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# Using Confocal Microscopy to Study the Effect of an Original Pro-Enzyme Se/Arabinogalactan Nanocomposite on Tissue Regeneration in a Skeletal System

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**Abstract**—The effect an original nanocomposite of elemental selenium and arabinogalactan heteropolysaccharide has on the rate of the calcification of calluses in the case of a traumatic injury is studied experimentally using confocal and fluorescence microscopy. With a local increase in the selenium concentration in the area of the traumatic injury, we observe low intensity of mineralization for the forming callus and a slowing down of bone regeneration. The difficulties of visualizing fluorescent labels introduced simultaneously are discussed.

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## INTRODUCTION

The problems of impaired mineralization of bone tissue have grown in recent decades, due to the widespread occurrence of osteoporosis, which is referred to as a silent epidemic due to its prolonged latent clinical course and manifestation only in the form of complications. Bone tissue mineralization is known to determine the strength of bone tissue in many ways.

Different methods are currently used to assess rates of bone mineralization: studying cross sections via backscattered electron analysis using Raman spectroscopy and Fourier transforms of IR microscopy with tetracycline labels introduced in vivo [1]; with 18F-fluorides in positron emission tomography [2]; and the fluorescence microscopy of bone tissue with fluorescent labels introduced in vivo. The labels introduced in vivo that reflect ossification are most often tetracycline. A single introduction of a label does not suffice to assess the dynamics of the process when it is necessary to estimate the time period of the deposition of calcium salts in the bone tissue, so tetracycline is either repeatedly introduced at certain intervals or it is alternated with other substances that form insoluble compounds with calcium ions. In this work, we investigate the possibility of simultaneously using two different labels introduced in vivo to assess the rate of ossification at a fracture site. In addition, we study the possibility of visualizing an original selenium preparation in the form of nanoparticles in arabinogalactan polysaccharide upon local administration and its effect on the ossification of the fracture site in the case of a bone tissue injury.

## EXPERIMENTAL

### *Synthesis and Characterization of the Selenium (0)–Arabinogalactan Nanocomposite*

An original nanocomposite of specially synthesized selenium and arabinogalactan heteropolysaccharide was used in this work. The composite was synthesized via oxidation in a water solution of bis(2-phenylethyl)sodium diselenophosphinate using hydrogen peroxide in combination with arabinogalactan as a stabilizing matrix of the resulting nanoparticles of elemental selenium(0). The selenium content determined by titrimetry in a nanocomposite sample was 0.54%. The composite was categorized via transmission electron microscopy using a TEM-410 microscope and by UV spectroscopy with a Shimadsu UV-1800 unit. The nanocomposite matrix, arabinogalactan, was also studied using optic spectroscopy.

### *Modeling a Traumatic Injury*

Experimental studies were conducted on two models of injury to bone tissue: a complete transverse fracture in the upper third of the femoral shaft with open external retrograde intramedullary osteosynthesis using a steel pin [3] (Wistar male rats, $N = 30$) and a hole fracture of the tibia (Chinchilla male rabbits, $N = 8$, including 5 that followed the natural course of

the reparative process (the control group) and 3 with the local intraoperative introduction to the fracture site of 50.33 μg/kg of nanoselenium, expressed in terms of selenium (the nanoselenium group)).

Our experiment was performed in accordance with the requirements for humane treatment with animals mandated by the Regulations for Studies Using Experimental Animals (and annex to Decree of the Ministry of Health of the USSR No. 755, August 12, 1977) as per the record approved by the Local Committee of Biomedical Ethics of Scientific Center of Reconstructive and Restorative Surgery of the Siberian Branch of the Russian Academy of Medical Sciences.

Markers of the rate of the regeneration of the bone tissue were an oxytetracycline hydrochloride solution (OOO Biokhimfarm; TU No. 9344-080-70972578-06), injected intramuscularly into the animals of all the groups on the 7th and 21st days of the experiment in doses of 250 mg/kg body weight, and a solution of Alizarin Red S, administered intraperitoneally on the 14th and 28th days in doses of 100 mg/kg body weight.

The rats were eliminated from the experiment on the 3rd, 5th, 9th, 14th, 21th, and 35th days; the rabbits, on the 35th day via the intrapleural introduction of a solution of sodium thiopental in doses of 167 mg/kg.

The material was fixed in a FineFix solution (Milestone, Italy).

The decalcification and subsequent preparing of bone tissue preparations for a histological study were done according to the method we developed in [4]. The material was prepared and embedded into paraffin blocks; multiple sections 7 μm wide were prepared, and the sections were deparaffinized and embedded in Fluoromount (Diagnostic Biosystems, REF K024, Lot P 939-B). The specific fluorescence of the fluorescent labels were visualized using a Zeiss LSM-710 laser confocal microscope and a Nikon Eclipse 80i microscope with an DIH–M epi-fluorescence attachment. Nikon B-2A (excitation at 450–490 nm, dichroic mirror 505 LP, emission at >515 nm) and TRITC (excitation at 528–553 nm, dichroic mirror 565 LP, emission at 590–650 nm) filters were used to select the required ranges of fluorescence. Recording was performed using a Nikon DS-Filc camera connected to a Nikon DS-U2 controller using the Nikon Elements program software.

Visualization of the oxytetracycline hydrochloride and Alizarin Red S preparations and that of the nanocomposite of elemental selenium on arabinogalactan were performed in the same manner.

## RESULTS AND DISCUSSION

The choice of arabinogalactan for the synthesis of selenium nanocomposite was due to this water-soluble heteropolysaccharide being known as a good basic biocompatible polymer in obtaining different nanocomposite systems for biomedical applications [5, 6].

It was found that the nanocomposite of elemental selenium and arabinogalactan synthesized was powder with a red-orange color and was very soluble in water. The optical absorption spectrum of the arabinogalactan used as a matrix for synthesizing the nanocomposite had pronounced peaks in the region of 199 and 287 nm that can be attributed to the end aldehyde groups of the polysaccharide's carbon units. Studying the optical absorption spectrum of the Se–AG nanocomposite, we see that the range of 190–1000 nm has a minimal increase (0.005 abs) at 926 nm and a gradual increase at 600–190 nm up to 0.772 abs with small plateaus in the regions of 230–221 nm and 204–197 nm, which is typical for nanoselenium(0) [7].

It was found during electron microscopy that the composite consisted of globules of arabinogalactan covered with selenium particles, the size of individual selenium particles being only 2–3 nm.

It was found in studying an isolated preparation of oxytetracycline hydrochloride that it consisted of needle-shaped crystals. Upon excitation by a laser with a wavelength of 405 nm, emissions in the spectral region of 490 to 565 nm were observed with a peak at 553 nm.

Upon laser excitation at 561 nm, the solution of Alizarin Red produced emissions in the range of 570–675 nm with a maximum at 605 nm.

Heavy calcium salt deposits in the calluses that had formed were observed in determining the ossification of the bones of the rats and rabbits of the control group; these were reflected as deposits of oxytetracycline and Alizarin Red introduced in vivo. On the 35th day after the injury, regenerated bone with the complete filling of bone defects was observed. It was found via epifluorescence that the sites of the deposits of both markers coincided, the use of confocal microscopy on the same samples having revealed both the isolated regions of the oxytetracycline and Alizarin deposits (overlapping in the projection) and the regions where ossification was observed at the time of injecting both Alizarin and oxytetracycline (the simultant deposition of both dyes). Confocal microscopy thus allowed us to localize the site of the deposits of Alizarin and oxytetracycline more precisely.

Studying the nanocomposite of elemental selenium and arabinogalactan via epifluorescence, it was found that the fluorescence in all regions was typical for our objects of study. Using our LSM–710 confocal microscope, it was found that the preparation had properties of quantum dots. The nanocomposite particles observed most often fluoresced over a wide range of the spectrum upon laser excitation at 405 nm with irradiation maxima at 480 nm, at 458 nm with a maximum at 538 nm, and at 514 nm with a maximum at 555 nm.

Larger particles absorbed only shortwave radiation and upon excitation by the laser at 405 nm produced an emission peak at 446 nm.

The animals injected with the selenium nanocomposite containing arabinogalactan had poorly regenerated bones at the fracture site. The trabecula of the bones were very thin. Barely visible deposits of oxytetracycline and Alizarin at the fracture site were observed using epifluorescence, with the oxytetracycline deposits predominating. A great many fluorescent amorphous masses outside the fracture site and intense luminescence of the fluorescence labels in the Haversian spaces were noted. Considering the emissions of these masses both in the regions of 515–560 nm (including the emission maximum of oxytetracycline) and 590–650 nm covering almost half of the radiation spectrum of Alizarin Red, it was difficult to make any conclusions as to their origin. These masses could have been either calcium complexes of oxytetracycline and Alizarin introduced in vivo or deposits of the nanocomposite itself fluorescing in vitro in the investigated region. The use of epifluorescence thus did not allow us to obtain clear answers to the questions raised.

Studying these preparations by confocal microscopy in the mode of spectral detection allowed us to see clearly that the fluorescent labels in the regenerated bone were almost entirely oxytetracycline.

The fluorescent amorphous masses outside the fracture sites and in the Haversian spaces in regard to the radiation spectrum were closer to the spectra of the nanocomposite of selenium and arabinogalactan (from 430 to 630 nm, with a maximum in the region of 490 nm).

## CONCLUSIONS

Our study allowed us to establish that the local application of nanoselenium at the fracture sites significantly impairs the reparative processes, slowing down bone regeneration and impairing mineralization in calluses that have formed. No such deviations in the natural course of the reparative process were observed in studies performed earlier on the biological effects of the arabinogalactan matrix in altering tissue. This allows us to exclude the development of the observed changes due to the toxic effect the matrix substance has on an organism's cells [8]. The local use of selenium nanoparticles associated with macromolecules of arabinogalactan on a fracture site seems to lead above all to significant diffusion impediments to the evacuation of the nanocomposite from this area and thus to pronounced prolonged local effects of nanoselenium on the site of the trauma.

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## REFERENCES

1. Misof, B.M., Gamsjaeger, S., Cohen, A., et al., *J. Bone Miner. Res.*, 2012, vol. 27, no. 12, pp. 2551–2561. doi: 10.1002/jbmr.1699.
2. Frost, M.L., Compston, J.E., Goldsmith, D., et al., *Calcif. Tissue Int.*, 2013, vol. 93, pp. 436–447. doi: 10.1007/s00223-01309778-7.
3. Shurygina, I.A., Shurygin, M.G., Rodionova, L.V., Lebedev, V.F., and Dremina, N.N., *Bull. East-Siberian Acad. Med. Sci.*, 2012, vol. 5, no. 2, pp. 120–122.
4. Shurygina, I.A. and Shurygin, M.G., RF Patent 2500104, 2013.
5. Shurygina, I.A., Sukhov, B.G., Fadeeva, T.V., et al., *Nanomedicine*, 2011, vol. 7, no. 6, pp. 827–833. doi: 10.1016/j.nano.2011.03.003.
6. Trofimov, B.A., Sukhov, B.G., Aleksandrova, G.P., et al., *Dokl. Akad. Nauk*, 2003, vol. 393, no. 5, p. 634.
7. Singh, S.C., Mishra, S.K., Srivastava, R.K., and Gopal, R., *J. Phys. Chem. C*, 2010, vol. 114, no. 41, pp. 17374–17384. doi: 10.1021/jp105037w.
8. Kostyro, V.V., Kostyro, Ya.A., Lepekhova, S.A., et al., *Bull. East-Siberian Acad. Med. Sci.*, 2013, vol. 1, pp. 106–110.

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