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Examining The impact of Chitosan and Nano-Chitosan Addition on The expression Levels of SOD and CAT Genes in Two Lines of Japanese Quail

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Abstract

THIS research aims to investigate the antioxidative potential of dietary chitosan (CH) and nano-chitosan (NCH) supplementation on the gene expression of pivotal antioxidant defense system components, namely SOD (superoxide dismutase) and CAT (catalase) genes. These genes are instrumental in safeguarding cells against the deleterious effects of reactive oxygen species. The study extends its scope to evaluating the hepatic and intestinal histology in two distinct lines of Japanese quail. Carried out as a fully randomized design experiment with 10 treatment groups, the research encompassed 840 one-day-old Japanese quails from two distinct lines: Sq, selected for body weight at 30 days after 42 generations of selection, and Jq, a control line without selection. Each line was further divided into five treatment groups, with three replicates per group and 28 unsexed chicks per replicate. The treatment groups included a baseline diet according to NRC (1994) guidelines, two groups supplemented with chitosan (CH) at 50 and 70 mg/kg diet, and two groups supplemented with nano-chitosan (NCH) at 30 and 50 mg/kg diet for both Sq and Jq lines. The study outcomes shed light on the varying concentrations of chitosan (30, 50, and 70 mg/kg) and their impacts on live body weight, CAT and SOD gene expression levels, as well as histopathological changes in the liver and intestine. The findings revealed discernible effects across all tested concentrations, elucidating the multifaceted influence of chitosan and nano-chitosan supplementation on the examined parameters.

Keywords: CAT, Chitosan (CH), Japanese quail, Nano-chitosan (NCH), SOD.

Introduction

The Japanese quail, renowned as the smallest domesticated bird species for egg and meat production, stands as a globally significant source of animal protein. Recognized for its early maturation, with females reaching maturity at five to six weeks of age, the Japanese quail has become a valuable model for research, allowing researchers to attain four to five generations per year. Advances such as the polymerase chain reaction and the elucidation of the Japanese quail genome structure [1] have propelled gene expression studies.

Chitosan, a versatile compound known for its antimicrobial, anti-inflammatory, antioxidant, anti-tumor, and immune-stimulating properties, has emerged as a promising feed additive, replacing antibiotic growth promoters in chicken diets. Derived from alkaline-deacetylated chitin obtained from shrimp waste, chitosan is a natural bio-poly amino-saccharide with biodegradable and non-toxic qualities (2;3). With documented immune-boosting and antibacterial effects (4), chitosan has found utility in promoting digestive health, removing toxins, and alleviating gastrointestinal ulcers and chronic constipation in animals [5].

Studies by Guan [6] and Hassan, [7] underscore the biological benefits of chitosan as a feed additive, including antibacterial, antioxidant, cholesterol-lowering, and immunomodulatory properties. Notably, supplementation with chitosan and nano-chitosan has been reported to enhance the antioxidative state of Japanese quails, elevating the activity of catalase (CAT), a key enzymatic antioxidant in poultry [7] Further, research by Xu [8] suggests that Chito oligosaccharide

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supplementation during the late laying season may contribute to improved serum lipid profiles, enhanced antioxidant activity, and heightened immunological function in hens. Chitosan-oligosaccharides (COS), a positively charged alkaline carbohydrate recognized for its antibacterial, anti-inflammatory, antioxidant, anticancer, immune-stimulating, and hypocholesterolemic characteristics, stands out as a potent alternative to antibiotic growth promoters [9]. Studies have demonstrated the growth-promoting properties of COS, showcasing its potential to enhance health status and reduce pathogen development in the small intestine. Dietary COS supplementation has been linked to improvements in the morphological and histological structure of the reproductive system and alimentary canal in farm animals [10].

Furthermore, chitosan nanoparticles have exhibited powerful antibacterial activity comparable to fosfomycin, making them a promising alternative to antibiotics for treating Staphylococcus aureus and Escherichia coli [11]. This study aims to investigate the antioxidant effects of dietary chitosan (CH) and nano-chitosan (NCH) supplementation on the gene expression levels of antioxidant enzymes SOD and CAT, along with an examination of liver and intestinal histology in two lines of Japanese quail.

Materials and Methods

Experimental Design and Birds

The study was conducted in poultry house at the Stino Quail Farm, Al-Hoda cooperative association, Cairo-Alexandria desert highway, 64 km, Egypt, involving 840 one-day-old Japanese quails from two lines: selected Japanese quail (Sq) and control or non-selected (Jq). The Sq line was selected for body weight at 30 days of age for 42 generations, while the Jq line served as the control. Birds were divided into five treatment groups for each line with three replicas per group (28 unsexed chicks/replica). The treatments included a basal diet according to NRC (1994) guidelines, Chitosan (CH) supplementation at 50 and 70 mg/kg diet, and nano-Chitosan (NCH) supplementation at 30 and 50 mg/kg diet.Housing and Environmental Conditions: Birds were housed in standard cages (50x30x50 cm^3) with unlimited access to water and food. A 24-hour light cycle was maintained, and environmental conditions, including temperature, were regulated throughout the experiment. Basal Diet Composition: Table (1) outlines the composition and calculated analysis of the basal diet, including ingredients and nutritional values.

TABLE 1. Provides the basal diets composition and calculated analyses.

Each1 kg contain Vit. A, 12000.000 IU; Vit. D3, 2000.000 IU; Vit E,10g; Vit. K2, 1g; Vit. B1, 1g, Vit. B2, 4g, Vit. B6, 1. 5g;Vit. B12, 10g; Pantathenic acid, 10g; Nicotinic acid, 20g; Folic acid, 1000 mg; Biotin, 50g; Choline chloride, 500g; Copper, 10g; Iodine, 1g; Iron, 30g; Manganese, 55g; Zinc, 55g; Selenium, 0.1g. 2Calculated according to NRC (1994).

Ingredients	**%**
Yellow corn (8.5%)	35.00
Soybean meal (44%)0	55.54
Corn gluten meal (62%)	
Dicalcium phosphate	0.80
Limestone	1.35
Salt NaCl	0.35
Vit-min premix1	0.30
DL-Methionine	0.05
L-Lysine	0.11
Total	100
Calculated analysis2	
Crude Protein %	24.02
Crude fiber%	3.87
Metabolizable energy, Kcal/kg	2900
Calcium%	0.81
Phosphorous (available)%	0.30
Methionine+Cystine %	0.75
Methionine %	0.50
Lysine%	1.30

Histopathological Analysis

Liver and intestine samples from each replicate were preserved in 10% formal saline, processed, and examined for histopathological changes. Tissue sections were prepared, deparaffinized, and stained with hematoxylin and eosin for microscopic analysis.

Whole Blood Sampling: Whole blood samples were collected using 70% isopropyl alcohol. Blood was divided into three tubes with different anticoagulants. Samples were diluted with RNase/DNase-free water and homogenized with TRIzol LS before RNA extraction [12].

RNA Isolation and Purification: Total RNA was extracted using the TRIzol LS procedure, and different purification methods were employed. RNA samples were assessed for quantity and quality using a NanoDrop 1000 Spectrophotometer and an Agilent 2100 Bioanalyzer. Purified RNA samples were stored at -80°C post-extraction.

cDNA Synthesis and Real-time PCR: Reverse transcription was performed using commercial reverse transcription kits. Real-time PCR was carried

out using a fast-start universal SYBR Green master (ROX) on a Q5 Real-Time PCR System. The efficiency of primers was determined, and relative expression levels were quantified using the comparative CT method.

Statistical Analysis

Data were subjected to a two-way analysis of variance using the General Linear Model (13). The statistical model considered the effects of line, supplementation, and their interaction. Standard errors (SE) and least square means (LSM) were reported, and significant differences between values were determined using Duncan's multiple range test at a 5% level of significance [14].

The following statistical model was used to examine all the results:

$$Yijk = Li + Sj + LSij + eijk$$

Where: Yijk= the kth observation of the jth treatment within the ith line; =the overall mean; Li=the ith line's influence; Sj=the jth supplementation's effect; and LSij=the ith line's and jth treatment's interaction

Results and Discussion

In contrast to the non-selected Quail line, the selected Quail line exhibited significantly higher Live Body Weight (LBW) at hatch and the ages of 7, 14, 21, 28, and 35 days after undergoing 42 generations of selection. As shown in Table (2) and illustrated in Figures 1 and 2, the average LBW at 5 weeks of age was 336.56 g for the selected Quail line and 204.44 g for the non-selected Quail line, indicating a substantial difference of 132.12 g attributed to generations of selective breeding for higher body weight at four weeks of age.

The body weight differences were evident from the hatch, with the selected line weighing 11.65 g compared to 9.12 g in the non-selected control line. At one week of age (BW1), the selected line exhibited a mean body weight of 42.66 g, surpassing the non-selected Quail line, which weighed 28.07 g. The selection for higher egg weight during the first 10 weeks of laying resulted in variations in body weight among different quail lines.

Studies by Abdel–Tawab [15] and Baylan *et al.* [16, 17] highlighted the impact of body weight selection procedures on various parameters, including body weight, weight gain, feed intake, and feed efficiency. The selected lines in these studies consistently demonstrated higher body weights at hatch and throughout subsequent generations.

Additional studies by Dobalova [18], Ursu [19], and Brah, [20] revealed variations in body weight among quail lines selected for specific traits such as body weight, egg production, and gender. Notably, female Japanese quail consistently showed higher body weights than males across all ages.

The two-way analysis of variance indicated significant differences between the selected and non-selected lines, emphasizing the impact of selective breeding on body weight. The results align with the findings of Ibrahim [21], who observed variations in body weight between different Japanese quail breeds subjected to selective breeding over 50 years.

Furthermore, the feeding experiment involving five treatment groups (basal diet control, CH supplementation at 50 and 70 mg/kg diet, and NCH supplementation at 30 and 50 mg/kg diet) demonstrated distinct trends in body weight across the two quail lines. The significant differences observed at 14 days and 35 days underscore the influence of dietary supplements on body weight. El-Ashram [22] similarly reported significant increases in final body weight and weight gain with chitosan supplementation. Based on Duffy [23], the antimicrobial properties of low molecular weight chitosan have proven to be highly effective against both viruses and bacteria. In the agricultural sector, chitosan has been widely used in beef and dairy cattle feed to enhance rumen fermentation and improve digestibility, as noted in Gandra [24]. This has led to the development of numerous feed additives for animal production, aimed at enhancing productivity.

Researchers have found that low molecular weight chitosan can serve as a natural substitute for synthetic antibiotics, as mentioned in Hashem [25]. Moreover, Rikta [26] highlights the recent utilization of chitosan in nanotechnology for its antiviral effects. This has paved the way for its application in animal nutrition and health management, as the unique nanoparticles of chitosan have proven to be beneficial.

Multiple studies have demonstrated noteworthy improvements in animal performance with the dietary supplementation of chitosan for weaned piglets [28] and rabbits [29]. Furthermore, Suthongsa [5] points out that chitosan enhances growth and nutrient absorption in pigs when used as a dietary supplement. Likewise, Liu [30], Magalhaes [31] and Pereira [32] indicate that growing piglets, lambs, chicks, and quail all exhibit improved growth performance with the introduction of chitosan into their diets [22]. Table 2 provides a summary of the Live Body Weight (LBW) Least Square Means (LSM) and Standard Errors (SE) at different ages for both selected and non-selected Japanese quail lines. The differences in LBW between treatments within each line are visually represented in Figures 1 and 2, emphasizing the significance of dietary

supplementation and genetic selection in shaping the body weight characteristics of Japanese quails.

Evaluation of Gene Expression Activity:

In Figures 3 and 4, the gene expression levels of Catalase (CAT) were assessed in Japanese quails fed with different dietary supplements. In the JQ feed, supplementation with 70mg of chitosan (CH), 30 mg of nano-chitosan (NCH), and 50mg of NCH led to an increase in CAT gene expression levels, while the dietary addition of 50mg of CH resulted in a decrease compared to the control group. Similarly, in the SQ feed, CAT gene expression levels increased with 50mg of CH and 50mg of NCH, while the supplementation of 70mg of CH and 30mg of NCH led to a decrease. These outcomes align with recent research by Hashem, [25], emphasizing the emerging role of nanotechnologies in cattle production, including nano antibiotics, nanophotonics, nano hormones, and nano minerals. Additionally, the study concurs with Hassan [7], demonstrating increased antioxidative status in Japanese quails with nano-chitosan supplementation and elevated catalase (CAT) activity, a key enzymatic antioxidant in poultry.

In the evaluation of Superoxide Dismutase (SOD) gene expression levels in Figures 3 and 4, the JQ feed supplemented with 70mg of CH and 50mg of NCH exhibited increased SOD gene expression compared to the control group. Similarly, in the SQ feed, SOD gene expression levels rose with 50mg of CH, 30mg of NCH, and 50mg of NCH compared to the control group. The JQ feed supplemented with 70mg of CH and 50mg of NCH demonstrated higher SOD gene expression levels compared to the control group. Conversely, in the SQ feed, SOD gene expression levels increased with 50mg of CH, 30mg of NCH, and 50mg of NCH compared to the control group. These findings align with previous studies emphasizing the beneficial effects of chitosan as a dietary supplement in animal feed. Chitosan has been reported to aid the digestive system by eliminating toxins, addressing gastrointestinal ulcers, and alleviating chronic constipation in animals [5]. Additionally, Guan [6] highlighted the advantageous biological benefits of chitosan, including antibacterial, antioxidant, cholesterol-lowering, and immunomodulatory properties.

The study by Hassan [7] further supports the positive impact of chitosan and nano-chitosan on the antioxidative state in Japanese quails, with enhanced CAT activity. Xu [8] also contributed to the literature by demonstrating that supplementing with chitooligosaccharide during the late laying season can lower serum lipids, boost antioxidant activity, and enhance immunological function in hens. The present findings align with Wan [33], who reported improved total antioxidant capacity, SOD, and CAT activities in pigs fed with dietary chitosan. Specifically, pigs fed with 100 mg/kg of chitosan exhibited enhanced antioxidant capacity, and supplementing with 300 mg/kg of chitosan increased mRNA expression in weaned pigs.

In the Liver

Control Group (First Group): No histopathological findings were observed. The central vein showed dilation, associated with intact surrounding hepatocytes in the parenchyma (Fig. 7). Focal circumscribed round aggregation of lymphoid cells was detected in the parenchyma (Fig. 8).

Second Group (50 mg/kg diet): Dilatation of the central vein was observed, associated with fatty changes in hepatocytes (Fig. 10).

Third Group (70 mg CH): Diffuse fatty changes were observed all over the parenchyma (Figs. 12).

Fourth Group (30 mg NCH): Hyperplasia in the lining epithelium of bile ducts and few inflammatory cell infiltrations were observed in the portal area. Fatty changes in a few hepatocytes were also noted (Figs. 14, 15).

Fifth Group (50 mg NCH): Focal lymphoid cell aggregation in the hepatic parenchyma, massive inflammatory cells in the portal area, and dilatation and congestion of sinusoids were observed (Fig.17). These results indicate the impact of chitosan feed additives on increased body weight.

The findings suggest that chitosan, as a feed supplement, stimulates the release of digestive enzymes and lessens diarrhea in animals while improving intestinal morphology, villus structure, microbiota, and nutritional digestion and absorption (12,15,17)).

In the Intestine

Control Group (First Group): No histopathological alterations were observed. The normal histological structure of the lining mucosal epithelium, underlying lamina propria, submucosa, muscular, and serosa was observed (Fig. 9).

Second Group (50 mg CH): Diffuse goblet cell formation in the mucosal lining epithelium and focal inflammatory cell infiltration in the underlying lamina propria were observed (Fig. 11).

Third Group (70 mg CH): Diffuse inflammatory cell infiltration in the lamina propria of the mucosa was observed (Fig. 13).

Fourth Group (30 mg NCH): Massive inflammatory cell aggregation and infiltration in the

lamina propria of the mucosal layer were noticed (Figs. 16).

Fifth Group (50 mg NCH): Inflammatory cell infiltration in the lamina propria of the mucosal layer was observed (Fig. 18). These results are consistent with studies suggesting that chitosan and nano-chitosan improve the ratio of villus height to crypt depth, reduce crypt depth, and enhance the antioxidative condition in animals (10; 7; 37 As stated in Enoka [1], chitosan nanoparticles exhibit antibacterial efficacy comparable to fosfomycin, presenting a robust alternative to antibiotics for addressing Staphylococcus aureus and *Escherichia coli* (*E. coli*) infections. Consequently, chitosan nanoparticles emerge as a promising substitute for traditional antibiotics. In a separate investigation, [8] conducted a study where quails were fed a diet supplemented with 150 mg of chitosan, leading to a noteworthy reduction in *E. coli* levels compared to the control diet. Similarly [39] reported a substantial decline in the *E. coli* population and a simultaneous increase in Bacillus population in broilers fed diets containing 1 and 2 g/kg of chitosan. Additionally, chitosan demonstrates positive effects on microbiota, villus structure, and apparent nutrient digestibility in both ruminants and monogastric animals, as highlighted [31]. In summary, the results indicate that the relative expression of the CAT and SOD genes was recorded for all experimental groups of J. Quail and S. Quail lines. The histopathological findings in the liver and intestine of quail administered chitosan and nano-chitosan were described, including goblet cell formation, inflammatory cell infiltrations, fatty changes in hepatocytes, hyperplasia in the lining epithelium of bile ducts, and lymphoid cell aggregation. according to the histopathological changes, the result does not mean at all that the additives led to an improvement in the condition of the liver or intestines, but on the contrary, inflammation increased, which may ultimately harm the food absorption processes as well as conversion rates. Therefore, we need more than the researcher on additive level of chitosan and nano-chitosan.

Conclusion

The study reveals the impact of selective breeding on Japanese quails, showing a significant weight difference between selected and non-selected lines. Additionally, chitosan and nano-chitosan affect gene expression and improve digestive enzymes and intestinal health, providing valuable insights for optimizing breeding and dietary strategies in birds.

Ethical Considerations

This study was conducted in accordance with ethical guidelines for animal experimentation, and all procedures were approved by The Institutional Animal Care and Use Committee (ARC-IACUC) Agricultural Research Centre Approval Number ARC APRI 57 23.

Conflict of interest

The authors declare that there is no conflict of interest.

Funding statement

There is no financing.

Acknowledgement

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Authors Contribution

All authors read and approved the final manuscript.

TABLE 2. Live body weight (g) LSM and SE at different ages of both selected and non-selected J.quail lines.

Strain	Hatch	7 DAYS	14 DAYS	21 DAYS	28 DAYS	35 DAYS
S. QUAIL	11.65[a]	42.66[a]	113.18[a]	201.81[a]	287.75[a]	336.56[a]
J. QUAIL	9.12[b]	28.07[b]	68.83[b]	115.96[b]	167.50[b]	204.44[b]
SE	0.506	1.720	4.440	7.752	11.106	13.198
Probability	0.00001	0.00001	0.00001	0.00001	0.00001	0.00001

a.b Means, within trait, with different letters, differ significantly ($p \leq 0.05$) from each other. N=420 per line.

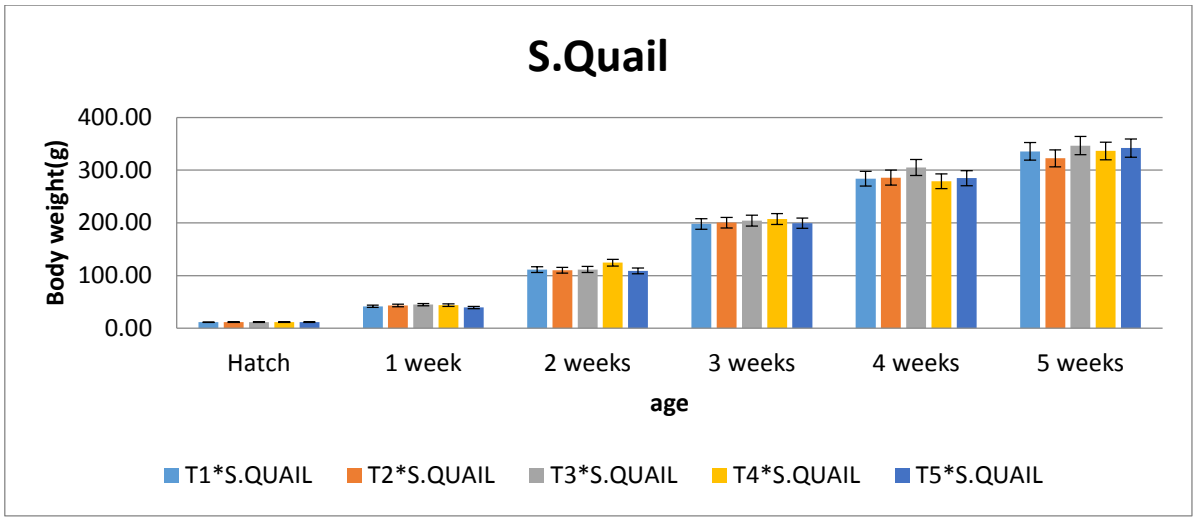

Fig. 1. Least Square Means and SE of LBW for 5 treatments in S. Quail line.

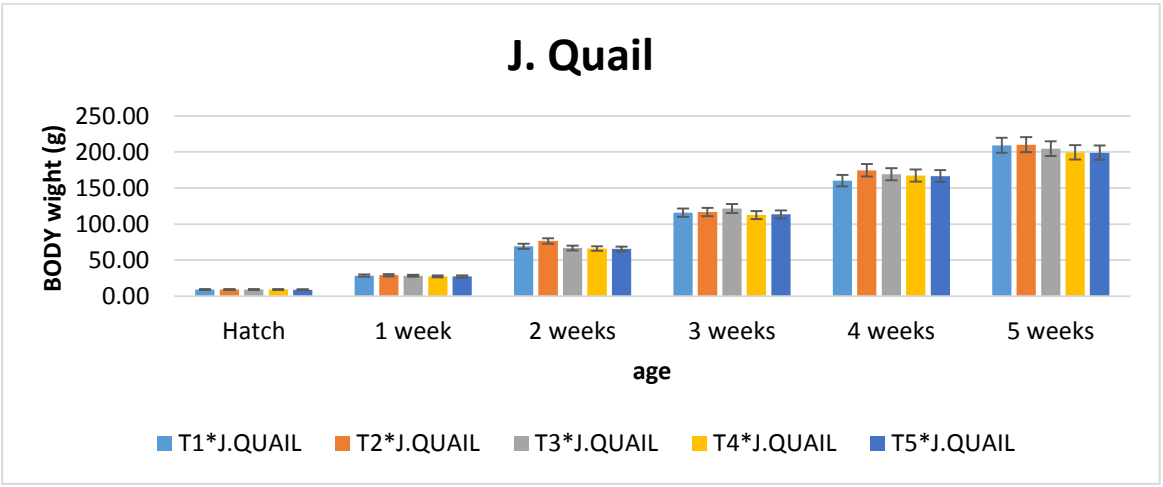

Fig. 2. Least Square Means and SE of LBW for 5 treatments in J. Quail line.

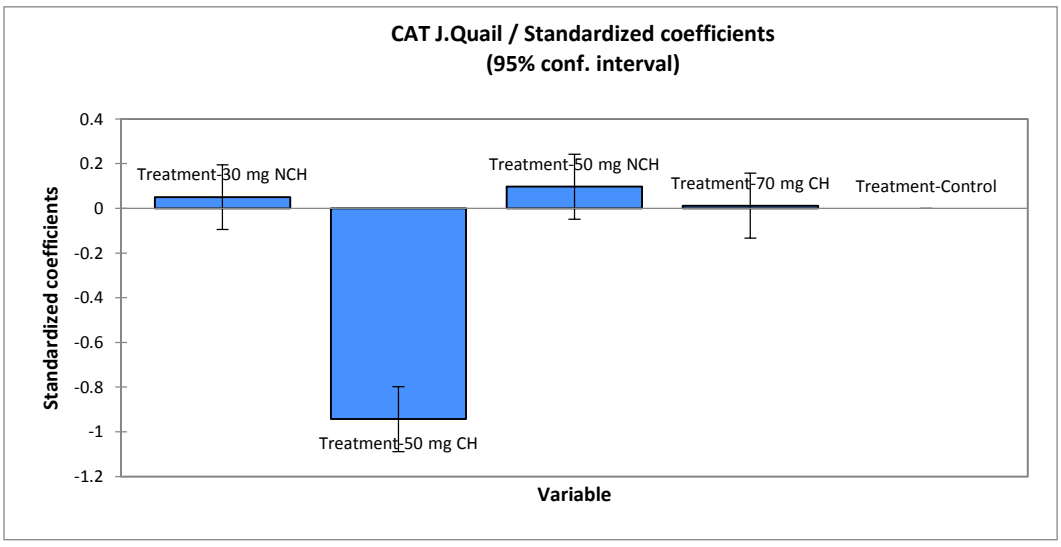

Fig. 3. Means of relative expression for CAT gene recorded for all experimental groups of Jq.

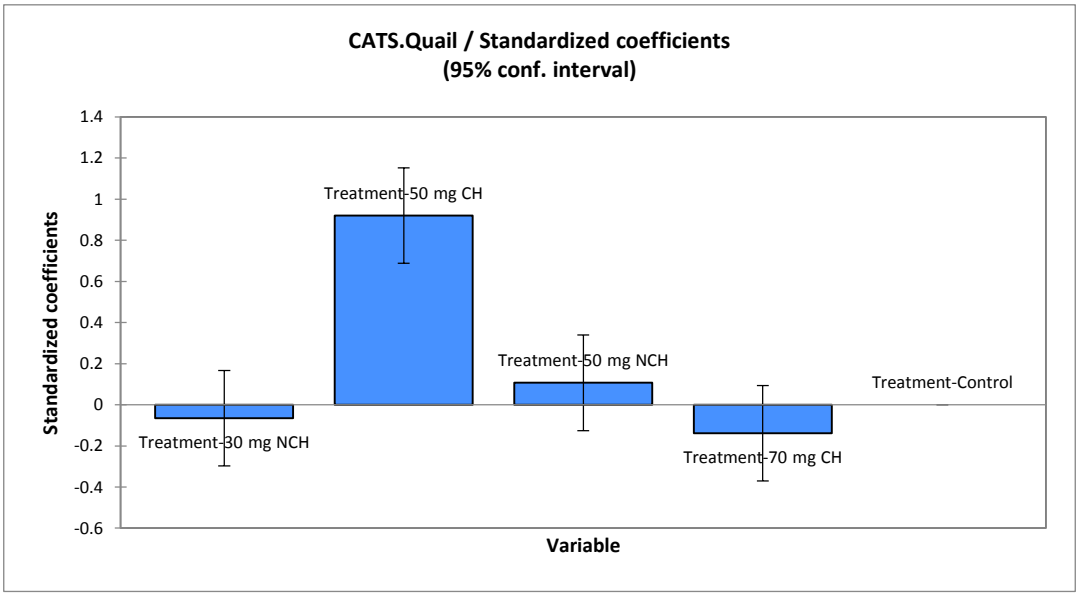

Fig. 4. Means of relative expression for CAT gene recorded for all experimental groups of Sq.

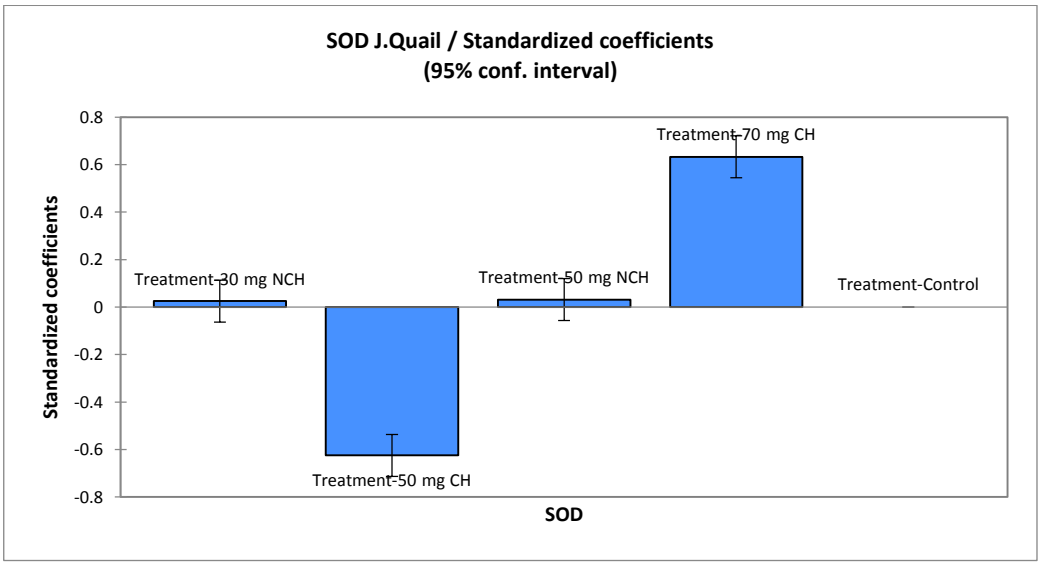

Fig. 5. Means of relative expression for SOD gene recorded for all experimental groups of Jq.

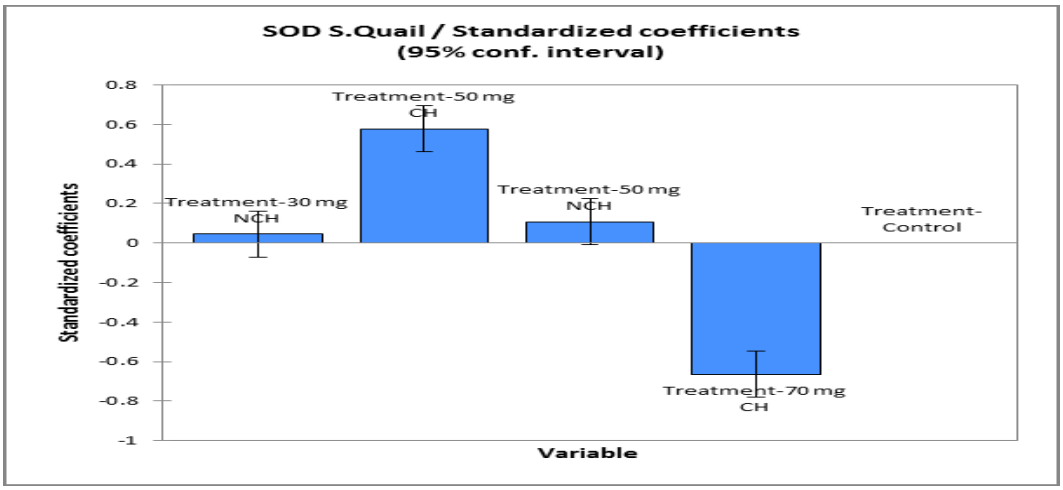

Fig. 6. Means of relative expression for SOD gene recorded for all experimental groups of Sq.

Histopathological Findings.

1. Group (1) of quail kept as control

Liver **Intestine**

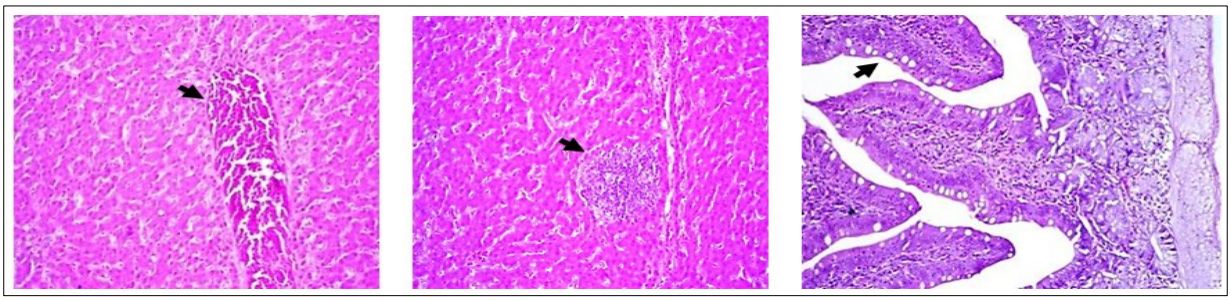

Fig.7. There was no histopathological findings and the central vein was dilated associated with intact surrounding hepatocytes in the parenchyma (arrow). H&E X 40.

Fig. 8. Focal circumscribed round aggregation of lymphoid cells was detected in the parenchyma (arrow). H&E X 40.

Fig. 9. There was no histopathological alteration and the normal histological structure of the lining mucosal epithelium with active goblet cells(arrow) and the underlying lamina propria, submucosa, muscularis and serosa were recorded. H&E X 40.

2. Group (2) of quail administrated 50 mg chitosan

Liver **Intestine**

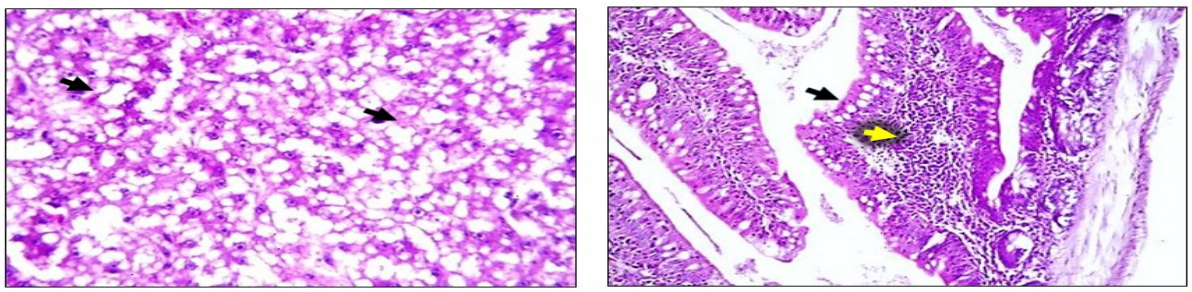

Fig.10. Severe diffuse fat infiltration in the hepatocytes of most of hepatic cords (arrows). H&E X 100.

Fig. 11. Diffuse and active goblet cells formation was observed in the mucosal lining epithelium (black arrow) while the underlying lamina propria showed focal inflammatory cells infiltration (yellow arrow). H&E X 40.

3. Group (3) of quail administrated 70 mg chitosan

Liver

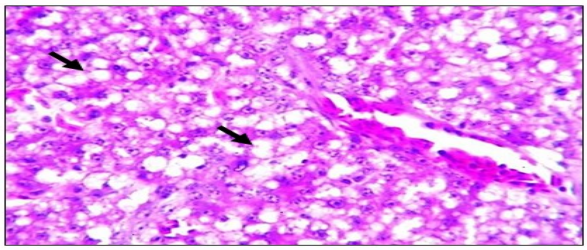

Fig. 12. The hepatocytes showed fatty change in diffuse manner all over the parenchyma (arrows). H&E X 100.

Intestine

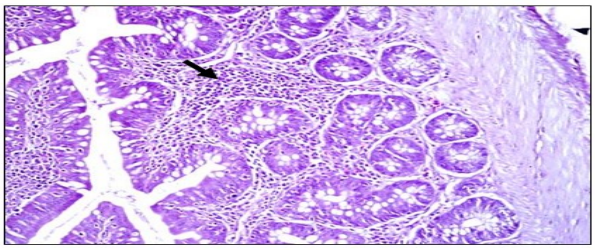

Fig. 13. The lamina propria of the mucosa showed diffuse inflammatory cells infiltration (arrow). H&E X 40.

4. Group (4) of quail administrated 50 mg nano-chitosan

Liver

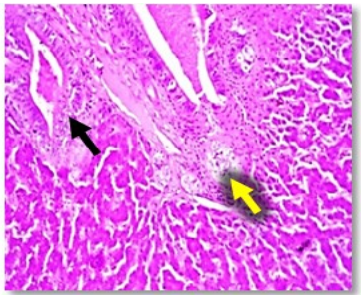

Fig. 14. Hyperplasia in the lining epithelium (black arrow) of the bile ducts with few inflammatory cells infiltration was detected in the portal area (yellow arrow). H&E X 40.

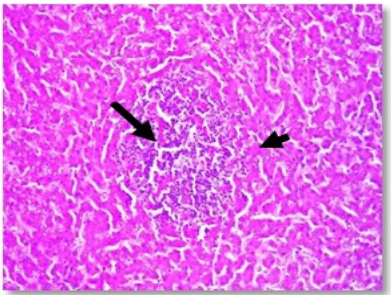

Fig. 15. There was focal aggregation of the inflammatory cells among the mild degenerated hepatocytes (arrow). H&E X 40.

Intestine:

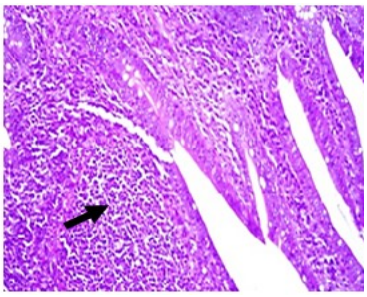

Fig. 16. Massive inflammatory cells infiltration was noticed in the lamina propria of the mucosal layer (arrow). H&E X 40.

5-Group (5) of quail administrated 70 mg nano-chitosan

Liver

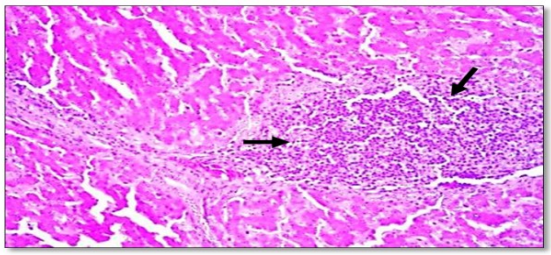

Fig. 17. The hepatic parenchyma showed focal lymphoid cells aggregation (arrow). H&E X 100.

Intestine

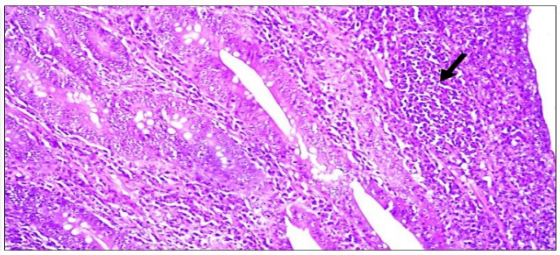

Fig. 18. The lamina propria of the mucosal layer showed inflammatory cells infiltration (arrow). H&E X 40.

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تأثير إضافه الشيتوزان والنانوشيتوزان كمواد مضاده للاكسده علي التعبير الجيني في خطين من السمان الياباني

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أُجريت هذه التجربة لدراسة تأثير إضافة الشيتوزان والنانوشيتوزان كمضادات للأكسدة على الأداء الإنتاجي والتعبير الجيني للسمان الياباني. تم استخدام 840 كتكوتًا في اليوم الأول من خطين مختلفين من السمان الياباني، حيث تم اختيار الخط الأول بناءً على وزن الجسم عند عمر 30 يومًا بعد 42 جيلًا من الانتخاب ، في حين كان الخط الثاني كمجموعة تحكم غير مختارة. تم تقسيم الكتاكيت إلى 5 مجموعات، حيث كانت المجموعة الأولى تتناول العليقة الأساسية بدون إضافات (مجموعة التحكم). بينما تمت إضافة 50 و 70 مجم/كجم من الشيتوزان إلى المجموعتين الثانية والثالثة على التوالي، وتم إضافة 30 و 50 مجم/كجم من النانوشيتوزان إلى المجموعتين الرابعة والخامسة على التوالي لكل من الخطين.تم تربية الطيور في أقفاص تربية مع توفير الماء والعلف بشكل حر طوال فترة التجربة، وكانت فترة الإضاءة على مدار 24 ساعة يوميًا. أظهرت النتائج زيادة في وزن الجسم في المجموعات التي تمت إضافة مضادات الأكسدة للخط المنتخب مقارنةً بالخط الغيرمنتخب . كما أظهرت زيادة في التعبير الجيني للكاتاليز في الخط الغيرمنتخب وعند تناول 30 و 50 مجم من النانوشيتوزان بالمقارنة مع المجموعة التحكم. وسجلت زيادة في مستوى (SOD) في الخط الغير منتخب المضاف إليه 70 و 50 مجم/كجم عليقة مقارنة بالمجموعة التحكم. توصي الدراسة بإضافة النانوشيتوزان إلى عليقة السمان الياباني بتراكيز 30 أو 50 مجم/كجم عليقة، حيث أدت إلى تحسين في وزن الجسم وزيادة في مستويات الإنزيمات المضادة للأكسدة SOD .

الكلمات الدالة : السمان الياباني ، الشيتوزان ، النانو-شيتوزان ، SOD، CAT.