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RESEARCH ARTICLE

Supplemental Selenium Nanoparticles-loaded to Chitosan Improves Meat Quality, Pectoral Muscle Histology, Tibia Bone Morphometry and Tissue Mineral Retention in Broilers

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ABSTRACT

The current study was conducted to evaluate the potential impact of dietary selenium nanoparticles (SeNPs) alone, in combination with chitosan or loaded to chitosan on pectoral muscle quality and tibia bone morphology in broilers. Two-hundred-and-forty day-old chicks were raised in five groups, each group having eight replicates (n=6/replicate). The control group received a basal diet whereas the other four groups received basal diet supplemented with selenium nanoparticles (SeNPs-0.5mg/kg), chitosan (COS-200mg/kg), SeNPs+COS (0.5mg/kg+200mg/kg) or SeNPs loaded to COS (SeNPs-L-COS-200mg/kg), respectively. On day 35, two birds/replicate were euthanized to collect blood, tibia bone, and pectoral muscle. Muscle pH, selenium and calcium concentrations were significantly higher ($P<0.05$) in broiler fed SeNPs+COS and SeNPs-L-COS while drip loss percentage was significantly lower ($P<0.05$) in broilers fed SeNPs-L-COS when compared with birds in the control group. Dietary SeNPs-L-COS resulted in significantly higher ($P<0.05$) water holding capacity, muscle, fiber diameter and cross-sectional area when compared with the control group. Bone weight and weight/length index were higher ($P<0.05$) in broilers fed SeNPs+COS and SeNPs-L-COS compared to birds in the control group. Diaphysis-diameter was higher ($P<0.05$) in broilers fed SeNPs and SeNPs-L-COS groups in comparison with birds in the control group. Phosphorus concentration was higher ($P<0.05$) in tibia bone in broilers fed SeNPs+COS and SeNPs-L-COS when compared to birds in the control group. Thus, we conclude that supplementation of SeNPs-L-COS improves breast muscle histology, tibia bone health, mineral retention in tissue, and resulting meat quality in broilers.

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INTRODUCTION

Poultry meat is a good source of protein, vitamins, and minerals and also has a low total fat content with higher percentages of unsaturated fatty acids compared to many other meats (Rehman *et al.,* 2018). Consumer acceptance of meat is largely driven by the physical factors such as color, tenderness, and juiciness. Most of these physical characteristics are a function of drip loss and pH of meat. These two factors are influenced by muscle oxidative status (Li *et al.,* 2018), which is a known indicator of muscle health. Producing high-yield broilers, however, can come at the expense of meat quality via deterioration of muscle oxidative status. Rapid growth of muscles has been associated with damage to weight bearing bones in commercial broiler as it creates an imbalance between size of the bird and weight-bearing capacity of the skeletal system (Rehman *et al.,* 2018). The

status of bone health can be reasonably assessed from the morphometric parameters and estimation of bone mineral content (Mohammed *et al.,* 2021). Higher growth rate of broilers tends to create an imbalance between size of the bird and weight-bearing capacity of the skeletal system (Rehman *et al.,* 2018). Additionally, improper mineralization during ossification is another factor leading to weakness and leg problems resulting in impaired mobility. Affected birds often have decreased access to feed and water, which further contributes to degradation of the meat quality.

Trace minerals play essential role in optimal muscle growth and bone development in broilers. Among these, selenium (Se) is an integral component of 25 different seleno-proteins critical for numerous physiological functions in broilers such as neutralization of free radicals and intra-cellular calcium storage. Across all tissues, skeletal muscle has highest capacity to retain selenium, which enhances its nutritive value of meat for consumers owing to higher bioavailability of selenium. Selenium deficiencies in broiler diet lead to degeneration of skeletal muscle as a result of increased oxidative damage to muscle cells and their membranes (Ibrahim *et al.,* 2019). Selenium deficiencies also result in impaired bone microarchitecture, increased osteoclastic activity resulting in bone resorption, and increased circulating concentration of calcium (Zeng *et al.,* 2013). Given the importance of selenium for optimal muscle and bone growth, supplementing diets with accessible selenium can support the growth requirements of rapidly growing broilers.

Inorganic sources of selenium, when added to broiler feed, have a narrow margin between beneficial and toxic doses and therefore require efficient delivery and consistent distribution of a minimal dose (Bai *et al.,* 2017). This challenge is being addressed through the use of selenium nanoparticles. Selenium nanoparticles (SeNPs) have higher bioavailability and catalytic efficacy, larger surface area, and lower toxicity (Hu *et al.,* 2012). Inclusion of SeNPs in broiler diets improves nutritional value and quality of meat through modulation of oxidative status (Mahmoud *et al.,* 2016). Additionally, dietary SeNPs enhance antioxidant capacity of the muscle, leading to an increase in the myoglobin content and thereby improved meat color (Li *et al.,* 2018).

The health promoting benefits of orally administered SeNPs to birds can be enhanced by loading them on biological carriers, which improves the SeNPs stability and biological availability (Bai *et al.,* 2017). Chitosan (COS) is a naturally occurring, non-toxic and biodegradable biopolymer, which allows transit drugs and trace minerals in gastrointestinal (GIT) fluids in their active forms and facilitates their controlled release and diffusion across the epithelium as well as their controlled release in GIT (Han *et al.,* 2012). Moreover, COS has prebiotic activity when administered to broilers. It reduces the dexamethasone-induced stress with positive effect on feed to gain (FG) ratios, intestinal morphology, and ileal digestibility (Osho and Adeola, 2020).

SeNPs can be loaded on the surface of COS thus producing stable selenium nanoparticles-loaded chitosan (SeNPs-L-COS). The resultant SeNPs-L-COS has a known protective role against oxidative stress in rats (Bai *et al.,* 2017). However, no published studies could be identified that measure whether SeNPs-L-COS improves the musculoskeletal health of broilers. Therefore, this study was conducted to investigate the possible impact of dietary SeNPs-L-COS on meat quality, muscle histology, bone morphometry, and retention of calcium, phosphorous and selenium in muscle, serum, and tibia bone in broilers.

MATERIALS AND METHODS

This experiment was approved by the Ethical Review Committee of the University of Veterinary and Animal Sciences, Lahore, Pakistan (DR393 dated 12/04/2019).

Synthesis of SeNPs-L-COS: The SeNPs-L-COS were prepared as described by Zhang *et al.* (2004). Briefly, 0.25M selenious acid solution was mixed with 0.5% COS solution. The reaction was initiated by adding ascorbic acid solution (0.05M) with change in color of solution from red to colorless. After 30 minutes of constant stirring, sodium hydroxide solution (1M) was added to it. The mixture was centrifuged for separating SeNPs-L-COS from the solution followed by washing with deionized water.

Experimental design, management and treatment: Two-hundred-and-forty day-old broiler chicks (Hubbard) were purchased from a local hatchery and randomly distributed into five groups with eight replicates per group (6 birds/replicate). The birds were kept in an environmentally controlled experimental shed. On day-1, the temperature was maintained at 35±1˚C and lowered gradually (2.8˚C per week) to achieve 26±1°C by the end of the third week and was maintained the same until day 35. Relative humidity was maintained at 65±5%. Chicks in the control group received a corn and soya-based basal diet (BD) (Table 1), while chicks in the remaining four groups received BD supplemented with SeNPs (0.5mg/kg), COS (200mg/kg), SeNPs+COS (0.5+200 mg/kg) or SeNPs-L-COS (200mg/kg). On day 35, two birds/replicate were randomly selected, humanely euthanized, and sampled for blood, tibia bones, and pectoral muscles.

Muscle parameters (pH, drip loss percentage, and histomorphometry): The pH of harvested muscle samples was measured by inserting a digital pH meter (Eutech, Singapore) 1 cm deep into the pectoral muscle. The pH was recorded at 0-hour and 24-hour post slaughtering and samples were refrigerated at 4˚C in between the two pH recordings. For drip loss percentage, Honikel's gravimetric method (Honikel, 1998) was used with minor modifications as reported by Rehman et al. (2018).

Three Hematoxylin and Eosin (H&E) stained sections were prepared from each muscle sample and were analyzed for histomorphometry using the microscope (Lx-400 Labomed USA) fitted with camera (iVu 1500) and software (Labomed pixel-pro 2.7.7) For muscle fascicle diameter (mm), images were captured at 4X and averaged over three fascicle measurements. For muscle fiber diameter (µm), images were captured at 10X. Averages of

Table 1: Ingredients and nutritive value of the basal diets

Ingredients	Ingredients %
Corn	58.50
Canola meal	8.00
Vegetable oil	1.50
Sunflower meal	3.50
Soybean meal 44%	25.0
Limestone	1.51
Dicalcium phosphate	0.90
Common salt	0.50
Vitamin Premix	0.13
D-L Methionine	0.21
L-lysine HCl	0.12
Micro min premix	0.13
Total	100
Molasses	4.00
Nutrient contents	
ME (MJ/kg)	12.2
CP (%)	20.72
Ca (%)	0.91
P (%)	0.61

Feed(kg) contained vitamins(vit) and minerals: Vit A, 11 000 IU; Vit B12, 0.0132 mg; vit D3, 2200 IU; vit E 22 IU; pantothenic acid(22mg);folic acid(1.1mg);Choline Cl(440mg); menadione(2.2mg); riboflavin(8.8mg); ethoxyquin(250 mg); thiamine(4.4mg); pyridoxine(4.4mg); biotin(0.22); Zn(200 mg); Fe(20 mg); Mn(240mg); Ca(170mg); Cu(20mg);I(0.91mg).

15 muscle fibers (five muscle fibers/fascicle; three fascicles/section) were reported. The muscle fiber cross-sectional area was determined using diameter of muscle fibers ($A=\pi r2$). Muscle fibers number was counted at 4X in 3 randomly selected fascicles. The count was initially carried out in 0.5mm radius circle and then reported for 1.0mm^2 as described by Rehman *et al*. (2018).

Bone morphometric parameters: The tibia bones were separated as drumsticks, boiled (10 minutes) to remove the flesh and subsequently dried overnight at room temperature. Bone weight was measured using weighing balance (BL 220H. Shimadzu Ltd. Japan). Bone length, outer diameter, and medullary canal diameter (mid-shaft) were measured using Vernier calipers (100-333-8B iGaging USA).

Bone weight/length index was calculated and reported (Seedor *et al.,* 2005). The robusticity index was calculated using the formula: Bone length (mm) / cube root of bone weight (mg). The tibiotarsal index was determined by the following formula: [Diaphysis diameter - Medullary canal diameter /Diaphysis diameter] ×100.

Mineral concentration in bone, muscle and serum: Acid digestion of dried bone fragments (1gm), pectoral muscle (1gm) and serum (1ml) was done according to method described by Saeed *et al*. (2017) and final volume of the solution was adjusted to 50 mL using distilled water. Selenium concentrations were measured through atomic absorption spectrophotometer (Model AA6501. Shimadzu. Ltd. Japan). Calcium levels were measured using flame photometry (Biotech Engineering Management Co. Ltd. UK). Phosphorus concentrations were determined using UV-spectrophotometer (V-1100, Thermo Fischer Scientific, USA).

Statistical design: Data were presented as mean ± standard error of mean (SEM). Group means were compared using one-way ANOVA (SPSS Version 20.0). Tukey's test was used for mean separation and comparisons across treatments. Differences were considered significantly different at $P<0.05$.

RESULTS

Muscle parameters (pH, drip loss percentage and histomorphometry): Effects of dietary supplementation of SeNPs loaded on COS on muscle characteristics in broilers are presented in Table 2. The 0-hour pH of the muscle did not differ among groups. At 24-hour, muscle pH was higher ($P<0.05$) in the COS, SeNPs+COS, and SeNPs-L-COS groups in comparison with the control group. Drip loss was lower ($P<0.05$) in all supplemented groups in comparison with the control group. Dietary supplementation of SeNPs-L-COS resulted in higher ($P<0.05$) muscle fiber diameter and their cross-sectional area in comparison with all other groups. The muscle fascicle cross-sectional area was higher ($P<0.05$) in the SeNPs-L-COS supplemented group in comparison with the control group. However, muscle fiber density did not differ among the groups.

Bone morphometric parameters: Effects of dietary supplementation of SeNPs loaded on COS on bone morphology characteristics in broilers are presented in Table 3. Bone weight and weight/length index were higher ($P<0.05$) in the SeNPs+COS and SeNPs-L-COS groups in comparison with the control group. Diaphysis diameter was higher ($P<0.05$) in the SeNPs-L-COS group in comparison with the control group. Tibiotarsal index was higher ($P<0.05$) in the COS group in comparison with other supplemented groups. Robusticity Index and tibia bone length did not differ among groups.

Mineral concentration in bone, muscle, and serum: Effects of dietary supplementation of SeNPs loaded on COS on mineral retention in broilers are presented in Table 4. In muscle tissue, retention of selenium was higher ($P<0.05$) in the SeNPs+COS group, whereas calcium retention was higher ($P<0.05$) in the SeNPs+COS and SeNPs-L-COS groups when compared with the control group. In serum, mineral concentrations did not differ among the groups. In bones, phosphorus content was higher ($P<0.05$) in all the supplemented groups when compared with the control group. Selenium and calcium concentrations in bone tissue did not differ among groups.

DISCUSSION

Due to rapid growth achieved in 35-day broiler production cycle, the previously reported selenium requirements based on a 42-day growth cycle (NRC, 1994) may not be sufficient for desired growth. Moreover, rapid growth can be a stressor for broiler, so selenium supplementation through its antioxidant role can prevent oxidative stress-led damages in muscle and bone tissues. With this understanding, the current research was conducted to access the effect of supplemental selenium on selected musculoskeletal parameters and tissue mineral retention in broiler.

To improve bioavailability, selenium was supplemented in nano-form in combination with COS. Supplementation of COS, SeNPs+COS, or SeNPs-L-COS resulted in a higher pH of pectoral muscle in comparison with the control group. The pH of broiler meat is positively

Table 2: Effect of Selenium nanoparticles-loaded to Chitosan on pectoral muscle of broiler chickens

Parameters	Control	SeNPs	COS	SeNPs+COS	SeNPs-L-COS	SEM	P-Value
pH (at 0 hour)	6.26	6.35	6.41	6.44	6.48	0.05	0.20
pH (at 24 hour)	5.69^{b}	5.76^{ab}	5.83^{a}	5.82^{a}	5.87^{a}	0.04	0.02
Drip loss (%)	3.74^{a}	2.88^{b}	2.98^{b}	2.86^{b}	2.57^{c}	0.20	0.04
MFD (µm)	38.80^{b}	39.50^{b}	39.90^{b}	40.10^{b}	42.60^{a}	0.80	0.03
MFCSA (μm^2)	1182^{b}	1225^{b}	1260^{b}	1250^{b}	1425^{a}	6.10	0.03
MFSD (mm^2)	0.74	0.74	0.75	0.82	0.85	0.11	0.06
MFSCSA (mm^2)	0.43^{b}	0.43^{b}	0.43^{b}	0.54^{ab}	0.58^{a}	0.03	0.03
MFDen	601	569	566	558	552	11.20	0.22

A significant difference (P<0.05) is represented using different superscripts ($^{a-c}$) in the same row. Different values illustrate the Mean±SEM of eight replicates. SeNPs-selenium nanoparticles, COS-Chitosan, SENPs-L-COS Selenium nanoparticles loaded to Chitosan, MFD-Muscle Fiber Diameter, MFCSA-muscle fiber cross sectional area, MFSD-muscle fascicle diameter, MFSCSA-muscle fascicle cross sectional area, MFDen-Muscle fiber density (Total number of muscle fibers/mm^2 of muscle area).

Table 3: Effect of Selenium nanoparticles-loaded to Chitosan on morphometric parameters of tibia bone of broiler chickens

Parameters	Control	SeNPs	COS	SeNPs+COS	SeNPs-L-COS	SEM	P-Value
Bone Length (mm)	85.60	85.70	86.40	85.20	85.00	0.80	0.80
Bone Weight (gm)	5.60^{b}	5.90^{ab}	5.80^{ab}	6.05^{a}	6.10^{a}	0.40	0.04
Diaphysis Diameter (mm)	8.30^{c}	8.40^{ab}	8.30^{bc}	8.30^{abc}	8.40^{a}	0.20	0.03
Medullary Canal Diameter (mm)	5.00^{ab}	5.10^{a}	4.70^{b}	5.20^{a}	5.14^{a}	0.10	0.04
Robusticity Index	4.83	4.74	4.80	4.68	4.64	0.06	0.14
Tibiotarsal Index	39.90^{ab}	39.00^{b}	44.00^{a}	37.80^{b}	38.80^{b}	1.50	0.04
Weight/length Index	65.50^{b}	68.90^{ab}	67.30^{ab}	71.00^{a}	72.10^{a}	1.60	0.03

A significant difference (P<0.05) is represented using different superscripts ($^{a-c}$) in the same row. Different values illustrate the Mean±SEM of eight replicates.

Table 4: Effect of Selenium nanoparticles-loaded to Chitosan on mineral content of broiler chickens

	Control	SeNPs	COS	SeNPs+COS	SeNPs-L-COS	SEM	P-Value
Muscle							
Se(µg/g)	0.08^{b}	0.18^{ab}	0.14^{b}	0.38^{a}	0.27^{ab}	0.03	0.04
Ca(mg/g)	0.21^{b}	0.24^{ab}	0.23^{ab}	0.27^{a}	0.28^{a}	0.01	0.02
P (mg/g)	6.90	7.14	6.80	7.01	6.98	0.10	0.43
Serum							
Se(mg/l)	0.21	0.51	0.48	0.36	0.32	0.01	0.30
Ca(mg/dl)	10.80	10.90	11.20	10.50	11.30	0.30	0.60
P(mg/dl)	6.73	6.90	6.50	6.40	6.43	0.30	0.90
Tibial bone							
Se(mg/kg)	0.33	0.38	0.34	0.41	0.70	0.20	0.06
Ca(gm/kg)	178	200	181	201	190	9.50	0.90
P(gm/kg)	110^{b}	114^{a}	112^{ab}	115^{a}	114^{a}	1.10	0.02

A significant difference (P<0.05) is represented using different superscripts ($^{a-c}$) in the same row. Different values illustrate the Mean±SEM of eight replicates. Ca-calcium, P-phosphorus; Se-selenium.

correlated with tenderness, juiciness, and redness and is negatively correlated with drip loss percentage (Khan *et al.*, 2018). After slaughtering, the cessation of blood flow prevents removal of lactic acid from muscle fiber and results in lowering of pH (Oliveira *et al.*, 2014). The SeNPs delay the metabolic conversion of glucose to lactic acid production and thus help to maintain the pH (Li *et al.*, 2018). Additionally, COS supports maintenance of meat pH through decreasing muscle glycogen glycolytic metabolism and lactate accumulation (Chang *et al.*, 2020). This explains the effectiveness of SeNPs and COS together in maintaining a higher pH after 24 hours of storage.

Supplementation of SeNPs-L-COS significantly increased water holding capacity (WHC) by decreasing the drip loss. This could have resulted from a higher pH favoring retention of water (Li *et al.*, 2018; Jin *et al.*, 2019). Reduced drip loss could also be attributed to increased bioavailability of SeNPs when bound to COS which increased the Se-associated antioxidant activity in breast muscle thus improving cell membrane integrity and viability (Cai *et al.*, 2012). Moreover, selenium supplementation is also known to prevent the denaturation of proteins in the muscle fiber. Taken together, selenium improves shelf life and meat quality by decreasing drip loss of broiler meat (Yang *et al.*, 2012).

Dietary supplementation of SeNPs-L-COS increased (P<0.05) muscle fiber diameter and cross-sectional area in comparison with all other groups. Increased muscle cell diameter and cross-sectional area may result from higher bioavailability of SeNPs when loaded on COS and SeNPs enhancing viability of satellite cells as reported by Jin *et al.* (2019). Satellite cells are present beneath basal lamina of skeletal muscle cells. These cells are capable of mitosis; subsequent to mitotic divisions, newly formed cells fuse with already existing muscle fibers thus increasing their diameter (Liu *et al.*, 2010). Furthermore, increased muscle fiber diameter is indicative of higher protein deposition in muscle cells. Higher protein deposition leads to increased protein-bound water and hence can further contribute to the improved WHC observed in the SeNPs-L-COS group.

Dietary SeNPs+COS and SeNPs-L-COS increased (P<0.05) the weight/length index when compared with the control group. Similarly, diaphysis diameter was higher in the SeNPs-L-COS group in comparison with the control group. We could not identify similar studies for comparisons; however, increased weight/length and diaphysis diameter could be attributed to selenium supplementation, which is reported to improve minerals absorption and promote bone development and growth (Jin *et al.*, 2019). The absorbed selenium might have contributed to increased formation of bone seleno-proteins, which support the skeletal development by mitigating reactive oxygen species (ROS) and supporting

osteoblastic differentiation of bone marrow stem cells (Zeng *et al.,* 2013).

Selenium and calcium retention in muscle was higher (P<0.05) in the SeNPs+COS and SeNPs-L-COS groups in comparison to the control group. Retention of trace minerals in tissues is an indicator of better absorption and availability of minerals for essential functions (Zheng *et al.,* 2020). SeNPs supplementation resulted in higher retention of selenium in breast muscle when compared with breast muscle of chickens supplemented with organic and inorganic selenium sources. This difference in retention could be due to adsorptive and receptor-mediated endocytosis of nano-selenium (Hu *et al.,* 2012; Ibrahim *et al.,* 2019), which is appears to be more efficient than absorption of non-nano forms of dietary selenium. Increase in calcium concentration in muscle could be attributed to the role of selenium in maintaining calcium homeostasis through increasing the intra-cellular storage of calcium in sarcoplasmic reticulum (Hu *et al.,* 2012; Bodnar *et al.,* 2016). Higher bioavailability of SeNPs might contribute to the optimal functioning of selenoproteins in breast muscle, thus protecting muscle against any oxidative stress.

In the current study, phosphorus (P) retention in bone was higher (P<0.05) in the SeNPs, SeNPs+COS and SeNPs-L-COS groups in comparison with the control group, whereas calcium concentrations remained unchanged. Although phosphorus concentration was higher in the supplemented groups when compared with the control group, the observed values were still within the normal range of phosphorus concentrations reported for long bone of chicken (104 g/kg to 167 g/kg) (Schuhmann *et al.,* 2014). Although we could not find the exact mechanism of interaction of selenium intake with bone phosphorus in the literature, selenium supplementation has been reported to positively affect bone mineral density and reduce bone fractures in aging men (Walsh *et al.,* 2021).

Conclusions: We concluded that dietary supplementation of SeNPs-Loaded to COS improved histo-morphometric characteristics of breast muscle and selected meat quality attributes in chickens. However, the effectiveness of SeNPs+COS supplementation was comparable to that of SeNPs loaded to COS for promoting the bone health and tissue minerals retention.

Authors contribution: HZ, SM and HUR planned and designed the experiment. RT helped in the synthesis of selenium nanoparticles loaded to chitosan. SA1, IK and HFR performed the experimental trial. IK, SR, SA2, and SD analyzed the data. IK, HZ, SA1 and HFR contributed to prepare the manuscript.

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