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UTILIZATION OF DIETARY ZINC OXIDE NANOPARTICLES ON PRODUCTIVE AND PHYSIOLOGICAL PERFORMANCE OF LOCAL LAYING HENS

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SUMMARY

The study aimed to evaluate the effect of dietary adding different levels of nano-zinc oxide (ZnO-NPS) on productive performance, nutrient digestion coefficient, physiological status, egg and bone quality and economic efficiency. One hundred and forty four (144) Gimmizah laying hens at 49 weeks of age were used, divided randomly to four similar experimental groups, each with 36 chickens divided into 3 replications (12 chickens / replicate). The dietary treatments included a corn-soybean meal-based diet (T_1; control diet - supplemented with premix which contain inorganic zinc as zinc oxide), and basal diet with free premix of Zn supplemented with 5, 15 and 25 mg/ kg nano zinc oxide (ZNO-NPS) for T2, T3 and T4, respectively. The results indicated a significant improvement in the rate of egg production, egg mass, and feed conversion rate by adding nano-zinc ($P \leq 0.05$). Birds fed on diets supplemented with zinc oxide nanoparticles recorded the highest significant improvement in egg index, egg shell quality, yolk and white quality and Haugh units in compared to control. The dietary treatments to which zinc nanoparticles were added had the highest digestibility of nutrients in compared to the control one. There was also a significant ($P<0.05$) improvement in some blood plasma parameters (total protein, albumin, liver and kidney enzymes, and high-density lipoprotein (HDL)), while the concentration of total cholesterol, total triglycerides, and low-density lipoprotein (LDL) decreased significantly with the increase in nano-zinc levels. The data showed that there was a significant effect of zinc nanoparticles on antioxidant status and zinc content. The addition of 25 mg of nano-ZnO/kg diet led to a significant increase in the content of glutathione peroxidase (GPx) and zinc content and a significant decrease in the concentration of malondial aldehyde (MDA). The best values for weight, fracture strength and ash percentage of leg bones of birds fed on diets supplemented with nano-zinc compared to the control group. The economic efficiency and relative economic efficiency improved by adding nano zinc, and the best values were for the third treatment (1.83 and 109.58, respectively), which was fed on the diet supplemented with nano zinc oxide (ZnO-NPS) at a rate of 15 mg/kg diet compared to the other treatments. Recommendations: The results indicated that feeding local laying hens on diets to which nano-zinc was added at a rate of 25 mg/kg ration (the fourth treatment) had the highest rate of productive performance - while the third treatment to which nano-zinc was added at a rate of 15 mg/kg was a better diet economic efficiency (1.83) and the highest relative economic efficiency (109.58).

Keywords: *Eggshell quality, Nano-zinc, Physiological status, Nutrients digestibility, Performance and Laying hens.*

INTRODUCTION

The overall economy of the poultry industry is assessed by its productivity and growth performance. In Egypt, one of the greatest challenges to efficient production of local chicken strains is reducing their performance. In view of the fact that, improving growth and feed conversion ratio has always been a top priority in the poultry industry. Researchers have used nutritional supplements, in poultry production to enhance production performance and to achieve some positive effects on maximizing egg production (El-Katcha *et al.,* 2018).

Zn is an essential trace mineral in poultry feeding as a form of inorganic (oxides and sulfates), involved in many physiological, metabolic and digestive processes in the body (Zhang *et al.,* 2018). It has three essential biological functions, namely, catalytic roles in the functioning of more than 300 enzymes, structural roles and regulatory roles. Moreover, it influences the immune system, nucleic acid synthesis, cell proliferation, protein synthesis, protein and carbohydrate metabolism, bone development, egg production, eggshell and enzymatic activities in poultry, moreover, a cofactor and/ or structural component of carbonic anhydras enzyme which is very important for supplying the carbonate ions needed during eggshell formatting (Tuzun *et al.,* 2018).

Zn content of the diet is very low compared to the Zn requirement of poultry (NRC, 1994); chicken diets are general supplemented with higher levels of Zn than the recommended by NRC. This result in high levels of Zn residue in the excreta of chickens, leading to environmental pollution thereafter enhancing Zn absorption can help alleviate both these issues. Zinc bioavailability is 6 - 11% in monogastric animals, this bioavailability and tissue accumulation of Zn depends upon various factors such as its chemical form, feed composition, age and physiological state of hens and interactions with other minerals (Lesson and summers, 2005).

Diets are commonly supplemented with inorganic sources such as oxide, carbonate, chloride, or sulfate salts due to its cost and commercial availability. However, due to the pH changes that naturally occur in the digestive tract of poultry, there may be antagonism and interactions among trace elements minerals, as well as with other compounds in diet formatting insoluble compounds, preventing their absorption in body (Aksu *et al.,* 2012), which in turn increases mineral excretion resulting in environmental pollution (Mohanna and Nys, 2004). Furthermore, higher inclusion levels of Zn may affect. The balance of other trace elements in the body, reduce the stability of vitamins and other nutrients and increase its accumulation inside the bird body (Zhao *et al.,* 2014). The reduced bioavailability of the inorganic mineral sources and environmental issue increase the interest in finding more available alternative such as nano sources.

Among the metal nano particles (NPS) annually produced in the world, zinc oxide nano particles (ZnO-NPS) are the third largest in the terms of size, shape, large surface area, high surface activity, high catalytic efficiency and strong absorbing ability (Reda *et al.*, 2021). With the emergence of nano technology, Zinc can be added as a feed supplements in many forms to improve the efficiency of trace minerals, productive performance, eggshell quality, antioxidant status, bone development and some blood biochemical of poultry (Geetha *et al.,* 2020 and Hussan *et al.*, 2021). Therefore, the purpose of this experiment was to study the effect of dietary zinc oxide nanoparticles (ZnO-NPS) on performance, nutrients digestibility, some physiological status, egg and bone quality and economic efficiency of Gimmizah laying hens.

MATERIALS AND METHODS

The present study was conducted at the Poultry Research Farm and the Poultry Nutrition Laboratory, Faculty of Agriculture, Menoufia University, Egypt from January to March, 2020 (12 weeks). All treatments and birds care procedures were approved by Institutional Animal Care and Use Committee (IACUC), Faculty of Agriulture, University of Menoufia (Ethical approval number: VUSC –04/2017).

Birds assay procedures:

One hundred and forty-four (144), 49 weeks old Gimmizah laying hens were used in this experiment. Hens were distributed at random into four similar experimental groups (36 hens/ group) and divided into 3 replicates of 12 layers each in a completely randomized design. Layers were housed in individual cages. Feed and water were provided *ad-libitum* during the experimental period. Artificial light was used beside the normal day light to provide 16 hour/ day photo period.

Experimental diets:

The composition of the basal diet is given in Table 1. Corn-soybean meal basal diet was formulated to contain adequate levels of all nutrients as recommended by the National Research Council's nutrients values for ingredients (NRC, 1994). Except for zinc, which was determined by the atomic absorption spectroscopy and was 34.82mg, Supplementation of Zn oxide was added to the basal diet (energy ME; 2747.6 kcal/ kg and crude protein CP; 16.18 %) to create the four experimental diets. T_1: Basal diet, (control group) supplemented with premix which contain inorganic zinc as zinc oxide and basal diet with free premix of Zn supplemented with 5, 15 and 25 mg/kg nano zinc oxide (ZNO-NPS) for T_2, T_3 and T_4, respectively. The zinc sources used included an inorganic zinc source (zinc oxide, ZnO, normal premix) purchased from Multimix Bruli-ER without choline (MV/Q C- F- 13) Ideco- 6 October, Gizza city, Egypt. Also, metal zinc oxide nanoparticles (ZnO-NPS) was purchased from Nano-Tech., Egypt for photo-Electronics communication center in front of the international school of chouei fat, El-wahaat Road, Dream land city, Entrance 3, city of 6 October, Gizza, Egypt. The product was a white powder with a measure

ZnO-NPS content of purity > 99.99 % and size of nanoparticles was 20 ± 5 nm. The morphological description of the ZnO-NPS was detected during transmissions electron microscopy (TEM) on JEOL (JEM-2100 high resolution transmission electron microscope at an accelerating voltage of 200 KV.

Measured parameters:

Productive performance and egg quality:

Egg production (EP), egg weight (EW) were recorded daily. Feed intake (FI) and feed conversion ratio (FCR) were recorded weekly. Based on the collected data, egg mass (EM) and FCR were calculated with the formulae EM= (EW × EP)/ 100 and FCR= FI/ EM, respectively. At 60 weeks of the experiment, nine eggs from each dietary treatment group were randomly for the determination of egg quality traits. Egg shape index was calculated from length and width, measure by digital tripod micrometer according to Romanoff and Romanoff (1949) as follows:

Table 1: Composition and chemical analysis of Gimmizah laying hens diet.

Ingredients	%
Ground yellow corn (8.5%).	65.24
Soybean meal (44%).	24.17
Vegetable oil.	0.20
Limestone ground.	7.57
Di-calcium phosphate.	2.13
Vitamin and minerals mixture[1].	0.30
Dl-methionine[2].	0.09
Salt (Sodium chloride).	0.30
Total	**100**
Calculated values[3]:	
Crude protein (%)	16.18
ME (kcal/ kg diet).	2747.6
C/P ratio.	169.81
Lysine (%).	0.89
Methionine (%).	0.37
Calcium (%).	3.42
Av. P (%).	0.48
Determined values:	
Dry matter (DM, %).	89.79
Crude protein (CP, %).	16.17
Ether extract (E.E, %).	2.90
Crude fiber (CF, %).	3.01
Calcium, (%).	3.40
Av. Phosphorus, (%).	0.49
Zinc (mg/ kg).	34.82

[1]*Vitamin and Mineral mixture at 0.30% of the diet supplies the following per kilogram of the diet: Vitamin A, 12,000 IU; vitamin D_3, 3,000 IU; vitamin E, 40 mg; vitamin K_3, 3 mg; vitamin B_1, 2 mg; vitamin B_2, 6 mg; vitamin B_6, 5 mg; vitamin B_{12}, 0.02 mg; niacin, 45 mg, biotin, 0.075 mg; folic acid, 2 mg; pantothenic acid, 12 mg; manganese, 100 mg; zinc, 50 mg; iron, 30 mg; copper, 10 mg; iodine, 1 mg; selenium, 0.2 mg; cobalt, 0.1 mg.*
[2]*DL – Methionine: 98% feed grade (98 % Methionine).*
[3]*Calculate according to NRC (1994), feed ingredient tables were used for calculation.*

Egg shape index = Width (mm)/ Length (mm) × 100. Eggshell percentage was calculated as; Eggshell percent = Eggshell weight, g/ Egg weight, g × 100.Eggshell strength was measured by using, break force machine, in Agricultural Engineering Department, Faculty of Agriculture, Menofia University, Eggshell thickness (ST): shell thickness (without its membranes) was determined according to Brant and shrader (1952) by using micrometer (to the nearest 0.01 mm) at the broad, narrow and the middle ends. Averages of shell thickness for all the three regions were calculated. The height of the albumen and yolk was determined using a standard tripod micrometerand the diameter of the yolk was measured by caliper. Egg yolk and albumen index (%) were measured according to Romanoff and Romanoff (1949). Haugh units, an indicator of albumen quality, was calculated using the following formula: Haugh units = 100 log (H + 7.57 – 1.7 $w^{0.37}$), Where: H = albumen height (mm) and W = egg weight (g) (Haugh, 1937).

Nutrients digestibility trail:

At the end of the experiment (60 weeks of age), three layer hens per treatment were randomly taken and kept in individual metabolic cages to conduct a digestibility trials. Diet was offered daily and water was available all the time. Consumed feed was recorded. After 3 days as a preliminary period, feces were collected quantitatively for 4 consecutive days for each bird. Fecal smples for each individual bird were stored at -20 ºC immediately after collection, bulked and dried at 70 ºC for 24 hours, thereafter; they ground and kept for chemical analysis.

The proximate analysis of tested materials, feed and dried excreta were carried out according to A.O.A.C (2005), using triplicate samples for each nutrient. The procedure of Jakobson *et al.* (1960) using trichloroacetic acid was adopted for estimating the fecal nitrogen. Urinary nitrogen was determined by difference (excreta N-fecal N). While, urinary organic matter was determined. Urinary organic matter (UOM) = urinary N × 2.62. The percentage of urinary organic matter in the feces was added to the sum of its other components (fecal CP % + EE % + CF % + Ash %). For feces it was: 100 – (fecal moisture % + CP % + EE % + CF % + Ash % + UOM %). The dry matters consumed and of excreta and their percentage were used to calculate the digestion coefficients of different nutrients.

Blood biochemical parameters:

During slaughtering of birds (60Wk), blood samples were collected in heparinized tubes from each bird, and then centrifuged at 3000 rpm for 15 minutes to separate plasma. The obtained plasma samples were stored at -20ºC until analysis. Plasma samples were analyzed for total protein, albumin (Henry *et al.*, 1974), Creatinine and activity of transaminase enzymes of plasma alanine amino transferase (ALT) and plasma aspartate amino transferase (AST) were determined by calorimetric method of Murray, 1984, total cholesterol and triglycerides (Stein and Myers, 1995), High–density lipoprotein (HDL) and low density lipoprotein (LDL); (Pisani *et al.,* 1995). For plasma zinc analysis, 1 ml of sample was dispensed into a porcelain crucible, oven- dried for 4 hours at 105ºC, and then ashed for 1h at 600ºC in a muffle furnace. Then dry ashed plasma blood was dissolved by adding 10 ml 50% HCL (V/ V) and kept covered overnight. The samples were filtered using Whatman filter in a 100 ml volumetric flask by washing crucibles 2 - 3 times and diluted with deionized distilled water and Zn concentrations were measured by ICP spectrometer (ICAP 5800 series; Thermo Scientific). These biochemical determinations of blood serum were performed calorimetrically by using commercial kits (spectrum diagnostics which was manufactured at 2006 by MDSS Gmbh, schiffgraben 41, 30175 Hannover, Germany).

Antioxidant status:

The glutathione peroxidase (GPx) activity was measured in plasma and liver accordance by using commercial analytical kits (Spectrum Diagnostics, Alobour, Cairo, Egypt) according to manufacturer's instructions. The GPx activity was expressed as units per milligram of protein (U/ mg protein) in tissue, and as units per milliliter (U/ml) in plasma. Liver malondialdehyde levels were spectrophotometrically (UV 4802, Unico Co, Dayton, USA) determined according to Ohkawa *et al.*, 1979 using the analytical kits (Spectrum Diagnostics, Alobour, Cairo, Egypt) and expressed as mg/ kg of the liver. Plasma MDA concentration was measured in accordance with Yagi, 1984 spectrophotometer at 520 nm and expressed as nmal/ ml thionbarbituric reaction substances (TBARS) index. For the oxidative stability determination, another set 12 eggs from each treatment group (3 eggs/ replicate) were preserved in the refrigerator at 4ºC for 7d. Lipid peroxidation in egg yolk samples at 7d was measured as TBARS according to the method described by Botsoglou *et al.* 1994 using commercial kits (Sigma–Aldrich St-Louis Mo, US). MDA was analyzed by a spectrophotometric method and TBARS concentration was expressed as the nano gram of MDA/ gram of egg yolk (mg/ g). GP_X activity was determined according to Paglia and valentine, 1967 using commercial kits (Sigma-Aldrich St-Louis Mo, US), according to the manufacturer's instructions. Glutathione peroxidase was expressed as units of activity per gram of egg yolk (U/ g).

Zinc content and tibia measurements:

At the end of the experiment, three hens/ each treatment group were slaughtered by cervical dislocation. Then liver and tibia bones were collected and frozen (-70ºC) until analysis. Zn content in the liver and eggs measured by atomic absorption spectrophotometer (AAS), (Flame technique), Model (SensAA: GBC scientific EQUIPMENT Spectrophotometer) at Animal Health Research Institute, Dokki, Gizza, Egypt, according to the method described by Sandoval *et al.* (1998). Briefly, the samples were dried at 105ºC for 12 hr and pre-digested in HNO_3 until charring was completed. Then, all the samples were drying ashed at 550ºC for 12 hr, solubilized in HCL, and filtered through 42 Whatman paper. To determine Zn concentration in tibia bones, the soft tissue was removed after 72 hr of extraction in diethyl ether. After that, the bones were dried for 12 hr at 105ºC and ashed in a muffle furnace at 550ºC. The bone ash was digested as previously described. Also liver was oven dried at 100ºC for 24 hr and finely group in a stainless-stell blade grinder and 1 g of liver and pancrease samples were dry ashed at 550 - 600ºC for 1 - 2h in a muffle furnace for mineral (Zn concentration) analysis.

Tibia bone breaking strength was determined according to the method of Crenshaw *et al.* (1981). Left tibia samples were crushed and defatted with petroleum ether for 24 hr using Soxhlet apparatus, and dried in the oven at 100°C for 24 hr. Dried bone samples were then burned (24 hr) into a muffle furnace preheated to 600°C for the determination of ash percentage (dry, fat free basis). The ash from tibia samples was solubilized with a mixture of nitric and perchloric acids, and the content of bone mineral Zn was determined according to the methods of Allen *et al.* (1997).

Economical efficiency:

Economic efficiency for egg production was calculated from the input-output analysis (Heady and Jensen, 1954) according to the price of the experimental diets and egg produced. Values of economic efficiency were calculated as the net revenue per unit of total costs (Soliman and Abdo, 2005).

Statistical analysis:

Data were statistically analyzed by the completely randomized design using SPSS 11.0 (2011) program and the differences among means were determined using Duncan's multiple range test (Duncan 1955). Percentages were transformed to the corresponding arcsin values before performing statistical analysis. Statistical model: $Y_{ij} = \mu + \alpha i + E_{ij}$

Where: Y_{ij} = Observed traits, μ = Overall mean, α i = Effect of treatment (i = 1, 2, 3 and 4) and E_{ij} = Experimental random error.

RESULTS AND DISCUSSION

Productive performance and egg quality:

Influence of nano zinc supplementation on productive performance and egg quality traits of Gimmizah hens are shown in Table 2. Egg production percentage, egg weight, egg mass were significantly ($P \leq 0.05$) influenced by different Zn nano-particular in compared to control group. Overall average egg production was 55.16 % for the basal diet with inorganic zinc (zinc oxide, T1), while greater improvement (60.67 % for egg production) was obtained at level of 25 mg ZnO-NPS/ kg diet, T4 compared to other dietary treatments (55.56 and 59.75 %) for T2 and T3 during the trial period (49 - 60 weeks of age) these values were significant, respectively. This finding is consistent with studies (Pathak *et al.*, 2021) who observed that positive effect of using the dietary supplement of ZnO-NPS on egg production in laying hens. The increase of egg production might be due the important role of Zn in the synthesis and secretion of luteing (LH) andfollicle stimulating (FSH) hormones (Bedwal and Bahuguna, 1994). Dietary zinc may influence egg production by interacting with the endocrine system since the hen is changing the production and secretion of reproductive hormones during sexual maturation (Renema *et al.*, 1999).

The highest value of average egg weight noted when laying hens fed 25 mg ZnO-NPS/ kg diet (T_4, 59.66 g) in compared to 57.35g for the control diet (T_1). Ismail *et al.* (2016), who obtained that dietary supplementation of nano form of zinc significantly ($P \leq 0.05$) increased egg weight compared to the control diet; with inorganic zinc. Birds fed the basal diet with 25 mg ZnO-NPS/ kg diet, (T_4) had the highest egg mass (36.72 g/ hen/ day) in compared to the control group (T_1) being 31.58 g/ hen/ day (Table 2). These results are supported by Fawaz *et al.* (2019) reported that addition of 20, 40 or 60 mg ZnO-NPS/ kg diet had significantly increased egg mass of laying hens. It was reported that Zn is required for the normal function of plentiful structural proteins, enzymes and hormones that is nessary for the growth and development (Bao and Choct., 2009), and has important roles in metabolism of energy and protein (Ibs and Rink, 2003). Thus, improvement in egg mass may be due to the role of Zn in many biochemical processes supporting life. Also, these improvements may be due to zinc supplementation which an essential nutrient requires for many physiological functions including antioxidant function, growth and fertility (Shay and Mangian, 2000). In contrast, the findings of Tsai *et al.* (2016) and Mao and Lien (2017), who reported that egg mass was not affected by nano Zn supplementation of laying hen diets.

Daily FI during the experimental period (49 - 60 wks) are shown in Table 2. Treated groups with nano zinc oxide recorded the lowest FI being116.93, 114.24 and 113.82 g/ hen/ day for T_2, T_3 and T_4, respectively in compared to the control diet (T1: 120.52 g/ hen/ day). They suggested that the superior performance of nanoparticles may be attributed to their smaller particles size and large surface area increased mucosal permeability, improved intestinal absorption and tissue deposition (Wang *et al.*, 2015).

However, feed conversion ratio (FCR) was significantly ($P \leq 0.05$) improved due to nano zinc supplementation at levels of 5, 15 and 25 mg/ kg diet; being 3.52, 3.23 and 3.15 g feed/ g egg mass for T_2, T_3 and T_4, respectively. In the absence of nano zinc, feed conversion ratio was 3.81 g feed/ g egg mass for the control, T_1 (basal diet with inorganic zinc). The results of most former studies confirmed that ZnO nanoparticles at 20 to 60 mg/ kg diet could be appropriate levels to achieve a better FCR laying hens (Abedini *et al.,* 2017 and Fawaz *et al.,* 2019). The positive effect of ZnO-NPS supplementation on productive performance and physiological process of poultry, as it is the main component of a large number of enzymes know as met all enzymes, which are involved in metabolism of energy, nucleic acids and protein (Attia *et al.*, 2019). These findings in the present study were disagreement with those obtained by (Olgun and Yildiz, 2017 and Abedini *et al.*, 2018) reported that adding nano zinc source had no significant effect on FCR. The differences between this study and other research papers may be attributed to differences in concentration levels, breed and environment and management procedures.

The results of this study on diets supplemented with different ZnO-NPS sources showed that egg shape index, eggshell percent, thickness and strength are improved in nano zinc groups in compared to control group (Table, 2). This finding is in agreement with the findings of Fawaz *et al.* (2019) who showed that eggshell percentage was linearly increased in laying hens by addition of zinc oxide nanoparticles. It could be supposed that nanoparticles of zinc oxide may provide a site of calcium deposition in uterus and consequently increase shell weight and percentage in compared to the control group. Moreover, in studies conducted by Abedini *et al.* (2018) and Pathak *et al.* (2020), a positive effect on eggshell thickness and strength were reported for nano zinc as compared to inorganic form. Improvement in the quality of eggshell may be due to the role of Zn in the processes of membrane and eggshell synthesis. Zn is a component of carbonic anhydrase enzyme which is essential for supplying carbonate ions during eggshell formation, and lack of this enzyme reduces shell quality (Nys *et al.,* 2004).

Table (2): Influence of dietary nano zinc supplementation on productive performance and egg quality traits of Gimmizah hens (Means ± S.E).

Items	Dietary treatment[1]				Sig[4]
	T_1	T_2	T_3	T_4	
	Productive performance (49- 60 wks of age)				
Egg production (%).	$55.16^{c} \pm 1.98$	$55.56^{b} \pm 0.91$	$59.75^{ab} \pm 1.80$	$60.67^{a2,3} \pm 2.02$	*
Egg weight (g).	$57.35^{b} \pm 0.93$	$58.74^{ab} \pm 1.13$	$59.24^{ab} \pm 1.02$	$59.66^{a} \pm 1.16$	*
Egg mass (g/ hen/ d).	$31.58^{d} \pm 1.23$	$33.26^{c} \pm 1.09$	$35.1^{b} \pm 1.17$	$36.72^{a} \pm 1.86$	*
Feed intake (g/ hen/ d).	$120.52^{a} \pm 2.12$	$116.93^{b} \pm 3.56$	$114.24^{c} \pm 3.04$	$113.82^{c} \pm 2.50$	*
Feed conversion (g / g).	$3.81^{a} \pm 0.19$	$3.52^{b} \pm 0.14$	$3.23^{c} \pm 0.14$	$3.15^{d} \pm 0.15$	*
	Egg quality week 60 of age				
Egg shape index (%).	$75.06^{d} \pm 0.53$	$77.64^{c} \pm 0.79$	$80.89^{b} \pm 1.03$	$82.89^{a} \pm 0.89$	**
Eggshell percent (%).	$10.13^{d} \pm 0.21$	$10.88^{bc} \pm 0.40$	$11.79^{b} \pm 0.27$	$13.29^{a} \pm 0.58$	*
Eggshell thickness (mm).	$0.379^{c} \pm 0.006$	$0.422^{b} \pm 0.007$	$0.37^{b} \pm 0.006$	$0.446^{a} \pm 0.008$	*
Eggshell strength (N/Cm3).	$32.89^{b} \pm 2.45$	$35.11^{ab} \pm 2.96$	$35.22^{ab} \pm 1.85$	$35.98^{a} \pm 2.43$	*
Albumen (%).	$51.96^{c} \pm 1.28$	$52.29^{b} \pm 0.78$	$54.01^{ab} \pm 0.88$	$54.97^{a} \pm 0.89$	*
Albumen index (%).	$16.27^{c} \pm 0.47$	$19.47^{a} \pm 0.62$	$17.46^{b} \pm 0.56$	$19.98^{a} \pm 0.66$	*
Yolk (%).	$34.25^{b} \pm 1.15$	$34.48^{b} \pm 0.81$	$36.84^{a} \pm 0.88$	$36.44^{a} \pm 0.88$	*
Yolk index (%).	$43.19^{c} \pm 1.76$	$46.58^{b} \pm 1.33$	$49.15^{ab} \pm 1.15$	$49.76^{a} \pm 1.39$	*
Haugh units.	$83.73^{d} \pm 1.73$	$90.01^{b} \pm 2.14$	$84.67^{c} \pm 1.93$	$91.91^{a} \pm 1.69$	**

[1]*Dietary treatments; T_1: basal diet with inorganic zinc (ZnO, the control diet; T_2: basal diet free zinc oxide + 5 mg ZnO-NPS/ Kg diet; T_3 basal diet free zinc oxide + 15 mg ZnO-NPS/ kg diet and T_4: basal diet free zinc oxide + 25 mg ZnO-NPS/ Kg diet.*

[2,3]*a, b, ……. etc. Means of the same raw (for treatments) with different super scripts are significantly different ($P \leq 0.05$).*

[4] *Sig: * significant and ** highly significant.*

Data for egg yolk, albumen quality traits (percent and index) as affected by different studied nano zinc oxide and Haugh units had highly significant increased by nano zinc supplementation of Gimmizah laying hens at 60 wks of age are displayed in Table 2. This study obviously indicates the beneficial effects of using Zn in larges, since Zn plays key role information of uterus during the deposition of albumen. So, all quality traits of eggs were affected by nanoparticles of zinc oxide at 20, 40 or 60 mg/ kg had improved Haugh unit in laying hens diets (Fawaz *et al.*, 2019). Also, Pathak *et al.* (2021) observed that the Haugh unit was significantly affected by supplementation of nano zinc at 60 mg/ kg in layer hens. The reason might be attributed to an increase in albumen height by nano zinc addition.

On the other hand, no significant affects were seen with values of Haugh units (HU) as receving of addition nano zinc (Abedini *et al.*, 2017 and Qin *et al.*, 2017) of laying hens.

Nutrients digestibility:

Experimental results presented in Table 3 showed the influence of dietary nano zinc oxide supplementation on nutrient digestibility of laying hens at 60 wks of age. Nano zinc in diets at 5, 15 and 25mg/ kg increased ($P \leq 0.05$) the nutrient digestibility of (DM, CP, CF, EE, and NFE) in compared to the control group.

Table (3): Influence of dietary nano zinc supplementation on the nutrients digestibility of Gimmizah hens (Means ± S.E).

Items	Dietary treatment[1]				Sig[4]
	T_1	T_2	T_3	T_4	
Dry matter (DM, %).	$65.36^{d} \pm 1.43$	$76.36^{c} \pm 1.26$	$83.41^{b} \pm 1.14$	$87.02^{a2,3} \pm 1.05$	**
Crude protein (CP, %).	$70.40^{c} \pm 1.56$	$74.66^{b} \pm 2.11$	$75.26^{b} \pm 1.82$	$77.89^{a} \pm 1.78$	*
Ether extract (EE, %).	$64.46^{b} \pm 1.48$	$66.02^{ab} \pm 2.02$	$66.80^{ab} \pm 2.11$	$67.29^{a} \pm 2.63$	*
Crude fiber (CF, %).	$21.76^{c} \pm 1.25$	$33.67^{b} \pm 2.30$	$33.96^{b} \pm 2.46$	$39.89^{a} \pm 2.25$	*
Nitrogen free extract (NFE, %).	$73.62^{d} \pm 1.65$	$80.72^{b} \pm 1.98$	$83.62^{a} \pm 2.13$	$77.41^{c} \pm 1.73$	*

[1]*Dietary treatments; T_1 basal diet with inorganic zinc (ZnO, the control diet; T_2: basal diet free zinc oxide + 5 mg ZnO-NPS/ Kg diet; T_3 basal diet free zinc oxide + 15 mg ZnO-NPS/ kg diet and T_4: basal diet free zinc oxide + 25 mg ZnO-NPS/ Kg diet.*

[2,3]*a, b, ……. etc. Means of the same raw (for treatments) with different super scripts are significantly different ($P \leq 0.05$).*

[4] *Sig: * significant and ** highly significant.*

These findings might be directly increased zinc retention associated with improvements in laying performance. The increase in nutrient digestibility might be due to the positive effects of nano zinc oxide on digestion and absorption of nutrients in the gastrointestinal tract (GIT) and higher bioavailability of zinc in the form of nanoparticles (Hussan *et al.*, 2021). The same results were obtained by (Fawaz *et al.*, 2019 and Kumar *et al.*, 2021) who showed that digestibility of CP, EE and CF increased by supplementation ZnO-NPS of layer hen diets. On the other hand, Tasi *et al.* (2016) found that supplementation 60 mg ZnO-NPS/ kg had no significant ($P > 0.05$) effect on dry matter, crude ash, crude protein and crude fat.

Blood biochemical parameters:

Data for blood plasma biochemical parameters of Gimmizah laying hens at 60 weeks of age as affected by different studied nano zinc oxide are displayed in Table 4. It is clearly shown that all groups of studied dietary nano zinc oxide had higher values of total protein, albumen, AST (aspartate amino transferase) and high-density lipoprotein (HDL)) than those of the control fed basal diet with inorganic zinc. Total protein as well as albumen and globulin were significantly ($P \leq 0.05$) higher in birds fed diets with different levels of zinc nanoparticles (Attia *et al.*, 2020). This increase might be attributed to the role of zinc in many physiological functions including growth and protein synthesis, nucleic acid synthesis and activity of many enzymes (Ibs and Rink, 2003). Its supplementation enhanced fat absorption, improved appetite, metabolism of carbohydrates, proteins, lipids and many essential biochemical processes of chickens (Attia *et al.*, 2013). In addition, Badawi *et al.* (2017) revealed that HDL-cholesterol was significantly increased by the addition of dietary nano-zinc in compared to inorganic zinc, control group.

Plasma levels of lipid parameters (total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL) and alanine amino transferase (ALT) were decreased by nano zinc supplementation at level of 25 mg/ Kg diet compared to the control group. This finding may be due to the improvement in calories and fat zinc in calories and fat intake after zinc supplementation and the fact that zinc is involved as a main building block in formation of several enzymes responsible for lipid digestion and absorption (Roberson and Edwards, 1994). Results obtained herein are in agreement with Mohamed (2019) who stated that dietary zinc decreased TG concentrations. While, plasma creatinine as mg/ dl was not significantly affected by different nano zinc oxide with may level that different nano zinc used in the present experiment were safe for birds and had no dangerous effects on kidney functions of treated birds. There were significant differences in Zn content of plasma among treatments by nano zinc oxide supplementation (Table 4). Simmilary, Zn level was linearly increased in plasma as the level of dietary Zn increased (Kumar *et al.*, 2021).

Table (4): Influence of dietary nano zinc supplementation on plasma biochemical parameters of Gimmizah hens at 60 weeks of age (Means ± S.E).

Parameters	Dietary treatment[1]				Sig4
	T_1	T_2	T_3	T_4	
Total protein (g/ dl).	6.00^{c} ± 0.29	7.20^{b} ± 0.10	8.80^{a} ± 0.06	$8.60^{a2,3}$ ±0.07	**
Albumin (g/ dl).	1.70^{c} ± 0.06	2.90^{b} ± 0.05	2.96^{b} ± 0.06	3.10^{a} ± 0.06	*
AST (U/ L).	58.95^{d} ± 0.69	59.75^{c} ± 0.84	67.76^{b} ± 0.79	69.92^{a} ± 0.86	*
ALT (U/ L).	30.79^{a} ± 0.84	30.04^{ab} ±0.42	28.37^{b} ± 0.35	28.11^{b} ± 0.36	*
Creatinine (mg/ dl).	2.66 ± 0.02	2.72 ± 0.01	2.24 ± 0.02	2.35 ± 0.01	NS
Triglyceride (mg/ dl).	95.45^{a} ± 0.87	94.50^{b} ± 0.87	83.62^{c} ± 0.77	77.21^{d} ± 0.58	*
HDL (mg/ dl).	28.00^{c} ± 0.76	43.00^{b} ± 0.28	49.02^{ab} ±0.29	52.11^{a} ± 0.29	**
LDL (mg/ dl).	64.90^{a} ± 2.29	52.89^{b} ± 2.58	48.60^{c} ± 2.57	43.90^{d} ± 2.27	**
Zn (mg/ dl).	1.50c ±0.057	1.63b ± 0.61	1.70^{ab}±0.061	1.91a ±0.057	*

[1]*Dietary treatments; T_1 basal diet with inorganic zinc (ZnO, the control diet; T_2: basal diet free zinc oxide + 5 mg ZnO-NPS/ Kg diet; T_3 basal diet free zinc oxide + 15 mg ZnO-NPS/ kg diet and T_4: basal diet free zinc oxide + 25 mg ZnO-NPS/ Kg diet.*

[2,3] *a, b, ……. etc. Means of the same raw (for treatments) and columns (for periods) with different super scripts are significantly different (P ≤ 0.05).*

[4] *Sig: * significant, NS. Non significant and ** highly significant.*

Antioxidant status:

Significant differences were observed in level of glutathione peroxidase (GPx) and malondialdehyde (MDA) in plasma, liver and egg yolk as presented in Table 5. GPx has antioxidative action and contributes to the oxidative defense by catalyzing the reduction of hydrogen peroxidase and lipid peroxidase to less harmful hydroxides (Arthur, 2020).

Table (5): Influence of dietary nano zinc supplementation on level of glutathione peroxidase (GPx) and malondialdehyde (MDA) in plasma, liver and egg yolk of Gimmizah hens (Means ± SE).

Parameters	Dietary treatment[1]				Sig[4]
	T_1	T_2	T_3	T_4	
Antioxidant capacity, GPx activity[5]					
Plasma (U/ ml).	3.4^{d} ± 0.27	3.97^{ab} ± 0.28	4.26^{b} ± 0.19	$4.87^{a2,3}$ ± 0.16	**
Liver (U/ mg of protein).	123.45^{d} ± 1.33	133.42^{c} ± 1.56	145.10^{b} ± 1.69	157.00^{a} ± 1.49	*
Egg yolk (mg/ g).	125.11^{d} ± 0.64	128.00^{c} ± 0.60	132.56^{b} ± 0.12	138.92^{a} ± 0.20	*
Oxidative stability MDA concentration[6]					
Plasma (nmol/ ml).	36.12^{a} ± 1.30	36.02^{a} ± 0.89	34.76^{b} ± 0.58	31.92^{c} ± 0.78	*
Liver (nmol/ ml).	2.46^{a} ± 0.014	2.02^{b} ± 0.012	1.61^{c} ± 0.0223	1.27^{d} ± 0.023	*
Egg yolk (mg/ g).	103.45^{a} ± 3.16	88.06^{b} ± 2.45	76.26^{c} ± 2.06	72.49^{c} ± 2.26	*

[1]*Dietary treatments; T_1 basal diet with inorganic zinc (ZnO, the control diet; T_2: basal diet free zinc oxide + 5 mg ZnO-NPS/ Kg diet; T_3 basal diet free zinc oxide + 15 mg ZnO-NPS/ kg diet and T_4: basal diet free zinc oxide + 25 mg ZnO-NPS/ Kg diet.*

[2,3]*a, b, ……. etc. Means of the same raw (for treatments) with different super scripts are significantly different (P ≤ 0.05).*

[4] *Sig: * significant and ** highly significant.* [5] *GPx activity; Glutathione peroxidase.* [6] *MDA; Malondealdihyde.*

Also, MDA is an important index of lipid peroxidation and oxidative damage caused by reactive oxygen species (Ros) (Nielsen *et al.*, 1997). These results agree with (Fathi *et al.*, 2016 and Ibrahim *et al.*, 2017) who revealed that adding ZnO-NPS significantly reduced MDA compared to the control. Zinc is apant of more than 240 enzymes that play a critical function in oxidative systems and protect cell from free radicals such as H_2O_2. Generally, the anti-oxidation role of zinc elements is to enhance the sensitivity of birds to some oxidative stresses (Zhao *et al.*, 2014). Also, Hassan *et al.* (2017) found that the addition of zinc oxide nanoparticles at a dose of 25 µg/ kg diet processes hepato-protective effect through scavenging of free radicals or by enhancing the activity of antioxidant, which then detoxify the free radicals. It has been well discussed that Zn is able to exert antioxidant effects by stimulating the expression of metallothioneins, as potent electrophilic scavengers and cell protective agents and activation of antioxidant proteins and enzymes, such as GPx (Jarosz *et al.*, 2017).

Zinc content and tibia bone characteristics:

The zinc concentrations in liver, egg yolk and tibia at 60 weeks of age of Gimmizah laying hens are depicted in Table 6. Data on Zn concentrations in liver of layer hens as affected by feeding with ZnO-NPS supplemented diets. Supplementing ZnO-NPS trace elements into bird's diets could modify mineral deposition given their high bioavailability compared to inorganic sources (Ibrahim *et al.*, 2017). Zn levels of liver in chickens were significantly increased by dietary inclusion of Zn (Akbari *et al.*, 2017 and Liu *et al.*, 2015). Also, the improvement of Zn concentration in liver may be due to the activity of metallo thionein, a cysteine- rich protein with the ability to bind divalent cations and control the pool and turnover of the micro elements (Coyle *et al.*, 2002). Another theory that may support the above results indicates that Zn in the form of nanoparticles is able to penetrate into the hepatic cells via blood or interstitial space. The increased uptake and interaction with biological tissues due to the size of the nanoparticles was reduced in the transitional zone between individual atoms or molecules and the corresponding bulk materials (Nel *et al.*, 2006). The amount of Zn in the egg yolk for birds that received 15 mg/ kg and 25 ZnO-NPS were more than the other groups. Abedini *et al.* (2018) reported that addition of ZnO-NPS had significantly increased the content of Zn yolk in laying diets. The higher content of nanoparticles is probably attributed their smaller panticle size, larger surface area and increased mucosal permeability, resulting in improved intestinal absorption and tissue depositions. The content of tibia Zn was significantly affected by supplementation of ZnO-NPS levels.

Table (6): Influence of dietary nano zinc supplementation on zinc content in liver, egg yolk and tibia and tibia bone characteristics of Gimmizah hens at 60 weeks of age (Means ± S.E).

Parameters	Dietary treatment[1]				Sig[4]
	T_1	T_2	T_3	T_4	
Liver Zn, (mg/ g).	$128.00^{d} \pm 0.12$	$146.80^{c} \pm 0.15$	$190.23^{b} \pm 0.12$	$197.10^{a2,3} \pm 0.10$	*
Egg yolk Zn (mg/ g).	$38.93^{b} \pm 0.12$	$39.20^{ab} \pm 0.14$	$41.36^{a} \pm 0.17$	$41.20^{a} \pm 0.16$	*
Tibia bone Zn (mg/ g).	$234.90^{c} \pm 2.26$	$275.00^{b} \pm 2.15$	$292.37^{ab} \pm 2.58$	$313.50^{a} \pm 2.60$	*
Tibia bone characteristic					
Tibia length (cm).	$10.43^{b} \pm 0.06$	$11.56^{a} \pm 0.17$	11.02 ± 0.07	$11.82^{a} \pm 0.18$	*
Tibia weight (fresh, g).	$7.19^{c} \pm 0.22$	$7.49^{bc} \pm 0.33$	$8.12^{a} \pm 0.46$	$7.95^{b2,3} \pm 0.39$	*
Tibia weight (dry, g).	6.98 ± 0.17	7.01 ± 0.13	7.33 ± 0.11	7.29 ± 0.12	NS
Tibia diameter (cm).	2.21c ± 0.06	2.40b ± 0.06	2.60a ± 0.10	2.48a ± 0.12	*
Tibia strength (N/ Cm^3).	$13.33^{c} \pm 0.06$	$14.50^{b} \pm 0.15$	$15.40^{ab} \pm 0.15$	$15.73^{a} \pm 0.14$	*

[1]*Dietary treatments; T_1 basal diet with inorganic zinc (ZnO, the control diet; T_2: basal diet free zinc oxide + 5 mg ZnO-NPS/ Kg diet; T_3 basal diet free zinc oxide + 15 mg ZnO-NPS/ kg diet and T_4: basal diet free zinc oxide + 25 mg ZnO-NPS/ Kg diet.*
[2,3] *a, b, ……. etc. Means of the same raw (for treatments) and columns (for periods) with different super scripts are significantly different ($P \leq 0.05$).*
[4] *Sig; significant and NS; non significant.*

Influnces of nano zinc oxide on tibia strength and morphometerics are presented in Table 6. Tibia was significantly longer for nano zinc oxide compared with the control group at 60 wks of age. A similar pattern was seen for weight and diameter measures, tibia breaking strength was significantly higher for 25 mg ZnO-NPS/ kg compared with the control group. In the present study, it is noted that values of tibia bone strength were increased being, 13.33, 14.50, 15.40 and 15.73 N/cm3 for T_1, T_2, T_3 and T_4, respectively.

This result agrees with (Alkhtib *et al.,* 2020) and Ghasemi *et al.*, 2020) who showed a significant increased in tibia strength and tibia length associated with feeding the chelated zinc or nano zinc supplements in compared to control. Trace minerals appear to play important roles in growth, development and maintenance of normal bone. Bone as a complex heterogeneous tissue is responsible for supporting muscle and therefore there is a close link between growth and development of bone with overall body growth (Loveridge *et al.,* 1993).

On the other hands, Cufader *et al.* (2019) showed that dietary nano zinc oxide powder with different levels (20, 40, 60, 80 and 100 mg Zn/ kg diet) and their interactions had no significant effects of on tibia weight and tibia breaking strength as tibia mechanical parameters.

Economical and relative economical efficiency:

Economic efficiency and relative economical efficiency data are shown in Table 7. The highest productive performance was in the 4th treatments that fed 25 ZnO-NPS/ kg diet. But the highest economic efficiency and relative economic efficiency was in 3rd the treatment ration which containing 15 mg ZnONPS/ kg diet (1.83 and 109.58, respectively) due to the high price of nano-zinc oxide compared to zinc oxide.

Table (7): Influence of dietary nano zinc oxide (ZnO-NPs) supplementation on economical and relative economical efficiency of Gimmizah laying hens.

Items	Dietary treatments[1]			
	T_1	T_2	T_3	T_4
Price of feed (L. E/ kg).	6.40	6.57	6.91	7.25
Total feed intake/ hen (kg).	4.34	4.21	4.11	4.10
Total feed cost hen (L. E).	27.78	27.66	28.40	29.37
Total number of eggs/ hen.	46.33	46.67	50.19	50.97
Total price of egg/ hen (L. E).	74.13	74.67	80.30	81.55
Net revenue hen (L. E) [2].	46.35	47.01	51.90	51.82
Economic efficiency [3].	1.67	1.69	1.83	1.74
Relative economic efficiency, (%)[4].	100	101.20	109.59	104.19

[1]*Dietary treatments; T_1 basal diet with inorganic zinc (ZnO, the control diet; T_2: basal diet free zinc oxide + 5 mg ZnO-NPS/ Kg diet; T_3 basal diet free zinc oxide + 15 mg ZnO-NPS/ kg diet and T_4: basal diet free zinc oxide + 25 mg ZnO-NPS/ Kg diet. Assuming the price of one-egg was 1.60 L.E. according to Egyptian market, 2020.*
[2]*Net revenue/ hen, (L. E) = Total price of eggs – Total feed cost.*
[3]*Economic efficiency = (Net revenue ÷ Total feed cost).*
[4]*Relative economic efficiency of control considered 100.*

A like with the current results (Badawi *et al.*, 2017 and Abd El-Haliem *et al.*, 2020) confirmed that a 40 ppm ZnO-NPS/ kg diet recorded the highest return and net profit values in spite of the high cost of ZnO-NPS feed additive. Additionally, Swain *et al.* (2015) reported that ZnO-NPS has been provided economic benefits in poultry. On the other hand, the cost of production and net profit of ZnO-NPS at different levels (0.0, 25, 50, 75 and 100 %) in broiler chicken diets were very similar (Asheer *et al.,* 2018).

CONCLUSION

The results indicated that feeding local laying hens on diets to which nano-zinc was added at a rate of 25 mg/kg ration (the fourth treatment) had the highest rate of productive performance - while the third treatment to which nano-zinc was added at a rate of 15 mg/kg was a better diet economic efficiency (1.83) and the highest relative economic efficiency (109.58).

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الإستفادة من إضافة النانو زنك على الأداء الإنتاجى والفسيولوجى للدجاج البياض

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هدفت الدراسة لتقييم تأثير إضافة مستويات مختلفة من النانو أكسيد الزنك (ZnO-NPS) على الأداء الإنتاجى، ومعامل هضم العناصر الغذائية، الحالة الفسيولوجية، جودة البيض والعظام والكفاءة الإقتصادية. استخدم عدد 144 دجاجة من سلالة الجميزة البياض في عمر 49 أسبوع، قسمت عشوائيا إلي أربع مجموعات تجريبية متشابهة في كل منها 36 دجاجة قسمت علي 3 مكررات (12 دجاجة/ مكررة) – كانت المعاملات التجريبية علي النحو التالي: المعاملة الأولي: عليقة الكنترول عبارة عن العليقة الأساسية التي تحتوي علي الزنك الغير عضوي (المعدني) بالمستوي الموصى به للسلالة، المعاملة الثانية والثالثة والرابعة تم استبدال الزنك المعدني في العليقة الأساسية بالنانو زنك بمستويات 5، 15، 25 مجم نانو أكسيد الزنك/ كجم عليقة علي الترتيب. أشارت النتائج إلى تحسن معنوي في معدل إنتاج البيض وكتلة البيض ومعدل تحويل الغذاء بإضافة النانو زنك ($P\leq 0.05$)، لوحظ أن أقل غذاء مأكول كان لطيور المعاملة الرابعة المضاف إليها النانو زنك بمعدل 25 مجم/ كجم عليقة مقارنة بباقي المعاملات. سجلت الطيور المغذاة على علائق مضاف إليها النانو أكسيد الزنك أعلي تحسن معنوي لدليل البيض، صفات جودة قشرة البيض، جودة صفار وبياض البيض ووحدات هاوف. كانت المعاملات الغذائية المضاف إليها النانو أكسيد الزنك أعلي معاملات هضم للعناصر الغذائية مقارنة بمعاملة الكنترول. كما لوحظ تحسن معنوي لبعض صفات بلازما الدم (البروتين الكلي، الألبيومين، إنزيمات الكبد والكلى والبروتين الدهني عالي الكثافة (HDL))، بينما انخفض معنويا تركيز الكوليسترول الكلي والدهون الثلاثية الكلية والبروتين الدهني منخفض الكثافة (LDL) بزيادة مستويات النانو زنك. أظهرت البيانات أن هناك تأثيرًا معنويًا للنانو أكسيد الزنك على حالة مضادات الأكسدة ومحتوى الزنك، أدت إضافة 25 مجم من النانو أكسيد الزنك/ كجم عليقة إلى زيادة معنوية في محتوى الجلوتاثيون بيروكسيديز (GPx) ومحتوى الزنك وانخفاض معنوى فى مستوى تركيز مالونداى الدهيد (MDA). كان أفضل قيم لوزن، قوة كسر ونسبة الرماد لعظام ساق الطيور المغذاة على العلائق المضاف إليها النانو زنك مقارنة بمجموعة الكنترول. تحسنت الكفاءة الإقتصادية والكفاءة الإقتصادية النسبية بإضافة النانو زنك وكانت أفضل قيم للمعاملة الثالثة (1.83 و109.58 علي الترتيب) التي غذيت علي العليقة المضاف إليها نانو أكسيد الزنك (ZnO-NPS) بمعدل 15 مجم/ كجم عليقة مقارنة بالمعاملات الأخري. **التوصيات:** أشارت النتائج إلي أن تغذية الدجاج البياض (دجاج الجميزة البياض) علي علائق مضاف إليها النانو زنك بمعدل 25 مجم/ كجم عليقة (المعاملة الرابعة) كانت أعلي معدل للأداء الإنتاجي - بينما كانت المعاملة الثالثة المضاف إليها النانو زنك بمعدل 15مجم/ كجم عليقة أفضل كفاءة اقتصادية (1.83) وأعلي كفاءة اقتصادية نسبية (109.58) وقد يرجع ذلك إلي إرتفاع سعر العليقة في المعاملة الرابعة مقارنة بباقي المعاملات التجريبية تحت ظروف التجربة.