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НОВИЙ ПОЛІМЕР-МІНЕРАЛЬНИЙ ZrO_2-Gd_2O_3 РЕНТҐЕНКОНТРАСТНИЙ НАНОКОМПОЗИТ, ФУНКЦІОНАЛІЗОВАНИЙ ГІАЛУРОНОВОЮ КИСЛОТОЮ ДЛЯ ВІДНОВЛЕННЯ КІСТКОВИХ ДЕФЕКТІВ В ЕКСПЕРИМЕНТІ

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Реферат

Мета. *Оцінка властивостей нового рентґенконтрастного полімер-мінерального композиту (Z1 і Z2) на основі наночастинок ZrO_2-Gd_2O_3, функціоналізованих гіалуроновою кислотою, створеного для відновлення штучно створеного дефекту хребця хвостового відділу щура.*

Матеріал і методи. *В експериментах використано 18 білих безпородних щурів-самок, яких поділили на 3 групи (по 6 тварин). Штучний дефект створювали у хребцях та заповнювали нанокомпозитами Z1 і Z2 різної консистенції. У контрольній групі дефект ушивали під кров'яним згустком. Рентґенологічне дослідження проводили у різні терміни (15 і 30 днів після операційного втручання).*

Результати й обговорення. *Запропоновані нові біоматеріали продемонстрували ознаки біологічної сумісності упродовж двох перших тижнів після хірурґічного втручання. Їхнє застосування не супроводжувалося нагноєнням на відміну від щурів контрольної групи, у яких загоєння під кров'яним згустком відбувалося повільно. Рентґеноконтрастність матеріалів Z1 і Z2 була виразною, хоча у всіх випадках через 30 днів простежували просочування матеріалу Z2 за межі дефекту вздовж тканин. Матеріал Z1 залишався у межах дефекту терміном на 15 і на 30 днів.*

Висновки. *Нова експериментальна модель in vivo, що базується на використанні хребців хвостового відділу, дозволила простежити за ефективністю дії досліджуваного нанокомпозиту для заміщення кісткових дефектів. Створений полімер-мінеральний нанокомпозит на основі наночастинок ZrO_2-Gd_2O_3 функціоналізованих гіалуроновою кислотою, посилює реґенерацію кісткового дефекту. Простежено клінічну біотолерантність і рентґенологічно - добру інтеграцію матеріалу Z1 з кістковою тканиною реципієнтного ложа.*

Ключові слова: *ZrO_2-Gd_2O_3, рентґеноконтрастний нанокомпозит, кістковий дефект, хребець хвостового відділу щура*

Abstract

A NOVEL POLYMER-MINERAL ZrO_2-Gd_2O_3 RADIOPAQUE NANOCOMPOSITE WITH HYALURONIC ACID COATING FOR REPAIR OF BONE DEFECTS IN EXPERIMENT

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*The **aim** of this study was to evaluate the ability of novel polymer-mineral radiopaque nanocomposites based on ZrO_2-Gd_2O_3 nanoparticles with polyelectrolyte-hyaluronic acid shell (Z1 and Z2) to enhance repair of artificially created defect in caudal vertebra bone of rats.*

Material and Methods. *18 white outbred female rats of 8-9 months of age and 300-350 g body weight were used in the experiments. The animals were divided into 3 groups, each including 6 rats. An artificial defect was produced in rat's vertebra and further regeneration of the osseous tissue was performed by using synthesized nanocomposites Z1 and Z2 of different consistency. In the control group, the bone defect was sutured below the blood clot. Radiological investigations were carried out in different terms of bone regeneration (15 and 30 days after surgical intervention).*

Results and Discussion. *Created biomaterials demonstrated biocompatibility as soon as in the two first weeks after surgery. Their application was not accompanied by inflammatory reaction and suppuration of the regenerate, opposite to formation of large areas of destruction of the osseous tissue during regeneration of bone defect in control group of rats in which regeneration of the defect was performed under blood clot. High density of Z1 material allows keeping it in the centre of the bone defect, opposite to Z2 material leaking from the defect area (15 and 30 days after surgical intervention).*

Conclusions. *Novel in vivo experimental model of caudal vertebra proved its efficiency in testing materials used for regeneration of the osseous tissue. Created polymer-mineral* *nanocomposite based on ZrO_2-Gd_2O_3 nanoparticles with the hyaluronic acid shell effectively enhanced regeneration of bone defect. The clinical biotolerance, radiopacity, as well as high integration of applied Z1 material with the osseous tissue of recipient bed have been demonstrated.*

Keywords: *ZrO_2-Gd_2O_3, radiopaque nanocomposite, osseous defect, rat's caudal vertebra*

Introduction

Search and development of new bioplastic materials which are effective in surgical intervention are a crucial issue of contemporary reconstructive medicine. This issue has been considered in numerous publications dedicated to new materials capable of effective stimulating of regeneration processes. Creation of new materials for replacement of bone defects of various aetiology, ways of improvement of their biocompatibility, osteogenous potential, as well as providing osteoinduction, osteoconduction and radiopacity, belong to the most important tasks of the orthopaedic and surgical practice. Various nanoscale materials possessing considerable mechanical strength, flexibility, and chemical stablility have proved their efficacy in reparative medicine [1]. However, evaluation of safety and efficacy of using nanocomposites in medicine requires additional experimental studies in order to prove their biocompatibility and functional efficacy in human body [2]. Many contemporary osteoplastic materials are based on bone collagen, hydroxyapatite, alpha- and betatricalcium phosphate. Significant attention is also paid to the nanoparticles (diameter 5-60 nm) of iron oxide, copper, zinc, silver, gold, and titanium. In particular, nanoparticles based on zinc oxide possess bacteriostatic and bactericidal properties, as well as healing activity, and they are effective remedies in regeneration of the osseous tissue [3].

Components of extracellular matrix, specifically, proteoglycans, glycoproteins and morphogenetically active proteins, participate in stimulation of osteogenesis. The proteoglycans composed of complex polysaccharides, mainly sulphate glycosaminoglycans such as chondroitin, heparan, dermatan, and keratan sulphates, support functioning of the osseous tissue. Other important components are the non-sulphate glycosaminoglycans, particularly hyaluronic acid (HA) - a natural polysaccharide binding water in the intercellular space and providing tissue resistance to compression. Hyaluronic acid fulfils the role of a barrier responsible for protective functions in the intercellular medium, and participates in transportation and distribution of water in tissues of the body [4,5].

The main goal of this study was to explore radiopague properties of novel polymer-mineral nanocomposites created on the basis of ZrO_2-Gd_2O_3 core functionalized with hyaluronic acid. The regeneration of osseous tissue defects in vivo experiments in rats was demonstrated under the effect of this osteoplastic material.

Material and Methods

Chemical Part. The radiopaque nanoparticles ZrO_2-Gd_2O_3 of 15 nm diameter containing 2% of Gd in their core were synthesized by sol-gel method at Donetsk O.O. Galkin Physical-Technical Institute of the National Academy of Sciences of Ukraine [6]. They were functionalized by grafting cationic polyelectrolyte and subsequent immobilization of hyaluronic acid (HA) on to their surface at Lviv National Polytechnic University [7-9].

ZrO_2-Gd_2O_3 nanoparticles were functionalized using a subsequent three-step process. At Stage 1 of functionalization oligoperoxide surfactant (OS) that is a copolymer of vinyl acetate (VA), 5-(tert-butylperoxy)-5-methyl-1-hexen-3-yne (VEP) and maleic anhydride (MA) (Fig. 1a), were synthesized, as described in [9]. OS contained 16 %, 48% and 36% (or 22.8%mol, 32.2%mol and 45.0%mol) links of VA, VEP and MA, respectively. Dimethyl aminoethyl methacrylate (DMAEMA) (Aldrich) was used without additional purification, and had following characteristics: nd20=1.439, d420=0.933 g/mL at 25°C. HA (poly(β-glucuronic acid-[1→3]-β-N-acetylglucosamine-[1→4]) (Aldrich, ≤1% protein impurity) was used without additional purification and dissolved in H_2O (5 mg/ml). Dimethyl formamide (DMF) solvent was purchased from Aldrich and used without additional purification. The nanoparticles were coated via adsorption of OS, and as a result, functional shell containing radical forming peroxide fragments was formed on the nanoparticles surface.

At Stage 2, the cationic polyelectrolyte chains consisting of the DMAEMA links were grafted to the nanoparticles via radical polymerization in the DMF initiated at 800°C (Fig. 1b) by the peroxide fragments from their surface. After thorough washing with isopropyl alcohol, the nanoparticles got 7.8% of grafted poly(DMAEMA). Washed samples of the nanoparticles were divided into 2 parts: sample Z1 which was dried and sample Z2 which was washed with distilled water

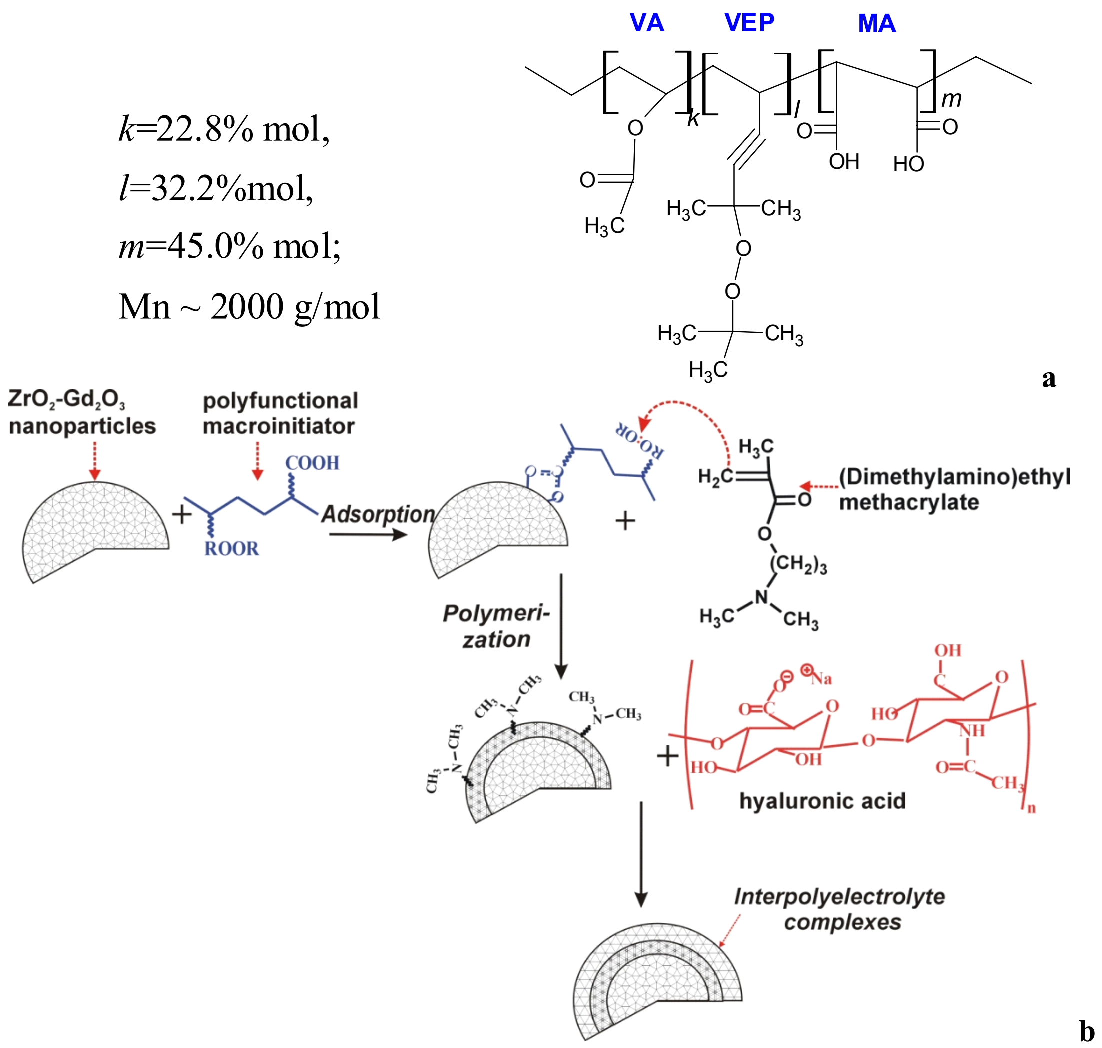

Fig. 1

Structure and scheme of formation of polymer-mineral radiopaque nanocomposites based on ZrO_2-Gd_2O_3 nanoparticles functionalized with hyaluronic acid: (a). Structure of superficial-active oligoperoxide modifier - copolymer vinyl acetate (VA), 5-(tert-butylperoxy)-5-methylhex-1-en-3-yne (VEP) and maleic anhydride (MA); (b). Scheme of modification of the surface of zirconium dioxide with oligoperoxide and formation of functional polymer membrane (poly(2-dimethylamino)ethyl methacrylate) on the surface of ZrO_2 using surface graft polymerization initiated from the surface of particle; formation of a interpolyelectrolyte complex under the influence of hyaluronic acid and poly(2-dimethylamino)ethyl methacrylate localized on the surface of ZrO_2 (Z1 and Z2 materials) particles

without prior drying and used for preparation of the paste with 70% concentration of particles in it.

At Stage 3, the nanoparticles were treated with 0.25 % water solution of HA. Nanoparticles/HA ratio equaled 3:1 by weight. As a result of HA deposition onto the nanoparticle surface with grafted cationic polyelectrolyte interpolyelectrolyte complexes, the poly(DMAEMA)/HA complexes were formed (Fig. 1b).

Animal study was carried out at the vivarium of the Danylo Halytsky National Medical University in Lviv. Eighteen white outbred female rats of 8-9 months of age and 300-350 g body weight were used in the experiments. Animals were treated according to the international principles of the European Convention for protection of vertebrate animals used for experimental and other scientific purposes (Strasburg, 1986) [10], and "General ethic principles of experiments on animals" approved by the 1st National Congress on Bioethics (Kyiv, Ukraine, 2001). Protocols of investigation were approved by the Commission on Bioethics issues at the Danylo Halytsky National Medical University in Lviv.

The animals were divided into 3 experimental groups, each including 6 rats. In the 1st group, Z1 material (thick paste-like mass of white colour) was used for grafting into the artificially created defects. In the 2nd group, Z2 material (white mass of creamy consistency) was used, while in the 3rd (control) group, bone defect was sutured below the blood clot.

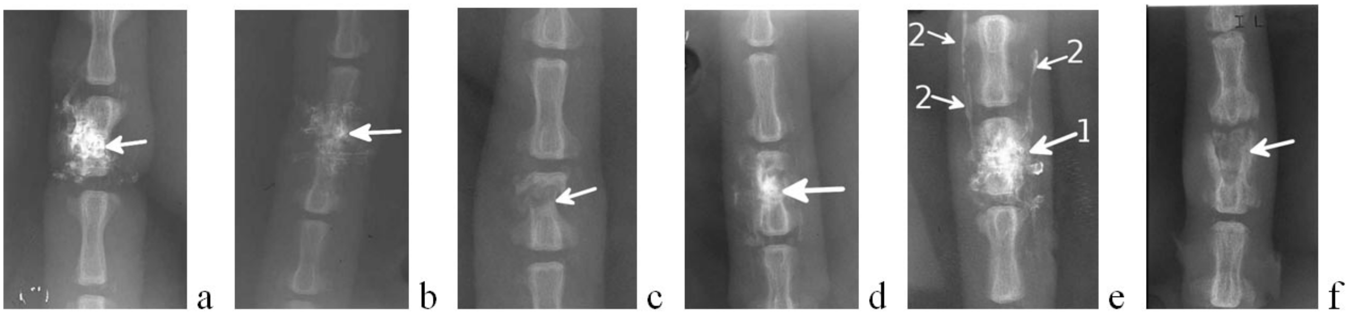

Fig. 2

Radiogram of the osseous defects: the osseous defect (shown with arrow) in rat's caudal division filled with Z1 (a), Z2 (b) material and the osseous defect sutured under the blood clot - control (c) in 15 days after surgical intervention; the osseous defect in rat's caudal division filled with Z1 (d), Z2 (e) and the osseous defect sutured under the blood clot - control (f) material in 30 days after the surgical intervention

An artificial bone defect was formed in rat's caudal vertebra using a procedure patented by the authors of the article [11]. Since the mentioned bone is similar in its structure and alveolar process to the osseous tissue of the periodontal complex, it was chosen for modelling of the pathological process accompanied by the osteoporotic process and, particularly, the formation of bony pockets. A linear longitudinal incision (10-15 mm) in the region of the upper third of the rat's tail was carried out in the study under aseptic conditions and local anaesthesia with 2% solution of novocain, with following desquamation of the tendon and soft tissues. Caudal vertebra was skeletonized with a raspatory into the area necessary for creation of the bone defect. The bone defect sized to 4-5 mm was formed in the centre of the vertebra with a round dental probe, and then filled with the material under study. The wound was sutured by means of the polyamide thread. Prevention of purulent complications was accomplished by adhering to the rules of asepsis and antisepsis.

Collection of the bio-material was carried out in 15 and 30 days after surgery. Skin reaction to the injected material was estimated; amputation of caudal division was performed under ether anaesthesia, and macro-preparations of vertebra regenerates were investigated radiologically (MINIDENT 55, Humenne, Chirana Stara Tura, Slovakia). Exertion on the radiological tube constituted 55 кV, current intensity - 10 mA, focal distance - 10 cm, and exposition time - 0.1-0.25 sec. For photographing, tissue fragments were placed on sheets of "Kodak" film (3×4).

Results and Discussion

In the 1st group, skin reaction to Z1 material was estimated in 15 days, and stable condition of the scar and absence of suppuration in the postoperative region were detected. In the 2nd group Z2 material, in contrast to Z1 material, caused a significant irritation in the area of intervention, and that effect was accompanied by an inflammatory reaction. In the 3rd (control) group, study of condition of the defect sutured under blood clot demonstrated a delayed healing and presence of the inflammatory reaction.

In 30 days after surgical intervention, skin reaction in animals of the 1st experimental group after grafting of Z1 material showed scab formation ringed with unchanged tissue, while there were no more signs of obvious inflammation. In the 2nd experimental group, after grafting of Z2 material, stabilization of skin reaction with formation of clear scab demonstrating insignificant signs of inflammatory reaction was observed. In the control group, skin reaction in the region of osseous defect sutured under blood clot showed haemorrhages and formation of scabs with signs of slight inflammatory process.

A radiopaque image of Z1 material in the form of large fluffy structure with uneven and indistinct contour was observed in 15 days after surgical intervention on radiograms of caudal fragments collected from rats of the 1st experimental group (Fig. 2a). This material completely filled the region of the defect. In the 2nd experimental group where Z_2 material was used, radiopacity of less intensity and some diffusion of investigated material (Z2) were observed in patterns obtained on the 15-th day after the applied surgical intervention (Fig. 2b).

Contours of marginal hollow osseous defects, as well as tiny and low-contrast structures, were properly visible in 15 days after surgical intervention on the radiograms of samples of the osseous tissue of vertebrae in the 3rd (control) group where the defects were sutured under blood clot (Fig. 2c).

In samples of the 1st experimental group, the image on the radiograms was distinct, and the radiopacity of Z1 material was maintained completely in 30 days after the surgical intervention (Fig. 2d). In 30 days in the 2nd experimental group treated with Z2 material, fluid consistency of the material caused an appearance of intensive radiopaque image on the radiogram not only in the centre of the defect; however, the leakage of the osteoplastic material was also observed (Fig. 2e shown with arrow - 1, 2). The margin filled with an unformed mass was detected along the periphery of the defect region. The radiologic image of the osseous defects sutured under blood clot remained unchanged in 30 days after surgical intervention (Fig. 2f).

Thus, trabecular structure of the bone was distinctly seen on the obtained radiograms, and osseous beams and their interrelation were well differentiated. Moderate radiopacity of image between soft tissues and background of films was also detected.

Discussion. Recently, we proposed a new universal model for the in vivo investigation of the efficacy of regeneration of the osseous tissue [11]. This experimental model implies a creation of defects in the caudal vertebra of experimental animals (rats) with subsequent filling of the defect with the osteoplastic material used for stimulation of the reparative osteogenesis. In present study, the above mentioned model was applied for evaluation of clinical consequences of using a new composite based on ZrO_2-Gd_2O_3 nanoparticles with a polymer shell functionalized with HA for filling regions of the osseous defect. It was found that this polymer-mineral nanocomposite used in the tenacious paste-like form (Z1 material) is biologically inert and completely fills artificially created osseous defects in rat's vertebra. Z1 material proved to be suitable for complete filling of the bone defect and initiating the reparative osteogenesis as soon as the first weeks after surgical intervention. Insignificant amount of PAS-positive substances and acid glycosaminoglycans were detected histochemically - a proof of effective processes of the reparative osteogenesis. Another composite, Z2 material of similar chemical structure but of different liquid consistency, caused irritation in the intervention region and did not fill the defect densely, appearing as a diffuse image on the radiograms.

We have established that functionalization of Z materials with HA not only prevented the re-infection, but also improved a course of postoperative period enabling a "controlled" inflammatory reaction. The results of our observation coincide with the literature data [4, 5], since the hydrophilic properties of HA promoted stabilization of coagulate, thus accelerating tissue regeneration without complications. Besides possessing antimicrobial properties, HA is also known to , inhance phagocytic activity of granulocytes, activate fibroblasts and endotheliocytes promoting their migration and proliferation, stimulate proliferative activity of the epithelial cells creating favourable conditions for the remodulation of connective tissue matrix [4, 5]. In another study (paper in preparation) histological and histochemical investigation confirmed high osteoplastic potential of the developed nanocomposite functionalized with HA.

Presence of the radiopaque core in both polymer-mineral Z_1 and Z_2 nanocomposites - Z_1 and Z_2 enables controlling their localization during regeneration of the osseous tissue. This property is a valuable advantage of created composites, since monitoring of various granular osteoplastic materials can be complicated because their radiopacity frequently resembles a natural bone.

Conclusions

High efficacy of applying the new experimental model proposed by the authors for investigation of processes of the reparative osteogenesis has been demonstrated. Regeneration of the osseous tissue of rat's caudal vertebra proved quite suitable for studying behaviour of new osteoplastic materials used in this work.

Polymer-mineral ZrO_2-Gd_2O_3 nanocomposites functionalized with HA are effective at regeneration of bone defect; they demonstrate bio-tolerance and high integration with the osseous tissue. High density of Z_1 material allows keeping it in the centre of the bone defect, opposite to Z_2 material leaking from the defect area by lymphogenic or venous ways. Due to the radiopaque core, the developed ZrO_2-Gd_2O_3 nanocomposites can be used for monitoring treatment course, since they can be easily detected by the X-ray method. The obtained results can be a basis for planning additional studies with prospects of their implementation in clinical periodontal practice, maxillofacial surgery, and implantology.

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