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Copper chitosan nanocomposite as antiviral and immune-modulating effect in broiler experimentally infected with chicken anemia virus

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Abstract

In the last few years, inorganic nanosystems, or nanometals, were of great interest to conventional therapy. In this study, Copper Chitosan Nanocomposite (CuCNP) was monitored for its antiviral, immune-stimulant, and lowering agent of Cu residue roles by using Chicken Anemia Virus (CAV) as a model. CuCNP is a metallic oxide nanocomposite with specific properties, such as sphere shape, no aggregation, and narrow size distribution 24.71±1.68 nm PdI: 0.691±0.02. We grouped 100 broiler chicks into four groups. Group 1 served as a regular negative control group. In drinking water, G2 was treated with CuCNP 1 mg/ml for five days. The G3 was infected with 0.2 ml I/M of CAV strain (MN339532) at one day old with CuCNP 1 mg/ml in drinking water for five days. G4 virus-positive control group with viral 3.987×10^6 virus copies/ml. Different serum and organ tissue homogenate samples were collected at different time intervals to measure residues, CAV viral concentration in organs, and serum to monitor cellular and humoral immunity. The excellent results of CuCNP are improving the innate immune response phagocytic activity, lysozyme, and NO, also cytokine levels mRNA of IFN-γ, IL-6, and IL-10 in G2 and G3 and elevating CAV antibody titers with decreases the CAV viral load in organs with a noticeable decrease of its residues in G2 and G3. The current study provides evidence of the immunostimulatory effect of CuCNP on CAV infection. It clarifies a constant reduction of CuCNP residues in broilers muscle and liver tissues, keeping its levels below Cu maximum residual limits (MRLs).

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Introduction

Copper is considered an essential element of animals' diet; cellular metabolism and enzyme activity are highly dependent on copper. Cu-Zn superoxide dismutase, cytochrome C oxidase, tyrosinase, and lysyl oxidase are involved in various essential processes for proper animal growth and maturation. Cu supplementing diets with organic and inorganic carbon-containing sources significantly impacts the overall production of poultry (1). The poultry industry is a base rock of the pillar economy. Thus, immunosuppressive diseases cause the most affecting factors of economic collapse. Chicken Anemia Virus (CAV) is one of the essential immunosuppressive agents affecting the poultry industry worldwide. The virus causes aplastic anemia and lymphoid atrophy, particularly in newly hatched chicks (2). CAV has been classified recently as one of the family *Anelloviridae*, genus *Gyrovirus* (3). The bone marrow hemocytoblasts and the thymus precursor lymphocytes are the significant targets cells for this virus. This disease can be spread vertically and horizontally due to clinical and subclinical infections, respectively (4). The top stage of anemia infection involving the thymus, the lymphoid organs, bursa of Fabricius, and spleen suffer from severe atrophy (5).

Nanomedicine is a new area of science combining nanotechnology with drugs, and it develops perfect techniques facing conventional therapy (6). Nanometal is one of the inorganic nanosystems. This could be attributed to their smaller size, penetrative ability to biological membranes, high physiological solubility, and physicochemical properties (7). Broiler chicken supplementation with copper chitosan nanoparticles improved growth performance and immunologic ability and enhanced the intestinal microbiota in addition to protein synthesis (8). Likely, copper nanoparticles supplementation in drinking water improves hematological profile, a pro-inflammatory cytokine, oxidant/antioxidant balance, behavioral patterns (ingestion, resting, movement activities, and comfort behavior), and productive performance (weight of the body, weight gain, intake of food and ratio of feed conversion) of broilers (9). The microorganism's cell membrane interacts with metal NPs, especially copper nanoparticles, resulting in an antimicrobial effect. The copper NPS action mechanism damages the interaction of enzymes and -SH groups. However, this leads to the production of oxidative stress (10). The virus deactivates through copper oxide nanoparticles and copper alloy surfaces due to the Cu^{2+} ions, indicating the effective inhibition of viral entry and replication, mRNA, and capsid protein degradation along the viral life cycle. Eventually, copper and copper complexes were found to have beneficially antiviral efficacy of ROS-mediated virus death, Cu complex, and Cu-chelation (11). Multiple factors control copper residues in the organs, such as the quantity of copper ingested, the interval of exposure, animal age, and breed (12). CuNPs are highly digestible in acidic environments than CuS. Meanwhile, the extended effect of CuNPs is attributed not only to the antimicrobial or antiviral action but also to Cu absorption and accumulation in different body organs (13).

The objective of the present study is to investigate copper chitosan nanocomposites' role as antiviral (decrease the CAV viral load) and immunomodulatory impact (like phagocytic activity, IFN- γ, IL-6, and IL-10 and CAV serum antibodies level) on challenged chickens with CAV and following up on its residues in different organs.

Materials and methods

Ethical statement

The Research Ethics Committee approved the animal studies for Environmental and Clinical Studies (protocol number:165569) at the Animal Health Research Institute (AHRI). They were performed according to the Guidance of the Egyptian Ethics Committee in compliance with the NIH Guidelines for Care and Use of Laboratory Animals.

Chemicals and reagents

Chitosan (medium molecular weight with 90% deacetylation), copper sulfate ($CuSO_4 \cdot 5H_2O$), and acetic acid were obtained from Sigma-Aldrich-USA. Copper nitrate ($Cu(NO_3)_2$) standard in HNO_3, 0.5 mol/l, 1000 mg/l was obtained from Merck, Germany. Acetonitrile (ACN) and methanol (MeOH) were acquired from HPLC grade (Fischer, Canada). All other chemicals and reagents were of analytical grade. *Candida albicans* (*C. Albicans*) was obtained from the Department of Mycology, AHRI. *Micrococcus lysodeikticus* bacteria, agarose, and Roswell Park Memorial Institute media 1640 media (RPMI) with L-Glutamine, fetal calf serum (FCS) was purchased from Sigma-Aldrich Co (Germany).

Preparation method

Preparation and characterization of copper chitosan nanocomposites (14). Briefly, ninety milliliters of copper sulfate solution (10 mM) were mixed with chitosan 0.2 g in 0.04% acetic acid (10 mL) and sonication for 30 min.

Cu-chitosan nano-composites characterization

The nanocomposite was characterized using Fourier Transmittance Infrared FT/IR-6100 Spectrometer and TEM (Transamination Electron Microscopy) ModelJEol JSM-6400 and done through Zeta sizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK).

Cytotoxicity evaluation of Cu-chitosan nanocomposites

Cytotoxicity of Cu-chitosan nanocomposites was evaluated using an African green monkey kidney cell (Vero cells) obtained from the Viscera (15). The used assay was sulforhodamine B (SRB) (16).

Experimental design

The experimental chicks were equally divided into four groups after the random examination of about five to ten chicks to the presence of CAV. The 1st group (g1) was a standard negative control group (untreated non-infected). The 2nd group (g2) was supplied at one day old with Cu-chitosan nanocomposite (1mg/ml) in drinking water for five days, which was added again after five days of rest (treated non-infected group). The 3rd group (g3) was inoculated at one day old with 0.2ml I/M of CAV strain (MN339532) After 24 hours post-infection, Cu-chitosan nanocomposite was added (1mg/ml) in drinking water for five days, followed by a five-day rest. Then, the treatment was repeated (treated infected group). For the 4th group (g4), chicks were inoculated one day with 0.2ml /chick I/M from the supernatant (organ suspension). They were prepared according to (17) from filed strain named Chicken anemia virus isolate Shark/1 (VP2) gene, Gen Bank: MN339532 with virus copies at 3.987×10^6 virus copies/ml, which served as the virus-positive control group (untreated infected group). They were then kept in the Avian Virology unit, Poultry Diseases Department, Animal Health Research Institute, Dokki, Giza, was used for experimental infection purposes.

Experimental birds

there is a total of one hundred and twenty commercial broiler chicks of one-day-old age free from CAV by PCR examination. According to the vaccination program, the experimental birds were housed separately in environmentally controlled isolation units (Hitchner B1vaccine at seven days old; LaSota vaccine at 14 days old, and Gumboro vaccine at 19 days old, all in drinking water) (18). Feed and water supplementation were given ad libitum until the end of the experiment.

Samples collection

Heparinized blood samples were collected for monocyte and phagocytosis assay for the seven days, 21, and 35 post-viral infections (dpi). Serum samples from chicks in groups 3 and 4 on 7, 14, 21, 28, 35, and 42 dpi were collected for an ELISA test and from all groups to measure lysozyme, NO, and copper serum levels. Three chicks from each group were used for necropsy.

Spleen samples were collected on the 7^{th} and 14^{th} dpi for IFN-γ, IL6, and IL10gene expression. We extracted RNA for Real-Time (RT) PCR for relative quantification of mRNA. The liver, liver, spleen, bone marrow, and thyroid organs were collected at 14^{th}, 21^{st}, and 42^{nd} dpi from group 3 (CAV-infected group and inoculated with copper chitosan) and group 4 (CAV-infected group) to detect CAV by RT-PCR (19). Liver and muscle tissues (3 chicks per group) were collected on the 1^{st}, 7^{th},21^{st},35^{th}, and 42^{nd} days post-medication to detect copper residue concentration by UV RP-HPLC. Tissue samples were kept at -70^0C till the analysis (20).

Detection of virus load

The CAV load was demonstrated through quantitative real-time PCR. The tissue's suspension was prepared for DNA extraction (21). Tissue suspension supernatants were treated with the PathoGene-spin™ DNA/RNA Extraction Kit following the manufacturer's instructions (iNtRON Biotechnology, Seongnam, Korea). CAV gene has been amplified by using its specific oligonucleotide sequence primers. The real-time PCR data acquisition and analysis were performed using the analysis computer system, V 2.2.2 software (AB Applied Biosystems) (22).

Assessment of the innate immune response

This was by the estimation of the following (1) According to Markowski-Grimsrud *et al.* (23), Boyum (24), Anthony *et al.* (25), the phagocytic activity of chicken peripheral monocytes and measuring serum nitric oxide was carried out on all serum samples (2,26), Lysozyme Assay was measured by agarose gel plate lysing assay (27).

Quantification of mRNA for IFN- γ, IL-6, and IL-10 by RT-PCR

RT-PCR evaluated the cytokine mRNA expression level. Their values were normalized to the endogenous control (28), and the fold changes in the target genes were determined using the $2^{-\Delta\Delta C}$ method (29).

Measurement of serum antibodies to CAV

a commercial ELISA kit for CAV, Biochek Company (Lot No. BPT431) was used to measure the antibody titer against CAV in infected groups (3 and 4).

Residue analysis

Copper serum level determination using BIOMED® kit spectrophotometry. Copper tissue residues were analyzed by the HPLC method. HPLC Agilent Series 1200 quaternary gradient pump, Series 1200 autosampler, Series 1200 UV visible detector, and HPLC 2D Chemstation software (Hewlett-Packard, Les Ulis, France) were used. Agilent C18 column (4.6mm i.d, 150mm, 5µm particle size) was utilized for chromatographic separation. Chromatographic conditions were adjusted (20). Briefly, the UV detector was set at 310 nm. The elution mixture consisted of ACN: ammonium acetate (50 mM) with 0.5 mg/ml EDTA (30:70 v:v) as the isocratic mobile phase. The flow rate was 1 ml/min with a specific retention time of 4.955 min. Sample preparation extraction was performed as the former (20). The calibration curve was set as a linear correlation between copper concentration and Area under the Curve (AUC) resulting from spiking blank tissue samples. The linear correlation was ranged within eight concentrations 0.1-15 µg/ml of the fortified solution by diluting with 1% nitric acid to ascertain the actual concentration of Cu in Cu-chitosan nanocomposite. The validation process and quantification were calculated, respectively (20,30).

Statistical analysis

The obtained results were statistically analyzed using IBM SPSS. Results were expressed as mean values±Standard Error (SE) and compared by one-way ANOVA ($P \leq 0.05$) and independent t-test.

Results

Cu-NPs Characterization: Particle Size, Morphology

TEM was used for determining the size and morphology of the Cu-chitosan nanocomposite. TEM showed sphere shape, no aggregation, and narrow size distribution 24.71±1.68 nm with a polydispersity index (PDI):0.691±0.02, indicating the remarkable homogeneity can be realized as demonstrated in (Figure 1).

Zeta potential

The zeta potential indicates unstable and stable suspensions. It is generally taken by using Dynamic Light Scattering (DLS). The zeta potential results illustrated that Cu-chitosan nanocomposite had a -12.93±0.67mV, viscosity 0.8872 cp (centipoise), and conductivity 0.0585 ms/cm, which were measured at pH 5. The concentration of copper in the final solution was 3000 mg/liter measured by atomic

absorption in the Central Laboratory at Ain Shams University and High-Performance Liquid Chromatography (HPLC) at the Animal Health Research Institute (AHRI).

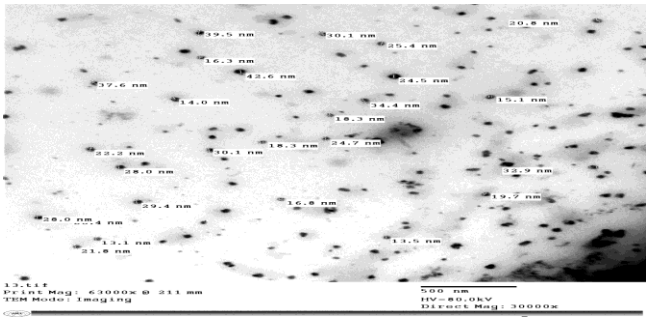


Figure 1: TEM of Cu-Chitosan nanocomposite showed 24.71±1.68 nm sphere shape and no aggregation (Central lab. in NRC). Direct Mag. 30000x

Chemical interaction

Fourier Transform Infrared (FTIR) spectroscopy analysis is a distinctive molecular fingerprint and detector of functional groups in compound comparison with pure compounds. FTIR analysis was conducted to determine the molecular interactions between chitosan and copper, as shown in figure 2. FTIR analysis showed the presence of bands at 3449.14cm^{-1} due to the overlap of O-H, 1627.91 cm^{-1} (NH_2 bending), 1383.89 cm-^{1} (C-H bending), 1198.41 cm^{-1}, and 1160.01 cm^{-1} (C-O-C stretching), while the values 995.15 and 961.06 cm^{-1} represented C-O ring skeletal stretching. The peaks at 660.61 and 603 (Cu-chitosan interaction), 585.61, and 516.9 cm^{-1} (N-H) were new exciting peaks in the fingerprint region in which Cu-O and Cu-N coordinated bound evidence for the interaction between copper and chitosan. Thus, NPS was capped by the polymer, as shown in (Figure 2).

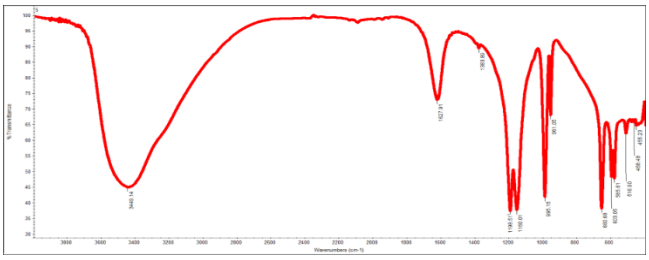


Figure 2: FT-IR spectra of Cu-Chitosan nanocomposite.

Cytotoxicity

On the confluent surface of Vero cells, specific concentrations of Cu-chitosan nanocomposites (0.01, 0.1, 1, 10, and 100 ug /ml) were inoculated. The effect on cell viability percentage was assessed after three days of inoculation by sulforhodamine B(SRB) assay. The viability percentage was 94.02% in 100 µg/ml and IC50> 100 µg/ml (Figure 3).

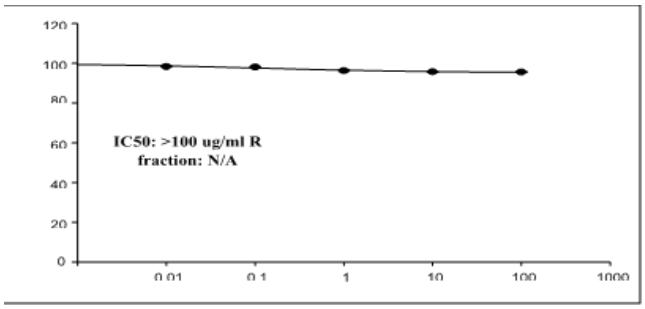


Figure 3: Viability percentage was assessed by SRB assay.

Real-Time PCR Results

The randomly examined chicks' one-day-old were CAV-free. Chicks were injected I/M by CAV at a one-day-old. The viral copies/ml was 3.987×10^{6} virus copies/ml/chick. The virus copies were statistically reduced ($P<0.05$) at 21 and 42 DPI in G3 than G4 (Table 1).

Table 1: The viral copies/ml in infected groups 3 and 4. by RT-qPCR (n=5)

Age/ Day	Group 3	Group 4
14 days	$5.295\pm0.02\times10^{3}$	$4.125\pm0.01\times10^{5}$
21 Days	$3.99\pm0.1\times10^{3*}$	$4.245\pm0.01\times10^{6}$
42 Days	$2.00\pm0.08\times10^{3*}$	$5.00\pm0.07\times10^{8}$

*The data are significant difference using independent t-test at $P<0.05$, group 3 (CAV infected group and inoculated with copper chitosan, group 4 (CAV infected group).

The phagocytic activity of peripheral blood monocyte cells

As shown in table 2, the highest amelioration was recorded in broilers treated with CU-CNPs (G2) in phagocytic percentage on 7 and 35 dpi and the phagocytic index at all-time intervals. On the contrary, the CAV infected group (G4) had reduced phagocytic percentage at all-time intervals and in the phagocytic index on seven dpi. Concerned records were presented in G3 (CU-CNP+CAV). It improved phagocytic percentage and index close to the values of the standard control group (untreated non-infected group) (G1). Moreover, records in G3 were reduced significantly than G2 in all intervals and increased significantly than G4 in phagocytic percentage at 7, 21, and 35 dpi and the phagocytic index at seven dpi.

Serum lysozyme and nitric oxide levels

Serum lysozyme and NO records were given in table 3. CU-CNP-treated group (G2) induced the highest levels at all-time intervals. At the same time, the reduction in lysozyme and NO levels was recorded in CAV-infected group (G4). CU-CNP treated with CAV-infected group (G3) modulated the deteriorated virus effect in G4 to a near the extent of the average values untreated non-infected group (G1).

The level of mRNA Gene Expression of IFN-γ, IL-6, and IL-10

Expression levels of transcripts for the signature pro-inflammatory (IFN-γ and IL-6) and the anti-inflammatory cytokine (IL-10) were evaluated by RT-qPCR as in (Figures 4 a-c). The used sequence primers are listed in (Table 4). The CU-CNP (G2) induced significantly higher mRNA of IFN-γ on 7, 14, and 21 dpi than the other groups and significantly higher levels than G4 of IL-6 and IL-10. While in CU-CNP + CAV (G3), all cytokine levels were increased than G4 at all-time intervals and approached the values of the CU-CNP group (G2).

Serum antibody titers to CAV

The used chicks were commercial broiler chicks of one-day-old age free from CAV by PCR examination. So, they were free of maternal antibodies against CAV. The antibody titers were evaluated to determine the efficacy of Cu-CNPs against CAV. As illustrated in (Table 4), antibody titers were significantly reduced in G3 than G4 on days 28 and 35 post-infection.

Plasma Cu-CNPs measurement by spectrophotometry

Plasma Cu concentration was significantly higher in the treated groups (G2 and G3) than in G1 and G4 and the other weeks of age, as shown in (Table 5). Analysis of variance of the result shows that G2 attained a much higher plasma Cu level than G3 except on day 21.

Table 2: Phagocytic activity of peripheral blood monocytes cells

Phagocytic parameters	Days post CAV infection	G1	G2	G3	G4
Phagocytic %	7	62.67±1.84^{b}	71.67±2.21^{a}	58.67±1.39^{b}	50.00±2.03^{c}
	21	67.33±4.41^{ab}	74.33±3.53^{a}	66.33±1.73^{b}	51.33±2.5^{c}
	35	68.00±3.41^{b}	76.66±6.25^{a}	68.33±5.69^{b}	59.67±3.5^{c}
Phagocytic Index	7	1.75±0.13^{b}	2.51±0.14^{a}	1.63±0.12^{b}	1.31±0.09^{c}
	21	1.86±0.07^{b}	2.96±0.12^{a}	1.89±0.11^{b}	1.46±0.2^{b}
	35	1.79±0.09^{b}	2.67± 0.17^{a}	1.72±0.21^{b}	1.62±0.2^{b}

Data are presented as mean ±SE. Means with different superscript small letters indicate significantly different at the same row between groups at P<0.05 using one-way ANOVA test. G1 control negative, G2 with copper chitosan group, 3 (CAV infected group and inoculated with copper chitosan), group 4 (CAV infected group).

Table 3: Serum lysozyme and nitric oxide levels

Phagocytic parameters	Days post CAV infection	G1	G2	G3	G4
Lysozyme (µg/ml)	7	14.90±2.1	15.90±3.77	14.02±2.3	13.70±2.01
	21	33.10±4.79^{b}	41.30±5.05^{a}	28.6±3.96^{bc}	24.10±2.46^{c}
	35	48.67±7.26	50.77± 10.2	47.95±11.4	47.20±6.3
Nitic oxide (µmol/ml)	7	6.91±0.06^{b}	7.69±0.35^{a}	6.5±0.56^{b}	6.23±0.47^{b}
	21	8..97±0.47^{a}	9.79±0.48^{a}	8.77±0.5^{a}	6.97±0.46^{b}
	35	12.02±0.15	12.50± 0.3	11.96±0.4	11.27± 0.5

Data are presented as means±SE. Means with different superscript small letters indicate significantly different at the same row between groups at P<0.05.

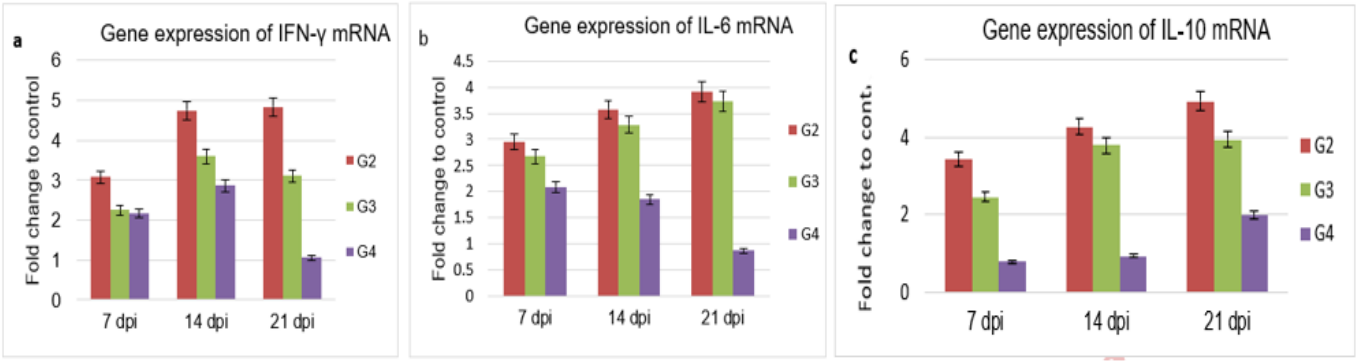


Figure 4: RT-qPCR for evaluation of mRNA expression of (a) IFN-γ gene; (b)IL-6 gene, and (c) IL-10 gene in broiler spleens.

Table 4: Geometric Mean (GM) of ELISA antibody titers in serum of the examined groups

Age/ Day	Group 3	Group 4
7 Days	341±16.3	755±11.5
14 Days	762±15.6	1215±18.9
21 Days	978±7.7	1786±8.9
28 Days	1034±16.1*	2154±49.1
35 Days	1419±9.5*	2532±27.9
42 Days	1726±32.4	2941±24.1

*The data are significant differences using the independent t-test at P<0.05. group 3 (CAV infected group and inoculated with copper chitosan, group 4 (CAV infected group).

Tissues CuCNPs detection by HPLC

Residue depletion of Cu in all tested groups was detected after treating Cu chitosan at 1mg/ml in drinking water for five consecutive days in G2 and G3. Under the specified HPLC conditions, Cu was determined as specific chromatograms in both liver and muscle, as shown in (Figures 5). There was a significant decrease in Cu residues of G3(Cu-chitosan + CAV) than G2 (Cu-chitosan) in liver tissues but significantly higher than G1(untreated non-infected group) and G4 on the 1st day,7th day, 21st day, 35th day, and 42nd days after the end of the treatment course. Moreover, the detected concentrations in G2 (Cu- chitosan) were increased significantly than other comparable groups throughout the experiment period, as detailed in (Figure 6).

On the other hand, the observed Cu residues concentrations in muscle tissues were significantly increased in G2 (Cu-chitosan) than the other comparable groups on different examination days. At the same time, G3 (Cu-chitosan + CAV) showed a significant decrease in Cu residue concentration than G2 (Cu-chitosan) in all tested days. Otherwise, a considerable increment in Cu level in G3 than G1 (untreated non-infected group) and G4 (CAV) on the 1st, 7th and 21st days of examination, but there were insignificant changes on the 35th and 42nd days (Figure 7).

Overall, Cu levels in G1 and G4 in liver and muscle tissues revealed no significant changes in all tested days. The mentioned results referred that CAV affects Cu residue levels in both liver and muscle tissues.

Table 5: Effect of Cu-Chitosan nanocomposite on plasma Cu concentration (Mean±SE) level (ppm) during the experiment

Age/ Day	G1(control)	G2(Cu-chitosan)	G3(Cu-Chitosan+CAV)	G4 (CAV)
14 Days	0.521±13.93^{b}	1.365±5.09^{a}	0.906±19.13^{b}	0.715±12.29^{b}
21 Days	0.773±21.11^{c}	1.219±1.69^{b}	3.259±12.39^{a}	0.526±11.27^{c}
28 Days	0.446±6.79^{c}	4.136±14.42^{a}	3.753±4.423^{b}	0.313±5.53^{c}
35 Days	0.426±3.56^{c}	4.436±10.46^{a}	3.913±7.35^{b}	0.485±8.04^{c}

Values in the same column with different superscripts are significantly different at P ≤ 0.05.

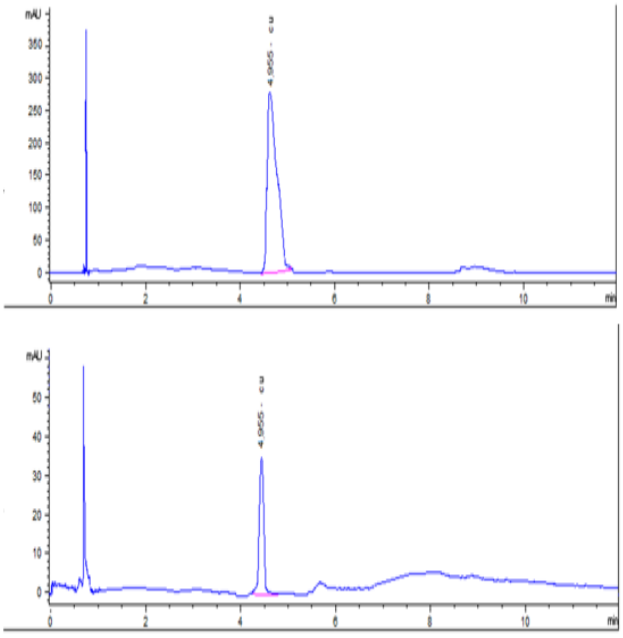


Figure 5: Chromatogram of Cu residues in the extracted liver sample with concentration 4.473 µg/g.

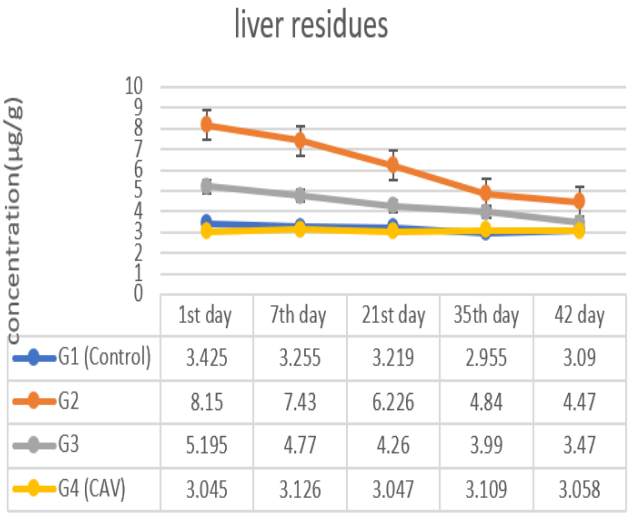
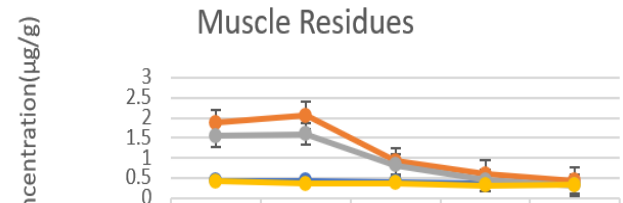


Figure 6: Effect of Cu-Chitosan nanocomposite on Cu liver residues concentration (µg/g) (Mean± SD) post-treatment.



	1st day	7th day	21st day	35th day	42 day
G1 (Control)	0.435	0.439	0.397	0.356	0.363
G2	1.875	2.059	0.922	0.601	0.438
G3	1.554	1.59	0.826	0.445	0.319
G4 (CAV)	0.414	0.357	0.374	0.313	0.326

Figure 7: Effect of Cu-Chitosan nanocomposite on Cu muscle residues concentration (µg/g) (Mean± SD) post-treatment.

Discussion

Nanotechnology has a wide range of applications in the poultry industry. Copper (Cu) NPs are the most studied SNPs in poultry field research, followed by zinc, zinc oxide, gold, silver, and selenium NPs, and then, to a lesser extent, chitosan and chromium NP formulations. The nanomaterials may be used as antimicrobials (antibacterial, antiviral, antiparasitic, and antifungal) and anti-mycotoxin agents, vaccine preparations, immune-stimulation, disinfectants, and product promotion (32,33).

The viral nanoparticles are a new and highly interdisciplinary field in which Viral Nanoparticles (VNPs) are used in various applications, including electronics, energy, and next-generation medical devices (34). The virucidal efficacy of gold/copper sulfide core-shell nanoparticles (Au/CuS NPs) against human norovirus (HuNoV) are variable, with viral capsid protein degradation and capsid damage appearing to be the mechanisms associated with inactivation (35).

In the present study, the Cu-chitosan nanocomposite was the first antiviral study on CAV, and it evoked a significant response. It has antiviral activity, immune-stimulant effect and was caused by lowering Cu residues in poultry. This may be attributed to their unique physicochemical characteristics, including the small size 24.71±1.68 nm, increasing their endocytic uptake by the GIT, and increasing their efficacy than bulk Cu at lower doses (36). Furthermore, their spherical shape, even with similar sizes (PDI: 0.691±0.02), helped them enter cells rapidly and quickly (37). In addition to their high negative charge -2.93±0.67mV, those can favor good stability since the Columbia repulsion forces arise from their surface charge. These forces overcome the Van der Waals attractive forces between them and prevent aggregation (38).

Based on this newly discovered type of host-virus interaction, the molecular mechanisms of this enigmatic interplay between viruses and cellular systems that manage Cu^{2+} or Cu^{1+} flux. Furthermore, ROS production was associated with the viral infection during the host-virus interaction, specifically with the induction of hallmarks of programmed cell death during the lytic phase of infection. Inhibition of ROS production by applying a peroxidase inhibitor or an H_2O_2 scavenger prevented host cell death and reduced viral production (39,40).

In this study, we evaluated the role of the prepared Cu-chitosan nanocomposite as an immune-stimulating compound to ameliorate the immunosuppressive effect of CAV infection. CAV infection in G4 caused a weak innate response (macrophage phagocytic activity, lysozyme, and NO assays) compared with the other groups.

These results are by Schat (41), who stated that CAV infection led to a suppressed immune response and decreased macrophages' phagocytic and bactericidal activity. In addition, the limited increase in cytokines (IFN-γ, IL-6, and IL-10) in G4 was agreed with Eldaghayes *et al.* (42), Kaiser *et al.* (43) who found a slight increase in the inflammatory cytokines in CAV infection in contrary to other previously recorded immunosuppressive viral infections as IBDV and MDV infections which evoked much higher levels of the inflammatory cytokines.

The gradual increase in virus copies after infection with CAV (G4) at examined age 21 and 42 dpi and remained high in the organs was agreed with Wani *et al.* (44), El-Samadony *et al.* (19), who reported that higher virus copies related to higher antibody levels. They suggested a high viral load in the organs as the complete virus clearance from infected cells depends on the generation of virus-specific CD8+ T cells, which may be depleted due to CAV infection (45). These weak responses in G4 may be attributed to the apoptosis induced by the nonstructural CAV viral protein, VP3 (apoptin), affecting the primary and secondary lymphoid organs (46).

Whereas Cu-chitosan nanocomposite treatment can regulate the immune responses induced by CAV infection (G3), as it increases the innate responses (phagocytic activity, NO, and lysozyme), antiviral (IFN-γ), pro-inflammatory (IL-6), and anti-inflammatory cytokines (IL-10). These actions subsequently increase the adaptive humoral response (CAV antibody titers) to remarkably higher levels with virus copy reduction in G3 than CAV positive control group (G4). These results were convenient with Wang *et al.* (8) who found that Cu-chitosan nanocomposite had an immune-stimulating effect on broilers through augmentation of lysozyme, immune organ indexes, immunoglobulins, and complement. The enhancement effect of Cu-chitosan nanocomposite may be attributed to the virucidal and immunostimulatory effects of both copper and chitosan in the Cu-chitosan nanocomposite. Copper augments the cellular and humoral immune response against viral infection by activating helper T lymphocytes (Th1 and Th2), antigen presentation process, and antibody production from activated B lymphocytes (47). Copper, as a pro-

oxidant, can also exert antiviral activity through ROS production induced by the free copper ions released from the nanoparticles, leading to oxidation of viral proteins or degradation of the viral genome (48).

Besides its antiviral activity, copper can hinder the apoptosis of hematopoietic and lymphopoietic tissues induced by CAV proteins by activating the antiapoptotic phosphoinositide-3-kinase/Protein Kinase B (PI3K/Akt) pathway (49).

Moreover, chitosan can change the immune status of the host to improve their ability to defend against viral infection as occurred in this study in G3, since chitosan can strongly stimulate the primary cells involved in immune responses, these have appeared clearly in the remarked decrease of viral loaded copies at the age of examination of this G3 in the comparative with infected G4 (50).

Additionally, chitosan triggers the production of IFN genes, supporting the induction and regulation of innate and adaptive antiviral mechanisms (44). These activities of chitosan may arise due to its mucoadhesive properties resulting from the electrostatic interaction between the positive charge of amine groups ($R\text{-}NH3^{+}$) and the negative charge on the mucosal surfaces (51). Moreover, chitosan in the nanoform exposes more amino and hydroxyl functional groups with other induced activities (8).

The increment in CAV antibody titers in G3 was directly proportional to IL-6 and IL-10 concentration increment. This might be due to the stimulation of B lymphocytes through direct interaction with nano-Cu or indirectly by cytokines of activated macrophages or other helper T cells under the effect of nano-Cu (7). Moreover, the decreased virus copies in G3 may be attributed to the increased level of IFN-γ, which is stimulated by CD4 (T-helper) and CD8 (T-cytotoxic) cells under the effect of copper preparation (40). IFN-γ plays an essential role during intracellular infection and inhibits virus replication in host cells (52).

Copper (Cu) is an essential trace element, even though it causes toxic effects at high doses. This study detected Cu residues by a precise, accurate, and selective RP-HPLC method. Herein, the detected residues concentrations of Cu were highly accumulated in broiler liver more than muscle. The same was reported by Khan *et al.* (53), Ahmed *et al.* (54). Moreover, Lee *et al.* (55) observed higher Cu concentrations in the organs (liver, kidneys, spleen, brain, lungs, and heart) of rats treated with CuNP than the CuMP treatments. Their observations followed the administration of Cu nanocomposites (CuNPs—25 nm) or microparticles (CuMPs-14-25 μm). According to the current observation, the serum's Cu-chitosan nanocomposite level is higher than average. This was assumed by Choi *et al.* (50). They reported that Cu-NP could transmit to blood with a strong binding capacity to ceruloplasmin protein than ordinarily supplied Cu sulfate. Moreover, Cu-chitosan nanocomposite traversed to brain, heart, kidney, spleen, liver, and gut, and these facts confirmed the current findings as follow, on the 42nd day, Cu levels in the liver were 3.09, 4.47, 3.47, and 3.058 μg/g for G1, G2, G3, and G4, respectively. These were higher than recorded by (56), as they found 1.35 ppm Cu in Pakistani poultry liver and 0.357 ppm in poultry meat, nearly detected in different tested groups. In the same line, Mroczek-Sosnowska *et al.* (57) confirmed that Cu and nano-Cu had an equal tendency to accumulate in liver tissues. Similarly, they found Cu and nano-Cu mean concentrations 4.14±0.46, 4.34±0.37 μg/g in liver samples and 0.30±0.03, 0.31±0.03 μg/g in muscles. Furthermore, they supported our results on Cu-chitosan cytotoxicity by concerning the histological effect of nano-Cu on different organs through their experiments and other research.

The mentioned trial results on the nano-Cu effect applied by different doses 50,100, and 200 mg/kg daily through five days confirmed that smaller doses had a slighter impact than the higher dose. On the contrary, higher levels of Cu residues were demonstrated by Khan *et al.* (53), as the mean concentrations in meat were 5.902±0.59 and 6.092±0.54 μg/g, respectively, of the two rearing groups of quails. Their recorded liver residue results were 8.32±1.8 and 8.88±1.32 μg/g, respectively. Cu residues recorded by Iwegbue *et al.* (58) were incompatible with our results. It ranged from 0.01 to 5.15 μg/g in chicken meat from seven different localities of Southern Nigeria. Their range was much lower or much higher than the one presented here.

Cu residues in chicken meat and liver tissues recorded by Akan *et al.* (59), Elsharawy (60) were lower than recorded in this study. Overall, this study aimed to apply Cu-chitosan nanocomposite as an immune-stimulant for broilers. The current vaccination programs have been set to layers and breeders, but there are no vaccines against CAV for broilers. CAV affects chicks during the first two weeks of age, which means that any immune-suppressant agent leads to tissue damage and deaths. Therefore, CAV leads to significant economic losses. So, Cu-chitosan nanocomposite application will be as antiviral and immune-stimulant agent.

Conclusion

The Cu-chitosan nanocomposite had improved innate immune response (phagocytic activity, lysozyme, and NO) and cytokine levels (mRNA of IFN-γ, IL-6, and IL-10) in regular broilers. Besides enhanced immunity expression in CAV infected broilers, increase the adaptive immune response against CAV, and decrease the CAV viral load in organs. The current study proved Cu-chitosan nanocomposite's immune-stimulant and antiviral activity on CAV infection, clarifying the constant decrease of Cu-chitosan nanocomposite residues in broiler muscle and liver tissues with keeping its residual levels below Cu MRLs.

Conflict of interest

The authors declare that they have no competing interests.

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تأثير جزيئات الشيتوزان النحاسية النانومترية كمضاد للفيروسات ومحفز مناعي في دجاج التسمين المصابة تجريبياً بفيروس أنيميا الدجاج

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الخلاصة

في الفترة الماضية ظهرت أنظمة النانو غير العضوية أو المعادن النانوية وهي ذات أهمية كبيرة للعلاج الغير تقليدي. وفي هذه الدراسة تم تجربه مركب النانو للنحاس الشتوزاني كمضاد الفيروسات في كتاكيت التسمين التجارية، وهو أيضا منبه للمناعة ولخافض للمتبقي من النحاس في المركب الشيتوزاني. مركز النانو للنحاس الشتوزاني والمركب عبارة عن أكسيد معدني نانوي له خصائص محددة وشكل كروي ولا يتجمع وحجمه ضيق ٢٤,٧١±١,٦٨ نانومتر، ٠,٦٩١±٠,٠٢. تمت دراسة هذا المركب النانوي كمضاد لفيروس فقر دم الدجاج في كتاكيت التسمين وذلك بتقسيم ١٠٠ كتكوت تسمين عمر يوم الي أربع مجموعات متساوية. المجموعة ١ بمثابة مجموعة سلبية للتجربة والمجموعة ٢ تمت معالجتها بمركب النانو للنحاس الشيتوزاني ١ ملغم/مل في مياه الشرب لمدة خمسة أيام والمجموعة ٣ تم حقنها في العضل ٠,٢ مل من فيروس فقر الدم الوبائي المعرف على بنك العترات بالرقم (MN339532) وأيضا مركب النانو للنحاس الشيتوزاني بنفس المعاملة السابقة في المجموعة ٢. المجموعة ٤ وهي المجموعة الإيجابية على للفيروس وهي تحتوي على عدد ٣,٩٨٧×١٠[٦] نسخة فيروسية/مل. تم جمع عينات مختلفة من الدم والمصل والأنسجة على فترات زمنية مختلفة لقياس المخلفات في الدجاج

اللاحم وأنسجة العضلات والكبد. وأيضا قياس تركيز فيروس فقر الدم الوبائي في الأعضاء والمناعة المصلية والخلوية والخلطية. ووجد أن مركب النانو للنحاس الشتوزاني يحسن من الاستجابة المناعية الفطرية مثل نشاط البلعمة، الليزوزيم وأوكسيد النترات ويحسن من مستويات السيتوكينات مثل الانترلوكين السادس والعاشر والانترفيرون كاما في المجموعة ٢ و ٣. علاوة أنه يرفع قرأت المناعة للأجسام المضادة لـلفيروس وأيضا يقلل من نسخ الفيروس في أعضاء الكتاكيت المصابة مع انخفاض ملحوظ في بقايا النحاس للمركب الشتوزاني. لذلك تقدم هذه الدراسة دليلًا على تأثير التحفيز المناعي لـهذا المركب على العدوى بفيروس فقر الدم الوبائي كما يقلل من الأثر المستمر لبقايا المركب في الدجاج اللاحم وأنسجة العضلات والكبد، مع الحفاظ على مستوياته أقل من الحدود القصوى المتبقية للنحاس.