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Development of novel ZrO_2-Gd_2O_3 nanocomposite functionalized with hyaluronic acid and its application for repair of bone defects in experimental animals

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ABSTRACT
The aim of this work was developing novel polymer-mineral radiopaque composites based on the nanoparticles of ZrO_2-Gd_2O_3 and functionalized with the hyaluronic acid, as well as their use for repair of bone defects in the experimental rats, and conduction of roentgenologic, histological and histochemical studies of the reparative osteogenesis. Roentgenologic study: 18 white outbred female rats of 8-9 months age and 300-350 g body weight were used in the experiments. The animals were divided into 3 groups, each including 6 rats. Artificial defect was done in rat's vertebra, and further regeneration of the osseous tissue was performed by using synthesized nanocomposites Z1 and Z2 of different consistency. In control group, bone defect was sutured below the blood clot. Radiological investigations have been carried out in different terms of bone regeneration (15 and 30 days after surgical intervention). Histological and histochemical study: Artificial bone defects were formed in the caudal vertebra of 24 female rats. Experimental groups: 1) novel ZrO_2-Gd_2O_3 nanocomposite with the hyaluronic acid in its coating; 2) synthetic «Easy-Graft™» material (Switzerland) prepared on the basis of β-tricalcium phosphate; 3) «Stimulus-Oss» material based on animal collagen with addition of 2% chlorohexidine and hydroxyapatite (Russian Federation); 4) control (bone defects were filled with blood clot). Vertebrae with the regenerate were isolated in 30 days by surgical intervention. Vertebra preparations were studied macroscopically and histologically. New experimental model (regeneration of bone defects artificially formed in rat's caudal vertebra) was proposed by the authors and it showed its advantages in the reparative osteogenesis. Created biomaterial demonstrated satisfactory bio-compatibility after surgery. Its 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 centre of bone defect, opposite to Z2 material leaking from the defect area (15 and 30 days after surgical intervention). Among 3 different osteoplastic materials used in the study, novel ZrO_2-Gd_2O_3 nanocomposite with the hyaluronic acid in its coating showed the highest effectiveness in stimulating regeneration of the osseous tissue. That effect was confirmed by the morphological, as well as by histological study of wound repair. New material has demonstrated the bio-tolerance and high integration with the osseous tissue. Novel *in vivo* experimental model of caudal vertebra proved its efficiency in testing materials used for regeneration of the osseous tissue. Created polymer-mineral radiopaque nanocomposite with ZrO_2–Gd_2O_3 core and the hyaluronic acid in its shell effectively enhanced regeneration of bone defect. The clinical, roentgenologic, histological and histochemical studies testify to bio-tolerance, radiopacity, as well as high integration of the applied Z1 material with the osseous tissue of the recipient bed.
Keywords: *osseous defect, rat's caudal vertebra, novel osteoplastic materials, ZrO_2-Gd_2O_3, radiopaque polymer-mineral nanocomposite, hyaluronic acid.*

1. INTRODUCTION
Creation of new bioplastic materials that are effective at surgical intervention is a crucial task in the contemporary reconstructive medicine. This issue was considered in numerous publications dedicated to such new materials affecting the regeneration processes [1-5]. Development of materials for the replacement of bone defects of various etiology, the ways for improvement of their biocompatibility, osteogenous potential, as well as providing osteoinduction, osteoconduction and radiopacity are the most important aims in the orthopedic and surgical practice. They are of special significance in the maxillofacial surgery, periodontology and implantology, since they are time-consuming and highly technological, should follow special clinical safety requirements and possess evident efficacy of a “final product” [6].

Various nanomaterials proved their usefulness in a reparative medicine, since they possess mechanical strength, flexibility, and chemical stability [7, 8]. However, bio-safety and efficacy requirements at using nanocomposites in medicine provoke additional experimental studies aimed at proving their biocompatibility and functional activity in the body [9]. Many contemporary osteoplastic materials are based on bone collagen, hydroxyapatite, or alpha- and beta-tricalcium phosphate [10]. Besides, big attention was paid to the nanoparticles (5-60 nm) of iron oxide, copper, zinc, silver, gold and titanium [11]. In particular, the nanoparticles based on zinc oxide possess the bacteriostatic and bactericidal properties, as well as healing activity, and they were shown to be effective in stimulating regeneration of the osseous tissue [12-14].

Components of extracellular matrix, in particular, proteoglycans, glycoproteins and morphogenetically active proteins, have been involved in the stimulation of osteogenesis. Proteoglycans composed of complex poly-saccharides, mainly sulphate glycosaminoglycans such as chondroitin, heparan, dermatan, and keratan sulphates, are important in functioning of the osseous tissue. Other important components are the non-sulphate glycosaminoglycans, particularly the hyaluronic acid - natural polysaccharide capable of binding water in the intercellular space and providing tissue resistance to a compression. The hyaluronic acid is a potential barrier responsible for protective functions in the intercellular medium, also participating in transportation and distribution of water in tissues of the body [15-18].

At early stage of application of the hyaluronic acid in the periodontal practice, an improvement of healing processes in tissues is accompanied by an increase in cellular infiltration and retardation of production of pro-inflammatory cytokines, while later on desquamation of cells and stimulation of mitosis take place. Besides, an increase in synthesis of the hyaluronic acid is induced, migration of cells by the hydrolyzed matrix of the osseous tissue is intensified, and new blood vessels are formed during healing process. Finally, a decrease in collagen matrix and shorter period of scarring are observed [19-23]. Glycosaminoglycans are capable of modulating metabolism of connective tissue cells and affect their differentiation [24]. The effect of glycosaminoglycans on the reparation of the osseous tissue of alveolar jaws was demonstrated [18, 25, 26], however, their role in that process has not been studied precisely.

The main goal of this study was to explore the ability of novel polymer-mineral composites created on the basis of ZrO_2–Gd_2O_3 nanoparticles functionalized with the hyaluronic acid to repair the osseous defects in the *in vivo* experiments, as well as to demonstrate the radiopacity of this material. The macroscopic estimation, histological and histochemical investigations of the reparative osteogenesis at different stages of that process have been also carried out.

2. EXPERIMENTAL SECTION

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, NAS of Ukraine [27]. They were functionalized by grafting the cationic polyelectrolyte and subsequent immobilization of the hyaluronic acid (HA) onto their surface at Lviv National Polytechnic University [28-30].

ZrO_2–Gd_2O_3 nanoparticles were functionalized using a subsequent three-step process. At the 1st stage of functionalization, the 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), was synthesized, as described in [37]. 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 the following characteristics: nd20 =1.439, d420 = 0.933 g/mL at 25°C. The 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 by adsorption of OS, and as a result, functional shell containing radical forming peroxide fragments was formed on the nanoparticles surface.

At the second stage, the cationic polyelectrolyte chains consisting of the DMAEMA links were grafted to the nanoparticles via radical polymerization in the DMF initiated at 800°C (Figure 1b) by the peroxide fragments from their surface.

At the third stage, the nanoparticles were treated with 0.25 % water solution of the HA.

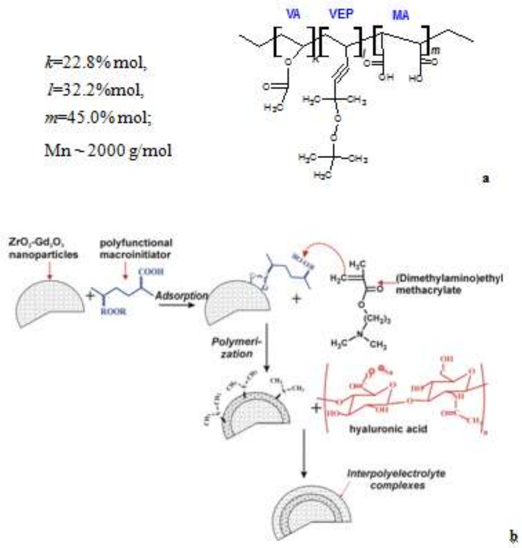

Figure 1. Structure and scheme of formation of polymer-mineral radiopaque nanocomposites based on ZrO_2–Gd_2O_3 nanoparticles functionalized with the hyaluronic acid: (a). Structure of a superficial-active oligoperoxide modifier – copolymer vinyl acetate (VA), 5-(tert-butylperoxy)-5-methylhex-1-en-3-yne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

After thorough washing with the isopropyl alcohol, the nanoparticles got 7.8% of grafted poly (DMAEMA). Washed samples of the nanoparticles were divided into 2 parts: sample Z1 that was dried and sample Z2 which was washed with distilled water without prior drying and used for preparation of the paste with 70% concentration of particles in it. Nanoparticles/HA ratio equaled 3:1 by weight. As a result of the HA deposition onto the nanoparticle surface with grafted the cationic polyelectrolyte inter-polyelectrolyte complexes, the poly(DMAEMA)/HA complexes were formed (Figure 1b). Animal study was carried out at the vivarium of Danylo Halytsky Lviv National Medical University. 18 white outbred female rats of 8-9 months age and 300-350 g body weight were used in the roentgenologic study. 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), and "General ethic principles of experiments on animals" approved by the 1st National Congress on Bioethics (Kyiv, Ukraine, 2001) [31]. The Protocols of investigation were approved by the Commission on the Bioethics issues at Danylo Halytsky Lviv National Medical University.

Animals were divided into 3 experimental groups, each including 6 rats. In the 1st group, Z1 material (thick paste-like mass of white color) 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. Artificial bone defect was formed in rat's caudal vertebra using a procedure patented by the authors of the article [32]. Since this bone corresponds in its structure and alveolar process to the osseous tissue of the periodontal complex, this object was chosen for modelling of the pathological process accompanied by the osteoporotic process and formation of the bony pockets, in particular. A linear longitudinal incision (10-15 mm) in the region of the upper third of rat's tail was carried out in the study at aseptic conditions under local anesthesia with 2% solution of novocaine, with following desquamation of the tendon and soft tissues. Caudal vertebra was skeletonized with a raspatory into the area necessary for creation of bone defect. Bone defect sized to 4-5 mm was formed in the centre of the vertebra with round dental probe, and then filled with a material under study. The wound was sutured by means of the polyamide thread. Prevention of the purulent complications was accomplished by keeping to the rules of asepsis and antisepsis. Collection of 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 done under ether anesthesia, and macro-preparations of vertebra regenerates were investigated roentgenologically (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 the sheets of "Kodak" film (3x4). For histologic and histochemical studies, 24 female rats of 8-9 months age and 300-350 g body weight were used. The animals were divided into 4 groups, each including 6 rats whose bone defects were treated with: 1) novel ZrO_2-Gd_2O_3 nanocomposite containing the hyaluronic acid in its coating; 2) commercial synthetic «Easy-Graft™» material (DS Dental, Switzerland) prepared on the basis of β-tricalcium phosphate; 3) bone regeneration «Stimulus-Oss» material (Russian Federation) based on animal collagen supplemented with 2% chlorohexidine bigluconate and hydroxyapatite; 4) control in which bone defect was filled with the blood clot. Artificial bone defect was formed in rat's caudal vertebra by using a procedure patented by the authors of the article [32]. Taking into account that this bone corresponds in its structure and alveolar process to the osseous tissue of the periodontal complex, it was used for modelling of the pathological process accompanied by the osteoporotic process and formation of bony pockets, in particular. A linear longitudinal incision (10-15 mm) in the region of the upper third of rat's tail was carried out at aseptic conditions under local anesthesia (2% solution of novocaine), with following desquamation of the tendon and soft tissues. Caudal vertebra was skeletonized with a raspatory into the area necessary for creation of bone defect. Bone defect sized to 4-5 mm was formed in a center of the vertebra with a round dental probe, and then filled up with a material under study. The wound was sutured by means of the polyamide thread. Prevention of the purulent complications was accomplished by keeping to the rules of asepsis and antisepsis. Isolation of the bio-material for histological and histochemical studies was carried out in 30 days after surgical intervention. Skin reaction to application of the injected material was estimated, amputation of the caudal division was done under ether anesthesia, and decalcinated macro-preparations of the vertebra regenerates were subjected to the macroscopic and histological investigation. Bone tissue fragments were fixed in Bouen's solution (saturated water solution of the picric acid – 75 ml, undiluted formalin – 25 ml, glacial acetic acid – 5 ml). Bone decalcification was carried out in 10% solution of disodium salt of the ethylenediaminetetraacetic acid (EDTA, Aldrich), dehydrated in the ethyl alcohol of increasing concentrations, and immersed in the melted paraffin. 7-10 μm thick histological sections colored with hematoxylin-eosin according to a standard technique, were prepared after the microtome slicing [33, 34]. Histological preparations were studied under a light optic microscope MBI-2 (LOMO, St-Petersburg, Russian Federation) and the microimages were taken by the "Olympus C 410" digital camera. Statistical calculations were performed using Microsoftt Office Excel 2003 program. Significance of difference between groups of data was determined by Student's t-criterion, and significance was defined as $p<0.05$.

3. RESULTS SECTION

Roentgenologic study. In the 1st experimental 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 opposite 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 the state of the defect sutured under blood clot demonstrated a delayed healing and a presence of the inflammatory reaction.

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

In 15 days after surgical intervention, the radiopaque image of Z1 material in the form of large fluffy structure with uneven and indistinct contour was observed on the roentgenograms of caudal fragments collected from rats of the 1st experimental group Fig. 2a). That material completely filled the defect region. On the 15-th day after applied surgical intervention, in the 2nd experimental group where Z2 material was used, a radiopacity of less intensity and some diffusion of investigated material (Z2) was observed in the obtained samples (Fig. 2b). In that term, the contours of marginal hollow osseous defects, as well as tiny and low-contrast structures, were properly visible on the roentgenogram of samples of the osseous tissue of vertebrae in the 3rd (control) group whose bone defect was sutured under the blood clot (Fig. 2c).

Figure 2. Roentgenogram 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 surgical intervention.

In 30 days after surgical intervention, in samples of the 1st experimental group, the image on the roentgenograms was distinct and the radiopacity of Z1 material was maintained completely (Fig. 2d). In that term in the 2nd experimental group treated with Z2 material, fluid consistency of this material caused an appearance of the radiopaque image on the roentgenogram beyond the borders of the center of the defect region due to a leakage of the osteoplastic material from that region (in Fig. 2e shown with arrows 1 and 2). The margins filled with unformed mass were detected along the periphery of the defect region. In 30 days after surgical intervention, the radiologic image of the osseous defects sutured under the blood clot remained unchanged (Fig. 2f). The trabecular structure of bone was distinctly seen on the obtained roentgenograms, and the osseous beams and their interrelation were well differentiated. Moderate radiopacity of the image between soft tissues and background of films was also detected.

Recently, we proposed a new universal model for *in vivo* investigation of the efficacy of regeneration of the osseous tissue [32]. This experimental model implies a creation of the defect in the caudal vertebra of experimental animals (rats) followed by filling that 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 new composite based on ZrO_2–Gd_2O_3 nanoparticles covered by a polymeric shell functionalized with HA for filling regions of the osseous defect. This polymer-mineral nanocomposite used in the tenacious paste-like form (Z1 material) was biologically inert and it completely filled artificially created osseous defects in rat's vertebra. Z1 material proved to be suitable for complete filling of bone defect and initiating the reparative osteogenesis as soon as in the first weeks after surgical intervention. Insignificant amount of PAS-positive substances and acid glycosaminoglycans were detected at the histochemical study, and that is a proof of effective reparative osteogenesis. Another nanocomposite (Z2) of similar chemical structure but of different liquid consistency, caused an irritation in the intervention region and it did not fill the defect densely, appearing as a diffuse image on the roentgenograms.

We have established that functionalization of Z materials with the HA prevented the re-infection, as well as improved a course of the postoperative period enabling a "controlled" inflammatory reaction. The results of our observation coincide with literature data [35, 36], since the hydrophilic properties of the HA promoted stabilization of the coagulate, thus, accelerating tissue regeneration without complications. Besides, the HA possesses the antimicrobial properties, enhances phagocytic activity of granulocytes, activates fibroblasts and endotheliocytes promoting their migration and proliferation, stimulates proliferative activity of the epithelial cells creating favorable conditions for re-modulation of the connective tissue matrix [35, 36]. In another study (our paper in press), histological and histochemical investigation confirmed high osteoplastic potential of the developed nanocomposite functionalized with the HA.

Presence of the radiopaque core in both polymer-mineral nanocomposites – Z1 and Z2 -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 frequently their radiopacity resembles that in a natural bone.

Taking into account the results of our roentgenologic study, it was concluded that the application of Z1 nanocomposite possessing higher density is more expedient for stimulation of bone repair than the application of less viscous Z2 material that was leaking into surrounding tissues via lymphogenic and venous ways. That is why only Z1 material was used in next histological and histochemical studies of regeneration of the bone defect.

Histological and histochemical studies. As mentioned above, application of Z1 material was accompanied by a positive effect on the repair of bone damage in rat's caudal vertebra model observed as soon as in the first days after surgical intervention. The results of evaluation of skin reaction in animals of various experimental groups are presented in Table 1.

In all animals of the 1st group with grafting of ZrO_2-Gd_2O_3 nanocomposite functionalized with the hyaluronic acid (Z1 material), the healing took place within a short period of up to 8-10 days. Surfaces cleaned up from the fibrous layers were observed in 8-10 days in rats of the 1st group, and a tendency to healing with initial tention was found in 83% ($P \leq 0.05$) of the treated animals.

Table 1. Criteria of the macroscopic investigation of the osseous tissue regenerates.

Criteria of macroscopic investigation	Duration of healing process (days)			Character of healing		Complication in the area of surgery	Presence and distinctness of defect contours		Appearance of new tissues in the center of the defect	
Groups and number of animals	8-10 days (accelerated)	11-14 days (moderate)	≥ 15 days (slow)	Initial tention	Secondary tention	Suppuration	Distinct	Indistinct	Compression of the defect	Absence of depression
Group 1, (n=6)	6 (100%)*	-	-	5 (83%)*	1 (17%)*	-	4 (67%)*	2 (33%)*	-	6 (100%)*
Group 2, (n=6)	1 (17%)	4 (67%)	1 (17%)	2 (67%)	4 (33%)	-	2 (33%)	4 (67%)	-	6 (100%)*
Group 3, (n=6)	-	4 (67%)	2 (33%)	4 (67%)	2 (33%)	-	3 (50%)	3 (50%)	1 (17%)	5 (83%)
Group 4, (n=6)	-	2 (33%)	4 (67%)	1 (17%)	5 (83%)	4 (67%)	1 (17%)	5 (83%)	3 (50%)	3 (50%)

In brackets - percentage of animals with noted criteria of the healing process
*$P \leq 0.05$ - compared to the control group (Group 4)

In 67% of animals of the 2nd (grafting of «Easy-Graft™» material) and 3rd (grafting of «Stimulus-Oss» material) groups, moderate healing rate was observed. However, there were also cases of accelerated healing (17%), as well as slow healing (17%), detected in the 2nd experimental group. While in 67% animals of the 4th group (control) in which the osseous defects were filled with blood clot, the healing process was slow. However, there were also cases of moderate healing process (33%), and in those cases the indicator of the initial tension equaled 17% and the indicator of secondary tension equaled 83% with formation of large shapeless scars in a rat's caudal region.

In the 1th experimental group, the wound surfaces cleaned up of the fibrous layers in 8-10 days in 83% ($P \leq 0.05$) of animals and had a tendency to healing with an initial tension. In the 2nd and 3rd groups, this process was less intensive showing similar indices in both groups with 67% ($P \leq 0.05$) of initial tension and 33% ($P \leq 0.05$) – of secondary tension. In the 2nd and 3rd groups, the absence of suppuration was caused by application of highly effective osseous plastic materials capable of preventing re-infection of the wound surface. The absence of suppuration in animals of the 1st group suggests good biocompatibility of the material due to a presence of the HA which possesses the antibacterial properties. Complications with the suppuration were observed in 67% cases of animals of the 4th group.

Distinct contours of the defect is an important criterion of assessment of the post-operative area. In the 1st group, this indicator constituted 67% ($P \leq 0.05$) of distinct margins versus 33% of indistinct margins, while in the 2nd group, the indicator of distinct margins of the post-operative defect constituted 33%, and of indistinct – 67%. In the 3rd experimental group, the ratio distinct to washed out contours of the defect was 50% to 50%, and in the 4th (control) group, the situation was the worst, since 83% of cases of the post-operative area had washed out margins and only 17% – distinct ones. In this group, 50% of animals had a compression of tissues of the post-operative wounds. Such compression was also observed in 17% of animals of the 3rd experimental group.

A size of the operative area is an important characteristic during the post-operative period, since a longitudinal linear incision done for skeletonization of the vertebra and sufficient access to the osseous tissue was rather big - 10-15 mm. In 2 weeks, the size of the postoperative area changed in some animals due to the development of the inflammatory processes and formation of the post-operative scar. In animals of the 1st group, this indicator constituted 88±8% ($P \leq 0.05$) of a primary size of the incision (Table 2). In animals of the 2nd and 3rd groups, it was 128±8% and 120±8%, respectively, while in the 4th control group, this index reached 144±16% due to the suppuration processes. Scales of the defect after surgery were increased in all experimental groups, comparing with the scales of the initial operation section (10-15 mm, 100%).

Table 2. Macroscopic characteristics of the defect size.

Macroscopic characteristics of the defect size	Size of the defect (mm)				% from the initial surgical incision
Surgery section	min	max	M	m	100±20
	10	15	12.5	2.5	
Group 1, n=6	10	12	11	1	88±8*
Group 2, n=6	15	17	16	1	128±8
Group 3, n=6	14	16	15	1	120±8
Group 4, n=6	16	20	18	2	144±16

*P≤0.05 - compared with the control group (Group 4)

Good integration of plastic material with an adequate number of newly formed vessels, slightly expressed vascular disorders, and moderate amount of the osteoblasts was observed on the 30th day of the morphological investigation of the vertebrae in the 1st experimental group (the osseous defects were filled up with novel Z1 material). A moderate amount of collagen fibers, absence of infiltration with the neutrophilic granulocytes, and absence of the suppuration on the periphery of the defects were observed (Figure 3 a, b).

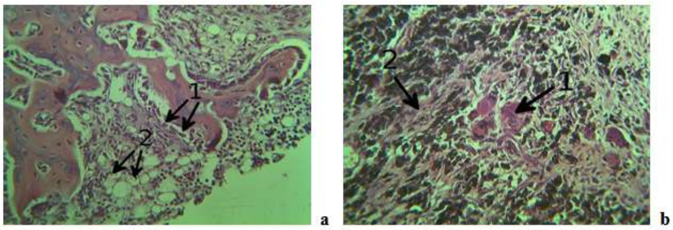

Figure 3. Grafting of Z1 material on the 30th day of monitoring: a) Active osteoblasts (1) on the surface of the osseous beams. Considerable amount of newly formed vessels (2) of regenerate; b) Integration of plastic material with the osseous tissue (1) (induction of osteogenesis around plastic material). Moderate amount of the connective tissue fibres (2) on the periphery of the defect. Hematoxylin-eosin, x200.

In animals of the 2nd group, a synthetic «Easy-Graft™» material prepared on the basis of β-tricalcium phosphate was applied. During examination of the histological preparations, round heterogeneous fragments of plastic material were detected in the center of the defects. The necrotizing remnants of the osseous tissue were also present there, and around those remnants, the infiltration of the polymorphous cells was detected. The lymphocytes and neutrophilic granulocytes constituted the basis of the infiltrate. Infiltration with the lymphocytes and single neutrophilic granulocytes was also found in the elements of plastic material located near the necrotizing osseous fragments. Fibroblasts and tiny collagen fibers having a concentric location accumulated on the periphery of those deposits. Hyperemia and stasis were observed in blood vessels of the reticulo-fibrous tissue. The foci of intensive infiltration of certain areas of this tissue with the neutrophilic granulocytes were also present in some regions.

A moderate amount of the reticulo-fibrous tissue with a significant number of newly formed vessels was present on the periphery of the defect in the 2nd experimental group. These vessels were found to be dilated and completely filled with the erythrocytes. In some cases, single haemorrhages into the reticulo-fibrous tissue were detected.

New formations of the osseous beams having a plate-like structure and penetrating into the reticulo-fibrous tissue were observed on the periphery of the maternal osseous tissue.

An insignificant number of active osteoblasts synthesizing components of the osseous matrix were detected on the surface of newly formed osseous beams. A moderate number of cells of the osteoblastic character and, on the periphery, of the fibroblastic differon, was accumulated around newly formed fragments of the osseous tissue (Figure 4 a, b).

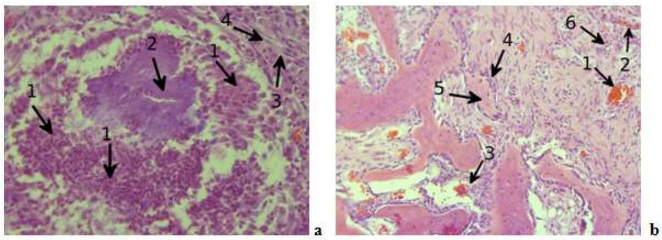

Figure 4. Grafting of «Easy-Graft™» material on the 30th day of monitoring: a) Polymorphous cellular infiltration (1) around the remnants of plastic material (2). The fibroblasts (3) and young connective tissue fibers (4) on the periphery of cellular elements; b) Hyperemia (1), stasis (2) in vessels of the reticulo-fibrous tissue. Single hemorrhages (3) into reticulo-fibrous tissue are observed. Moderately expressed osteoblastic reaction (4) on the surfaces of the osseous beams (5). Polymorphous nuclear infiltration (6) of reticulo-fibrous tissue; Hematoxylin-eosin, x200.

In animals of the 3rd group treated for bone repair with «Stimulus-Oss» material based on animal collagen supplemented with 2% chlorohexidine bigluconate and hydroxyapatite, on the 30th day after surgery single tiny heterogeneous fragments of plastic material in the center of the defect were detected in samples of the applied material. The connective tissue was moderately infiltrated with the lymphocytes, histocytes and tiny osteogenous elements and located around those fragments. Some regions of the necrotizing remnants of the osseous tissue were also visualized. Tiny osteogenous elements accumulated there. Besides, active osteoblasts synthesizing osteoid components were found in samples obtained from animals of this group in central regions of the defects, along with tiny fragments of the plastic material. The newly formed vessels appeared in the reticulo-fibrous tissue that filled the defects. A majority of blood vessels were dilated and completely filled with the erythrocytes, and hemorrhages into the reticulo-fibrous tissue were found in some cases.

Newly formed osseous beams branched from the margins of the maternal osseous tissue around which small amount of active osteoblasts accumulated. The osteoclasts that engulf the elements of the osseous tissue were also present there. Thus, in animals of the 3rd group, reconstruction of the osseous tissue occurred with participation of the osteoclasts with a simultaneous resorption of this tissue. There was a moderate amount of collagen fibres observed in the reticulo-fibrous tissue, and the amount of the connective tissue elements considerably exceeded the amount of newly formed osseous beams. In some areas, a polymorphous cellular infiltration of basic substance of the connective tissue was detected. The lymphocytes are dominating in the infiltrates, and plasma cells and histocytes were also detected, but only single neutrophilic granulocytes were found (Figure 5 a, b).

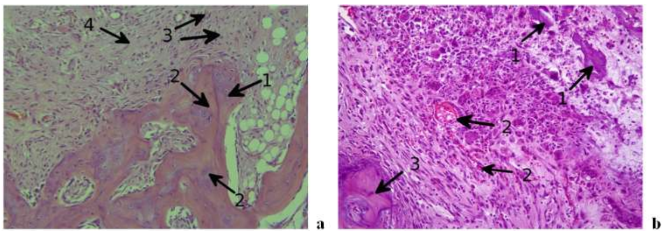

Figure 5. Grafting of «Stimulus-Oss» material on the 30th day of monitoring: a) Formation of the osseous tissue (1) on the periphery of the maternal bone (2). Significant amount of the fibroblasts (3) and young collagen fibres (4) in the reticulo-fibrous tissue; b) Grafting of «Stimulus-Oss» material on the 30th day of monitoring. Tiny fragments of the osseous plastic «Stimulus-Oss» material (1). Dilated and overfilled with blood newly formed capillaries (2). Necrotizing remnants of the osseous tissue (3); Hematoxylin-eosin, x200.

The erythrocytes of blood deposit were stuck, deformed, and some of them were destroyed (Figure 6a). Cells of the histocyte-macrophage system whose cytoplasm contained a significant amount of hemosiderin in the form of brown-yellow granules were located on the periphery of bone defect.

In addition to a significant number of erythrocytes, a moderate number of the neutrophilic granulocytes accumulated in the defect area. Besides, single eosinophils were present there. A considerable number of lymphocytes, plasma cells, young cells of the connective tissue were detected. In some areas, the fragments of half-destructed osseous tissue were observed whose osteoid was intensively basophilic, spongy and porous. The osteoclasts were observed around the area of destruction of the osseous tissue. Moderate granulation and reticulo-fibrous tissues were located on the periphery. Most vessels were dilated and overfilled with the erythrocytes. The erythrocytes were deformed and stuck (stasis), and flow of blood outside the vessels with formation of tiny hemorrhages was observed in some areas (Fig. 6 b).

The osseous tissue located on the periphery of bone defect contained some areas deprived of cells, cores of matrix destruction, and lacunae with uneven margins where active osteoclasts were found. In these areas, a significant number of lacunae with a marked destruction of the osseous tissue was visualized (Fig. 6 c). Destructive fissures passing through cement lines were also detected there. In some areas, damaged osseous trabeculae were found, their margins were uneven, and the osteoid was partly destructed. The re-absorption of the osseous defects by means of the osteoclastic and, to less extent, by smooth resorption took place both on the surface and inside vascular canals of the osseous tissue. In the preserved osseous lacunae, osteocytes with irregular elongated polygonal form were located. The nucleus of most osteocytes was oval and it occupied a considerable space in cell body. It was moderately basophilic, and the nucleolus was usually not visualized. Cytoplasm became narrow with slight basophilic tint.

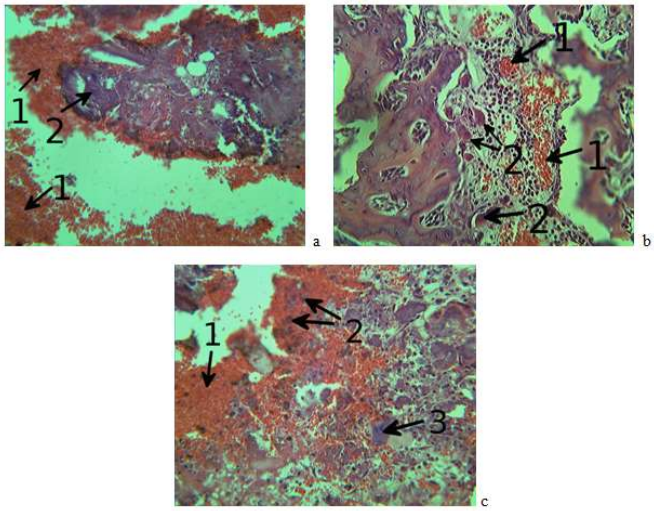

Figure 6. Osseous defect filled under the blood clot on the 30th day of monitoring: a) Elements of blood clot (1), fragments of destructed osseous tissue in the centre of the defect (2), surrounded with the polymorphous nuclear leukocytes and lymphocytes. Hemosiderin is seen in cell cytoplasm of the histocyte-macrophage system. b) Stasis in vessels of the granulation tissue (1). Osteoclastic resorption of the osseous tissue (2). c) The erythrocytes of blood clot (1), hemosiderin (2) in cell cytoplasm of histocyte-macrophage system. Half-destructed osseous beams are seen (3). Hematoxylin-eosin, x200.

The microfissures, basophilia, and irregular swellings of cement lines were recorded in the osseous trabeculae distant from the defect. In these areas, the osseous matrix was unevenly colored. Yellow bone marrow was found in the intertrabecular spaces of the preserved spongy osseous tissue, and quite rarely, elements of red bone marrow were seen there.

Summarizing, novel ZrO_2-Gd_2O_3 nanocomposite functionalized with the HA (Z1 material) exceeded in its capability to enhance bone repair such known osteoplastic materials as «Easy-Graft™» and «Stimulus-Oss».

In the present study, we evaluated the biological and clinical consequences of using novel composite based on ZrO_2–Gd_2O_3 nanoparticles with a polymeric shell functionalized with the HA for filling the osseous defect. For comparison, known osteoplastic materials such as «Easy-Graft™» (DS Dental, Switzerland) and «Stimulus-Oss» (Russian Federation) were applied. A new experimental model for *in vivo* investigation of the efficacy of regeneration of the osseous tissue was used [32]. It implies a creation of the defect in the caudal vertebra of experimental animals (rats) with following filling of that defect with the osteoplastic material used for stimulation of healing and optimization of the reparative osteogenesis. It was found that this polymer-mineral nanocomposite used in a tenacious paste-like form (Z1 material) is biocompatible and completely fills the artificially created osseous defects in rat's vertebra.

The main ways for eliminating bone tissue defects are as following: 1) introduction of special gels or pastes, that are hardening in the cavity of cracks or small defects; 2) application of three-dimensional blocks (inserts) with porous structure for repair of defects of large scale. Synthetic materials based on β-tricalcium phosphate and integrated with different compounds such as calcium hydroxyapatite, calcium sulfate, calcium carbonate, alumina, silica, zirconia, or organic and polymeric components (hyaluronic acid, chondroitin sulfate, collagen protein) were created for replacement of damaged bone [37].

There were several reasons for using ZrO_2-Gd_2O_3-based nanocomposite as bone replacement material. It provides an adequate structural strength during the repair of bone tissue, ensures micro- and nanoporosity of certain degree and morphology, eliminates or significantly minimizes the adverse effects of treatment of bone defects, accelerates the recovery process, and provides a sufficient quality of the displaced bone area. Zirconia demonstrates special requirements for the materials used for bone regeneration in the orthopedics of prosthetics joints and in dentistry for dental implants [38]. Functionalized zirconia nanoparticles proposed here for bone regeneration were additionally modified by GdO_2 in order to provide the radiopacity. Using a radiopaque bone substitute is a big advantage since it allows monitoring the process of bone regeneration (our paper in press).

We have established that functionalization of Z1 material with the HA prevented the re-infection, as well as improved a course of the postoperative period enabling a "controlled" inflammatory process. The hydrophilic properties of the HA [15-18, 37] promoted coagulate stabilization, thus, accelerating bone tissue regeneration without complications. The HA possesses antimicrobial properties; enhances phagocytic activity of granulocytes, activates fibroblasts, and endotheliocytes, also promoting their migration and proliferation. Besides, it stimulates proliferation of the epithelial cells, creating favorable conditions for re-modelling of the connective tissue matrix [15, 17, 18, 24, 37].

The absence of significant number of the immunocompetent cells near the dispersed particles of the applied material in bone defect area proves a proper integration of these particles and their biocompatibility, as well as a lack of their antigenic effects causing a rejection. There were no neutrophilic reaction and suppuration of the regenerate that often take place at trials of new nanomaterials. An increased number of blood vessels suggested a proper blood flow and supply of the regenerate with oxygen and nourishments. In the control group, suturing of the defect under blood clot was accompanied by a formation of large regions of the destructed osseous tissue. A resorption of the osseous defects both on the surface and inside (vascular canals) the osseous tissue also took place.

Thus, application of novel ZrO_2-Gd_2O_3-based nanocomposite functionalized with the HA improves significantly bone repair in rat`s caudal vertebra. It also possesses better characteristics at bone repair, comparing with such materials as «Easy-Graft™» and «Stimulus-Oss» proposed by the market.

4. CONCLUSIONS

New experimental model - rat's caudal vertebra - proposed by the authors, was used for investigation of the effect of novel osteoplastic material on the repair of bone defects. The polymer-mineral ZrO2–Gd2O3 nanocomposite functionalized with the hyaluronic acid demonstrated its higher effectiveness at the repair of bone defect, comparing with such materials available at the market as «Easy-Graftтм» and «Stimulus-Oss». This composite also demonstrated bio-tolerance and high integration with the osseous tissue Due to the radiopaque core, the developed ZrO2–Gd2O3 nanocomposite can be used for monitoring the treatment course, since it can be easily detected with X-ray method. The perspectives of implementation of new material in clinical periodontal practice, maxillofacial surgery and implantology have been discussed.

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