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Optimal Nano Zinc Oxide Supplementation Enhances Growth, Antioxidant Status, and Reproductive Performance in Japanese Quail (*Coturnix japonica*)

Nada S. Eltafahny*, Mohammed T. Ibrahim, Hayam M. Samy, Manal M. Mahmoud, Heba A. Alian

Suez Canal University, Faculty of Veterinary Medicine, Department of Nutrition and Clinical Nutrition, Ismailia (41522), Egypt.

Abstract | This study evaluated the impact of different concentrations of nano zinc oxide (ZnNPs) on growth performance, antioxidant defense, bone characteristics, serum mineral levels, intestinal morphometrics, and some aspects of fertility, including sex hormones, ovarian parameters, and testicular parameters of Japanese quails. 144 one-week-old quail chicks were distributed into four groups. T_0 served as the control group received only the basal diet (corn-soya based diet with 24% CP and 2900 kcal). T_1, T_2, and T_3 were given the basal diet enriched with ZnNPs at a level of 25, 50, and 100 mg/kg diet, respectively. The results showed that 25 and 50 mg /kg diet of ZnNPs showed non-significant improvement in growth performance by 4.3% and 6.5%, respectively. However, they significantly improved antioxidant defense, tibial weight, zinc content of tibia, intestinal morphometrics, and serum Zn-Ca level. In addition, there is a significant increase in serum testosterone, Follicle-Stimulating Hormone, and Luteinizing Hormone of both males and females, especially at 6 weeks old in T_1 and T_2 *($P < 0.05$)*. Also, nano zinc induced early puberty and improved primary and Graafian follicles of females in T_1 and T_2. Moreover, it increased the zinc content of the whole egg and eggshell in all treated groups *($P < 0.05$)*. It was revealed that T_1 significantly augmented the thickness of the seminiferous tubules and the number of Leydig cells *($P < 0.05$)*. In conclusion, 25–50 mg/kg diet of nano zinc oxide enhances growth, antioxidant status, and reproductive performance in Japanese quail, with 25 mg/kg diet being the most economically viable. However, 100 mg/kg diet impairs intestinal health and antioxidant defense, highlighting the risks of excessive supplementation.

Keywords | Performance, Fertility, Japanese quail, Nano zinc oxide

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***Correspondence** | Nada S. Eltafahny, Suez Canal University, Faculty of Veterinary Medicine, Department of Nutrition and Clinical Nutrition, Ismailia (41522), Egypt; **Email:** nada_samir@vet.suez.edu.eg
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INTRODUCTION

In Egypt, the Japanese quail (*Coturnix japonica*) has gained importance in the agricultural and nutritional sectors. They grow rapidly, mature, and begin laying eggs within five to six weeks, allowing farmers to quickly reap returns In addition, quail eggs are esteemed for their significant nutritional profile, which includes essential minerals, vitamins, and antioxidants. Also, their meat is low in fat and high in protein (Vali, 2008).

Zinc is considered a vital trace mineral needed for optimal growth and performance of the body systems (Roohani *et al*., 2013). It is involved in many biological functions, including protein synthesis, wound healing, immune defense, and enzyme activity. Various enzymes, such as superoxide dismutase (SOD), play a crucial role in the antioxidant defense mechanism, depending on zinc for their structural integrity (Saber and Alian, 2024). Supplementation with dietary zinc picolinate has been linked to a decrease in serum malondialdehyde, which is a measure of lipid peroxidation (Prasad and Bao, 2019).

Zinc also contributes to reproductive health, skin integrity, and the maintenance of feathers. It is required for male and female sexual functions. In males, it helps in testicle growth, testosterone metabolism, sperm production, and motility while decreasing the excess estrogen found in male reproductive tissue. A lack of this element in females could lead to an unusual increase in prolactin secretion, as it is necessary for progesterone synthesis. Trace elements like zinc are known to affect eggshell quality by directly interacting with calcite crystals during development or by acting as essential enzymes in the production of membranes and eggshells (Abbasi *et al*., 2022).

In modern animal production, sustaining proper zinc levels is crucial for animal health, productivity, and food quality. Therefore, balanced zinc nutrition is important for sustainable livestock management. The nanoscale of minerals exhibits superior bioavailability due to their greater surface area, enhanced surface activity, elevated catalytic activity, and improved adsorption capacity (Hassan *et al*., 2020). In animal nutrition, nano zinc oxide is being utilized increasingly to improve zinc bioavailability and promote livestock growth, immunity, and gut health (Yusof *et al*., 2022). In addition, nano zinc improves growth performance of broilers including body weight gain, feed intake, FCR, carcass traits, and meat quality (Hussan *et al*., 2022).

There is an awareness of changing inorganic sources of minerals with nanoparticles to satisfy animal needs and enhance zinc bioavailability (Alian *et al*., 2022). Therefore, the current study aim is to assess different concentrations of nano zinc oxide (ZnNPs) impact on growth performance, antioxidant defense, bone characteristics, serum mineral levels, intestinal morphometrics, and some aspects of fertility, including sex hormones, ovarian parameters, and testicular parameters of Japanese quails.

MATERIAL AND METHODS

Birds and management

A total of 144 one-week-old quail chicks with an initial body weight of about 32 g were used. The study was conducted on a private farm in Port-Said under supervision of professors of nutrition department. The birds were raised in a battery cage system with modifications to meet the size and behavior of chicks with semi-automated drinkers and feeders. We raised the chicks for 6 weeks in an aseptic, environmentally controlled room. The temperature was ranged from 22 ºC-31ºC to ensure bird comfort (Yusuf *et al*., 2014). The chicks were given unlimited access to water and feed. Birds were checked for health condition, mortality, and the onset of sexual maturity three times daily (8 am, 2 pm, and 6 pm). Lighting was maintained continuously during the brooding phase (1–2 weeks). After that, the birds were exposed to light for 23 hours and darkness for one hour (Kar *et al*., 2017).

Experimental design

Quail chicks were randomly distributed to four experimental groups. Each group was allocated into three replicates of 12 quail chicks. The groups were as follows: T_0 fed the basal diet (corn-soya based diet) without zinc supplementation as a control diet. T_1, T_2, and T_3 were fed the basal diet enriched with nano zinc oxide at a level of 25, 50, and 100 mg Zn/kg diet, respectively.

Formulation and Characterization of zinc oxide nano particles (ZnNPs)

The ZnNPs were prepared at Nano Gate Company for the Synthesis of Nanomaterials, Technology and Development, Nasr City, Egypt. The nano zinc oxide was prepared from zinc acetate dihydrate by the chemical method as described by Beek *et al*. (2005), and Pacholski *et al*. (2002). X-Ray Diffraction (XRD) and HRTEM have been used to investigate and determine the structure and particle size of the powders. HRTEM performed on Talos F200i (Thermo-Scientific) high resolution transmission electron microscope at an accelerating voltage of 200 kV. An XRD pattern has been performed using XPERT-PRO Powder Diffractometer system, with 2 theta (20°-80°), with a minimum step size 2Theta: 0.001, and at wavelength ($K\alpha$) = 1.54614° Figure 1.

General properties

Batch number: Zn20232; Appearance (Color): White to yellow color; Appearance (Form): Powder; Shape (TEM): Spherical like shape; Size: 18±3 nm; Molecular weight: 81.408 gm/mol

Experimental diets

One basal experimental corn-soybean-based diet was prepared to meet the nutrient requirements of growing Japanese quails according to NRC (1994). The basal diet contained 24% CP and 2900 Kcal ME/kg diet (Table 1). The diets were fed as dry mash during 7 weeks of age. Proximate chemical analysis was performed according to the standard methods of analysis (AOAC, 2002) for the experimental diet and feed ingredients.

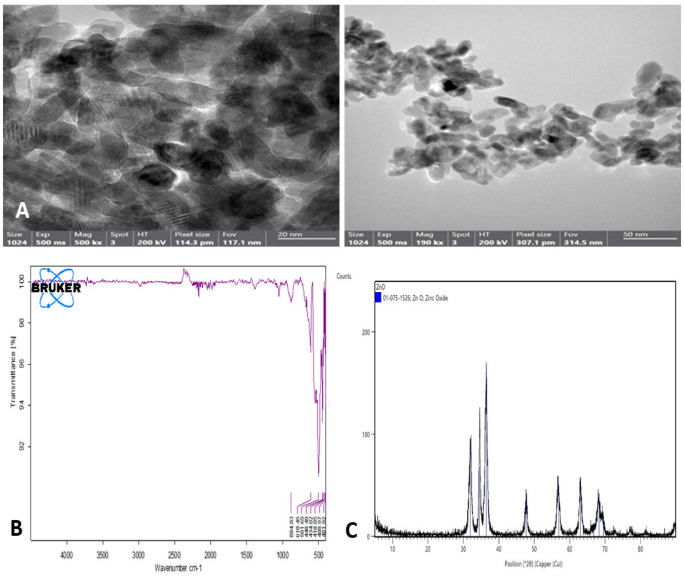

Figure 1: A: Shows the TEM image of ZnO nanoparticles and SAED (selected area electron diffraction).
The TEM image of Zinc oxide revealed that the prepared zinc oxide is in the nanoscale with an average size of 18±3 nm and spherical-like shape. B: shows the FTIR spectrum of ZnO NPs. C: Fourier-transform infrared (FT-IR) spectra were conducted Vertex 70 RAM II IR (Bruker, Germany).

Studied parameters

Growth performance

Body weight (BW) and weight gain (WG), feed conversion ratio (FCR), and feed intake (g) for each replicate were evaluated weekly according to Reda *et al.* (2021). The performance index (PI) was estimated as live BW (kg)/ FCR×100. The protein efficiency ratio (PER) was calculated as WG (g)/protein intake (g) according to El-Kazaz *et al.* (2020).

Sampling

At 5, 6, and 7 weeks old, two female and two male quail were randomly selected per replicate. During the night, they were allowed only water. After weighing, each bird was manually slaughtered. Serum samples were collected, centrifuged at 4000 rpm for 10 minutes, and stored at -18 °C for future biochemical assessment.

At 6 weeks, samples from the intestine, liver, and spleen were kept in 10% formalin for histopathological examination (Khoobbakht *et al.*, 2020). Testes and ovarian external parameters, tissue zinc content analysis, and histopathology were done at 7 weeks old after maturation (Hafez *et al.*, 2023). At 7 weeks old, the egg that was produced was stored in the refrigerator for up to a week for zinc content analysis (Roberts and Graham, 2004).

Table 1: The calculated values and composition of the experimental basal diet.

Ingredients %	Diet (1-7 wks.)
Ground yellow corn (7%) [a]	55.0326 Kg
Soya bean meal (43.3%) [a]	33.825 kg
Corn gluten (62) [a]	5.742 kg
Poultry byproduct meal (64.71) [a]	3.00 kg
Dicalcium. Phosphate (& 19%P & 22% Ca)	0.5668 kg
Limestone (38% Ca)	1.340 kg
DL-Methionine (99%)	0.080 kg
L-Lysine (99%)	0.095 kg
Sodium chloride	0.200 kg
Minerals and vitamins premix[b]	0.150 kg
Total	100.00 kg
Calculated values	
Crude protein (CP%)	24 %
ME (kcal/kg)	2900 (kcal/kg)
Calorie/ Protein ratio (C/P)	120.83
Calcium (%)	0.80 %
Available Phosphorus (%)	0.40 %

(a) Chemical analysis and determined values were according to (AOAC, 2002). (b) Each 1 kg contains: Vit. A 4 mIU, vit. D_3 1 mIU, vit. E 13333mg, vit. K_3 1333 mg, vit. B_1 1333 mg, vit. B_2 5000mg, vit. B_{12} 10mg, Vit. B_6 1666 mg, biotin 100 mg, pantothenic acid 6666 mg, nicotinic acid 20000 mg, folic acid 1000mg, zinc sulfate 33000mg, mangesium sulfate 300000 mg, iron sulfate 26666 mg, copper sulfate 10000 mg, potassium iodide 1000 mg, sodium selenate 66mg, cobalt sulphate33mg, carrier (CaCO3) to 1kg. (Broiler Premix Alpha Company, Egypt. patches No. 190828, production 9-2023). *Analysis of feed staff was done at the Animal Health Research Institute, Doki, Egypt.

Bone parameters (Tibia weight and bone ash)

At 6 weeks old, the tibia was removed, and the surrounding soft tissues were cleared before weighing it. The bones were kept in a freezer at -18°C (Lee *et al.*, 2023). Then ashed in a muffle furnace overnight at 600°C (Hall *et al.*, 2003).

Antioxidant status

At 6 weeks old superoxide dismutase (SOD) activity, was quantitatively assessed by the colorimetric method using purchased kits according to Ōyanagui (1984). Malondialdehyde (MDA) was quantitatively measured by the colorimetric method by using purchased kits according to (Ohkawa *et al.*, 1979).

Serum mineral levels

Serum Ca level was determined as the procedure of Leary *et al.* (1992). Serum P level was measured according to Goldenberg and Fernandez (1966). Serum Zn level determined as (Song *et al.*, 1976).

Histopathological examination

Tissue samples from the intestine, testis, and ovary of all groups (one chick/replicate) were selected and directly fixed in 10% formalin (natural pH) for histopathological investigation.

After fixation, samples were dehydrated in rising grades of ethyl alcohol, cleared in xylene, and inserted in paraffin. Five-micron-thick portions were cut and stained with hematoxylin and eosin (Bancroft and Gamble, 2008). Finally, the specimen was explored and photographed using a digital camera fitted with a Leica microscope (DM 1750, version 3.6.0, United States).

Intestinal morphometry

After 6 weeks of feeding, the morphological parameters of the intestine were measured. Villus length (μm) from the villus tip to the bottom, not including the intestinal crypt. Villus width (μm) at the midpoint of each villus. Crypt depth (μm) from the top of the crypt to the inner edge of the muscularis mucosae. Absorption area (villi length x villi width). Number of goblet cells as well as thickness of the intestinal wall (μm) from the inner edge of muscularis mucosae to the outer edge of serosa. All parameters were measured using Image J analysis software. Each slide was taken from ten readings from all four groups, following (Aptekmann *et al*., 2001).

Reproductive traits

Male fertility parameters

Testosterone hormone levels were measured at 5 weeks old (the week before maturity), 6 weeks old (at maturity), and 7 weeks old (one week after maturity) (Hafez *et al*., 2023). It was measured by Enzyme-Linked Immunosorbent Assay (ELISA) kits (Wu, 2006).

Testicular histology: After 7 weeks of feeding, the morphological parameters of the testis were measured by using Image J analysis, and the epithelial heights, luminal and tubular diameters of seminiferous tubules were determined from a HE stained section. Using a light microscope attached to a camera at 20x magnification, at least 4 random microscopic fields per bird for each group (Ibrahim *et al*., 2024).

Cloacael gland parameters: Two birds from each replicate at the age of 7 weeks were used to determine the gland width (lateral) and gland height (dorsoventral) of each bird, measured to the nearest 0.01 mm using a caliper (Khoobbakht *et al*., 2020). Cloacael gland index: Obtained by the width * height of the gland (Siopes and Wilson, 1975).

Female fertility parameters

FSH-LH hormone levels were measured at 5, 6, and 7 weeks old by Enzyme-linked immunosorbent Assay (ELISA) kits (manual protocol/version of December 1998 for Prolactin, LH, FSH) according to (Amballi *et al*., 2007). The onset of maturity was identified when the first hen in the group produced an egg (Reddish *et al*., 2003).

Ovarian histology: The number of ovarian follicles was measured by using Image J from six random slides from each bird in all four groups treatments Petersen *et al*. (2015).

Zinc content of the tissue

At the end of the experimental period, zinc content determination in testes, ovary, whole egg, eggshell, and tibia bone was estimated by the microwave as a closed system for the digestion of samples according to the European Committee for Standardization (CEN, 2014). Analysis for investigated minerals (heavy metals) was performed at the Food Toxicology and Contaminants Department, National Research Centre, using an Atomic Absorption Spectrophotometer ICE 3500 series (Thermo) according to Abdel-Rahman *et al*. (2018).

Statistical analysis

Using SPSS 16, the collected data from the treated groups were statistically examined for mean and standard error compared to the T_0 group (Coakes and Steed, 2009). One-way ANOVA with Duncan multiple comparison tests were used. Statistical differences were considered significant at $P < 0.05$ among all groups Snedecor and Cochran (1980).

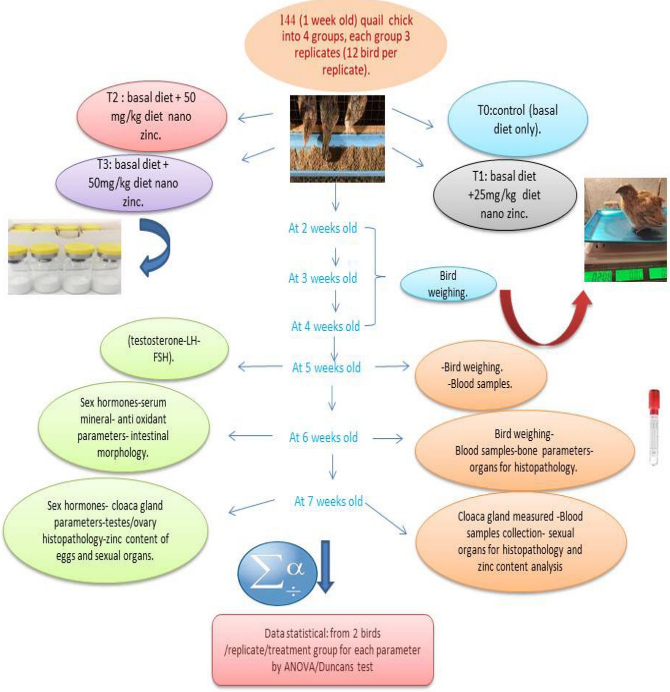

Figure 2: Experimental design and time-scheduled parameters.

RESULTS

Growth performance

Groups supplemented with nano zinc showed nonsignificant improvements in overall growth parameters as final B.W, cumulative W.G, FCR, and FE with the increase of nano zinc level. 4.3%, 6.5% and 8.7% improvements especially in final body weight gain were observed among T1, T2 and T3, respectively. T_2 recorded a numerical increase in cumulative FI and protein intake compared to other groups. Also, nano zinc-supplemented groups demonstrated non-significant improvement in performance index (PI) and PER (Table 2). All experimental groups showed no significant differences in final BW, cumulative WG, cumulative FI, FCR, performance index, protein intake, or protein efficiency ratio.

Bone parameters, serum mineral and antioxidant status

T_2 and T_3 had significantly higher tibial weights than T_0 *($P < 0.05$)*. There was a numerical increase in bone ash in nano zinc-supplemented groups compared to T_0. However, there was no significant difference between groups. T_3 had the highest significant zinc content of the tibia, followed by T_2, then T_1, compared to the T_0 group ($P < 0.05$). T_2 had the highest significant Ca value, followed by T_1, then T_0, and finally T_3 ($P < 0.05$). The highest significant reduction of serum P level was noticed in T_2 and T_1 compared with other groups ($P < 0.05$). All nano zinc-supplemented groups had the highest zinc level. The greatest, significant elevation in serum Zn level was noticed in T_2, followed by T_3 and then T_1 ($P < 0.05$) compared to T_0. The highest, significant improvement in SOD activity was noticed in T_2 followed by T_1 and then T_3 ($P < 0.05$) compared to the T_0 group. The excessive significant reduction of malondialdehyde (MDA) serum level was observed in T_2, followed by T_1 ($P < 0.05$) compared to T_3 and T_0 (Table 3).

Intestine morphometric

The intestine of the T_0 group showed normal architecture. It consists of three layers (mucosa, lamina propria, tunica muscularis). Intestines from quails of T_1 and T_2, respectively, showed a normal simple columnar epithelial cell layer with elongation of villi length and presence of numerous goblet cells. However, the T_3 group showed mild histopathological alterations such as desquamated epithelium with disarrangement of intestinal villi and presence of tissue debris in the lumen (Figure 2).

Table 2: The overall growth performance indices of quail chicks at the end of 6 weeks old.

Groups/ Parameters	T_0	T_1	T_2	T_3
Initial B.W. (g)	32.11 ±1.17^a	32.06 ±0.74^a	32.11±0.53^a	32.31 ±1.17^a
Final B.W. (g)	230.08±14.22^a	240.41±8.30^a	245.54±2.78^a	250.59 ±10.13^a
Cumulative W.G.(g)	197.96 ±14.06^a	208.35±8.82^a	213.43 ±3.25^a	218.28±9.52^a
Cumulative FI (g)	972.36 ±27.63^a	974.91 ±5.89^a	1000.53 ±10.56^a	977.75 ±12.55^a
FCR	4.95±0.28^a	4.69 ±0.21^a	4.69 ±0.05^a	4.49 ±0.17^a
Performance index (PI)	4.07±0.51^a	5.16±0.43^a	5.24 ±0.11^a	5.61 ±0.42^a
Protein intake (g)	233.37 ±6.63^a	233.98 ±1.41^a	240.13 ±2.53^a	234.66±3.01^a
Protein efficiency ratio	0.847 ±0.04^a	0.89 ±0.04^a	0.88 ±0.01^a	0.93 ±0.03^a

The table value is mean ± SE. Values in the same row that have different superscripts are significantly different at $P < 0.05$ among groups. T_0: basal diet only. T_1, T_2, and T_3: basal diet + ZnNPs (25, 50, and 100 mg Zn / kg diet, respectively).

Table 3: Impact of nano zinc oxide on bone parameters, serum minerals, and antioxidant status of Japanese quail.

Groups/ Parameters	T_0	T_1	T_2	T_3
Tibial weight (g)	1.33±0.11^b	1.46±0.08^{ab}	1.82±0.12^a	1.79±0.06^a
Bone Ash%**	29.87±1.79^a	30.13±1.75^a	30.36±1.29^a	30.97±1.33^a
Zinc content of tibia (mg/kg)	77.43±1.72^d	87.25±1.49^c	96.11±0.8^b	103.39±1.41^a
Ca (mg/dL)	7.13±0.01^c	7.31±0.01^b	7.45±0.02^a	6.71±0.04^d
P (mg/dL)	4.06±0.01^b	3.96±0.01^c	3.89±0.01^d	4.38±0.02^a
Zn (µg/dL)	50.64±0.19^d	64.02±0.62^c	84.83±0.60^a	71.50±0.74^b
SOD (pg/ml)	230.23±3.28^d	331.06±4.97^b	359.18±3.01^a	250.36±2.50^c
MDA (nmol/L)	2.61±0.04^a	2.06±0.04^b	1.59±0.03^c	2.76±0.15^a

The table value is mean ± SE. Values in the same row that have different superscripts are significantly different at $P < 0.05$ among groups. T_0: basal diet only. T_1, T_2, and T_3: basal diet + ZnNPs (25, 50, and 100 mg Zn / kg diet, respectively). *The parameter percentage was related to body weight. ** The parameter percentage was related to sample weight.

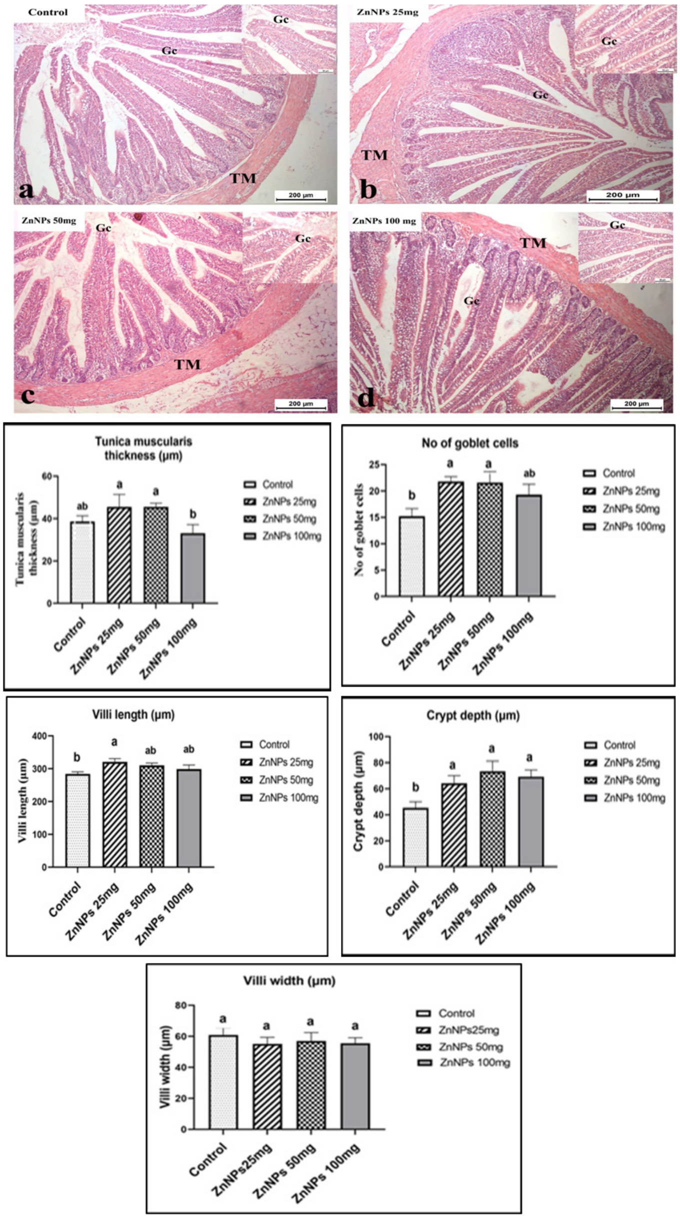

Figure 2: Photomicrograph of the intestine stained with hematoxylin-eosin (HE) and the average of intestinal parameters of the quail after feeding a supplemented diet for 6 weeks. (a) Control T0; (b) 25mg ZnNPs T1; (c) 50mg ZnNPs T2; (d) 100mg ZnNPs T3. GC: Goblet cells, TM: Tunica Muscularis. Scale bar: 200 µm. Data showed in the other images (Tunica Muscularis, nom of goblet cells, villus width, villus height and crypt depth) is measured and is the mean ± SE (n=4). Columns in the figure with different superscripts refer to significant differences (P < 0.05) among the groups.

Male and female fertility parameters

Male testosterone hormone

At 5 weeks old, the T_1 and T_2 groups exhibited numerically higher testosterone levels compared to the T0 group. However, no significant differences were noticed in serum testosterone levels among all groups. At 6 weeks old, a significant rise in serum testosterone levels was noted in the T_1 and T_2 groups when compared to T_0 and T3 (*$P < 0.05$*). By 7 weeks old, there were again no significant differences in serum testosterone levels among the groups, although T_1 and T_2 levels continued to be numerically higher than those of the T_0 group (Table 4).

Female FSH hormone

At 5 weeks old, there were no significant differences in serum FSH levels among groups. By 6 weeks old, there was a significant increase in serum FSH levels in T_1 compared to T_0 *($P < 0.05$)*. At 7 weeks old, T_3 showed the most significant increase in serum FSH levels among all groups *($P < 0.05$)*. Furthermore, the value of T_2 was found to be significantly greater than that of T_0 *($P < 0.05$)*; however, no significant difference was observed between T_1 and T_2 (Table 5).

Female LH hormone

At 5 and 6 weeks old, there were no significant differences in serum LH levels among all groups. However, at 7 weeks old, a significant increase in LH levels was observed in group T_1 compared to groups T_2 and T_3 *($P < 0.05$)*. Additionally, there was no significant difference between T_1 and T_0, although numerically, the LH level in T_1 was higher than that in T_0. At 5 and 6 weeks of age, there were no significant differences in serum luteinizing hormone (LH) levels among the groups. However, in 7 weeks, group T_1 showed a significant increase in LH levels compared to groups T_2 and T_3 (*$P < 0.05$*). While there was no significant difference between T_1 and T_0. Nevertheless, it is important to note that the LH level in group T_1 was numerically higher than that observed in group T_0 (Table 4).

Male cloaca gland parameters

T_1 and T_2 were numerically higher than the T_0 group in the cloacal gland length and index. T_1 and T_2 showed a significant improvement in the cloacal gland width compared to T_0 *($P < 0.05$)* (Figure 3).

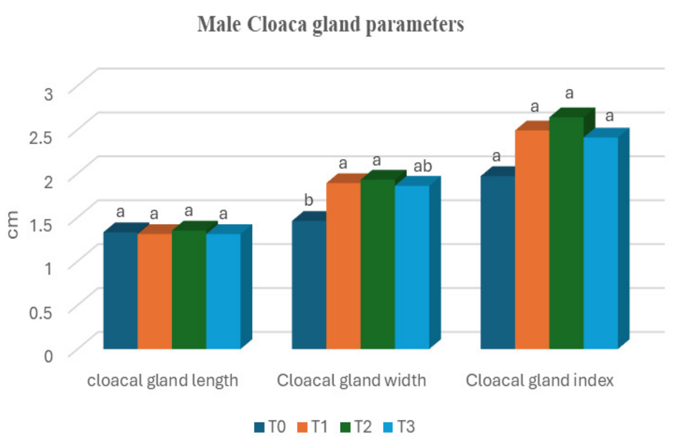

Figure 3: Impact of nano zinc on the cloacal gland parameters. T_0: basal diet only. T_1, T_2, and T_3: basal diet + ZnNPs (25, 50, and 100 mg Zn / kg diet, respectively). Columns with different superscripts refer to significant differences (*$P < 0.05$*) among the groups.

Table 4: Changes in sex hormone levels at 5, 6, and 7 weeks of male quail.

Groups Hormone/age	T_0	T_1	T_2	T_3
Testosterone (ng/mL) at 5 weeks.	0.89 ± 0.53^{a}	1.82 ± 0.30^{a}	1.13 ± 0.44^{a}	0.77 ± 0.25^{a}
Testosterone (ng/mL) at 6 weeks.	0.12 ± 0.04^{b}	0.98 ± 0.26^{a}	1.48 ± 0.20^{a}	0.27 ± 0.12^{b}
Testosterone (ng/mL) at 7 weeks.	0.76 ± 0.38^{a}	0.93 ± 0.20^{a}	0.85 ± 0.20^{a}	0.38 ± 0.18^{a}
FSH (mIU/ml) at 5 weeks old	0.07 ± 0.14^{a}	0.10 ± 1.38^{a}	0.08 ± 1.12^{a}	0.07 ± 0.20^{a}
FSH (mIU/ml) at 6 weeks.	0.08 ± 0.02^{b}	0.37 ± 0.14^{a}	0.24 ± 0.02^{ab}	0.17 ± 0.05^{ab}
FSH (mIU/ml) at 7 weeks	0.30 ± 0.01^{c}	0.45 ± 0.09^{bc}	0.54 ± 0.06^{b}	0.77 ± 0.06^{a}
LH (mIU/ml) at 5 weeks	0.35 ± 0.05^{a}	0.40 ± 0.05^{a}	0.35 ± 0.05^{a}	0.27 ± 0.02^{a}
LH (mIU/ml) at 6 weeks	0.50 ± 0.04^{a}	0.43 ± 0.08^{a}	0.53 ± 0.04^{a}	0.60 ± 0.09^{a}
LH (mIU/ml) at 7 weeks	0.70 ± 0.04^{ab}	1.05 ± 0.27^{a}	0.4 ± 0.05^{b}	0.47 ± 0.03^{b}

The table value is mean ± SE. Values in the same row that have different superscripts are significantly different at $P < 0.05$ among groups. T_0: basal diet only. T_1, T_2, and T_3: basal diet + ZnNPs (25, 50, and 100 mg Zn / kg diet, respectively).

Table 5: Impact of nano zinc oxide on tissue zinc of Japanese quail.

Groups/ Parameters	T_0	T_1	T_2	T_3
Zinc content in the ovary (mg/kg)	24.54 ± 0.93^{d}	29.01 ± 0.34^{c}	33.10 ± 0.64^{b}	37.34 ± 0.36^{a}
Zinc content of testis (mg/kg)	14.39 ± 0.37^{d}	18.63 ± 0.44^{c}	22.08 ± 0.39^{b}	30.06 ± 1.01^{a}
Zinc content in whole egg (mg/kg)	10.55 ± 0.51^{d}	14.47 ± 0.20^{c}	16.72 ± 0.32^{b}	20.91 ± 0.66^{a}
Zinc content in egg shells (mg/kg)	5.56 ± 0.54^{c}	7.65 ± 0.35^{bc}	9.25 ± 0.14^{b}	13.66 ± 1.42^{a}

The table value is mean ± SE. Values in the same row that have different superscripts are significantly different at $P < 0.05$ among groups. T_0: basal diet only. T_1, T_2, and T_3: basal diet + ZnNPs (25, 50, and 100 mg Zn / kg diet, respectively).

Testicular morphology

The testis of the T_0 group showed a normal structure of seminiferous tubules. However, the T_1 and T_2 groups showed improvement as seminiferous tubules lined by spermatogonia cells (primary and secondary) with elongated spermatids in the lumen of seminiferous tubules, and the tubules were closely attached, and the interstitial space and Leydig cells appeared normal. While the seminiferous tubules of T_3 showed some histopathological alterations, including vacuolation of spermatogonia and Sertoli cells, with sloughing of cells into the lumen. Some seminiferous tubules showed a reduction of sperm cells (Figure 4).

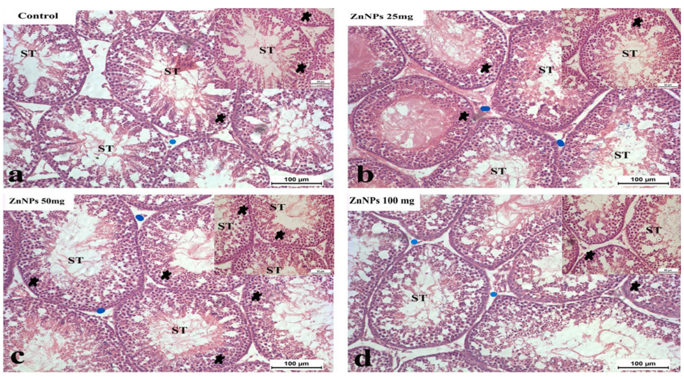

Figure 4: Photomicrograph of testis stained with hematoxylin-eosin (HE). (a) Control T_0; (b) 25mg ZnNPs T_1; (c) 50mg ZnNPs T_2; (d) 100mg ZnNPs T_3. Asterisk: Spermatogonia cells, ST: spermatid, blue mark: Leydig cells. Scale bar: 100 µm.

Onset of female puberty

Nano zinc-supplemented groups produced their first egg at five weeks old, followed by the T_0 group three days later.

Zinc content of tissue

Significant differences in ovarian zinc content were noted among all *groups (P < 0.05)*. It was significantly higher in the nano zinc-treated groups when compared to the T_0 group *(P < 0.05)*. The zinc content of the testis differed significantly among groups (P < 0.05). There was a significant elevation in the zinc content of the testis in nano zinc-supplemented groups when compared to the T_0 (P < 0.05). It increased with the increasing level of nano zinc. The zinc content of whole eggs was significantly enhanced in the groups supplemented with nano zinc compared to the T_0 group (*P < 0.05*) and exhibited an increase in correlation with the higher levels of nano zinc added. The maximum zinc content in the eggshell was observed in group T_3 (*P < 0.05*). Groups T_1 and T_2 also demonstrated significantly elevated zinc levels compared to the T_0 group (*P < 0.05*) (Table 5).

Ovarian morphology

The ovary in the T_0 group exhibited a normal structure. The T_1 and T_2 groups presented numerous primary and Graafian follicles, characterized by a normal interstitial space and an increased thickness of the granulosa cell layer, along with the presence of a yolk sphere in the lumen. Meanwhile, the ovary in the T_3 group showed mild sloughing of the yolk sphere and mild vacuolation of the granulosa cell layer (Figure 5).

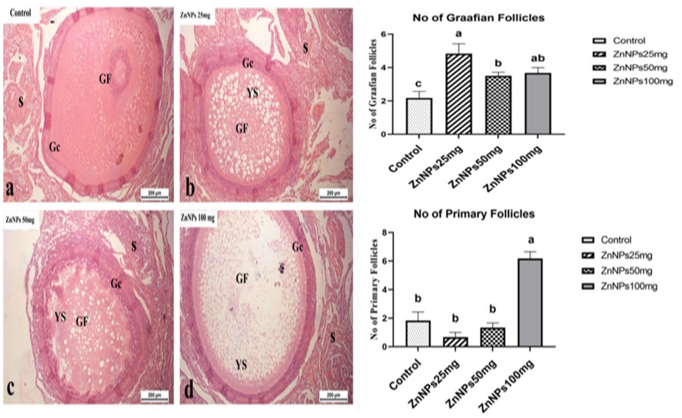

Figure 5: Photomicrograph of an ovary stained with hematoxylin-eosin (HE). a) Control T_0; (b) 25mg ZnNPs $T_{1;}$ (c) 50mg ZnNPs $T_{2;}$ (d) 100mg ZnNPs T_3. GF: Graafian Follicles, GC: Granulosa cells, S: Stroma, YS: Yolk spheres. Scale bar: 200 µm.

DISCUSSION

Nano zinc-supplemented groups revealed non-significant improvement in overall growth parameters. Which agreed with Ramiah *et al*. (2019) who noticed that the addition of nano zinc oxide at the levels of 40 mg/kg diet, 60 mg/kg diet, and 100 mg/kg diet had no significant effect on growth performance, especially in weight gain, but there is a slight numerical increase mentioned in the results. Our results were inconsistent with (El-Katcha *et al*., 2017) who discovered that the addition of nano zinc oxide to broiler chicken diets at levels of 15 mg/kg diet significantly decreased feed efficiency parameters and broiler performance. Our results differ among the reports due to many factors such as the amount of zinc in the control diet, duration of the experiment, and environmental factors.

It was noticed that the 50 and 100 mg/kg diet nano zinc significantly had the highest tibial weights ($P < 0.05$). Nano zinc-supplemented groups had significantly higher tibia zinc content than the T_0 ($P < 0.05$). This may be because nano zinc increases the collagen matrix, as there is no increase in bone ash in our results (Molenda and Kolmas, 2023). These observations agreed with Mohd Yusof *et al*. (2022) who concluded dietary ZnNPs at 40, 70, and 100 mg/kg diet enhanced the morphological characteristics of broiler tibia bones, including weight but tibia zinc content improved especially with 100 mg/kg diet. Although these findings disagreed with Varun *et al*. (2017) who said nano zinc supplementation into broiler diet did not significantly affect bone morphometric parameters such as bone weight. Also disagreed with Cufadar *et al*. (2020) who supplemented the feed of 44-week-old laying hens with 20-40-60- 80 and 100 mg ZnNPs /kg diet. They found no significant effect on tibia Zn content and tibia weight.

There was no significant difference among all groups in bone ash percent. These results agreed with Hassan *et al*. (2023) who concluded that 35, 70, or 105 ppm of nano zinc in the diet of layers does not affect the bone characteristics. In contrast, El-Rayes *et al*. (2019) showed that tibia bone ash content increased with increasing nano-zinc form 0 or 30 mg/kg in the diet of one-day-old chicks.

The highest significant value in serum Ca level was observed in the 50 mg/kg diet-supplemented group, followed by the 25 mg/kg diet-supplemented group then the T_0 ($P < 0.05$). This may be because zinc is essential for the enzyme system that produces vitamin D3, which regulates calcium and phosphorus levels by enhancing calcium absorption (Guyton and Hall, 2006). This agrees with Dukare *et al*. (2021) who published that 80 ppm of market or green nano zinc increases serum Ca, P, and zinc. But disagreed with Abedini *et al*. (2018), who discovered that supplementation of the layer diet with an 80 mg/kg diet of nano zinc oxide did not affect Ca serum levels.

The highest significant reduction of serum P level was noticed in the 50 and 25 mg /kg diet-supplemented group compared to all groups ($P < 0.05$). This may be due to decreased retention of phosphorus due to increased bone zinc content (Stewart and Magee, 1964). This was consistent with Zaboli *et al*. (2013). They reported that the addition of 20 and 40 mg/kg diet of nano zinc decreases serum phosphorus levels numerically in goats at 35 days. In contrary Dukare *et al*. (2021) concluded that higher serum phosphorus levels were noticed in broiler chicks fed 80 ppm of market nano zinc or green nano zinc source compared to lower levels of nano zinc or inorganic source.

All nano zinc-supplemented groups had higher serum zinc levels compared to the T_0 ($P < 0.05$). The higher zinc content is possibly because zinc nanoparticles are more bioavailable and more effective than traditional zinc sources (Swain *et al*., 2016). That agrees with El-Katcha *et al*. (2018) when used 30 and 60 ppm ZnNPs in the diet of laying hens. However, this disagreed with Cufadar *et al*. (2020) who supplemented the feed of 44-week-old laying hens with 20, 40, 60, 80, and 100 mg ZnNPs /kg diet and found no significant effect on serum zinc.

There was a significant improvement in serum SOD and MDA in nano zinc-supplemented groups compared to T_0, especially in 25 and 50 mg/kg diet-supplemented groups ($P < 0.05$). The significant upgrading in serum SOD and decrease in MDA levels in nano zinc group, suggests improved antioxidant capacity and reduced oxidative stress. This can be attributed to the role of zinc for the activity of antioxidant enzyme (SOD) and MDA (Hidayat *et al*., 2021). This agreed with Reda *et al*. (2021) who used 0.1–0.3 g/kg diet nano zinc and gave a positive impact on blood SOD and MDA. In contrast Fathi (2016), showed that ZnNPs at higher concentrations (40 mg/kg diet) did not significantly increase Cu-Zn-SOD activity in serum.

Intestines from quail fed on 25mg ZnNPs and 50 mg ZnNPs, respectively, showed a dramatic histological improvement compared to the T_0 and T_3 groups. As it showed a standard simple columnar epithelial cell layer with elongation of villi length and an increase in goblet cells. These results agreed with El-Sawy *et al*. (2021), who said the same with 20mg /kg diet ZnNPs for broiler diet. But we were inconsistent with Mohammed *et al*. (2023), who added 6, 8, 10, and 12 mg zinc oxide nanoparticles/liter to the drinking water of the broiler. They found that crypt depth did not differ significantly between the ZnNPs and the control. T_3 had some intestinal impairment and may have zinc toxicity thresholds, which reported by Fatima *et al*. (2024) who said nano zinc may has toxicological effects, which vary according to size, shape, and concentration. And concluded, more studies are necessary to clarify the detrimental effects of ZnNPs.

The 25 mg and 50 mg ZnNPs groups showed a dramatic histological improvement in seminiferous tubules compared to the T_0 and 100 mg/kg diet nano zinc-supplemented group.

The presence of primary and secondary spermatogonia, along with elongated spermatids, suggested effective sperm production. Additionally, healthy seminiferous tubules and normal Leydig cell appearance indicated preserved testicular architecture and hormonal function. These findings were parallel with Khoobbakht *et al*. (2020). In contrast, Khoobbakht *et al*. (2018) who concluded that zinc oxide nanoparticles had a limited effect on male Japanese quail reproduction.

The 25 mg and 50 mg ZnNPs groups showed a histological improvement in ovarian structure compared to the T_0 and T_3 groups. As there were numerous primary follicles and Graafian follicles with normal interstitial space and increased thickness of the granulosa cell layer, with the presence of a yolk sphere in the lumen. This may be due to an increase in the level of FSH (Hisaw, 1947). These findings align with those of Hazim and Amen (2011), who reported a significant improvement in sex hormones (FSH-LH) in a zinc-containing diet (50-75-100 mg/ diet) of broiler breeder chicken. However, Jafari *et al*. (2021) said that there were no significant differences observed among groups with nano zinc in serum levels of sex hormones in the broiler breeder.

There was a significant increase in zinc content of testis and ovary in nano zinc supplemented groups when compared with the T_0 ($P < 0.05$), with an increase in the level of supplementation. This was compatible with Hong *et al*. (2022) and Zhao *et al*. (2017). In contrast, Ahmed *et al*. (2024), found that testis zinc concentration was not affected by zinc oxide nanoparticles in the diet of rats. Also, Zhao *et al*. (2016) showed that the addition of nano zinc into the diet of hens did not affect the ovarian zinc concentration.

At 6 weeks old, 25 and 50 mg/kg diet of nano zinc supplemented groups created a significant increase in serum testosterone level than T_0 and T_3. This agreed with Amen and Al-Daraji (2011), who found a significant increase in male broiler plasma testosterone at 0, 50, 75, 100 mg/kg of diet of zinc. This may be due to zinc is a vital mediator in the binding of hormones and is involved in the conversion of testosterone to dihydrotestosterone, a zinc-dependent enzyme process (Baltaci *et al*., 2019).

At 6 weeks old, there was a significant increase in FSH serum level in a 25mg ZnNPs/kg diet than the T_0. But at 7 weeks old, 50 and 100 mg ZnNPs /kg diet created a significant increase in serum FSH than the T0. This may be due to zinc has critical role in the female endocrine system (sex hormones) (Baltaci *et al*., 2019). This agreed with Hazim and Amen (2011) found that feeding broiler breeder chickens a zinc-containing diet (50–75–100 mg Zn/diet) resulted in notable improvements in sex hormones (FSH–LH).

At 7 weeks old, the 50 and 100 mg/kg diet nano zinc supplemented group showed a significant increase than the T_0, which agreed with Hazim and Amen (2011).

There was no significant difference between groups in cloacal gland length and cloacal gland index, which agrees with Khoobbakht *et al*. (2018). This result disagreed with Sachs (1967), who stated that cloacal gland size is androgen-related and highly positively correlated with sexual activity in pre-pubertal Japanese quail, which was compatible with our study, as there was an improvement of testosterone level (sexual activity) in male quail.

In our study, cloacal gland width had a significant increase in the 25 and 50 mg/kg diet of the nano zinc supplemented group than the T_0 group. This agreed Biswas *et al*. (2007) who concluded that the cloaca gland in Japanese quail was positively related to the level of testosterone, and in our study, there is an increase in male sex hormone. But disagreed with Khoobbakht *et al*. (2020) who studied an experiment on day-old Japanese quail chicks supplemented with 25 mg/kg diet of nano zinc oxide and found that there was no effect on cloacal gland index.

In the first week of starting egg production, nano groups produced the first egg 4 days before the T_0 group. This means there was early puberty in birds fed a nano zinc diet. In our study, there was an improvement in female sex hormones in groups fed a nano zinc diet. This was compatible with Bhagat and Singh (2022), who stated that any discrepancies in the mineral composition of the diet could lead to delayed puberty and a decrease in sex hormones. Also, Baltaci *et al.* (2019) mentioned that zinc is an important mineral that may induce puberty by stimulating the reproductive endocrine system.

Nano zinc-supplemented groups had a significantly higher level of zinc content in eggshells and whole eggs. This compatible with Mao and Lien (2017) who stated that layers fed meals containing 80 mg of ZnNPs/kg feed had greater eggshell zinc concentration than the non-supplemented group *($P \leq 0.005$).* This may be due to increased zinc retention as a result of the improvement of Zn bioavailability from a nano zinc-supplemented diet (Tsai *et al.*, 2016).

Nano zinc is costly and supplementation of nano zinc into the poultry diets must be considered. So in the current study we showed that 25 and 50 mg/kg diet of nano zinc have a positive effect on growth performance and reproductive traits. However from economical point we should focus only on level 25 md/kg diet, as reported by Saber and Alian (2024) who told that nano zinc at the level of 5 and 10 mg/ kg diet of broiler where significantly the best in economic evaluation than control and other groups including group that fed diet supplemented with 20 mg/kg of nano zinc. And concluded, addition of nano zinc at level of (5 mg Zn/kg diet) is a new trending feed additive in the broiler industry. Also Almeida *et al.* (2024) said that nano zinc required only at a small doses which resulting usually in reduced feed costs.

CONCLUSION AND RECOMINDATION

In conclusion, 25–50 mg/kg diet of nano zinc oxide enhances growth, antioxidant status, and reproductive performance in Japanese quail, with 25 mg/kg diet being the most economically viable. However, 100 mg/kg diet impairs intestinal health and antioxidant defense, highlighting the risks of excessive supplementation. These findings support the use of ZnNPs as a sustainable alternative to inorganic zinc in quail diets, though further research is needed to optimize mineral balance and assess long-term impacts.

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NOVELTY STATEMENT

This study provides novel insights into the dose-dependent effects of nano zinc oxide (ZnNPs) on reproductive performance, antioxidant status, and bone characteristics in Japanese quail, with a focus on economically optimal supplementation levels (25–50 mg/kg). Unlike previous studies, we demonstrate that excessive ZnNPs (100 mg/kg) negatively impacts intestinal morphometrics and antioxidant defense, emphasizing the need for balanced supplementation**.**

AUTHOR'S CONTRIBUTION

The research strategy and document were created by all the researchers. While NS, MM, and HA composed the article and completed the statistics, NS conducted the trial and managed the experimental conditions. The finished manuscript was examined and revised by MT.

Ethical approval

All experimental guidelines were followed by the Animal Welfare and Experimental Ethics Committee, Suez Canal University, Faculty of Vet. Med., Egypt (Approval No.2023042).

Generative AI and AI-assisted technology statement

All authors of this work declare that generative AI technologies including large language models (e.g, ChatGPT, Copilot) and text-to-image generators were not utilized in any capacity during the preparation, writing, or editing of this manuscript.

Conflict of interest

There are no conflicting interests.

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