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Chitosan Hydroxyapatite: Physic-chemical Properties and its Effect on the Growth and Development of Broiler Chickens

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ABSTRACT

The current study aimed to obtain a calcium-containing, biocompatible drug based on chitosan *Bombyx mori*. Composites of Chitosan (CS) *Bombyx mori* with hydroxyapatite (HA) in the ratio of CS/HA = 50:50 mass percentage were synthesized *in situ* conditions at Ca/P = 1.67 mol% with intensive stirring for one hour at a speed of 1400 rpm and a temperature of 40 ± 2ºC. It was revealed that the components form an intermediate complex through –N-Ca, O-Ca, O (glycosidic bond)–Ca, H–O-bonds interacted by electrostatic forces. Atomic force microscopy studies indicated particles in the 100-50 nm size range on the polymer matrix surface. The polymer matrix prevented the growth of HA crystals and particle agglomeration. It was also determined that the CS/HA composite was non-toxic, and the LD_{50} was more than 5000 mg/kg. The composites were introduced into the chickens' diet in groups for 30 days at 25 to 40 mg/kg doses. The findings indicated an increased survival rate of chickens by 100%, improved the morphological parameters of the blood, and enhanced the contents of calcium, phosphorus, and hemoglobin. The addition of CS/HA=50:50 mass percentage contributed to an increase in the number of erythrocytes in the blood of broilers and hemoglobin by 11-12%. It should be noted that CS/HA did not adversely affect other morphological parameters of chicken blood. Therefore, CS/HA is recommended for the prevention of osteoporosis and osteomalacia in broiler chickens.

Keywords: Broiler chicken, Composites of chitosan Bombyx mori, Hydroxyapatite, *In situ*, Osteoporosis

INTRODUCTION

Biopolymer chitosan is a linear polysaccharide consisting of linked residues of N-acetyl-2-amino-2-deoxy-D-glucose (45-5%) and 2-amino-2-deoxy-D-glucose β(1→4) (55-95%; Lv, 2016; Mittal et al., 2018). Chitin is contained in crustacean skeletons, insect cuticles, and fungal cell walls (Suneeta and Kishor, 2020; Jiménez-Gómez and Antonio, 2020). Accordingly, chitin contents obtained from these sources differ in morphological parameters and physical and mechanical properties (Sampaio et al., 2005; Paulino et al., 2006; Suneeta and Kishor, 2020; Jiménez-Gómez and Antonio, 2020). Due to protonated amino groups, CS can interact with DNA, proteins, lipids, charged organic substances or synthetic polymers. These properties of CS increase the possibilities of its use in combination with various inorganic and organic compounds, where they are successfully used in bone tissue engineering (Aguilar et al., 2019; Hassan et al., 2022).

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is a member of the calcium phosphate class, with bioactivity and osteoconductivity close to natural bone with regard to its chemical composition (Mehdi et al., 2013; Sherif, 2019). Hybrid (nano) composites of hydroxyapatite (HA) can be derived based on biopolymers (Gašparič et al., 2017; Heshmatpour et al., 2018). There are several methods for synthesizing CS/HA composites, of which the most common methods are *in situ* synthesis, coprecipitation, freezing, lyophilization, and biomimetic (Venkatesan and Kim, 2010; Li et al., 2013; Szatkowski et al., 2015).

Composites CS/HA has been introduced in traumatology, dentistry, and veterinary medicine, as well as in wastewater treatment and as an agent that absorbs toxins (Le et al., 2012; Rita et al., 2021). The use of chitosan for this purpose is of particular importance due to its good matrix formation and bioactive properties. According to Shakir et al. (2017) and Sharifianjazi et al. (2022), materials used to fill bone defects should have four main properties, including osteoconduction, osteoinduction, osseointegration and osteogenesis, which are characteristic of chitosan.

Undoubtedly, chitosan compounds with HA and other calcium phosphates are distinguished by high biodegradation, bioactivity, and antimicrobial properties in the correction of defects in bone tissue cells (Wasso et al., 2020; Walaa et al., 2020; Widnyana et al., 2021; Mohamed et al., 2022). Calcium phosphate is effectively used in nutrients to prevent osteoporosis and osteomalacia in chickens and cattle (Uhl, 2018).

Currently, oral administration of bioactive substances based on CS is successfully considered in veterinary practice (Adams and Hewison, 2010; van de Graaf et al., 2015). In modern poultry farming, several new diseases of the bone tissue in fast-growing chickens have increased. The effective action of nano-HA in treating such diseases can be obtained using *in ovo*, which has a positive effect on increasing body weight and improving the condition of the bones. However, when taken orally, the size and high degree of crystallinity of HA impede body absorption (Oshida, 2015; Matuszewski et al., 2020). In CS/HA composites, the CS macromolecule prevents the growth of HA crystals and controls the size of their particles, which contributes to the absorption of the drug through the cells.

Depending on the synthesis conditions, the chemical composition of calcium-phosphorus compounds changes. For example, at a ratio Ca/P > 1.67 mol, calcium phosphate is formed with a pH > 7. It also leads to the formation of insoluble unstable composites when interacting with water or physiological fluids (Ca/P < 1.67, Eliaz and Metoki, 2017).

In addition to HA-based materials, regenerating or completely resorbable materials have been developed. They are based on the use of a porous resorbed matrix carrying proteins and bone cells in tissue engineering. Calcium phosphates at Ca/P < 1.67 ratio include tricalcium phosphate (TCP-$Ca_3(PO_4)_2$), calcium pyrophosphate (CPP-$Ca_2P_2O_7$), calcium polyphosphate ($(Ca(PO_3)_2)_n$), carbonat hydroxyapatite (KHA, Na_2O-CaO-P_2O_5, Na_2O-CaO-P_2O_5-SiO_2, K_2O-CaO-P_2O_5; Georgiou and Knowles, 2001).

Calcium phosphate-based preparations, such as HA, tricalcium phosphate (TCP), and carbonate-substituted carbonate-hydroxyapatite (KHA), have effective bioactive properties, high protein adsorption and osteoblast formation in tissues where cells are involved in bone regeneration (Meyer et al., 2004).

The effect of a phosphorus source on the balance of calcium in the body and on the bones of broilers was studied by Kaveh et al. (2020). It has been established that the inclusion in the diet of chickens of an inorganic source of phosphorus-containing dicalcium phosphate (14% active phosphorus and 23% calcium) has a more effective effect on the growth rates and productivity of chickens than the addition of an organic source - roasted bone meal (14 ± 2% of available phosphorus and 32% calcium).

Chitosan is a natural polycation and is extracted from the exoskeletons of crabs, fungi, and insects (Swiatkiewicz et al., 2015). Chitosan and its derivatives have bacterial, fungicidal, insecticidal, biostimulating, immuno-modulating properties (Amar Cheba, 2020). Recently, there has been a trend in the use of chitosan and its modifications in veterinary practices (Kobayashi et al., 2002; Shi et al., 2005; Raphaël and Meimandipour, 2017). Due to polyelectrolyte interaction, chitosan interacts with lipase, including fat is absorbed in the small intestine, reducing the amount of cholesterol in the blood (Lokman et al., 2019). On the practical effect of 0.2-0.3% solution of chitosan on the value of eggs, it was established that chitosan-containing feed lowered the cholesterol of brioler chickens (Kobayashi et al., 2002; Nogueira et al., 2003; Swiatkiewicz et al., 2015). Unlike chitosan at a rate of 0.5 g/kg, a comparative study showed that chitin effectively stimulated the growth, quality of the carcass, state of the organs of broilers, compared to the chickens in the control group fed the standard that diet gained weight and had more fat accumulation (Lokman et al., 2019).

The effect of dietary chitosan on growth, productivity, and protein retention in broilers was investigated. Results indicated that broilers fed a diet containing 0.5 or 1.0 g/kg of chitosan contributed to increased growth and feed absorption (Shi et al., 2005).

Poultry farming is a dynamically developing animal husbandry field, which solves many agro-industrial problems (Gržinić et al., 2023). However, there is also a deterrent to the development of poultry farming, which is the availability of forage (Vincenzo et al., 2018). There are many uses for new types of feed additives that are cost-effective and improve the diet balance (Gerber et al., 2015). In addition, they can improve the quality of products and physiological state of chickens (Hafez and Attia, 2020). In recent years, such feed additives mainly include natural feed bio-additives. These non-toxic and biocompatible dietary supplements have adsorption and ion exchange properties and cleanse the body of toxic substances (Abudabos et al., 2013).

Under experimental conditions, the current study aimed to investigate the effect of chitosan/HA on safety and weight gain, the morphological composition and leukocyte blood formula, and acute toxicity of broiler chickens. This study aimed to obtain chitosan-apatite composites with controlled particle sizes and study their physicochemical and bioactive properties.

MATERIALS AND METHODS

In this study, CS *Bombyx mori* with a molecular mass of 2×10^5 and a degree of deacetylation (DD) of 90%. To obtain HA, $CaCl_2$, and KH_2PO_4 (98.0%) were used (Sigma-Aldrich, USA).

Ethical approval

All procedures were approved by the Animal Care Committee of Veterinary Medicine, Animal Husbandry and Biotechnology, Samarkand, Uzbekistan. The principles of laboratory animal care were followed, and specific international rules and regulations were observed.

Morphology of the films Chitosan/Hydroxyapatite

The morphology of CS/HA was studied with an Atomic Force Microscopy (AFM) Agilent 5500 atomic force microscope (USA) at 22°C using silicon cantilevers with a stiffness of 9.5 N/m and a frequency of 145 kHz. The maximum scanning area on AFM was 25×25 µm^2 for X and 1 µm for Z.

Acute toxicity of Chitosan/Hydroxyapatite

Acute toxicity composites CS/HA was studied at the Scientific Center for Standardization of Medicines Samarkand, Uzbekistan on 24 white mice of mixed sex weighing 20 ± 1 g, divided into four groups. Chitosan hydroxyapatite was dissolved in 2% acetic acid to gain a gel state. It was then intragastrically administered to groups one to four once at doses of 1000 mg/kg, 2000 mg/kg, 4000 mg/kg, and 5000 mg/kg, respectively (Habriev, 2005). Experimental mice were under continuous hourly observation during the first day of the test. The general condition of mice and their behavior, the intensity and characteristics of motor activity, the presence of convulsions, coordination of movements, response to external stimuli and skeletal muscle tone, appetite, body weight, quantity, and the consistency of feces as the indicators of their functional state were taken into account. The clinical states of the animals, including the presence/absence of signs of poisoning, the time of their appearance, and the death of mice, were monitored during the study. All experimental animals were kept under standard conditions, on a general diet with free access to water and food. After the completion of the experiment, the average lethal doses (LD_{50}) were determined (Tisserand and Young, 2014).

Study design

Due to the fact that broiler chickens were kept in cramped quarters and under artificial lighting. Due to the inferiority of the diet, they were prone to hypovitaminosis, diseases, immunodeficiency, namely calcium, and phosphorus deficiency. Consequently, this negatively affected the increase in live weight and egg production of broiler chickens, leading to osteoporosis and osteomalacia in chickens in some cases. To improve the immune system of chickens and ensure a stable Ca-P balance in the body, the effect of additives of CS/HA on the physiological state of broiler chickens was studied. To do this, the test duration and the CS/HA powder dose added to their diet were controlled. Biologically, the active properties of CS/HA were studied in laboratory conditions for 100 one-day-old chickens of the breed Ross-308, adding 25 30, 35, and 40 mg/kg of composite powders to their diet. Then, the chickens were divided into 10 groups, and changes in their weight were recorded 30 days. During the 30-day experiment, the control group of chicken was fed a standard diet (Barekatain et al., 2021, Table 1).

Table 1. Standard diet for broiler chickens (Percentage of additives relative to 100 kg of feed)

Number	The composition of the diet	Volume
1	Soy flour	20 kg
2	Corn	32.6 kg
3	Wheat	25.6 kg
4	Sunflower flour	7.3 kg
5	Cotton flour	2 kg
6	Vegetable oil	2 liters
7	Methionine	51 g
8	Calcium orthophosphate	1780 g
9	Limestone	8.22 g
10	Salt	50 g

In the experimental groups (2-10), instead of vitamins and premixes, CS/HA powders were added to the diet for 15-25 days, and the results were obtained on day 30. The effectiveness of the composites used in the experiments was evaluated by the survival rate of chickens and their live weight gain.

Blood parameters

Hematological studies were performed on days 10, 20, and 30 of the experiment. Blood for the analysis of experimental and control chickens was taken from the axillary vein. Hemoglobin concentration was determined in the presence of acetone cyanohydrin by the hemoglobin-cyanide method on the KFK2 device (Whitehead et al., 2019). The number of erythrocytes, leukocytes, and platelets in 1 mm^3 of blood was counted in the Goryaev chamber after staining them based on Romanovsky-Giemsa and dye-methyl violet according to the method by Campbell (1988).

The leukocyte formula in blood smears was determined after double staining according to Papenheim with the Filipchenko three-field method (Alturkistani et al., 2015; Sohair et al., 2017; Sufiriyanto et al., 2018; Kolesnik et al., 2020). Preliminary laboratory experiments were conducted to determine the optimal dose of chitosan hydroxyapatite and the timing of their addition to chicken feed. The prepared diet did not include vitamins and minerals. From days 10 to 20, the chickens were fed the supplements of CS/HA were added in modules of 0, 25, 30, 35, and 40 mg per 1 kg of feed, respectively. After that, blood samples of experimental chickens were obtained and the content of Ca/P in the composition of their blood and blood serum was determined. Biochemical parameters of blood samples were determined on Automatic Chemical Analyzer CC-T180, indicating the hematological composition of blood. Acute toxicity of CS/HA preparations was determined under *in ovo* conditions (Yair and Uni, 2011; Shokraneh et al., 2020). During the experiment, the indicators of the effectiveness of drugs, the survival rate of chickens, and the average live weight of one head of chickens were determined according to the following formula.

$$P = \frac{W_t - W_0}{W_0} x100$$

Where, P is the percentage of live weight gain, Wt denotes the live weight of 1 chicken head at the end of the experiment, Wo determines live weight of 1 chicken at the beginning of the experiment, 100 is coefficient. The data from this study were transferred to SPSS software version 23 for statistical analysis and were compared using the Kruskal-Wallis test. The Mann-Whitney test was used to compare the groups. In this study, a p-value less than 0.05 was considered statistically significant.

Obtaining Chitosan/Hydroxyapatite composites with the variation of synthesis conditions

The CS/HA composites were obtained under *in situ* conditions using 2% acetic-water solution of CS, 1 M aqueous solutions of $CaCl_2$ and KH_2PO_4. First a solution containing Ca/P = 1.67 mol% was mixed with a solution of chitosan atmassratio CS/HA = 50:50 mass percentage and intensively mixed on a magnetic stirrer. Then, with dropwise addition 6% NaOH precipitates into the reaction mixture. The target product is CS/HA (Nikpour et al., 2012), where the precipitate in solution NaOH was kept for 8 hours. After that, the obtained samples of CS/HA were washed with distilled water up to pH = 7 and dry up freeze-dried to constant weight (Figure 1).

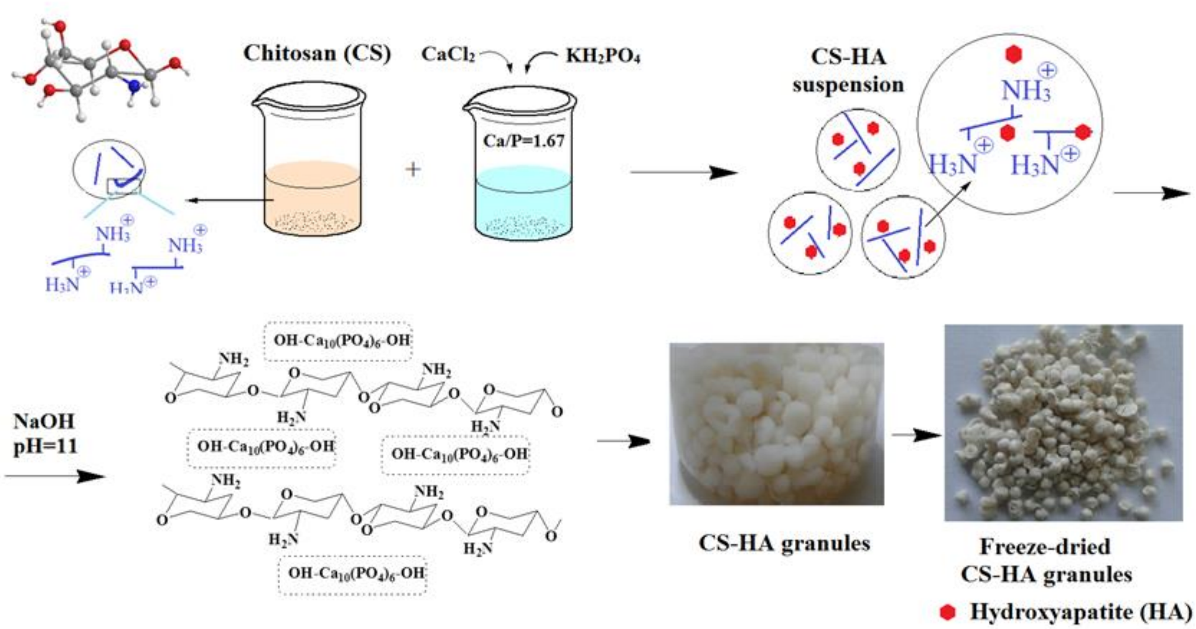

Figure 1. Preparation of Chitosan/Hydroxyapatite composites under *in situ* condition

RESULTS AND DISCUSSION

Experimental samples of hydroxyapatite chitosan *Bombyx mori* have been produced under laboratory conditions and identified by physicochemical research methods. The elemental composition and some characteristics of CS and its composites are determined with HA (Table 2).

The results indicated that with the introduction of 50 mass percentage HA into a chitosan macromolecule, its features of water absorption decreased approximately by three times. Accordingly, the degree of its solubility in acetic acid fell and formed a suspension. The content of calcium ions (Ca^{2+}) in the composite and the ash content of the preparation were 9.16% and 59.9%, respectively.

Under the chosen synthesis conditions, apatite nanoparticles stabilized by the polymer matrix were formed when the intermediate complex of chitosan with hydroxyapatite was stored at pH >7 for 8 hours. Hydroxyapatite nanoparticles stabilized by a chitosan macromolecule have a hexagonal structure corresponding to the interplanar distances of HA with hexagonal syngony (JCPDS-No.-00-09-0432; Vokhidova et al., 2022). Thus, a preliminary structure of the CS/HA composite was proposed based on experimental and theoretical research methods (Figure 2).

Table 2. Some physicochemical characteristics of Chitosan/Hydroxyapatite samples

Number	Samples	N_{total} (%)	Ca^{2+} (%)	Ash content (mass percentage)	Humidity (%)	Solubility in 2% CH_3COOH (%)
1	Chitosan	8.27	-	3.6	1.87	98
2	Chitosan/Hydroxyapatite = 50:50	4.10	9.2	59.9	0.65	Suspension

Ca^{2+}: Calcium

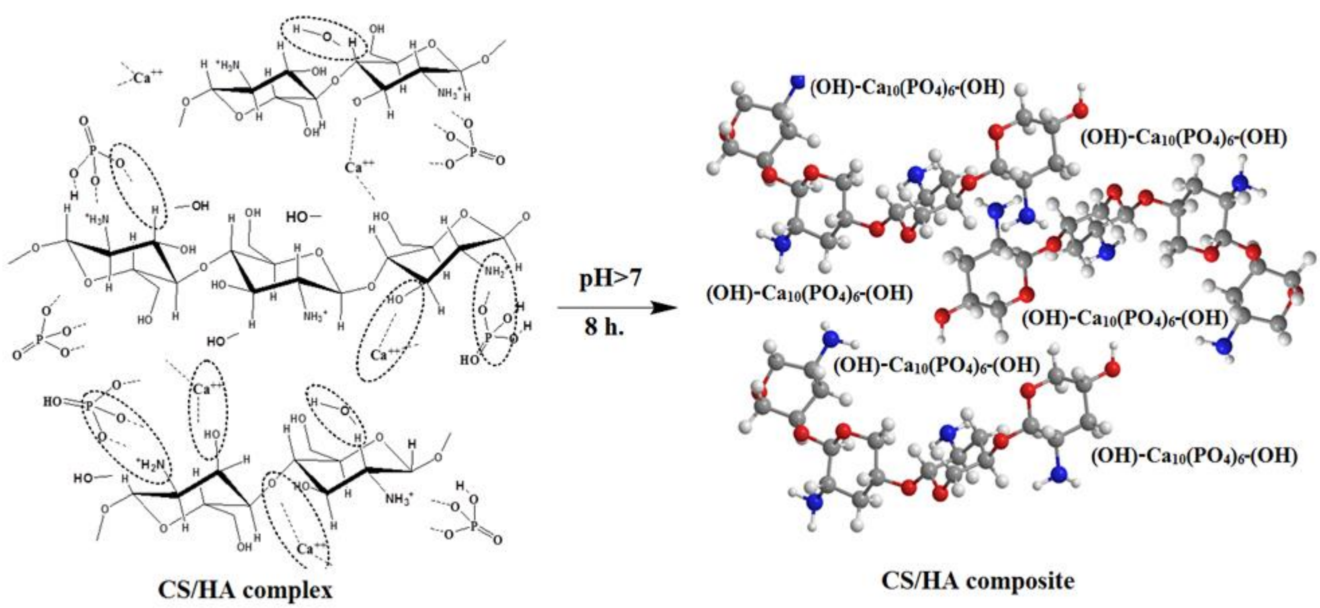

Figure 2. Preliminary structure of the resulting Chitosan/Hydroxyapatite composite (Source: Vokhidova et al., 2022).

Atomic force microscopy analysis

The morphology of the chitosan/HA films was studied by the AFM method. It was found that nanoparticles within the range of 100 to 450 nm were formed on the surface of the films of the studied composites (Figure 3).

The AFM micrographs showed that HA crystallites were formed on the surface of the films under *in situ* conditions. It was found that with a synthesis duration of up to 8 hours, polydisperse particles were formed in the 100-50 nm size range, where the average size was 280 nm

(25%). Moreover, the formed particles were unevenly distributed over the polymer matrix.

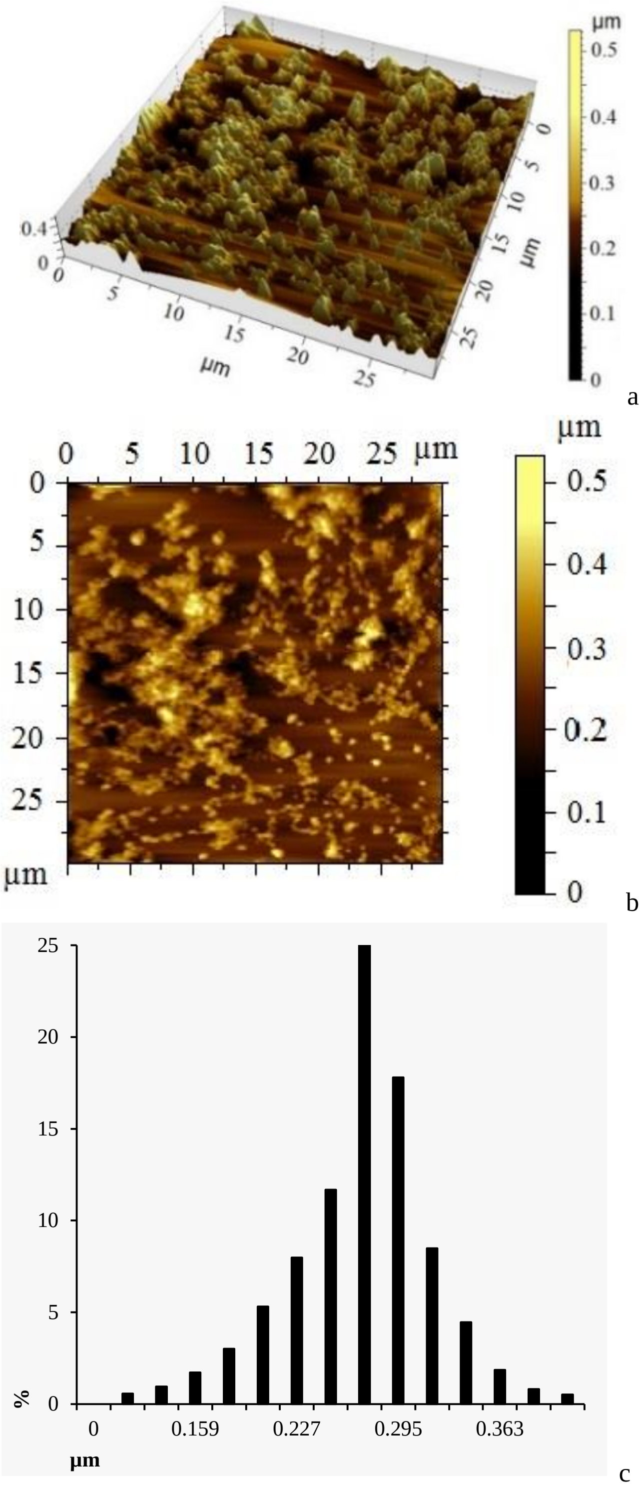

Figure 3. Phase (a) and topographic (b) atomic force microscopy images of Chitosan/Hydroxyapatite (50:50 mass percentage) and particle distribution histogram (c)

Acute toxicity of composite Chitosan/Hydroxyapatite

Composite acute toxicity study showed that after a single intragastric administration CS/HA = 50:50 mass % at doses of 1000 mg/kg, 2000 mg/kg, 4000 mg/kg, and 5000 mg/kg. The general condition of the mice remained stable, and no visible changes were observed in their behavior (Table 3).

During the observation of the experimental mice, it was revealed that the coordination of movements and the tone of the skeletal muscles were normal, and there were no convulsions. Mice responded to tactile, pain, sound, and light stimuli. The condition of the skin and hair was unchanged, and the color of the mucous membranes and the size of the pupil were without deviations. Mice in all groups indicated no loss of appetite, increase in water consumption, change in body weight. Moreover, the amount and consistency of feces were normal. Within 14 days, the death of mice was not observed. Thus, as a result of the experiments, it was found that the data obtained showed that LD_{50} CS/HA = 50:50 mass percentage was > 5000 mg/kg; the drug was non-toxic.

Table 3. Determination the acute toxicity of chitosan hydroxyapatite

Number of mice	Chitosan hydroxyapatite *Bombyx mori*			
	Weight (g)	**Dose (mg/kg)**	**Way introductions**	**Fatal outcome**
1	21	1000	intra-gastric	Not
2	19			Not
3	20			Not
4	21			Not
5	19			Not
6	20			Not
1	20	2000		Not
2	21			Not
3	19			Not
4	21			Not
5	21			Not
6	20			Not
1	20	4000		Not
2	20			Not
3	21			Not
4	19			Not
5	20			Not
6	20			Not
1	20	5000		Not
2	20			Not
3	19			Not
4	20			Not
5	19			Not
6	19			Not
LD_{50}	>5000 mg/kg			

Live body weight and survival rate

The results show that when 25-40 mg/kg of chitosan apatite was added to the feed composition, 100% safety was observed, and the weight gain was 71.5-75.5% (Table 4). Bioadditives chitosan/HA effectively influenced the morphological parameters and leukocyte blood count, contributing to a significant increase in erythrocytes and hemoglobin ($p \leq 0.05$). As a result of the study, the survival rate of chickens in the control group was 90%, and the survival rate of chickens in experimental groups (2-10), where CS/HA was added to the diet at a dose of 25-40 mg/kg, reached 100%.

During the test period, the live weight of one broiler from the control group increased from 310 to 1350 g. At the same time, the survival rate reached 90%, and the increase in the average live weight of chickens was 67.5%. In the experimental groups, the live weight increased from 311 ± 5 to 1200-1510 g, with an efficiency of 100%, and the average live weight of chickens increased to 75.5%. Therefore, 40 mg/kg of chitosan hydroxyapatite and 15 days were selected as the optimal dose and duration of CS/HA introduction into the chickens' diet. During the experiments, the live weight of the experimental chickens increased slightly by only 8%. This is probably due to the lack of vitamin and mineral supplements in the diet of chickens. However, it should be noted that the addition of chitosan hydroxyapatite, regardless of the dose, could lead to an increase in the immunity of chickens, and no clinical symptoms of infectious diseases were observed.

Table 4. The effect of chitosan *Bombyx mori* hydroxyapatite on the live weight gain of chickens and their safety during a 30-day study

No.	Groups	Dose of Chitosan/Hydroxyapatite (mg/kg)	Duration of adding Chitosan/Hydroxyapatite (day)	Average live weight of 1 chicken at the beginning of the experiment (g)	Live weight of 1 chicken after 30 days (g)	Survival rate (%)	Increase in average live weight of chickens (%)
1	Control	-	Standard ration	310	1350	90	67.5
2	Chitosan/Hydroxyapatite	25	10-20	310	1200	100	60.0
3		30		313	1250	100	62.5
4		35		309	1290	100	64.5
5		40		311	1350	100	67.5
6		25	15-25	313	1250	100	62.5
7		30		309	1340	100	67.0
8		35		312	1430	100	71.5
9		40		311	1510	100	75.5

Ca-P balance in blood serum

With an increase in the dose of the composite in the diet of chickens, the amount of calcium and phosphorus naturally increased. Calcium content in the control group chickens was 2.2 ± 0.415 mmol/l, while the content of Ca increased to 3.5 ± 0.354 mmol/l at the same time as in the study group fed dose of CS/HA 40 mg/kg. It should be noted that the content of phosphorus increased in proportion to the dose of CS/HA supplements. At a dose of CS/HA 40 mg/kg, the phosphorus content compared to the control sample increased by approximately 1.3 times (Table 5). In fact, calcium ions can increase intestinal pH and reduce the solubility and availability of minerals (Adedokun et al., 2004; Olukosi and Fru-Nji, 2014; Gautier et al., 2017).

It was found that minor additions of CS/HA to the standard diet of broiler chickens contributed to an increase in the amount of Ca and P in the blood serum, but had little effect on the amount of K^+ and Na^+. According to studies on the effect of CS/HA on the biochemical parameters of blood serum, the amount of Ca and P in the experimental groups was higher than in control chickens (Xu et al., 2021). This is due to the fact that Ca and P from the composition of CS/HA are effectively absorbed by the body of chickens. The data obtained in the current study are in good agreement with the results of Sharaviev (2015) as they investigated the probiotic Bacell-M and its effect on the biochemical parameters of the blood of chickens. Thus, CS/HA at a dose of 35-40 mg/kg in the diet of chickens increases their safety and weight gain in broiler chickens. There is no negative effect on the hematological parameters of their blood.

Table 5. The effect of different doses of Chitosan/Hydroxyapatite on the serum biochemical parameters in broiler chickens

Number	Dose (mg/kg)	Ca^{2+} (mmol/l)	P (mmol/l)	Na^{+} (mg/%)	K^{+} (mg/%)
1	Control	2.20 ± 0.42	1.4 ± 0.87	364 ± 11.8	22.0 ± 10.7
2	25	2.31 ± 0.38	2.0 ± 0.37	371 ± 20.5	22.0 ± 10.6
3	30	2.44 ± 0.43	1.7 ± 0.69	365 ± 20.2	22.3 ± 11.1
4	35	2.75 ± 0.29	2.2 ± 0.44	370 ± 20.3	22.4 ± 8.11
5	40	3.51 ± 0.35	2.3 ± 0.51	364 ± 20.1	22.8 ± 11.3

Ca^{2+}: Calcium, P: Phosphorus, Na^{+}: Sodium, K^{+}: Potassium

Morphological parameters and leukocyte count

The morphological blood parameters of broiler chickens were studied and the effect of CS/HA on the content of retinol in the liver and blood serum was investigated. Four groups of broiler chickens were selected for the study. The first group was the control and were fed a basal diet. For the first 10-20 days, the diet of the second group included basal diet and CS/HA powders at a dose of 25 mg/kg. During 20-30 days, the diet of the third group entailed CS/HA at a dose of 30 mg/kg. Finally, between days from 30 to 40, chitosan hydroxyapatite supplements were added at a dose of 35 mg/kg to the diet of chickens in the fourth group. After that, the leukocyte formula was determined in all groups of chickens on days 20, 30, and 40 (Tables 6 and 7).

Compared with the experimental control groups, erythrocytes, and hemoglobin increased by 7-8%, 9-10%, and 11-12%, in the blood of chickens in the second, third, and fourth groups, respectively. At the same time, the number of leukocytes and platelets in the blood of chickens in all experimental groups practically did not differ from that of the control group ($p < 0.05$, tables 6, 7). The results were in an agreement with the literature data (Abudabos et al., 2013). It was found that the addition of powders of the CS/HA = 50:50 mass percentage composite to the diet of broiler chickens, regardless of the period of administration, contributed to an increase in the number of erythrocytes and hemoglobin in the blood of chickens. It can be inferred that the composite based on chitosan and hydroxyapatite positively affected the morphological parameters of blood due to the synergistic effect. The inclusion of CS/HA in the diet of chickens stimulates the activity of hematopoietic organs.

Table 6. The influence of Chitosan/Hydroxyapatite on the number of erythrocytes (10^{-12} g/l) and leukocytes (10^{-9} g/l) in broiler chickens

Number	Dose of Chitosan/Hydroxyapatite, (mg/kg)	Erythrocytes (10^{-12} g/l)			Leukocytes (10^{-9} g/l)		
		Experiment period (day)					
		10	20	40	10	20	40
1	Control	2.62 ± 0.07	2.64 ± 0.10	2.68 ± 0.11	29.3 ± 1.09	29.7 ± 1.10	28.8 ± 1.11
2	25	2.83 ± 0.09	2.85 ± 0.12	2.89 ± 0.05	28.5 ± 1.20	28.5 ± 1.25	28.1 ± 2.30
3	30	2.61 ± 0.08	2.87 ± 0.13	2.94 ± 0.01	28.1 ± 1.12	28.2 ± 1.03	28.3 ± 2.77
4	35	2.60 ± 0.05	2.65 ± 0.10	3.00 ± 0.06	27.9 ± 1.21	28.5 ± 1.15	28.1 ± 1.55

Table 7. The effect of Chitosan/Hydroxyapatite supplements with different composite on platelets count (10^{-9} g/l) and hemoglobin (g/l) in broiler chickens

Number	Dose of Chitosan/Hydroxyapatite (mg/kg)	Platelets (10^{-9} g/l)			Hemoglobin (g/l)		
		Experiment period (day)					
		10	20	40	10	20	40
1	Control	37.2 ± 2.01	38.5 ± 2.03	37.5 ± 2.55	79.3 ± 1.11	85.5 ± 1.13	90.0 ± 1.12
2	25	35.6 ± 1.19	36.7 ± 1.17	37.0 ± 2.06	84.8 ± 1.17	91.4 ± 1.00	97.2 ± 0.06
3	30	36.4 ± 1.12	37.3 ± 1.20	37.1 ± 2.00	79.8 ± 0.09	93.2 ± 0.06	99.0 ± 0.09
4	35	36.7 ± 1.33	37.0 ± 1.35	36.8 ± 0.95	78.2 ± 1.15	85.8 ± 0.05	100.6 ± 1.2

CONCLUSION

To conclude, the optimal conditions for the synthesis of the formation of chitosan hydroxyapatite Bombyx mori nanocomposites in the ratio of 50:50 mass percentage. It has been shown that chitosan apatite is a non-toxic preparation, and the LD_{50} of the CS/HA composite is 5000 mg/kg. In the current study, powders of CS/HA were introduced into the diet of broiler chickens, and their positive effect on the physiological state of chickens, as well as on the morphological and some biochemical parameters of their blood, were analyzed. It was found that the addition of CS/HA in a dose of 40 mg/kg to the diet of chickens could maintain the balance of Ca and P and was easily absorbed by their organism.

DECLARATION

Funding

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Authors’ contributions

Noira R. Vokhidova, the head of project, conceived the presented idea and verified the analytical methods. Sayyora Sh. Rashidova supervised the project. Kandiyor Kh. Ergashev synthesised the samples of composites, prepared figures and tables. Davletbay I. Ibragimov conducted experiments on the bioactivity of the composite. All authors checked the statistical results and contributed to the final version of the manuscript.

Competing interests

There are no conflicts to declare.

Ethical considerations

The authors follow the required ethical standards for publishing all available data related to this study for the first time without copying any data from other published papers.

Availability of data and materials

The full data of the present study will be sent according to the reasonable request that will be sent to the corresponding author.

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