CHITOSAN HYDROXYAPATITE BOMBYX MORI AND ITS EFFECT ON THE TREATMENT OF OSTEOPOROSIS

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Abstract

In this work, composites of chitosan *Bombyx mori* (CS) with hydroxyapatite (HA) and their composites structure (CS/HA=50:50 wt. % and Ca/P=1.67%) studied by X-ray diffraction analysis and FITR spectroscopy. The acute toxicity of the composite was studied; after a single intragastric administration of the composites CS/HA=50:50 wt.% at doses from 1000 to 5000 mg/kg, the general condition of the mice remained stable and no visible changes were observed in their behavior. The LD_{50} of the chitosan hydroxyapatite preparation was >5000 mg/kg. Long-term administration of the drug into the body of animals with experimental osteoporosis led to an increase in the mass of the femur, bone compaction and an increase in the calcium content in the ashes of animal bones. The data obtained are of applied interest in the use of hydroxyapatite chitosan for the prevention and treatment of osteoporosis and the replacement of bone tissue in living organisms.

***Keywords**:* chitosan *Bombyx mori*, hydroxyapatite, *in situ*, non-toxicity, osteoporosis

Introduction

Currently, non-toxic preparations based on CS and its derivatives are widely used in medicine and veterinary medicine [1].

Hydroxyapatite is chemically bound to the inorganic component of the bone matrix as a complex structure - $Ca_{10}(OH)_2(PO_4)_6$. The advantages of hydroxyapatite nanocrystals are osteoconductivity, bioresorption and close contact. The osteoporotic bone crystal is calcium phosphate hydroxide with the chemical formula $Ca_{10}(OH)_2(PO_4)_6$. A normal bone crystal is a sodium-calcium bicarbonate hydrate with the chemical formula $Ca_8H_2(PO_4)_6{\times}H_2O–NaHCO_3–H_2O$. The main elements of hydroxyapatite crystals, consisting of atomic minerals in a specific geometric order, and their relationship with the biological activity of bone cells have opened up opportunities for the use of hydroxyapatite crystals as a supplement in osteoporosis[2].

Osteoporosis is by far the most common metabolic disease affecting bones [3]. One innovative approach to enhance osteoporosis therapy and prevent potential side effects is the development of bone-targeted drug delivery technologies. It minimizes systemic toxicity and also improves the pharmacokinetic profile and therapeutic efficacy of chemicals. Early studies have shown that nanoparticles (NPs) HA promotes the biological activity of osteoblasts, enhancing bone regeneration [4–5]. In this regard, the nanocarrier itself promotes the growth of bone tissue and increases the deposition of bone mass.

Bone crystals are extremely small, with an average length of 50 nm (in the range of 20–150 nm), an average width of 25 nm (in the range of 10–80 nm), and a thickness of only 2–5 nm. The apatite phase contains 4–8% carbonate by mass, called dalit. The mineral composition will change with age and is always dependent on calcium deficiency when carbonate and phosphate ions are in the crystal lattice [6]. Formula $Ca_{8.3}(PO_4)_{4.3}(CO)_{3x}(HPO_4)_y(OH)_{0.3}$reflects the average bone composition, where y decreases and x increases with age, while the x+y addition will be constant at 1.7. The growth of miner-

al crystals occurs at a certain orientation, in which the c-axis of the crystal is approximately parallel to the length axis of the collagen fiber, where the crystal is deposited [7].

One of the advantages of a nanocomposite is its ability to assemble with other molecules. These properties will improve the quality of osteoporosis treatment, although they require further study. At 12 weeks postoperatively, the nano-HA/CS group showed complete healing of the segmental bone defect, while the defect was still visible in the CS group, although the depth of the defect had decreased. These observations suggest that nano-HA/CS injectable scaffolds are potential candidate materials for bone tissue regeneration [8].

The studies have been carried out to investigate the osteoregenerative ability of collagen/CS/GA scaffolds in rats with hormone deficiency caused by experimental bilateral oophorectomy. Forty-two rats were divided into neovariectomized (-NO) and ovariectomized (O) groups, divided into three subgroups: a control (blank defect) and two subgroups treated with Coll/CS/HA scaffolds prepared using different methods of incorporating hydroxyapatite, *in situ* (Coll/CS/HA-1) and ex situ (Coll/CS/HA-2). The defect areas were subjected to macroscopic, X-ray and histomorphometric analysis. Inflammatory processes in the area of the tibial defect, indicating immune rejection of matrices, were not detected, which confirms the biocompatibility of biomaterials. Bone formation, starting from the edges of the bone defect, was observed in all rats, with higher volume in the -NO groups, especially in the Coll/CS/HA-2 group. Less bone formation was found in O subgroups compared to -NO. In conclusion, collagen/CS/HA scaffolds stimulate bone growth in vivo, but pathological conditions of bone fragility caused by gonadal hormone deficiency can delay the process of bone repair [9].

The hybrids consist of a collagen/CS/hyaluronic acid biopolymer matrix and aminofunctional silica particles adorned with apatite to which alendronate molecules have been coordinated. They can be accurately injected into diseased tissue as a viscous sol or partially cross-linked hydrogel, where they can serve as scaffolds for locally controlled bone regeneration/remodeling, supporting osteoblast formation/proliferation and maintaining an optimal level of osteoclasts. These materials do not exhibit systemic toxicity. They can be especially useful for the restoration of small osteoporotic bone defects, for combined use in the treatment of osteoporosis and bone tissue [10].

An analysis of the literature shows that despite the fact that a huge amount of research is being carried out on CS/HA composites, each work is unique in its own way and is of fundamental and applied interest.

Materials and methods

Research objectsis a natural polysaccharide - CS, obtained from chitin of silkworm pupae *Bombyx mori*, as well as calcium hydroxyapatite.

Synthesis of calcium hydroxyapatite under "in situ" conditions. Composites CS/HA=50:50 wt.% obtained by adding CS to the solution *Bombyx mori* aqueous solutions of $CaCl_2$ and KH_2PO_4, at a constant molar ratio Ca/P=1.67. The reaction mixture is intensively stirred at a speed of 1400 rpm for 1 hours at40±2°C.[11-13]. Granules CS/HA obtained by drop precipitation of the target product with a 6% solutionNaOH [14].

*IR spectroscopic studies*CS and CS/HA were carried out on an IR-Tracer-100 spectrometer from SHIMADZU (Japan), the device is characterized by a high signal-to-noise ratio of 60,000:1, a resolution of 0.25 cm-1 and the ability to operate in a fast scanning mode with registration of up to 20 spectra per second .

X-ray studies samples were performed on the basis of diffraction patterns, which were taken on an XRD-6100 instrument (Shimadzu, Japan) controlled by a computer. We used CuKα radiation (β-filter, Ni, 1.54178 current mode and tube voltage were 30 mA and 30 kV) and a constant detector rotation speed of 4 deg/min with a step of 0.02 deg. ($\omega/2\theta$-coupling), and the scanning angle varied from 4 to 80°. According to the Debye-Scherrer formula, the interplanar distances of crystals and the average size of crystallites were calculated [15]:

$$D=0,89\lambda/(\beta_{1/2}\cos\theta) \quad (1)$$

Acute toxicity composites CS/GA was studied at the Scientific Center for Standardization of Medicines on 20 white mice of mixed sex and weighing 20 ± 1 g. CS/HA was dissolved in2% acetic acid to a gel state and administered to mice once intragastrically at doses of 1000 mg/kg, 2000 mg/kg, 4000 mg/kg, and 5000 mg/kg [16]. Animals were monitored continuously for the first hour, then hourly during the first day of the experiment, and once a day for the next 13 days of the experiment. As indicators of the functional state of animals, the general condition of mice and their behavior, the intensity and nature of motor activity, the presence of convulsions, coordination of movements, response to external stimuli and skeletal muscle tone, appetite, body weight, quantity and consistency of fecal masses were taken into account. During the experiment, the clinical condition of the animals was monitored: the presence/absence of signs of poisoning, the time of their appearance, and the death of mice. All experimental animals were kept under standard conditions, on a general diet with free access to water and food [16]. After the completion of the experiment, the average lethal doses (LD_{50}) were determined.

*For simulation experimental osteoporosis*used ovariectomy and to speed up the process - the introduction of glucocorticosteroids. For example, dexamethasone at a dose of 0.3 mg/kg, administered to ovariectomized rats even once every 2 weeks for at least 1 month, significantly reduces bone density in the vertebrae, and after 3 months, in the pelvic bones [17]. The experiments were carried out on mature white female rats, the animals were subjected to ovariectomy, since females develop osteoporosis faster than males. The animals were divided into 4 groups of 6-7 animals each. The first group - intact, who did not undergo oophorectomy and did not inject dexamethasone. The second group was the control group, in this group, bilateral ovariectomy was performed and dexamethasone was subsequently administered without treatment of osteoporosis.

Animals of the third and fourth groups - experimental, also underwent bilateral oophorectomy and then dexamethasone was administered.

A month after the modeling of experimental osteoporosis, the animals of the experimental groups (Groups 3 and 4) were injected with the compound in the form of a suspension of *Bomby xmori* CS/HA and calcium gluconate produced by OJSC Borisov Medical Preparations Plant, Republic of Belarus, series 49 31820822, expiration date 08 2027) in dose of 250 mg/kg intragastrically for a month. After the administration of the compound and the drug, blood was collected to determine the level of calcium in the blood of the animals. To determine the mass, density and calcium content in the femur, the femurs of animals were taken after euthanasia. Bone density (HU-Hounsfield) [18] of animals was examined on the IQON SPECTRAL Magnetic Resonance Imaging Scanner in the Department of Radiology and PET/CT(Positron emission tomography/Computed tomography)diagnostics clinic named after M. Fedorovich. The content of calcium in the ashes of animal bones was studied by the complexometric method in the laboratory of defoliants of the Institute of General and Inorganic Chemistry of the Academy of Sciences of the Republic of Uzbekistan [19].

Results and its discussion

Structural studies of CS and its composites were studied by FITR spectroscopy (Fig. 2).The results obtained show that two high-intensity groups of modes in the region 1090-980 $cm^{-1}$and 600-560 cm^{-1} belong to hydroxyapatite ($-PO_4^{3-}$) group. The vibration bands observed at 3359 cm^{-1} and 709 cm^{-1} correspond to the hydroxyl group (-OH). In the region of 877 cm^{-1}, absorption was observed corresponding to the $-HPO_4^{2-}$ groups (Fig. 1, line 1). It can be seen from the IR spectrum that absorption bands characteristic of structurally unbound water are visible in the region of 1625 cm^{-1} [20].In the IR spectrum of CS, a wide band is observed in the region of 3368-3289 cm-1 of wave numbers, which refers to the stretching vibrations of –OH and $–NH_2$ groups, at 2921-2877 cm^{-1}vibration bands of $–CH_2$ groups appear.

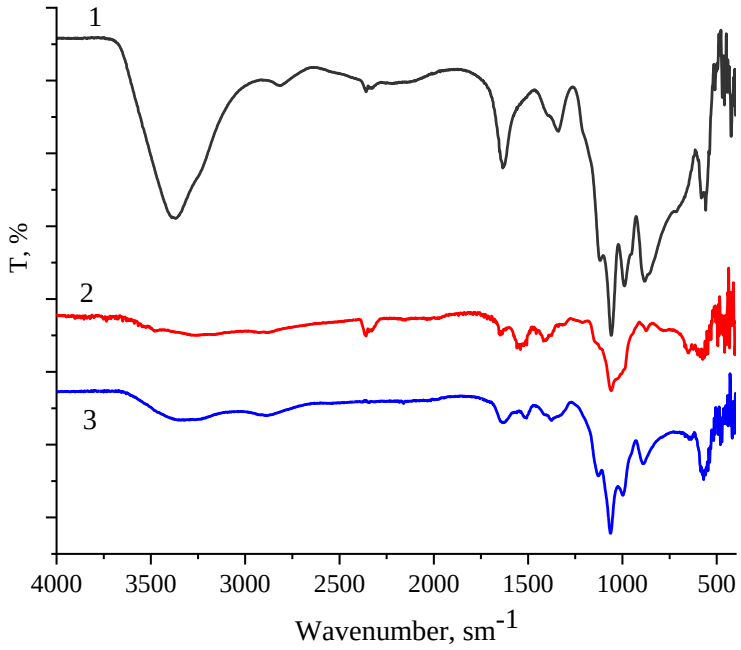

Figure 1. FTIR spectra of samples 1) HA 2) CS. 3) CS/HA=50:50 wt.%

In the region of 1653-1575 cm^{-1}, bending vibrations of $-NH_2$ groups are observed, and in the region of 1423-1321 cm^{-1} -OH, -CH groups, at 1150-1070 cm^{-1}, stretching vibrations of ether bonds - C-O-C - (Fig. 2, line 2).

The structure of the bands in the regions of 1700-920 cm^{-1} for samples of CS and CS/HA differs significantly (Fig. 1, line 3). The IR spectra of all obtained CS-HA samples are identical and are characterized by absorption bands at 1150–920 cm^{-1} and 561–602 cm^{-1}, which are related to antisymmetric stretching and bending vibrations of PO bonds in the phosphate group (PO_4^{3-}). It should be noted that intense absorption bands of A-type carbonate at 1425 cm-1 and B-type carbonate at 879.54 cm^{-1} appear in the IR spectrum of CS/HA-50:50 mass % sample. In the spectra of samples with different ratios of CS/HA, the absorption bands -OH (CS) andPO_4^{3-}($Ca_3(PO_4)_2$ groups overlap at 1027 $cm^{-1}$and 1120 cm^{-1} [21-22].

X-Ray Structural Analysis of CS/HA Composites

Comparative X-ray diffraction studies of CS and its composites with HA have been carried out. The results of X-ray diffraction studies of CS macromolecules show the appearance of characteristic peaks at 2Ɵ 10° and 20°(Figure 3).

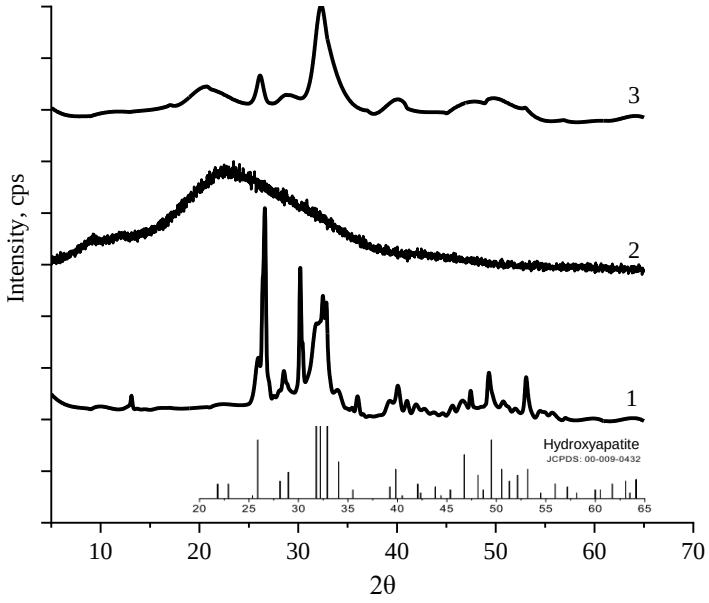

Figure 2. X-ray diffraction samples:1) HA 2) CS 3) CS/HA-50:50 wt.%

Line 1 in Fig. 2. shows intense peaks of apatite crystals in the composite at angles 2Ɵ20.36º, 26.14º, 29.11º, 32.205º, 40.05º, 46.95ºand50.18º, which are in good agreement with literature data[23]. It should be noted that upon receipt of hydroxyapatite CS, the peaks characteristic of CS are observed to completely disappear, and, accordingly, an increase in the content of apatite in composites leads to the appearance of new peaks corresponding to HA at angles of 2Ɵ 30-50º, in which an increase in the intensity of peaks at angles of 2Ɵ32 ,2°,46.95º and 50.18º [11, 21]. The data obtained are reproducible, and the developed method makes it possible to obtain CS/HA composites with a controlled composition and structure.

Study of Acute Toxicity of Composite CS/HA

According to the developed method, experimental batches of CS/HA were obtained and their acute toxicity was studied. The studies show that after a single intragastric administration CS/HA=50:50 wt. %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. The coordination of movements and the tone of the skeletal muscles were normal, there were no seizures, the reaction to tactile, pain, sound and light stimuli was pronounced, the condition of the hair and skin was unchanged, the color of the mucous membranes and the size of the pupil were virtually without deviations, appetite was pronounced , water consumption has not increased, body weight is unchanged, the amount and consistency of fecal matter are fixed in the norm. The death of mice within 14 days was not observed. drug LD_{50}CS/HAamounted to more than 5000mg/kg[24].

The obtained data show that LD_{50}CS/HA=50:50 wt. %is more than 5000 mg/kg and belongs to practically non-toxic drugs. Due to morphological characterization and MTT analysis, CS showed no toxicity to human fetal osteoblast cells (hFOB). It has also been shown that CS reduces the viability of human osteosarcoma by 96% compared to control samples [25]. This suggests a pro-apoptotic mechanism against osteosarcoma, as well as a potential clinical application of CS as a drug in ceramic scaffolds at tumor resection sites. MTT results for os-

teoblasts (hFOB) and osteosarcoma (MG-63) on days 3 and 6. The presence of CS showed no toxic effect on the viability of osteoblast cells on days 3 and 6 compared to pure HA samples.

Although the toxicity/biocompatibility of hydroxyapatite nanoparticles (HA NPs), a promising nanobiomaterial, has been widely studied, their effects on the reproductive system after exposure are less used [26]. In the present study, male rats were exposed to HA NPs (300 mg/kg body weight) to determine their possible reproductive toxicity. In addition, the protective effect of CS NPs nanoparticles CS NPs, 280 mg/kg body weight) and/orcurcumin (Kur.) (LF Cur., 15 mg/kg body weight) against reproductive toxicity caused by HA NPs. Animals were dosed daily by gavage at appropriate doses for 45 consecutive days. The results obtained showed that GA NPs cause a significant decrease in sperm count, sperm motility, testosterone hormone, steroidogenic enzymes (17-ketosteroid reductase and 17-β-hydroxysteroid dehydrogenase) and antioxidant enzymes (glutathione peroxidase, glutathione-S-transferase, catalase and superoxide dismutase) in addition to total antioxidant capacity and reduced glutathione. LH and FSH, abnormal spermatozoa, oxidative stress parameters (substances reactive with thiobarbituric acid, nitric oxide (NO) and δ-hydroxydeoxyguanosine, p53, TNFα and interleukin-6 were significantly higher.

DNA damage was also analyzed by analyzing the level of 8-OHdG, which is considered as an indicator of genotoxicity, as well as suppression of mtTFA gene expression, UCP2 induction. Similarly, the histopathological score also changed after exposure to GA NPs. Antioxidant activity of NPsCS and Cur demonstrated a mitigating effect against HA NP-induced reproductive impairment by improving sex hormone characteristics, inflammatory factors, and antioxidant status. The present study concluded that HA NPs induce reproductive toxicity, and it is important to use nanoantioxidants of Cur and CS as protective agents. HA showed high biocompatibility and non-toxicity while maintaining excellent osseointegrative and osteoconductive characteristics [27].

Study of the effect of the CS Bombyx mori/HA compound on experimental osteoporosis in an experiment on white rats

To confirm the development of osteoporosis in experimental animals, indicators such as calcium content were used, bone mineral density and femur mass.

To determine the mass, density and calcium content in the femur, the femurs of animals were taken after euthanasia. The bone density (HU-Hounsfield) [18] of the animals was examined using the IQON SPECTRAL Magnetic Resonance Imaging Scanner at the Department of Radiology and PET/CT Diagnostics of the M. Fedorovich Clinic. The content of calcium in the ashes of animal bones was studied by the complexometric method [19].

According to the table, the development of osteoporosis in ovariectomized rats in the control group is accompanied by a significant decrease in the weight of the dried femur, as well as a decrease in the level of calcium in the blood. In addition, there was a pronounced decrease in the density of the bone tissue of the femur and an increase in bone fragility compared to the intact group (Fig. 3, 4).

With the introduction of the compound HA CS *Bombyx mori*, the mass of the femur increased by 23.6%, the density of the femur increased by 12.1% compared with the control group. Serum calcium levels did not change significantly. When determining the calcium content in bone ash, compared to the control group, it increased by 16.4%, which does not differ significantly from the results of the intact group (Fig. 5).

In the comparator group of calcium gluconate, the femoral bone mass increased by 31.6%, the density of the femur increased by 21.7% compared with the control group. Serum calcium levels did not change significantly. With the introduction of calcium gluconate, the calcium content in bone ash increased by 8.9% compared to the control group, which does not differ significantly from the results of the intact group (Fig. 6).

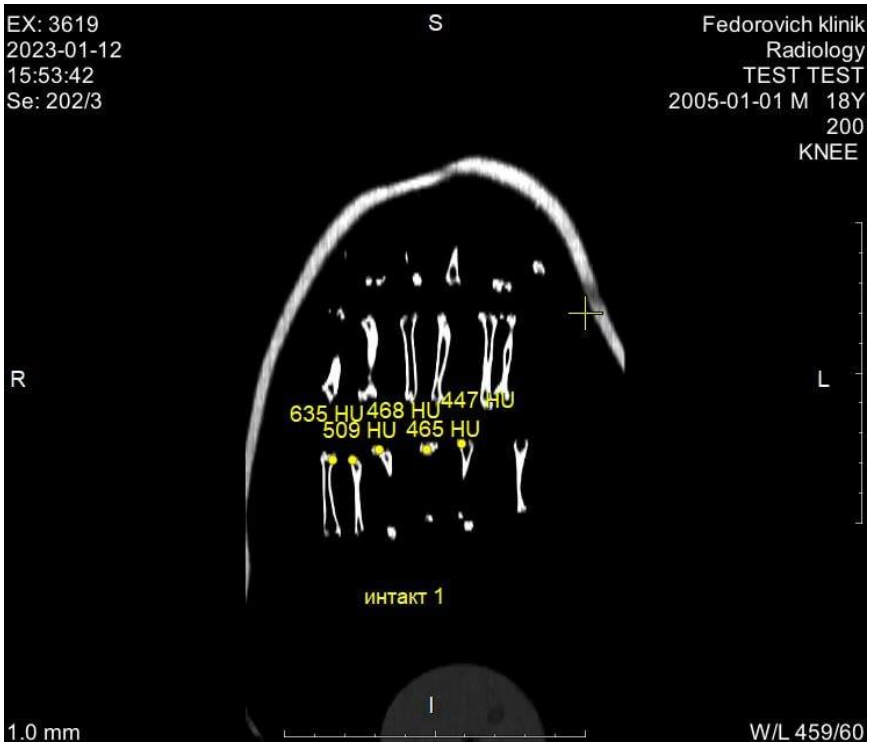

Figure 3. MRI image of the femurs of animals after euthanasia andcontact group

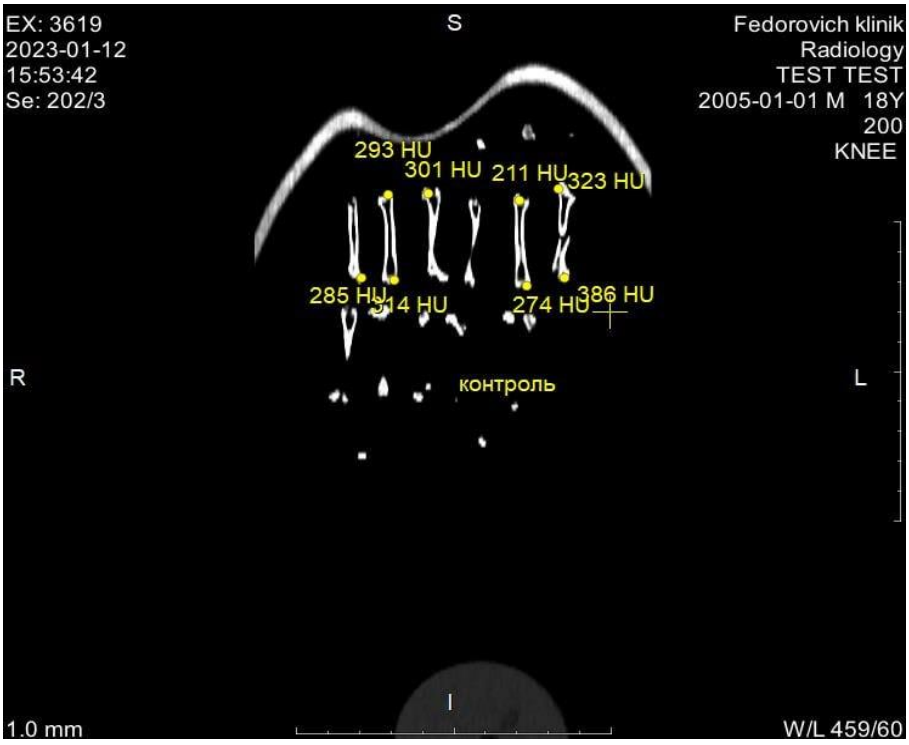

Figure 4. MRI image of the femurs of animals after euthanasia tocontrol group

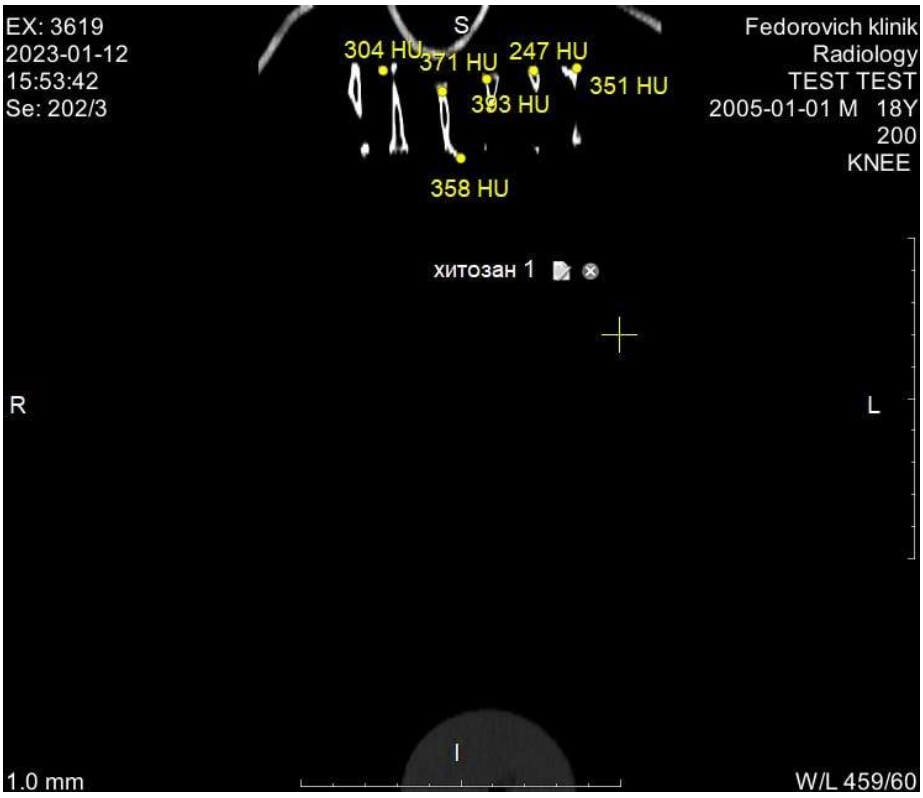

Figure 5. MRI image of the femurs of animals after euthanasia, taking CS/HA *Bombyx mori*

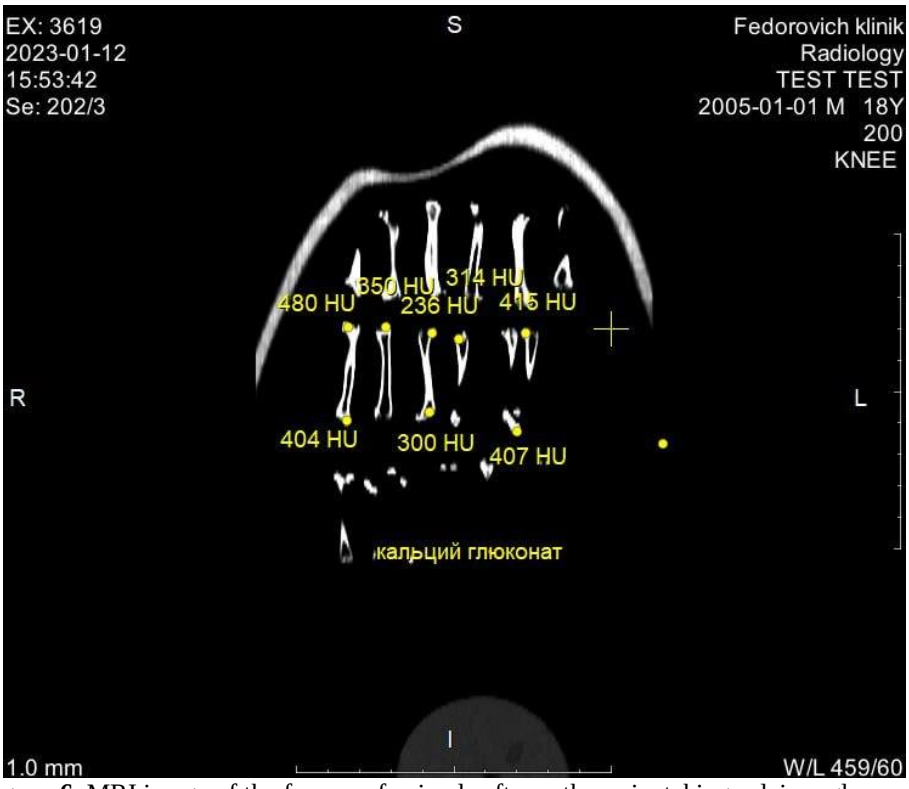

Figure 6. MRI image of the femurs of animals after euthanasia, taking calcium gluconate

Comparison of the results shows that CS *Bombyx mori* HA at the tested dose promotes an increase in the mass and density of the femur, as well as an increase in the calcium content in the bones of animals. The results of the experiment are shown in Table 1.

Table 1.

Effect of HA CS *Bombyx mori* and calcium gluconate preparation on experimental bone osteoporosis

No	Groups	Femur mass (per 100 g of body weight)	Blood calcium level	Femur Density, HU	Calcium content (%) in bone ash
1	Intact	0.233±0.02	2.60±0.09	493.1±27.6	7.23±0.24
2	Control	0.161±0.01x	2.24±0.10 x	298.4±17.4 x	6.52±0.13 x
3	CS/HA 250mg/kg	0.199±0.01y	2.23±0.06 x	334.6±18.8 x	7.59±0.21 y
4	Calcium gluconate, 250 mg/kg	0.212±0.01 y	2.30±0.05 x	363.3±27.6 x	7.10±0.17 y

Note:
^{X}- statistically significant in relation to the intact group at P<0.05;
^{at}- statistically significant in relation to control indicators at P<0.05.

Thus, ovariectomy in female animals and the subsequent administration of glucocorticosteroids leads to a violation of mineral metabolism and the development of osteoporosis, as can be seen from the results obtained. When comparing the results of the control group with the intact group, a statistically significant decrease in the mass of the femur, the level of calcium in the blood of animals, the density of the thigh tissue, as well as the total calcium content in the ashes of animal bones is observed. These indicators in this model indicate the development of osteoporosis in experimental animals.

When comparing the obtained indicators with the results in the group of animals that were injected with the drugcalcium gluconate no statistically significant difference was observed. The positive effect of the HA CS *Bombyx mori* compound on the course of experimental osteoporosis is consistent with the results obtained by other researchers in the treatment of osteoporosis with various hydroxyapatite compounds [2, 28].

It has been shown that CS is suitable for use in IT as a bone graft, in the manufacture of porous matrices, films for superficial wounds, drug release, and in formulations for the production of hydrogels [29, 30]. However, the disadvantage of CS is its poor mechanical properties [31], therefore, to improve this, several studies report the development of CS/HA based composites, since both biomaterials have excellent biocompatibility with human cell tissue [32], this composite is reported to have improved mechanical properties due to the binding of amino and hydroxyl groups of CS with calcium ions present in HA [33-34].

Osteoblasts play a critical role in bone formation. Previously, it was shown that chitosan nanofibers can stimulate the proliferation and maturation of osteoblasts. This translational study used an animal model of bone defects to evaluate the effect of chitosan nanofiber scaffolds on bone healing and possible mechanisms. In this study, homogeneous CS nanofibers with a fiber diameter of about 200 nm were investigated.

A bone defect was surgically created in the proximal femur of male C57LB/6 mice, and then CS nanofiber scaffolds were implanted in the left femur on day 21 and compared with the right femur, which served as a control. Histological analysis showed that implantation of chitosan nanofiber scaffolds did not lead to hepatotoxicity or nephrotoxicity. The results indicated the potential of CS nanofiber scaffolds for the treatment of bone diseases, including bone defects and bone fractures [35].

Conclusions

Thus, we have developed a method for obtaining CS/HA *Bombyx mori* -50:50 wt.% composites with a controlled composition and structure. The results of X-ray diffraction studies of CS/HA composites show the appearance of characteristic peaks at 2Ѳ 20.36º, 26.14º, 29.11º, 32.205º, 40.05º, 46.95º and50.18º. IR spectroscopic studies show that the interaction of CS macromolecules with a hydroxyapatite molecule occurs due to electrostatic and hydrogen bonds of the functional groups of CS and HA.

The acute toxicity of CS/HA *Bombyx mori*composites has been studied and it has been found that the LD_{50} of the drug is >5000 mg/kg and it belongs to practically non-toxic drugs.Long-term administration of the CS *Bombyx mori*/HA compound to animals with experimental osteoporosis led to an improvement in the previously given indicators, that is, in comparison with the control group, in this group of animals, the femoral bone mass, bone density and calcium content in the ashes of animal bones were higher. The results obtained are of scientific and applied importance in the creation of a drug for the prevention and treatment of osteoporosis in veterinary medicine.

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