EFFECT OF DIETARY DIFFERENT LEVELS OF (SILVER VS GRAPHENE) NANOPARTICLES ON BONE CHARACTERISTICS AND GASTROINTESTINAL MICROFLORA OF BROILER CHICKENS

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SUMMARY

Three hundred and sixty unsexed day-olds of Indian River (IR) broiler chicks were used to study the effect of using different types and levels of nanoparticles (silver vs. graphene) in broiler diets on bone traits and microbiological characteristics of the small intestine and cecum contents of broiler chicks. This experiment contained nine treatments and 40 chicks in each. The experiment lasted from 1 to 35 days of age and included 3 treatment diets (starter, grower and finisher) supplemented with 2.5, 5.0, 7.5, and 10.0 ppm/kg of both types of nanoparticles, plus a control (basal diet), throughout the 3 different feeding stages. Results of this experiment showed that:

1. All physical and chemical bone measurements weren't significantly affected by different types or levels of nanoparticles, except tibia width and ash%, which were significantly affected by nanoparticle levels.
2. The count of *Lactobacillus spp.*in both the small intestine and ceca was significantly affected by different types and levels of nanoparticles.

On the other hand, *E.coli* counts in both the small intestine and ceca decreased significantly with increasing nanoparticle levels. However, *E.coli* was not significantly affected by nanoparticle type in the small intestine, but it was significantly affected by type in the ceca.

Keywords: *Nanoparticles, Silver, Graphene and Broiler Chicks*

INTRODUCTION

One of the most widely used nanomaterials is silver nanoparticles (SNaPs), because of their purifying ability and antiseptic properties (Chen *et al.,* 2007). Silver compounds are considered a potential alternative to some food additives such as oligosaccharides, organic acids, plant extracts, etc. The main objective of their use as additives in poultry feed is their effective anti-microbial role, which acts against potential pathogens but not against symbiotic microbial communities (Fondevila *et al.,* 2009).

Silver nanoparticles have high effectiveness as antimicrobial and can be used as feed additives because they are compatible with the biological system in the body. It is known that silver nanoparticles have a broad-spectrum effect against harmful microbes such as *E. coli*, *Vibrio cholerae*, *Salmonella typhi*, *Pseudomonas* aeruginosa (Ahmadi *et al.,* 2009).

Graphene nanoparticles, graphite nanoparticles, or charcoal nanoparticles are different structural forms of carbon nanoparticles. Graphene is a single- atom-thick substance consisting of carbon atom bound in a β-sp^2 structure arranged in a honeycomb lattice. Graphene reduces cell adhesion when it enters the cytoplasm and nucleus (Wang *et al.,* 2011).

The growth of Gram-positive, Gram-negative and Escherichia coli bacteria was significantly affected by the sharp edges of the reduced graphene nanoparticles (Akhavan and Ghaderi, 2010). Park (2010) also found that graphene is non-cytotoxic to mammalian cells.

This study aimed to investigate the effect of using different types and levels of nanoparticles (graphene or silver) in broiler feed on chemicals and physical bone measurements, and microorganism in the digestive tract.

MATERIALS AND METHODS

The present study was carried out at the Poultry Nutrition Farm, Poultry Production Department, Faculty of Agriculture, Ain Shams University, Shalakan, Kaliobia, Egypt. This experiment was conducted to evaluate the effect of using nanoparticles) silver or graphene) as supplementation in broiler diets on some bone measurements and microorganisms in the digestive tract. A total of 360 unsexed one-day-old of IR (Indian river) broiler chicks were used in this study divided into nine treatments. Each treatment comprised 40 chicks, divided into eight replicates of five chicks. The tested nanoparticles treatments were 0 nanoparticlesas as a control treatment, and 2.5, 5.0, 7.5 and 10.0 ppm/Kg feed of either silver or graphene nanoparticles in starter, grower and finisher diets, respectively.

Three diets in mash form were formulated for the experiment, including a starter from 1 to 14 days, a grower from 15 to 28 days and finisher from 29 to 35 days. The nutrient requirements were supplied according to, the strain catalog. Chicks were fed experimental corn – soybean diets, the composition and calculated analysis of which are shown in Table (1).

Table (1): Composition and calculated analysis of starter, grower and finisher diets.

	Diets		
Ingredients	**Starter***	**Grower***	**Finisher***
Yellow corn	55.76	59.70	63.70
Soybean meal 48%	37.84	33.10	28.22
Soy oil	2.44	3.40	4.42
Bone meal	2.91	2.60	2.26
Limestone	0.24	0.35	0.50
HCL Lysine	0.00	0.04	0.08
DL Methionine (99%)	0.21	0.21	0.22
Salt	0.30	0.30	0.30
Premix**(Vit+Min)	0.30	0.30	0.30
Total	**100.00**	**100.00**	**100.00**
Calculated analysis* **			
Crude protein (%)	23.01	21.04	18.99
M E (kcal / kg)	3003	3102	3204
C\P ratio	130	147	168
Calcium (%)	1.00	0.95	0.90
Available phosphorus (%)	0.50	0.45	0.40
Methionine (%)	0.63	0.60	0.58
Methionine + Cysteine (%)	0.95	0.90	0.85
Lysine (%)	1.35	1.25	1.15

** Starter (1-14 days), Grower (15-28 days) and finisher (29-35 days).*
*** Each 3 kg contains: Vit A 12 000 000 IU, Vit D3 2 000 000 IU, Vit E 10g, Vit K3 2 g, Vit B1 1 g, Vit B2 5 g, Vit B6 1.5 g, Vit B12 10 mg, Nicotinic acid 30 g, Pantothenic acid 10 g, Folic acid 1 g, Biotin 50 mg Choline 250 g, Iron 30 g, Copper 10 g, Zinc 50 g, Manganese 60 g, Iodine1 g, Selenium 0.1 g, Cobalt 0.1 g and carrier (CaCO3) to 3 kg.*
**** Calculated analysis according to NRC (1994).*

Microbiology measurements of digestive tract content:

At the end of the experiment, four birds from each group were randomly selected for digestive sampling in the lower ileum and cecum (2 cm from Meckel's diverticulum to ileocecal junction). The contents of the small intestine and cecum were collected to determine the microbiological flora in (microbiological laboratory, Faculty of Science, South Velia University) for enumeration of total bacteria, *E. coli* and *lactobacillus spp.* Samples were taken in falcon tubes and cooled until incubation. The samples were processed quickly after collection. The microbial counts were determined as logarithmic colony forming units (CFU) per gram of sample.

Bone measurements and analyses:

Bone dry matter:

The tibia was dried in a drying oven at 60° C overnight, weighed (AOAC, 2012).

Bone dimensions:

The tibia was first thawed at room temperature for about one hour and then measured for its length (from proximal to distal end) and width using a Hardened Stainless Steel digital micrometer according to the method described by Samejima (1990).

Seedor index (SI) (g/mm):

The Seedor index is a value that expresses bone mineral density (BMD). It is calculated by dividing tibia dry weight (grams) by its length (mm), as proposed by Seedor *et al*. (1991). This index gives an absolute figure and does not have a unit. It represents an indication of bone density: the higher the value, the denser the bone.

Tibia breaking strength (TBS):

Tibia breaking strength (TBS) was determined on tibiae on a wet-basis following the method of Crenshaw *et al.* (1981) by applying the simple three-point bending concept. This determination was made at the Research Center of Properties and Testing of Materials and Quality Control, Engineering Consulting Center, Faculty of Engineering, Ain Shams University, with an Instron Universal Testing Machine, which was set at a maximum load of 50 Kg and a crosshead speed of mm/ min. TBS was defined as the upper limit force that the tibia bone can afford when applied perpendicularly on the longitudinal axis of this bone and before the bone itself is broken. This potency is determined universally by the unit of area which is presented as Newton. Tibiae were first removed from a -20° C deep freezer, kept overnight in a 10° C fridge to be regularly thawed, and then left for about 1 h at room temperature prior to the TBS procedure.

Data and statistical analysis:

Statistical analysis of both experiments was conducted using the general linear model (GLM) procedure of base SAS® (SAS instituted, 2004). Factors test using two ways ANOVA. Means were compared using Duncan´s multiple range test (Duncan, 1955) where the level of significance was set at minimum ($P \leq 0.05$). Treatments were assigned as the main factor, the statistical model performed as follow:

$$Y_{ijk} = \mu + T_i + L_j + (T*L)_{ij} + E_{ijk}$$

Where
Y_{ijk}= is the effect of the observation
μ = overall mean.
T_i = the effect of i^{th} type of nanoparticles.
Lj= the effect of the j^{th} level of nanoparticles.
$(T*L)_{ij}$= interaction between types and levels of nanoparticles.
E_{ijk} = random error.

RESULTS AND DISCUSSION

Tibia bone measurements and chemical composition:

Tibia bone measurements:

The results in Table (2) show the effect of dietary nanoparticles (types and levels) on tibia physical measurements. Tibia width was affected significantly by different levels of nanoparticles (silver vs. graphene) but it was not affected by the type of nanoparticles. Broiler chickens fed diets supplemented with (2.5 ppm/ kg diet) nanoparticles showed significantly lower values compared with those fed control diets (6.27 Vs. 7.79 mm respectively).

Other physical measurements (tibia length (mm), seedor index, wet tibia weight (g), dry tibia weight (g) and tibia breaking strength (Newton)) were not significantly affected by different types or levels of nanoparticles.

Bone chemical composition:

As shown in Table 3 the effect of different types or levels of nanoparticles as feed additives in broiler diets on bone chemical composition was evaluated. Data in Table (3) indicate that Ash % increased eith increasing levels of nanoparticles compared with the control group. The corresponding figures were (41.23, 42.12, 45.07 and 41.96 vs. 35.06 %), respectively, with significant differences between treatments.

It is worth noting that broiler chicks fed control diets during the bexperimental period (35 days) had the lowest tibia ash% compared with those fed different dietary treatment. In addition, chicks fed diets supplemented with SNaPs showed lower ash% than those fed GNaPs diets (39.52 and 42.66 respectively). However, the differences were not significant.

Phosphorous % showed the same trend since chickens fed control diets have the lowest compared with those fed different levels of nanoparticles without significant differences. On the contrary, supplementation of (10 ppm/ kg diet) nanoparticles in broiler diets resulted in a non-significant reduction in calcium % compared with those fed other dietary treatments.

Microbiology measurements of digestive tract content:

Data in Table 4 showed the effects of different types or levels of nanoparticles (silver vs. graphene) on microorganisms in the small intestine and ceca of broiler chicks. Results showed a significant increase in *Lactobacillus spp.* count in the small intestine with increasing nanoparticle levels in a diet where birds fed the diet with 5.0 ppm/ kg diet recorded highest *Lactobacillus spp.* count and control group was the lowest (2.79 $X10^4$and $0.68X10^4$ respectively).

On the other hand, *E.coli* decreases significantly in the small intestine with increasing nanoparticles levels in a diet where birds fed control diet recorded highest *E.coli* count and those fed the diet with 7.5 ppm/ kg diet nanoparticles recorded the lowest (1.50 $X10^4$and $0.20X10^4$ respectively).

These results agree with Pineda *et al.* (2012) who found that there were significant effects on total count bacteria, *lactobacillus ssp.* and *E.coli* bacteria. The study showed a decrease in harmful bacteria such as *E.coli* compared with the control group.

Similarly, there was a significant difference between the different types of nanoparticles in *lactobacillus ssp.* in the small intestine where the birds fed diets supplemented with graphene nanoparticles recorded *lactobacillus ssp.* count higher than the birds fed diets supplemented with silver nanoparticles (2.24 $X10^4$ and 1.49 $X10^4$respectively). *E. coli* wasn't affected significantly by different types of nanoparticles in the small intestine where the two types have the same effect on *E.coli* count in the small intestine ($0.54X10^4$ and $0.56X10^4$ respectively).

In ceca *Lactobacillus spp.* count increased significantly with increasing nanoparticles levels where the birds fed diets supplemented with 7.5 ppm/kg diet was recorded highest *Lactobacillus spp.* count and control group was the lowest (5.04 $X10^4$and0.44 $X10^4$respectively). *E. coli* was significantly affected by treatments where the control group recorded the highest *E.coli* count and birds fed diet supplemented with 7.5 ppm/kg diet was recorded lowest *E.coli* count (1.62 $X10^4$ and 0.08 $X10^4$respectively).

On the other hand, there were significant differences between the different types of nanoparticles in *Lactobacillus spp.* and *E. coli* where the groups fed diets supplemented with graphene nanoparticles recorded a higher count than the groups fed diets supplemented with silver nanoparticles ($4.86X10^4$ vs. $2.71X10^4$) and ($0.46X10^4$ vs. $0.42X10^4$) respectively.

The mechanism of the inhibitory effects of nanoparticles was stronger in the case of Gram-negative bacteria. This might be related to the thickness of the peptidoglycan layer in Gram-positive bacteria cell wall, which may prevent to some extent and the increasing in to some extent and the increasing in Gram-negative bacteria (Kout-Elkloub *et al.,* 2015). The increase in *Lactobacillus ssp.* may be related to the competitive relationship between harmful bacteria and beneficial bacteria in digestive tract and the increase in total count bacteria may be related to the increasing in beneficial bacteria in the digestive tract. Singh *et al*. (2008) reported higher sensitivity of Gram-negative bacteria to nanoparticle treatment. This result agrees with Sawosz *et al.* (2007), who examined the effects of silver nanoparticles (SNaPs) on microbial profile of caecum of Japanese quail. However, Pineda *et al.* (2012) found that different levels of SNaPs did not affect microorganisms in the cecum of broiler chicks. These results may be related to the antimicrobial effect of SNaPs and its role as a regulatory factor of microorganism in the digestive tract where the pathogenic bacteria count is low (Fondevila, 2009).

CONCLUSION

In conclusion, used different levels of nanoparticles (silver vs. graphene) as feed additives in broiler diets has a positive effect on small intestine and cecum.

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Table (2): Effect of nanoparticles (silver and graphene) in broiler diets on some bone measurements.

Item	Type	Control	Level				Overall	Sig.			
			2.5 PPM	5 PPM	7.5 PPM	10 PPM		MSE	T	L	T*L
Tibia length (mm)	SNaPs	87.15	85.44	84.54	85.26	85.20	**85.52**	**3.51**	**NS**	**NS**	**NS**
	GNaPs	87.15	84.35	82.08	86.90	83.23	**84.74**				
	Overall	**87.15**	**84.89**	**83.31**	**86.08**	**84.21**					
Tibia width (mm)	SNaPs	7.79	6.28	7.11	6.37	6.95	**6.90**	**0.77**	**NS**	******	**NS**
	GNaPs	7.79	6.26	6.81	7.62	7.50	**7.20**				
	Overall	**7.79^{a}**	**6.27^{b}**	**6.96^{ab}**	**6.99^{ab}**	**7.22^{a}**					
Seeder index	SNaPs	0.86	0.84	0.83	0.92	0.87	**0.86**	**0.01**	**NS**	**NS**	**NS**
	GNaPs	0.86	0.77	0.97	0.75	0.87	**0.85**				
	Overall	**0.86**	**0.81**	**0.90**	**0.84**	**0.87**					
Wet tibia weight(g)	SNaPs	14.00	15.00	14.00	17.00	16.00	**15.20**	**3.57**	**NS**	**NS**	**NS**
	GNaPs	14.00	13.50	13.50	13.00	12.50	**13.30**				
	Overall	**14.00**	**14.25**	**13.75**	**15.00**	**14.25**					
Dry tibia weight(g)	SNaPs	7.11	6.66	6.83	8.30	7.77	**7.33**	**1.04**	**NS**	**NS**	**NS**
	GNaPs	7.11	5.61	7.49	6.13	6.36	**6.54**				
	Overall	**7.11**	**6.14**	**7.16**	**7.21**	**7.07**					
Tibia breaking strength (Newton)	SNaPs	354.08	277.25	237.00	239.41	330.65	**287.68**	**69.18**	**NS**	**NS**	**NS**
	GNaPs	354.08	251.42	285.60	382.25	280.15	**310.70**				
	Overall	**354.08**	**264.34**	**261.30**	**310.83**	**305.40**					

*a,b: Means in the same row or column with the same letters are not significantly different. MSE: Mean standard error NS: Non-significant **: (P≤ 0.01) SNaPs= silver nanoparticles, GNaPs = graphene nanoparticles, T*L= the interaction between type and level of nanoparticles.*

Table (3): Effect of nanoparticles (silver and graphene) in broiler diets on some bone chemical composition.

Item	Type	Control	Level				Overall	Sig.			
			2.5 PPM	5 PPM	7.5 PPM	10 PPM		MSE	T	L	T*L
% Ash	SNaPs	35.06	36.22	42.15	43.45	40.69	**39.52**	**5.35**	**NS**	*	**NS**
	GNaPs	35.06	46.23	42.09	46.69	43.22	**42.66**				
	Overall	**35.06[b]**	**41.23 [a]**	**42.12 [a]**	**45.07[a]**	**41.96[a]**					
% Organic matter	SNaPs	65.14	63.55	55.50	56.22	58.87	**59.86**	**4.15**	**NS**	**NS**	**NS**
	GNaPs	65.14	54.24	58.93	57.70	57.82	**58.77**				
	Overall	**65.14**	**58.90**	**57.22**	**56.96**	**58.35**					
% Calcium	SNaPs	15.46	15.89	15.89	15.13	15.57	**15.59**	**0.97**	**NS**	**NS**	**NS**
	GNaPs	15.46	15.02	15.78	16.00	15.24	**15.50**				
	Overall	**15.46**	**15.46**	**15.84**	**15.57**	**15.40**					
% Phosphorus	SNaPs	8.27	9.32	8.20	9.40	9.15	**8.87**	**0.62**	**NS**	**NS**	**NS**
	GNaPs	8.27	8.69	9.00	8.55	8.35	**8.57**				
	Overall	**8.27**	**9.00**	**8.60**	**8.98**	**8.75**					

*a,b: Means in the same row or column with the same letters are not significantly different. MSE: Mean standard error NS: Non-significant *: (P≤ 0.05) SNaPs= silver nanoparticles, GNaPs = graphene nanoparticles, T*L= the interaction between type and level of nanoparticles.*

Table (4): Effect of nanoparticles (silver and graphene) in broiler diets on digestive microorganism in digestive tract.

	Item	Type	Control	Level				Overall	Sig.			
				2.5 PPM	5 PPM	7.5 PPM	10 PPM		MSE	T	L	T*L
Small intestine	***Lactobacillus ssp.***	SNaPs	0.68 $X10^4$	1.29 $X10^4$	1.60 $X10^4$	1.74 $X10^4$	2.13 $X10^4$	**1. 49^b $X10^4$**	**$0.10X10^4$**	**	**	**
		GNaPs	0.68 $X10^4$	1.50 $X10^4$	3.84 $X10^4$	1.94 $X10^4$	3.26 $X10^4$	**2.24^a $X10^4$**				
		Overall	**0.68^d $X10^4$**	**1.39^c $X10^4$**	**2.72^a $X10^4$**	**1.84^b $X10^4$**	**2.69^a $X10^4$**					
	E.Coli	SNaPs	1.50 $X10^4$	0.37 $X10^4$	0.26 $X10^4$	0.24 $X10^4$	0.35 $X10^4$	**$0.54X10^4$**	**$0.13X10^4$**	**NS**	**	*
		GNaPs	1.50 $X10^4$	0.45 $X10^{4b}$	0.55 $X10^{4b}$	0.15 $X10^{4c}$	0.15 $X10^{4c}$	**$0.56X10^4$**				
		Overall	**1.50^a $X10^4$**	**0.41^b $X10^4$**	**0.39^b $X10^4$**	**0.20^c $X10^4$**	**0.25^{bc} $X10^4$**					
Ceca	***Lactobacillus ssp.***	SNaPs	0.44 $X10^4$	2.87 $X10^4$	1.05 $X10^4$	4.99 $X10^4$	4.21 $X10^4$	**2.71^b $X10^4$**	**$0.08X10^4$**	**	**	**
		GNaPs	0.44 $X10^4$	5.81 $X10^4$	7.71 $X10^4$	5.09 $X10^4$	5.27 $X10^4$	**4.86^a $X10^4$**				
		Overall	**0.44^d $X10^4$**	**4.34^c $X10^4$**	**4.38^c $X10^4$**	**5.04^a $X10^4$**	**4.74^b $X10^4$**					
	E.Coli	SNaPs	1.62 $X10^4$	0.32 $X10^4$	0.17 $X10^4$	0.00 $X10^4$	0.00 $X10^4$	**0.42^bX10^4**	**$0.04X10^4$**	*	**	**
		GNaPs	1.62 $X10^4$	0.20 $X10^4$	0.17 $X10^4$	0.15 $X10^4$	0.17 $X10^4$	**0.46^aX10^4**				
		Overall	**1.62 $X10^{4a}$**	**0.26^b $X10^4$**	**0.17^c $X10^4$**	**0.08^d $X10^4$**	**0.07^d $X10^4$**					

*a,b,c: Means in the same row or column with the same letters are not significantly different. MSE: Mean standard error NS: Non-significant *: (P≤ 0.05) SNaPs = silver nanoparticles, GNaPs = graphene nanoparticles, T*L= the interaction between type and level of nanoparticles.*

تأثير المستويات الغذائية المختلفة من الجسيمات النانوية (الفضة مقابل الجرافين) على خصائص العظام والميكروبات المعوية لدى دجاج التسمين

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تم استخدام ثلاثمائة وستين كتكوت تسمين من سلالة الانديان ريفر IR عمر يومً غير مجنسة لدراسة تأثير استخدام أنواع ومستويات مختلفة من الجسيمات النانوية (الفضة والجرافين) في علائق دجاج التسمين على بعض صفات العظم ، وعدد الكائنات الحية الدقيقة في الأمعاء الدقيقة والأعور. تضمنت هذه التجربة 9 معاملات و 40 كتكوت تسمين لكل معاملة. استمرت التجربة من يوم إلى 35 يومًا وغذيت الكتاكيت على 3 علائق (بادئ ونامي وناهي) تحتوي على 2.5 و 5.0 و 7.5 و 10.0 جزء في المليون / كجم من كلا النوعين من الجسيمات النانوية بالاضافة الي عليقة الكنترول خلال الثلاث مراحل المختلفة ، على التوالي. أظهرت نتائج هذه التجربة ذلك:

1. لم تتأثر جميع القياسات الفيزيائية والكيميائية للعظام بشكل كبير بالأنواع أو المستويات المختلفة من جسيمات النانو باستثناء عرض الساق والرماد٪ حيث تأثرت بشكل كبير بالمستويات المختلفة من جسيمات النانو.
2. تأثر عدد *Lactobacillus ssp.* في كل من الامعاء الدقيقة والاعور معنويا بالأنواع والمستويات المختلفة من جسيمات النانو. من ناحية أخرى ، انخفضت *E.coli* في كل من الامعاء الدقيقة والاعور معنويا مع زيادة مستويات جسيمات النانو.

الخلاصة:؟؟؟؟؟؟