in duodenum, jejunum and ileum was observed in 20 mg/kg of ZnONPs supplemented groups.

Intestinal Tight Junction protein gene expression:

The effects of ZnONPs on ZO-1, mucin-2 and claudin-3 gene expression in intestine were presented in Fig. 11. Dietary supplementation of ZnONPs did not affect the mRNA expression of Zo-1 ($p>0.05$) compared with other groups. However, mucin-2 and claudin-3 intestinal tight junction protein genes were significantly ($p<0.05$) upregulated (4.18 and 3.63-fold respectively) in broiler chicks fed with 20 mg/kg of ZnONPs.

Discussion

In the present study, zinc oxide nanoparticles (ZnONPs) were synthesized, characterized and evaluated to use as an effective feed additive in broiler chicken diet. ZnONPs are specially prepared and having the particle size of 1 to 100 nm (Swain et al., 2015). Average particle size of ZnONPs was 4.4 ± 1.45 nm in Dynamic Light Scattering (DLS) analysis which is comparable with the findings of Melk et al. (2021) who reported that the average particle size distribution of ZnONPs was 10.58 ± 3.350 nm. The average zeta potential value obtained was - 35.4 mV and the negative values detected by zeta potential were responsible for stabilization of the nanoparticles. The value present in this study is in agreement with the earlier study of Barzinjy et al. (2020) who reported the average zeta potential value of −40 mV. In the present study Inductively Coupled plasma mass spectrometry analysis revealed that nano zinc contained 74.78 % elemental zinc. Zinc oxide mainly crystallizes in two forms, hexagonal wurtzite and cubic zincblende. The wurtzite structure is most common and most stable at ambient conditions (Rai et al. 2021). XRD analysis of ZnONPs showed that all the diffraction peaks corresponded to the hexagonal wurtzite structure (JCPDS Data Card No: 36-1451). These results were in agreement with the findings of Yedurkar et al. (2016) who reported the wurtzite structure of ZnONPs. The average crystallite size of ZnONPs was in the nanometric range which is comparable with the particle size obtained in DLS technique. HR-TEM analysis also confirmed that most of the ZnONPs are in nano meter scale and are hexagonal in shape which is in conformity with the findings of Ezealisiji et al. (2019) who observed s spherical particles. The intensive peak at 448.42 cm^{-1} in FTIR analysis clearly indicates the characteristic absorption of Zn – O bond. As a thumb rule, the absorption peak maximum for ZnONPs ranges between 300 and 380 nm. Here, ZnONPs exhibited the absorption peak between 350 – 360 nm which is close to the findings of Talam et al., 2012 who observed that ZnONPs exhibits a strong absorption band at about 355 nm. In our study, diffuse reflectance spectra of ZnO nanoparticles revealed characteristic absorption edge near 380 nm with high reflectance of 50 % in the visible region (Fig. 6b) which is in agreement with the results of Zeferino et al. (2011). The results indicated that ZnONPs does not produce any toxicity effects on cells in all the concentrations. Reduction of cell viability by more than 30 % was considered a cytotoxic effect (ISO 10993-5). The results of the present study were in agreement with Masud et al. (2020) who carried out the i*n vitro* cytotoxicity analysis of the ZnO bio nanocomposite using the BHK-21 cell and the Vero cell line for 48 hours and reported more than 95% of cell viability which indicated the cytocompatibility of the bio nanocomposite of ZnO. In contrast, Saranya et al. (2017) reported that the ZnONPs showed improved cell viability at lower concentration (10 µg/100 µl) in Vero cell line. The present study suggested that zinc oxide nanoparticles were exhibited no cytotoxicity up to the concentration of 100 µg/ml in cell lines and primary culture.

Due to higher bioavailability of Zn from nano-zinc than inorganic and organic sources, they have a better absorption than other two sources resulting in better growth. (Pathak *et al*., 2020). In overall trial period, significantly higher body weight gain and FCR was obtained in 20 mg/kg of ZnONPs when compared to control. This finding was consistent with Zhao et al. (2014), who reported that 20–60 mg/kg ZnONPs could improve body weight gain and feed conversion ratio in broiler chicks compared to 50 mg/ kg traditional ZnO. Similarly, Fawaz et al. (2021) reported that 20 - 40 mg/kg ZONPs improved growth performance in broilers compared to other concentrations (10 & 30 mg/kg). There was no significant difference ($p>0.05$) among the treatment group in SFV and WHC in this study. Similarly, Hussan et al. (2020) found that there was no significant difference ($P > 0.05$) observed in WHC of meat by the supplementation of nano ZnO at graded levels (2.5,5,10 and 20 ppm of nano zinc and 40 ppm of ZnO). Whereas, 60 mg/kg of zinc oxide nanoparticles supplemented birds had significantly lower ($p<0.05$) meat pH. This finding of present study in agreement with the results of Eskandani *et al*. (2021) who found lower pH value of breast meat in chickens fed with ZnONPs (30, 50, 70 & 90 mg/kg) and 70 mg Zn amino acid complex. ZnONPs fed groups had significantly higher ($p<0.05$) dressing percentage compared with other treatment groups in this study. However, Hatab et al. (2022) suggested that ZnONPs at 40 mg/kg increased carcass yield in comparison with other groups (0&60 mg/kg ZnONPs).

There was no significant difference ($p>0.05$) in the glucose, total protein, albumin, cholesterol, ALT, uric acid, calcium and phosphorus values between the treatment groups. Total Protein, Total Bilirubin, Aspartate aminotransferase (AST) and Alanine Transaminase (ALT), ALP, uric acid levels, creatinine and globulin were not significantly affected by ZnONPs supplementation (Abdel-Monem et al., 2021; Mahmoud et al., 2021). Zinc concentration in serum was elevated dose dependently. This finding was consistent with Hatab et al. (2022) who reported that supplementation of nano form of Zn into broiler diets (40 and 60 mg/kg diet) increased the concentration of Zn in serum dose dependently. Such amelioration of zinc can be ascribed to enhanced bioavailability of zinc in its nano form leading to improved absorption in the digestive tract. It might be attributed to increased villi length in the intestine of the broiler chickens due to the supplementation of zinc oxide nanoparticles.

The highest villi length, crypt depth and villi length to crypt depth ratio in duodenum, jejunum and ileum was observed in 20 mg/kg of ZnONPs supplemented groups which is in conformity with Fawaz *et al*. (2021) who supplemented chicks with different ZnONPs concentrations (10, 20, 30, and 40 mg/kg ration) showed a significant increase in villus height and villus/crypt ratio in small intestine. Dietary supplementation of ZnONPs did not affect the mRNA expression of Zo-1 ($p>0.05$) compared with other groups but mucin-2 and claudin-3 intestinal tight junction protein genes were significantly ($p<0.05$) upregulated (4.18 and 3.63-fold respectively) 20 mg/kg of ZnONPs group indicating the enhanced intestinal barrier function thereby improving the nutrient absorption in broiler chicks. However, Zhang *et al*. (2022) reported that ZO-1 and claudin-2 mRNA expressions were higher in 160 mg/kg ZnO

nano particle group than that in the Negative Control group. In contrast, Zhang *et al.* (2012) observed that inclusion of zinc in the diet of chicks did not affect the mRNA levels of Mucin-2.

It can be concluded that ball milling technique economical method for the preparation of nano zinc oxide with respect to simplicity as well as it has high yield of 96.7 % and can be used for large scale industrial production of zinc oxide nanoparticles. The present study results showed that the supplementation of Zinc in the forms of nanoparticles at the lower concentration of 20 mg/kg could be optimal supplementation level in broiler diets and could be used as an effective feed additive in broilers with beneficial effects on the growth performance, meat quality and intestinal barrier functions of broiler chicken.

Declarations

Acknowledgement

The author is thankful to the Department of Animal Nutrition and Department of Animal Biotechnology, Madras veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, for providing necessary funding, technical and instrumental facility to carry out research work.

Authors' contribution

V. Mozhiarasi: Optimization of Synthesis of ZnONPs, Conducting the feeding trial, Growth and carcass characteristics studies, serum biochemistry and statistical analysis, draft manuscript writing; R. Karunakaran: Conceptualization, design and supervision of complete study, fund mobilization, growth performance data analysis, manuscript reviewing editing and finalizing; P. Raja; Characterization and *In vitro* cytotoxicity assay of ZnONPs, Real time PCR analysis, manuscript correction; L. Radhakrishnan; Feed formulation, monitoring of feeding trial and manuscript reviewing and editing; N. Pazhanivel: intestinal morphometry and manuscript reviewing and editing. All authors approved the final version of the manuscript.

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript

Data availability:

The data are available by the corresponding author upon a reasonable query

Ethical approval

The experimental procedures and animal handling were approved by the Institutional Animal Ethical Committee (IAEC) for the use and care of animals at Madras Veterinary College, TANUVAS, Chennai -7 (Lr.No. 508/DFBS/IAEC/2022 dated on 05.05.2022).

Consent for publication

All authors gave their consent and accredited this study for research publication.

Consent to participate

All authors approved the final version of the manuscript.

Conflict of interest

The authors do have not any conflict of interest to declare

References

1. Abdel-Monem N, Elsebai A, El-Hady A and Mohamed A (2021). Impact of Dietary Zinc Oxide Nano-Particles on Antioxidant Status, Liver and Kidney Functions in Alexandria Chickens. Egypt Poult Sci J 41(3):675-690.
2. Abdelnour SA, Alagawany M, Hashem NM, Farag MR, Alghamdi ES, Hassan FU, Bilal RM, Elnesr SS, Dawood MA, Nagadi SA and Elwan HA (2021). Nanominerals: fabrication methods, benefits and hazards, and their applications in ruminants with special reference to selenium and zinc nanoparticles. Animals 11(7):916.
3. Alyamani, AA, Albukhaty S, Aloufi S, AlMalki FA, Al-Karagoly H and Sulaiman GM (2021). Green fabrication of zinc oxide nanoparticles using phlomis leaf extract: characterization and in vitro evaluation of cytotoxicity and antibacterial properties. Molecules 26(20):6140.
4. Asheer M, Manwar SJ, Gole MA, Sirsat S, Wade MR, Khose KK and Ali SS (2018). Effect of dietary nano zinc oxide supplementation on performance and zinc bioavailability in broilers. Indian J Poult Sci 53(1):70-75.
5. BAHS, 2019. Basic Animal Husbandry Statistics Department of Animal Husbandry and Dairying, Government of India
6. Barzegar M, Zaghari M, Zhandi M and Sadeghi M (2021). Effects of zinc dosage and particle size on gut morphology, tight junctions and TNF-α expression in broiler breeder hens. J Anim Physiol Anim Nutr 1–11.

7. Barzinjy AA and HH Azeez (2020). Green synthesis and characterization of zinc oxide nanoparticles using Eucalyptus globulus Labill. leaf extract and zinc nitrate hexahydrate salt. SN Applied Sciences 2(*5*): 1-14
8. BIS, 2007. Bureau of Indian Standards, Indian Standards for Poultry Feeds - Specifications. 5th ed. New Delhi.
9. Cheng J, Kornegay ET and Schell T (1998). Influence of dietary lysine on the utilization of zinc from zinc sulfate and a zinclysine complex by young pigs. J Anim Sci 76:1064-1074.
10. Eskandani M, Janmohammadi H, Mirghelenj SA, Ebrahimi M and Kalanaky S (2021). Effects of zinc nanoparticles on growth performance, carcass characteristics, immunity, and meat quality of broiler chickens. *Iran J Appl Anim Sci* 11(1):135-146.
11. Ezealisij KM, Siwe-Noundou X, Maduelosi B (2019). Green synthesis of zinc oxide nanoparticles using *Solanum torvum* (L) leaf extract and evaluation of the toxicological profile of the ZnO nanoparticles–hydrogel composite in Wistar albino rats. Int Nano Lett 9: 99–107.
12. Fawaz R, Ibrahim R, Ali N, Mahmoud U And Amen O, Effect of Dietary Zinc Oxide Nanoparticles On Growth Performance and Clostridium Perfringens Infection In Broiler Chickens. Bulg. J. Vet. Med.
13. Ghaffari SB, Sarrafzadeh MH, Fakhroueian Z, Shahriari S and Khorramizadeh MR (2017). Functionalization of ZnO nanoparticles by 3-mercaptopropionic acid for aqueous curcumin delivery: Synthesis, characterization, and anticancer assessment. Mater Sci Eng C *79*:465-472.
14. Hafez A, Hegazi SM, Bakr AA and Shishtawy HE (2017). Effect of zinc oxide nanoparticles on growth performance and absorptive capacity of the intestinal villi in broiler chickens. Life Sci J 14(*6*): 67-72.
15. Hatab MH, Rashad E, Saleh HM and El-Sayed ESR (2022). Effects of Dietary Supplementation of Zinc Oxide Nanoparticles on Productive Performance, Physiological, Histological Changes and Tissues Zn Concentration in Broiler Chicks. https://doi.org/10.21203/rs.3.rs-1523659/v1
16. Hussan F, Krishna D, Preetam VC, Reddy PB and Gurram S (2022). Dietary supplementation of nano zinc oxide on performance, carcass, serum and meat quality parameters of commercial broilers. Biol Trace Elem Res 200(1):348-353.
17. Hussien KRA., Ismail ZSH and Abdel-Wareth AAA (2021). Application of zinc oxide nanoparticles as feed additive in broiler chicken nutrition under hot environmental conditions. SVU-Int J Agric Sci 3(3):159-169.
18. ISO 10993-5, Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity, ISO 2009
19. Jayarambabu N, Kumari BS, Rao KV and Prabhu YT (2014). Germination and growth characteristics of mungbean seeds (Vigna radiata L.) affected by synthesized zinc oxide nanoparticles. Int J Curr Eng Technol 4(5):3411-341628.
20. Khah MM, Ahmadi F and Amanlou H (2015). Influence of dietary different levels of zinc oxide nano particles on the yield and quality carcass of broiler chickens during starter stage. Indian J Anim Sci 85(3):287-290.
21. Kumar VP, Manikandan N, Nagaprasad N, LetaTesfaye J and Krishnaraj R (2022). Analysis of the Performance Characteristics of ZnO Nanoparticles' Dispersed Polyester Oil. Adv Mater Sci Eng *2022*.
22. Mahamuni PP, Patil PM, Dhanavade MJ, Badiger MV, Shadija PG, Lokhande AC and Bohara RA (2019). Synthesis and characterization of zinc oxide nanoparticles by using polyol chemistry for their antimicrobial and antibiofilm activity. Biochemistry and biophysics reports, 17:71-80.
23. Mahmoud MA, Yahia D, Abdel-Magiud DS, Darwish MH, Abd-Elkareem M and Mahmoud UT (2021). Broiler welfare is preserved by long-term low-dose oral exposure to zinc oxide nanoparticles: preliminary study. Nanotoxicology 15(5): 605-620.
24. Masud RA, Islam MS, Haque P, Khan MNI, Shahruzzaman M, Khan M, Takafuji M and Rahman MM, 2020. Preparation of novel chitosan/poly (ethylene glycol)/ZnO bionanocomposite for wound healing application: effect of gentamicin loading. *Materialia*, 12:100785.
25. Melk MM, El-Hawary SS, Melek FR, Saleh, DO, Ali, OM, El Raey, MA and Selim, NM (2021). Nano zinc oxide green-synthesized from plumbago auriculata lam. alcoholic extract. Plants 10(11):2447.
26. Pathak, SS; Reddy, KV and Prasoon, S (2016). Influence of different sources of zinc on growth performance of dual-purpose chicken. J Bio Innov., 5:663-72.
27. Patra AK & Lalhriatpui M, (2020). Progress and prospect of essential mineral nanoparticles in poultry nutrition and feeding - a review. Biol Trace Elem Res 197: 233-253
28. Rai H, Prashant & Kondal N (2021). A review on defect related emissions in undoped ZnO nanostructures. Materials Today: Proceedings. 48(5): 1320-1324
29. Saranya S, Vijayaranai K, Pavithra S, Raihana N and Kumanan K (2017). In vitro cytotoxicity of zinc oxide, iron oxide and copper nanopowders prepared by green synthesis. *Toxicology Reports*, 4:427-430.
30. Schmidt CW (2009). Nanotechnology-related environment, health, and safety research: examining the national strategy 117(4). https://doi.org/10.1289/ehp.117-a158
31. Swain PS, Rao SB, Rajendran D, Dominic G and Selvaraju S (2016). Nano zinc, an alternative to conventional zinc as animal feed supplement: A review. Anim Nutr 2(3):134-141
32. Talam S, Karumuri SR and Gunnam N, 2012. Synthesis, characterization, and spectroscopic properties of ZnO nanoparticles. International Scholarly Research Notices, *2012*
33. Yedurkar S, Maurya C and Mahanwar P (2016). Biosynthesis of zinc oxide nanoparticles using ixora coccinea leaf extract—a green approach. Open J Synth Theor Appl *5*(1):1-14.

34. Youssef FS, El-Banna HA, Elzorba HY, Galal AM (2019). “Application of Some Nanoparticles in the Field of Veterinary Medicine.” Int J Vet Sci Med 7 (1): 78–93.
35. Zaboli K, Aliarabi H, Bahari AA and ABBAS AKR (2013). Role of dietary nano-zinc oxide on growth performance and blood levels of mineral: A study on in Iranian Angora (Markhoz) goat kids. J Pharm Health Sci 2(1):19-26
36. Zeferino RS, Flores MB and Pal U (2011). Photoluminescence and Raman scattering in Ag-doped ZnO nanoparticles. J Appl Phys 109(1):014308.
37. Zhang B, Shao Y, Liu D, Yin P, Guo Y and Yuan J (2012). Zinc prevents Salmonella enterica serovar Typhimurium-induced loss of intestinal mucosal barrier function in broiler chickens. Avian Pathol 41(4):361-367. http://dx.doi.org/10.1080/03079457.2012.692155
38. Zhang J, Li Z, Yu C, Liu H, Zhou B, Zhang X, Wang T and Wang C (2022). Efficacy of using zinc oxide nanoparticle as a substitute to antibiotic growth promoter and zinc sulphate for growth performance, antioxidant capacity, immunity and intestinal barrier function in broilers. Ital J Anim Sci 21(1):562-576.
39. Zhao CY, Tan SX, Xiao XY, Qiu XS, Pan JQ and Tang ZX (2014). Effects of dietary zinc oxide nanoparticles on growth performance and antioxidative status in broilers. Biol Trace Elem Res 160(3):361-367.

Tables

Table 1 Experimental design for *in vivo* feeding trial to evaluate the effect of supplementation of zinc oxide nanoparticles in broiler chicken

T	Experimental Design	No. of birds	R	Total
T_1	Basal diet + inorganic zinc (80 mg/kg)	10	3	30
T_2	Basal diet + organic zinc (60 mg/kg)	10	3	30
T_3	Basal diet + nano zinc (60 mg/kg)	10	3	30
T_4	Basal diet + nano zinc (40 mg/kg)	10	3	30
T_5	Basal diet + nano zinc (20 mg/kg)	10	3	30
		Total		150
Experimental duration (*in days*): 35; T – Treatments; R – Replications				

Table 2 Feed ingredients composition (%) and nutrient content of broiler chicken pre-starter (1-7 days), starter (8-21 days) and finisher (22-35 days) feeds

Ingredients	Inclusion level (%)		
	Pre-starter (*1 - 7 days*)	Starter (*8 - 21 days*)	Finisher (*22 – 35 days*)
Maize	49.56	50.35	54.25
Soya bean meal	42.50	40.09	35.00
Palm Oil	3.80	5.40	6.50
Calcite	1.22	1.22	1.30
Dicalcium Phosphate	1.80	1.84	1.81
Salt	0.30	0.30	0.30
Methionine	0.15	0.14	0.12
Threonine	0.10	0.10	0.15
Vitamin Premix	0.05	0.05	0.05
Trace Mineral Mixture	0.10	0.10	0.10
Choline chloride	0.15	0.15	0.15
Probiotic	0.05	0.05	0.05
Liver tonic	0.04	0.04	0.04
Toxin binder	0.10	0.10	0.10
Coccidiostat	0.05	0.05	0.05
Antioxidant	0.01	0.01	0.01
Total	**100.00**	**100.00**	**100.00**
Nutrient composition			
CP (%)	23.05	22.08	20.02
ME (kcal/kg)	3003.00	3103.00	3198.00
Lysine (%)	1.56	1.48	1.31
Methionine (%)	0.52	0.50	0.45

Table 3. The sense and antisense primer sequences of intestinal tight junction proteins and reference gene used for real time PCR

Gene	Orientation	Primers Sequence	Product size (bp)
MUC-2	Forward	5′-TTCATGATGCCTGCTCTTGTC-3′	93
	Reverse	3′-CCGTAGCCTTGGTACATTCTTGT-5′	
ZO-1	Forward	5′-GCCTGAATCAAACCCAGCAA-3′	197
	Reverse	3′-TATGCGGCGGTAAGGATGAT-5′	
Claudin-3	Forward	5′-GAAGGGCTGTGGATGAACTG-3′	221
	Reverse	3′-GAGACGATGGTGATCTTGGC	
16S rRNA	Forward	5′-GTAACGCAAGCGATCNCG-3′	130
	Reverse	3′-AACCGCGACGCTTTCCAA-5′	

Table 4 Effects of dietary zinc oxide nanoparticles (ZnONPs) supplementation on feed intake, weight gain, feed conversion ratios (FCR) in broiler chickens from 1 to 35 Days of age (Mean # ± SD)

Treatments	Feed Intake (g)				Body Weight Gain (g)				FCR (g/g)			
	Age in days				Age in days				Age in days			
	1 - 7	8-21	22-35	1-35	1-7	8-21	22-35	1-35	1-7	8-21	22-35	1-35
T_1 (Control)	144.71 ± 4.14	1125.26 ± 30.13	1797.53 ± 66.04	3076.80 ±148.17	108.46 ±12.42	694.66 ±79.78	1061.53^{a} ± 141.26	1856.00^{a} ± 196.27	1.36^{bc} ±0.02	1.63 ±0.03	1.69^{b} ±0.01	1.65^{b} ±0.01
T_2 (Zn met-60 mg/kg)	147.57 ± 1.58	1131.70 ± 13.70	1832.06 ± 55.98	3142.75 ± 70.09	111.69 ± 9.56	712.96 ±61.48	1068.26^{a} ± 134.54	1889.59^{ab} ± 176.90	1.38^{c} ±0.09	1.60 ±0.02	1.72^{b} ±0.01	1.66^{b} ±0.01
T_3 (ZnONP-60mg/kg)	152.00 ± 4.88	1144.43 ± 54.41	1840.56 ± 74.61	3152.30 ± 75.4	114.80 ± 9.09	718.73 ±60.77	1153.33^{b} ± 123.12	1972.54^{b} ± 196.60	1.32^{ab} ±0.10	1.59 ± 0.06	1.61^{a} ±0.04	1.60^{a} ±0.01
T_4 (ZnONP - 40 mg/kg)	153.11 ± 8.34	1147.36 ± 22.86	1856.50 ± 18.47	3143.87 ± 36.37	114.19 ± 9.17	716.06 ±50.43	1074.5^{a} ± 120.95	1904.76^{ab} ± 140.97	1.34^{bc} ±0.03	1.62 ± 0.02	1.72^{b} ±0.07	1.66^{b} ±0.04
T_5 (ZnONP - 20 mg/kg)	154.37 ± 3.00	1158.70 ± 31.09	1860.76 ± 38.42	3130.94 ± 64.25	111.47 ±12.05	708.33 ±67.66	1118.6^{ab} ± 152.99	1945.07^{ab} ± 189.17	1.29^{a} ±0.01	1.58 ±0.01	1.66^{a} ±0.02	1.60^{a} ±0.02

[#]Each value represents three replicates with ten birds per replicate

Means bearing different superscript in a column differ significantly with ($p < 0.05$)

Table 5 Effects of zinc oxide nanoparticles on carcass characteristics (Mean [#] ± SD)of broiler chicks

Treatment	pH	SFV (kg/cm$^{2)}$	Dressing Percentage	WHC (%)
T_1 (control)	6.21^{c} ± 0.01	0.76 ± 0.09	69.9^{a} ± 1.33	1.43 ± 0.35
T_2 (Zn met - 60 ppm)	6.22^{c} ± 0.07	0.76 ± 0.08	69.66^{a} ± 2.39	1.44 ± 0.33
T_3 (ZnONPs - 60 ppm)	5.96^{a} ± 0.06	0.71 ± 0.07	73.13^{b} ± 2.09	1.53 ± 0.41
T_4 (ZnONPs - 40 ppm)	6.10^{b} ± 0.04	0.74 ± 0.1	72.67^{b} ± 1.20	1.49 ± 0.1
T_5 (ZnONPs - 20 ppm)	6.16^{b} ± 0.01	0.74 ± 0.08	71.55^{b} ± 2.00	1.52 ± 0.35

[#]Each value represents the mean of six observation

Means bearing different superscript in a column differ significantly with ($p < 0.05$)

Abbreviation: SFV- Shear Force Value; WHC- Water Holding Capacity

Table 6 Effects of zinc oxide nanoparticles on Serum Biochemistry value (Mean [#] ± SD) of broiler chicks

Treatment	Glucose (mg/dl)	Cholesterol (mg/dl)	Total protein (g/dl)	Albumin(g/dl)	Uric acid (mg/dl)	ALT (U/L)	Calcium (mg/dl)	Phosphorus (mg/dl)
T_1 (Control)	282.83 ± 54.43	120.66 ± 31.48	2.96 ± 0.41	1.48 ± 0.24	9.24 ± 0.86	19.00 ± 4.28	12.80 ± 0.57	8.28 ± 1.28
T_2 (Zn met-60 mg/kg)	304.00 ± 18.74	185.33 ± 68.43	3.03 ± 0.18	1.71 ± 0.11	9.45 ± 1.32	18.16 ± 3.76	13.17 ± 1.13	8.15 ± 1.77
T_3 (ZnONPs-60mg/kg)	269.00 ± 37.15	113.83 ± 28.10	2.83 ± 0.32	1.55 ± 0.24	9.95 ± 1.95	18.50 ± 4.59	12.96 ± 2.34	7.22 ± 1.25
T_4 (ZnONPs - 40 mg/kg)	300.66 ± 37.66	169.33 ± 70.30	2 .63 ± 0.41	1.73 ± 0.45	9.54 ± 0.93	22.33 ± 6.40	13.29 ± 2.84	7.76 ± 2.23
T_5 (ZnONPs - 20 mg/kg)	307.83 ± 26.09	151.83 ± 71.43	3.03 ± 0.22	1.65 ± 0.15	8.39 ± 3.18	20.83 ± 5.63	11.93 ± 1.42	8.87 ± 0.70

#Each value represents the mean of six observation

Table 7 Effects of zinc oxide nanoparticles on serum zinc concentration (Mean# ± SD) of broiler chicks

Treatment	Zinc (mg/L)
T_1 (Control)	1.39^{a} ± 0.1
T_2 (Zn met-60 mg/kg)	1.37^{a} ± 0.06
T_3 (ZnONPs- 60mg/kg)	2.18^{b} ± 0.12
T_4 (ZnONPs - 40 mg/kg)	1.97^{b} ± 0.31
T_5 (ZnONPs - 20 mg/kg)	1.81^{b} ± 0.22

#Each value represents the mean of six observation

Means bearing different superscript in a column differ significantly with ($p < 0.01$)

Table 8 Effect of supplementation of zinc oxide nanoparticles on intestinal morphology of 35-day old broiler chicken (Mean# ± SD)

Treatment	Villi length (µm)			Crypt depth (µm)			Villi length: Crypt depth		
	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
T_1	554.3^{a} ± 134.3	548.41^{a} ± 77.02	338.82± 80.36	38.50^{a} ± 15.31	39.15^{a} ± 12.29	32.85^{a} ± 7.22	15.08^{a} ± 4.21	13.83 ± 3.11	8.98^{a} ± 2.30
T_2	614.46^{ab} ± 110.77	544.42^{a} ± 141.51	346.79 ± 55.48	41.37^{ab} ± 8.76	39.47^{a} ± 7.42	33.30^{a} ± 5.80	15.70^{a} ± 3.24	13.47 ± 3.17	9.23^{ab}± 2.13
T_3	681.40^{b} ± 149.91	591.54^{ab} ± 71.91	369.48 ± 97.35	43.93^{ab} ± 11.12	44.73^{b} ± 6.75	37.73^{b} ± 6.78	18.63^{b} ± 4.25	14.37 ± 3.11	9.94^{ab}± 3.27
T_4	655.02^{b} ± 132.53	616.40^{bc} ± 115.19	349.07 ± 84.78	42.07^{ab} ± 8.79	43.44^{ab} ± 9.67	36.83^{b} ± 7.71	18.16^{b} ± 6.29	14.24 ± 3.12	9.65^{ab}± 3.32
T_5	847.20^{c} ± 138.32	650.27^{c} ± 94.61	372.43 ±103.82	45.53^{b} ± 9.73	44.82^{b} ± 9.92	39.69^{b} ± 8.00	19.01^{b} ± 5.04	14.66 ± 3.13	10.72^{b} ± 2.81

#Each value represents the mean of thirty-six observation

Means bearing different superscript in a column differ significantly with ($p < 0.05$)

[T_1 - Control; T_2 - Zn met-60 mg/kg; T_3 - ZnONPs- 60mg/kg; T_4 - ZnONP - 40 mg/kg; T_5 - ZnONP - 20 mg/kg]

Figures

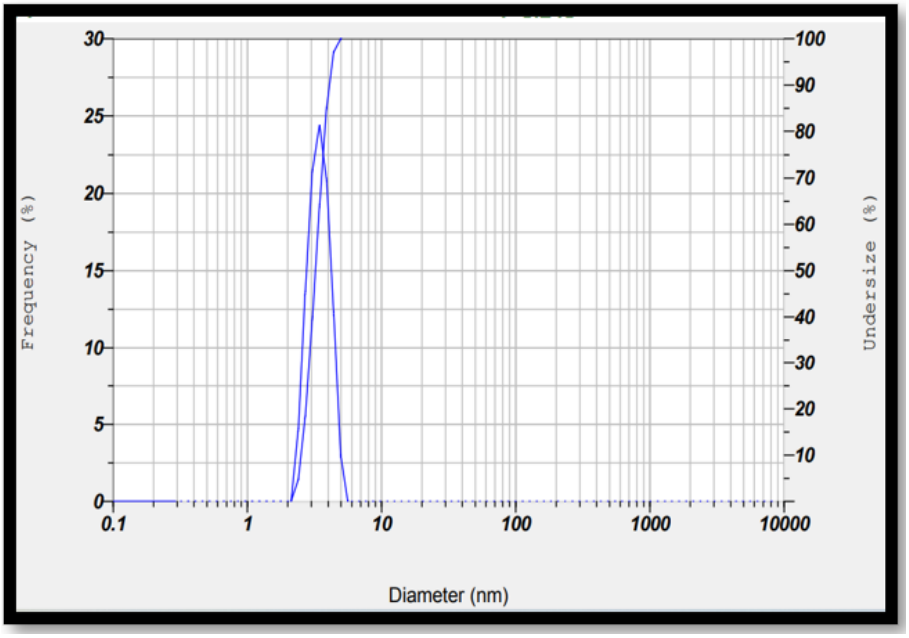


Figure 1

Particle size analysis of zinc oxide nanoparticle

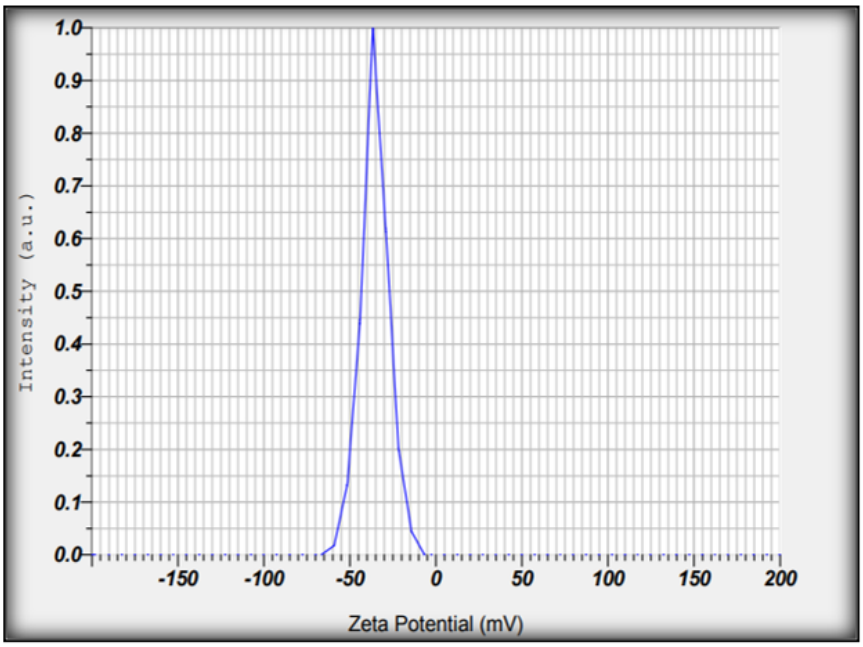


Figure 2

Zeta potential analysis of zinc oxide nanoparticle

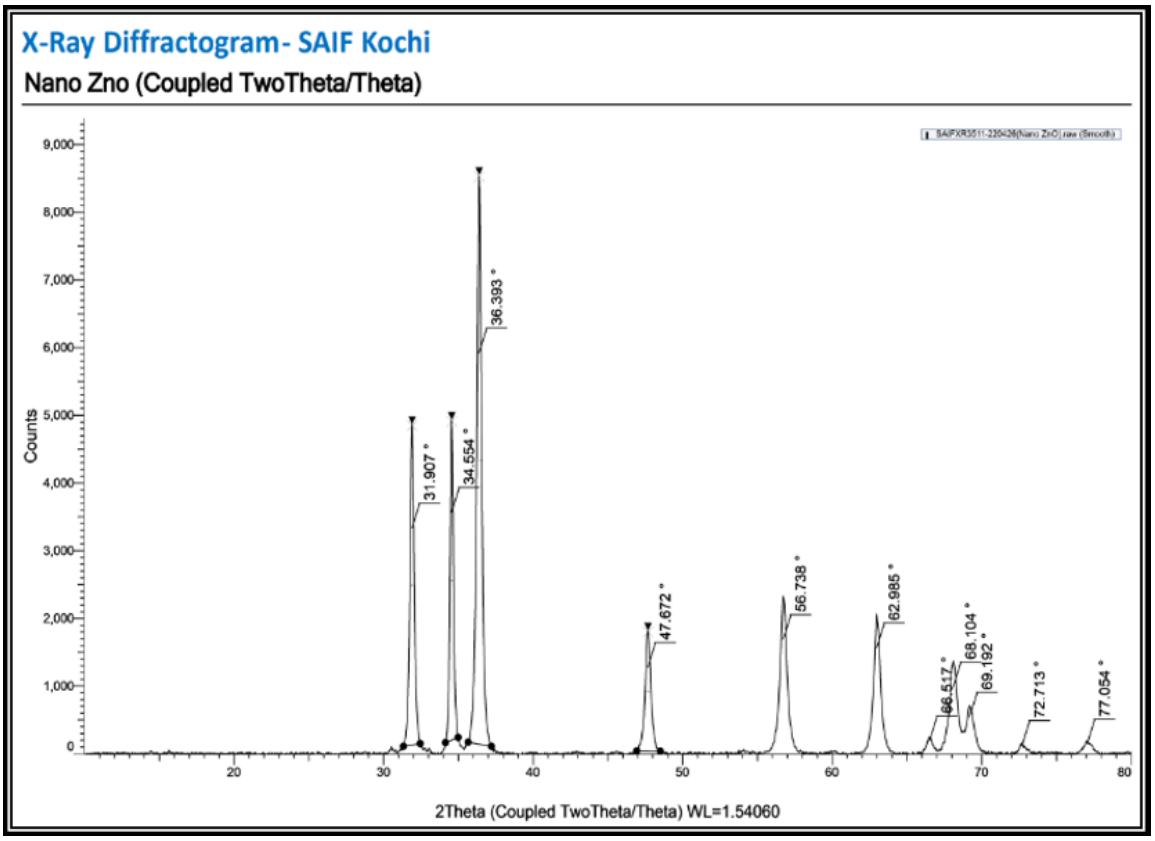


Figure 3

XRD analysis of zinc oxide nanoparticles

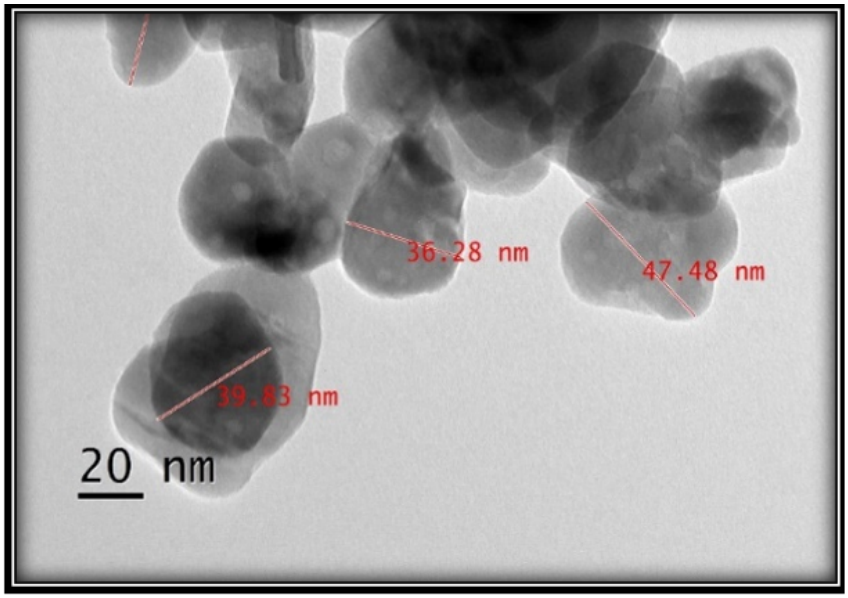


Figure 4

TEM analysis of zinc oxide nanoparticles

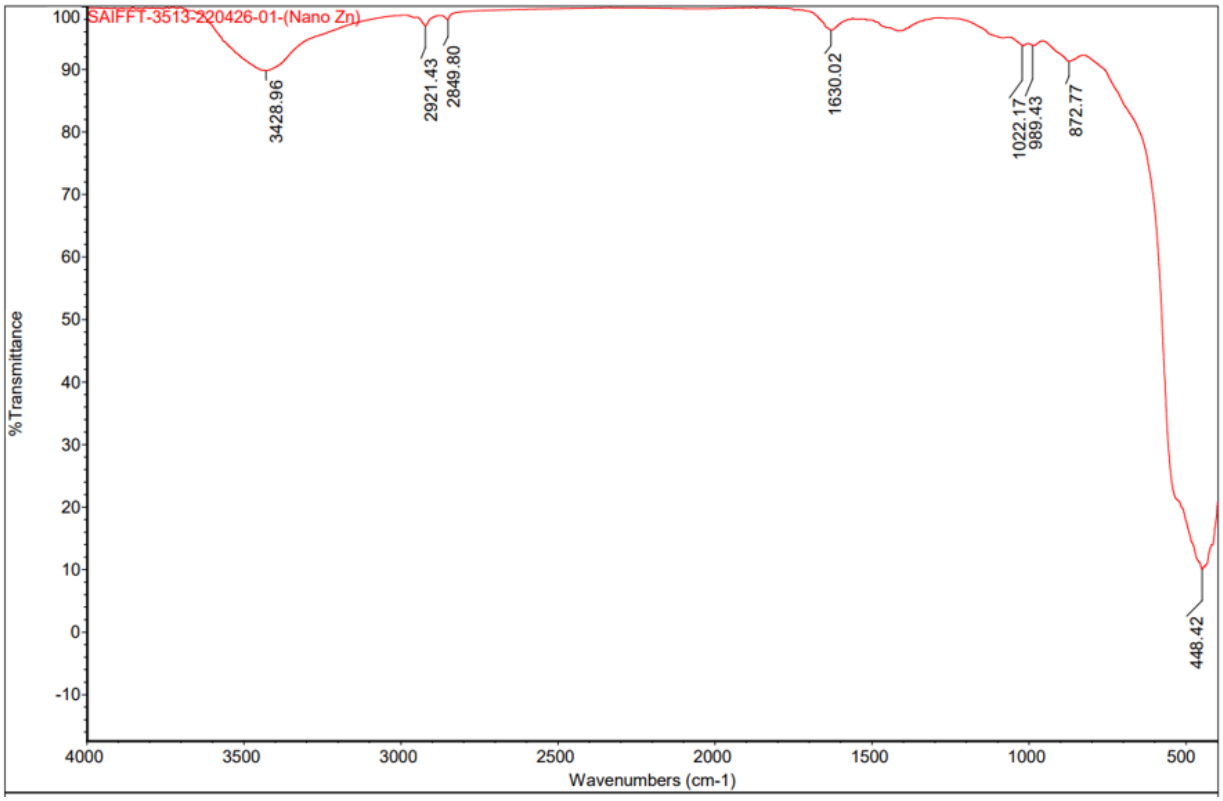


Figure 5

FTIR analysis of zinc oxide nanoparticles

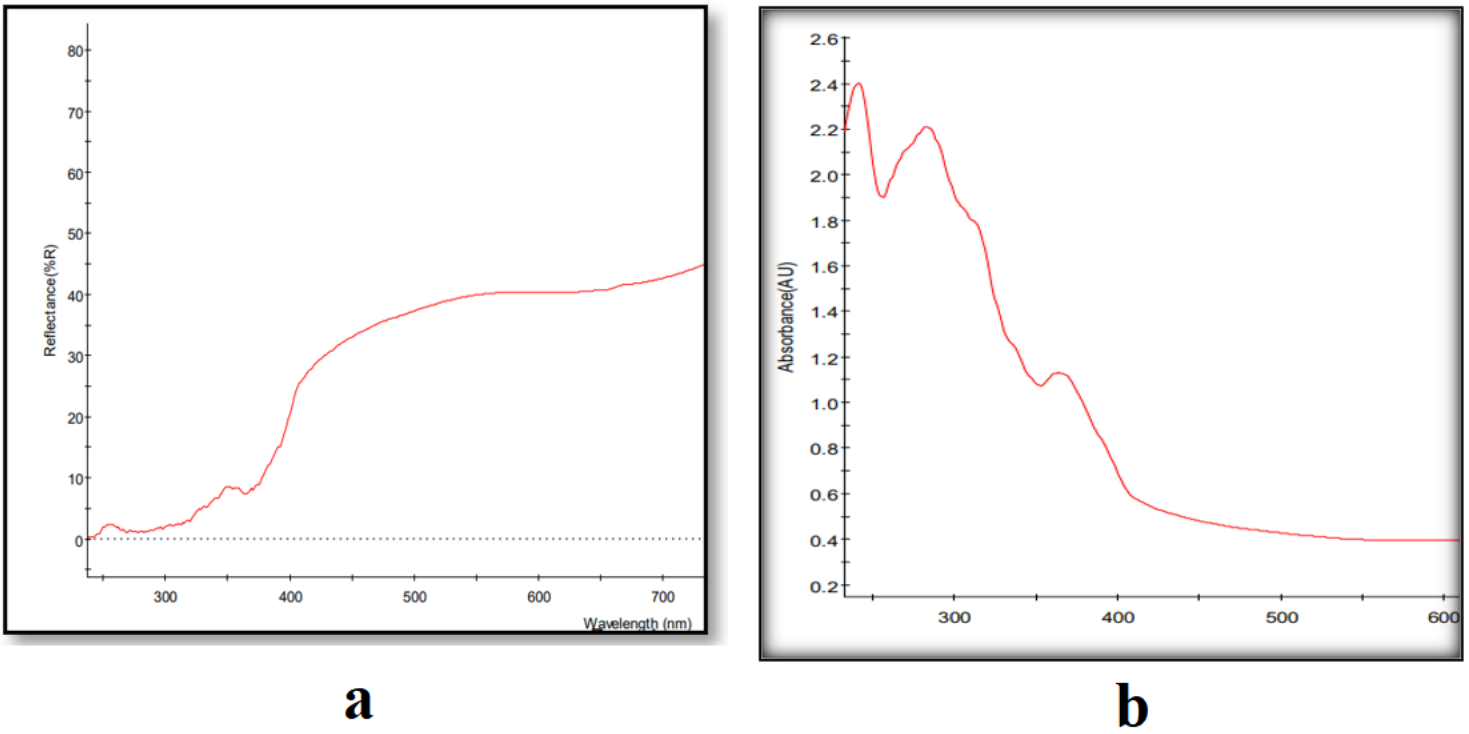
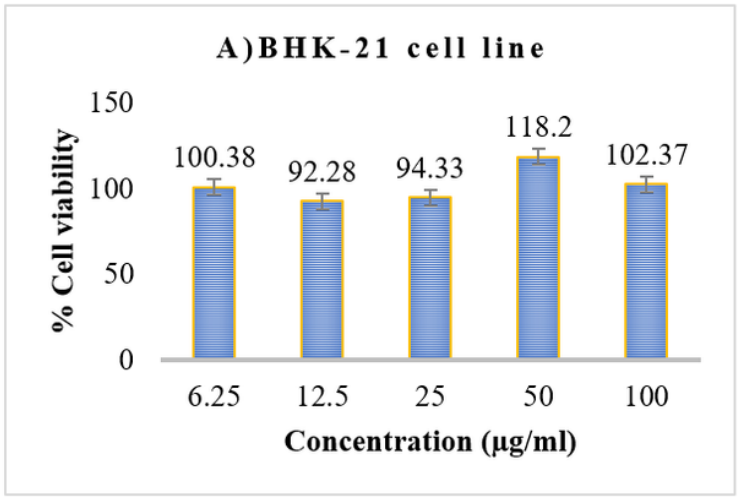
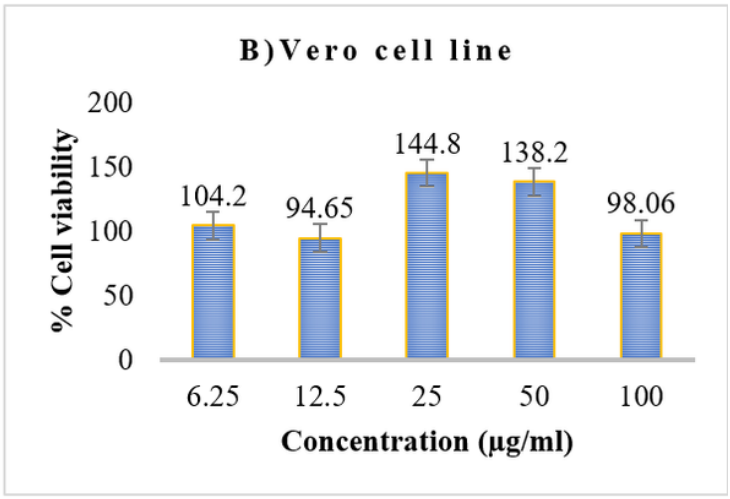


Figure 6

a UV-Vis Reflectance spectrum of ZnONPs

b UV-Vis absorbance spectrum of ZnONPs





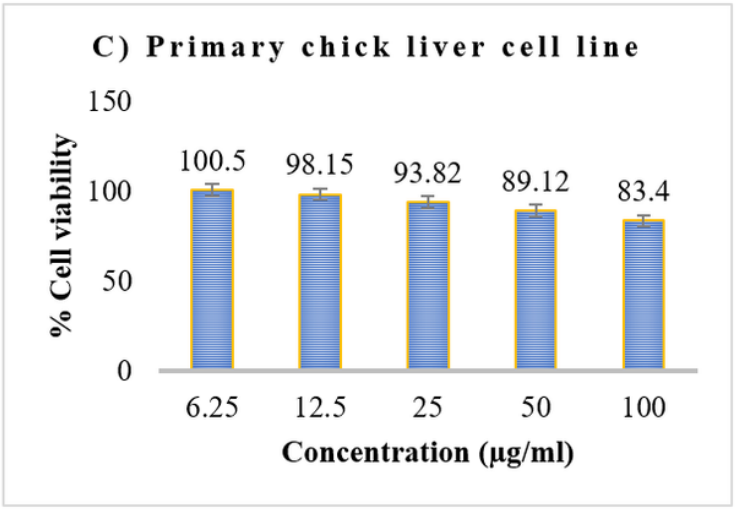
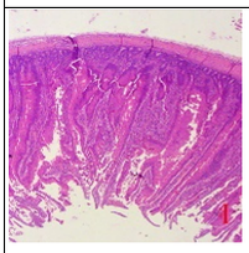
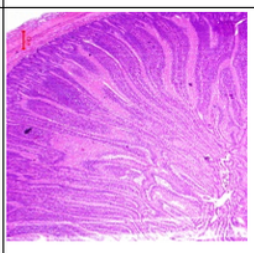
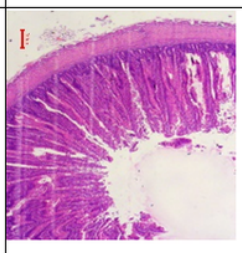
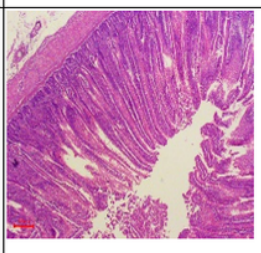
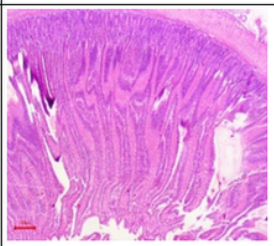


Figure 7

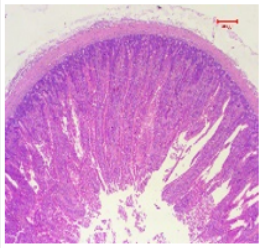
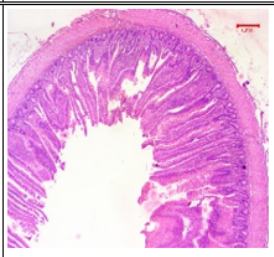
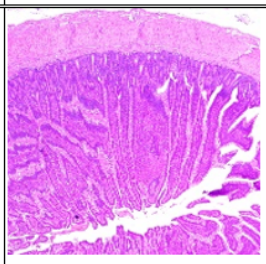
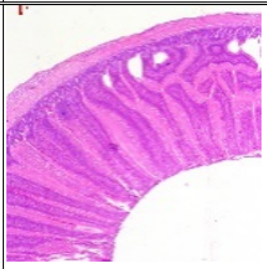
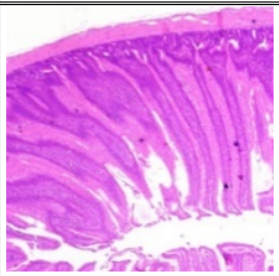
Cell viability (%) observation. Each cell line was treated with indicated amount of ZnONPs and measured after 72 h of exposure period. A) BHK – 21, B) Vero and C) Primary chick liver culture

Control	T_2	T_3	T_4	T_5
				
Normal mucosal layer, villi and crypt	Normal mucosal layer, Villi and crypt	Normal mucosal layer, Villi and crypt	Normal mucosal layer, Villi and crypt	Significantly increased villi length and crypt depth

[T_1 - Control; T_2 - Zn met-60 mg/kg; T_3 - ZnONPs- 60mg/kg; T_4 - ZnONP - 40 mg/kg; T_5 - ZnONP - 20 mg/kg]

Figure 8

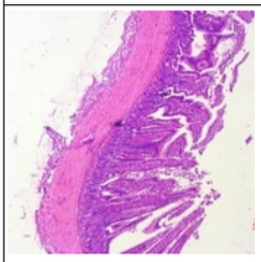
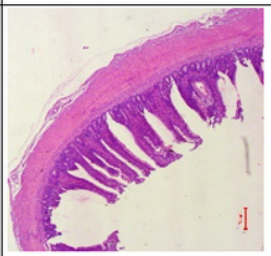
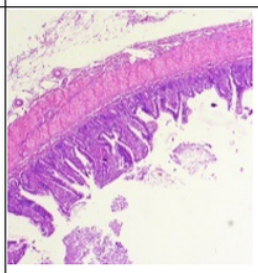
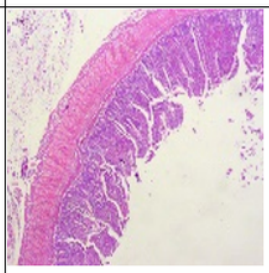
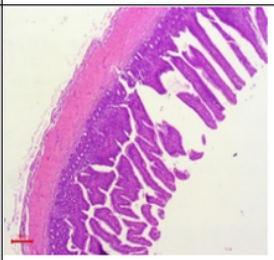
Histomorphometry analysis of duodenum of broiler chicks fed on Control, Organic zinc (Zn met) and zinc oxide nanoparticles at 35 days of age (H&E×400)

Control	T_2	T_3	T_4	T_5
				
Normal mucosal layer, Villi and crypt	Normal mucosal layer, Villi and crypt	Normal mucosal layer, Villi and crypt	Normal mucosal layer, Villi and crypt	Significantly increased villi length and crypt depth

[T_1 - Control; T_2 - Zn met-60 mg/kg; T_3 - ZnONPs- 60mg/kg; T_4 - ZnONP - 40 mg/kg; T_5 - ZnONP - 20 mg/kg]

Figure 9

Histomorphometry analysis of jejunum of broiler chicks fed on control, Organic zinc (Zn met) and zinc oxide nanoparticles at 35 days of age (H&E×400)

Control	T_2	T_3	T_4	T_5
				
Normal mucosal layer, Villi and crypt	Normal mucosal layer, Villi and crypt	Normal mucosal layer, Villi and crypt	Normal mucosal layer, Villi and crypt	Significantly increased crypt depth

[T_1 - Control; T_2 - Zn met-60 mg/kg; T_3 - ZnONPs- 60mg/kg; T_4 - ZnONP - 40 mg/kg; T_5 - ZnONP - 20 mg/kg]

Figure 10

Histomorphometry analysis of ileum of broiler chicks fed on control, Organic zinc (Zn met) and zinc oxide nanoparticles at 35 days of age (H&E×400)

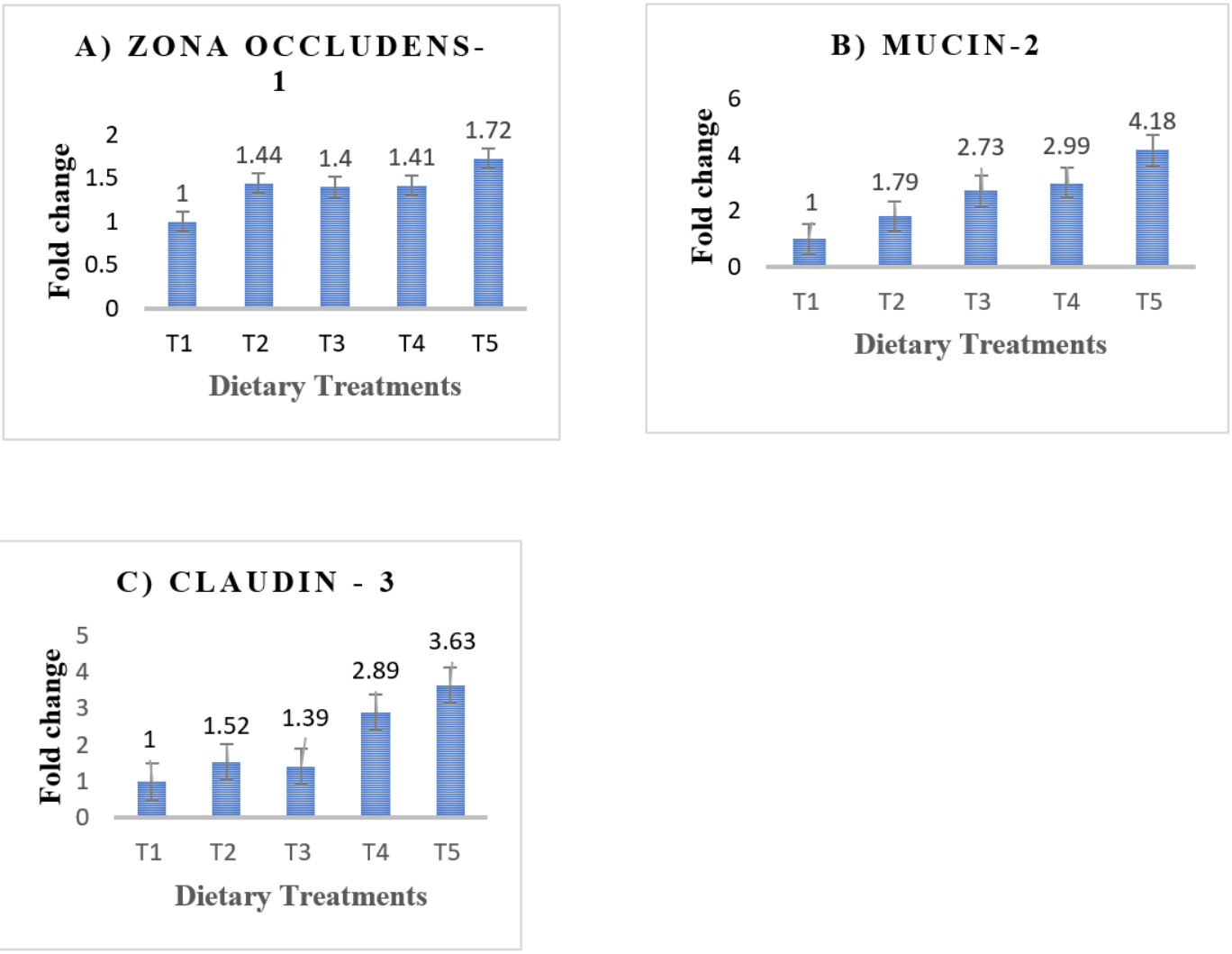


Figure 11

Effects of Zinc Oxide Nanoparticles on intestinal tight junction protein gene expression of broiler chicks A) Zona Occludens-1, B) Mucin-2 and C) Claudin-3