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EFFECT OF BIOACTIVE GLASS-BASED COMPOSITE AND LOW ENERGY LASER ON BONE REGENERATION IN AN EXPERIMENTALLY INDUCED BONE DEFECT

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Sergey Konovalenko[1]✉, Volodymyr Protsenko[2], Yevgen Solonitsyn[2], Taras Osadchuk[2], Volodymyr Konovalenko[1], Taras Omelchenko[3]

[1] RE Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine, Kyiv, Ukraine
[2] State Institute of Traumatology and Orthopaedics, National Academy of Sciences of Ukraine, Kyiv, Ukraine
[3] Bogomolets National Medical University, Kyiv, Ukraine

✉ *ip15@ukr.net*

ABSTRACT — In vivo experimental study was conducted to investigate the effect of bone regeneration under the use of a bioactive glass-based composite as well as the use of laser irradiation in the presence of the bioactive glass-based composite. In 16 white non-inbred rats, with a body weight of 300–350 g the bioactive glass-based composite was implanted into the tibial metaphysis. Besides, 90 female rats of the Wistar line weighing 250–270 g underwent laser irradiation on an artificially created tibial bone defect filled with the bioactive glass-based composite. The period of observation lasted respectively 2 and 4 weeks. It was found that application of the bioactive glass promotes bone regeneration, which is noticeably enhanced with the use of low energy laser. The combining effect leads to a significant increase in bone regeneration and the expression of bone tissue metabolic markers: osteocalcin and alkaline phosphatase. The most pronounced effect of the laser on bone regeneration occurred at a wavelength of 810 nm, which opens up prospects for the use of this technique in clinical practice.

KEYWORDS — bone regeneration, bioactive glass (BG)-based composite, low energy laser, osteocalcin (OC), alkaline phosphatase (ALP), bone tumor.

INTRODUCTION

The problem of filling in bone defects due to tumor excision and specific diseases, and the search for optimal materials for this purpose, is a topical issue in orthopaedics, traumatology, oncology, maxillo-facial surgery and neurosurgery [11]. Current stage of development in reconstructive surgery raises the urgent task of designing materials that can function as a framework for the regeneration of a damaged bone, whilst being gradually split and replaced by the patient's own tissue, thus stimulating osteogenesis. At the same time, there are still many unclear issues related to the effects of biomaterials on osteogenesis [12]. Implant materials and structures should be biocompatible, osteoinductive, and, preferably, osteoconductive without causing toxic reactions, suppuration, rejection, and metallosis [16]. Among implantation materials, bioactive glass biocomposites and their modifications are widely used [17]. Bioactive glass biocomposites are completely replaceable by bone tissue without the formation of a fibrous layer. They actively stimulate bone formation and significantly enhance reparative processes in damaged tissues, which contributes to rapid bone fusion and restoration of the bone structure [17]. When applied, such biocomposites can firmly bond to the recipient's bone tissue due to the formation of a hydroxycarbonate-apatite layer, promote the activation and proliferation of osteogenic cells, and vascularisation due to the release of three biologically active ions (silicon, calcium and phosphates) in the physiological environment. The introduction of calcium-phosphate compounds into the composition of a bioglass makes it possible to obtain a material with specific properties that satisfy its use in skeletal regions under stress [14, 15]. Various techniques for intensifying repair processes in damaged tissues, including those in bone tissue, are reported in the literature [5]. One of the techniques of intensification of repair processes of damaged tissues, which is actively studied in the world today, is the effect of low energy laser radiation (LLLT — low level laser therapy) [5]. Light in the red to near infra-red range of 600–1,070 nm has the greatest influence on the biochemical activity of different cells [1]. There is also a substantial amount of data on the effectiveness of light energy in the treatment of cancer pathology: from surgery using high-power lasers to safer and, most importantly, organ-saving photodynamic therapy using photosensitizers and photobiomodulation [2, 3, 4, 13]. Due to its proven biostimulatory effects, LLLT at different wavelengths (660, 810, 940 nm) has been successfully used to improve cell proliferation and healing in bone tissue [6, 7, 8, 9]. Enhancement of osteogenesis occurs due to the increased activity of osteoblasts induced by

laser exposure: in cultured human hypoxic osteoblasts LLLT enhances the expression of bone morphogenic protein-2 (BMP-2), which acts as a transforming growth factor-β (TGF-β) for osteocalcin, type I collagen and alkaline phosphatase [10]. Considering the above, we proceeded with a series of experimental works to study the combined application of a bioactive glass-based composite and laser radiation on bone regeneration.

Aim: to study the effect of photobiomodulation on bone regeneration in the context of bone formation markers: osteocalcin and alkaline phosphatase.

MATERIALS AND METHODS

The research was carried out in accordance with international requirements for the humane treatment of laboratory animals in the framework of the "European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes" (Strasbourg, 1986) and the Law of Ukraine No. 3447-IV of 21.02.2006 "On protection of animals against cruel treatment".

The experiment was conducted on 16 animals (white non-inbred rats, with body mass of 300–350 g) obtained from the vivarium (RE Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, Ukraine). We evaluated porous samples (pellets) of a bioactive glass biocomposite when implanted into the metaphysis of the tibia.

The bioactive glass-based composite ("Biocomposite synthetic bone") was synthesied in laboratory of Prof. V. Dubok at Institute of Materials Research, National Academy of Sciences of Ukraine). The composition of the bioactive glass-based composite: bioactive glass 50–65%, hydroxyapatite 14–17%, tricalcium phosphate — 14–17 %, wollastonite — 7–9%.

The introduction of animals into the experiment, surgical intervention and withdrawal from the experiment was performed under general thiopental anaesthesia. The period of observation was determined by the objectives of the respective study (2 and 4 weeks). 16 rats underwent surgery on a section of the proximal cnemis (proximal area of the tibia), a scalpel was used to cut through the skin on the inner surface of the cnemis above the metaphysis of the tibia, the soft tissues were crossed, the periosteum was debrided with a raspator, then the bone was marked with an awl and a 2 mm drill was used to drill the bone plate to the medullary canal, the marrow in the area of the hole was exhumed with the Folkman spoon, then the bone cavity in the area of the trepanation hole was filled with the BG-based composite pellets, the soft tissues were tightly sutured over the hole, the skin was sutured and the surgical suture was treated with brilliant green or iodine.

On the 2nd and 4th weeks after the surgery, the laboratory animals were withdrawn from the study to investigate the intensity of bone tissue regeneration at the site of BG implantation by means of light and electronic microscopy.

For morphological studies with a light microscope, a segment or a whole rat tibia with the implanted bioglass composite was removed and fixed in 10% neutral formalin solution, decalcified in 4% nitric acid solution, dehydrated in alcohol of increasing concentration and immersed in paraffin. Serial histological slices with a thickness of 7–9 μm were made on a microtome and stained with Weigert's iron haematoxylin and eosin as well as with Van Gasson's pyrofuchsin. The stained slices were analysed using an Olympus BX-63 microscope.

A fragment of the rat tibia with the implanted BG-based composite was removed for morphological studies using an electron microscope. Samples were fixed in 10% formalin solution (phosphate buffer, pH=7.4). Decalcification was performed in Osteofast 2 solution (BioGnost, Croatia). After decalcification, bone fragments were excised at the defect level. The samples were then dehydrated in isopropanol and embedded in paraffin (Leica Surgipath Paraplast Regular, Leica Biosystems, Germany). Slices of 7–8 μm thickness were obtained from paraffin blocks on a Thermo Microm HM 360 microtome (Thermo Fisher, USA). After deparaffinisation, the microslices were stained with haematoxylin and eosin and encased under coverslips in balsam (Merck, Germany). Microslides were examined and microphotographs were taken using an Olympus BX-51 microscope (Japan).

In addition 90 female rats of the Wistar line with a weight of 250–270 g were treated with laser irradiation on an artificially created tibial bone defect filled with the bioactive glass-based composite. Animals were allocated into 5 groups: "Control" (N=15), "Bio Glass" (N=15), "Laser 660" (N=15), "Laser 810" (N=15), "Bio Glass+Laser 660" (N=15), "Bio Glass+Laser 810" (N=15). "Lika Surgeon" and "Lika therapeut M" lasers manufactured by "Fotonika Plus" company (Cherkasy, Ukraine) were used in the experiment. Irradiation of the rat tibial defect filled with the pellets of the bioactive glass biocomposite (3–5 mg) was performed with the laser at wavelength of 660 and 810 nm, 50 mW power and 5 minutes exposure. Levels of osteocalcin and alkaline phosphatase in the blood serum were measured the next day after the intervention, as well as on the 22nd day. The second control point was not chosen by chance because the highest

levels of serum markers of osteogenesis after bone injury are usually observed on the 14th day [9, 10]. Therefore it was decided to postpone the analysis by 7 days to make the measurement results more relevant.

RESULTS AND DISCUSSION

The morphological examination of bone tissue microslides in experimental animals after the implantation of pellets of the BG-based composite into the bone cavity using a light microscope detected: in the areas where biocomposite pellets had got into lacunes of cancellous bone, active osteogenesis was observed around this material (Fig. 1 a, b).). The biocomposite was partially surrounded by a connective tissue, mostly by bone tissue. Morphogenesis corresponded to the formation of lamellar bone tissue. There was isolation of the biocomposite from the surrounding bone marrow.

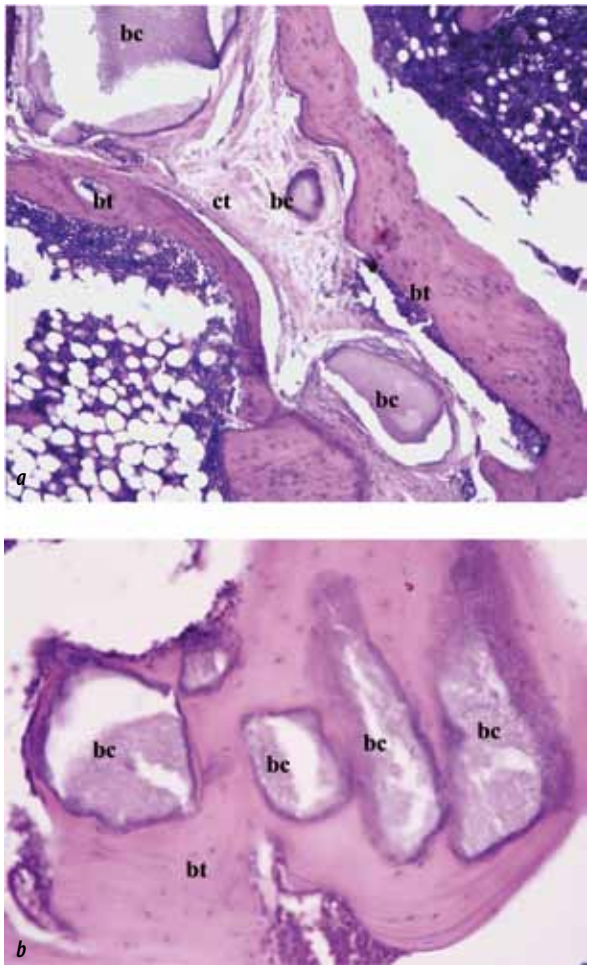

Fig. 1. *(a, b) Active osteogenesis around the biocomposite. Notes: bc — biocomposite; ct — connective tissue; bt — bone tissue. Hematoxylin-eosin, a: ×100; b:×200*

Thus, active osteogenesis was observed around the BG-based composite, which was surrounded by newly formed bone tissue.

Morphological examination of bone tissue microslides in experimental animals after implantation of BG-based composite pellets into the bone cavity using an electron microscope revealed heterogeneous ultrastructural changes in selected samples. The examined samples were dominated by areas of heterogeneous cell populations, among which there were osteoblasts and fibroreticular cells, with far fewer macrophages. The extracellular matrix contained few fibrous components, only single bundles of collagen fibres. There were also small non-cellular areas with no signs of osteogenesis (see Fig. 2). The formation of a new bone matrix was detected only in individual small loci, in the form of trabeculae. Clusters of activated osteoblasts are concentrated along the contour of these trabeculae (see Fig. 3). The bone matrix contained dense clusters of fibrous elements and was electron-dense, which indicates its mineralization. In some loci, lacunas with osteocytes were found that had a well-developed endoplasmic reticulum and euchromatin dominated in the nucleus (see Fig. 4). Such ultrastructural characteristics of osteocytes indicate a functional state, i.e. their continued involvement in the formation of the bone matrix.

Thus, a weak reparative osteogenesis was detected around the BG-based composite, and areas of cellular organization contain activated osteoblasts as well as fibroreticular cells. Overall, the changes can be assessed as an initiatory stage of osteogenesis.

The following osteocalcin (OC) (see Table 1) levels were obtained in laboratory animals treated with laser irradiation on an artificially created tibial bone defect filled with the BG-based composite.

Analysis of osteocalcin expression at the control points indicates that both the BG-based composite and laser irradiation have a positive effect on bone regeneration: in comparison to the control group, where the index was 67.9 ng/ml on day 22, in animals treated only with the BG-based composite — osteocalcin expression was 74.6 ng/ml; the photobiomodulatory effect of the λ660 nm laser stimulated osteocalcin level up to 67.1 ng/ml and the λ810 nm laser up to 69.9 ng/ml.

However, it should be emphasised that the highest level of osteocalcin expression in the second control point was observed with the combination of the bioactive glass with the photobiomodulatory exposure to the light at a wavelength of 810 nm: 84.4 ng/ml, whereas in the "Bioglass+Laser 660" group the figure amounted to 79.9 ng/ml. (see Fig. 5).

We also observed positive effects using both bioactive glass and laser irradiation on the expression level of alkaline phosphatase (AP) (see Table 2).

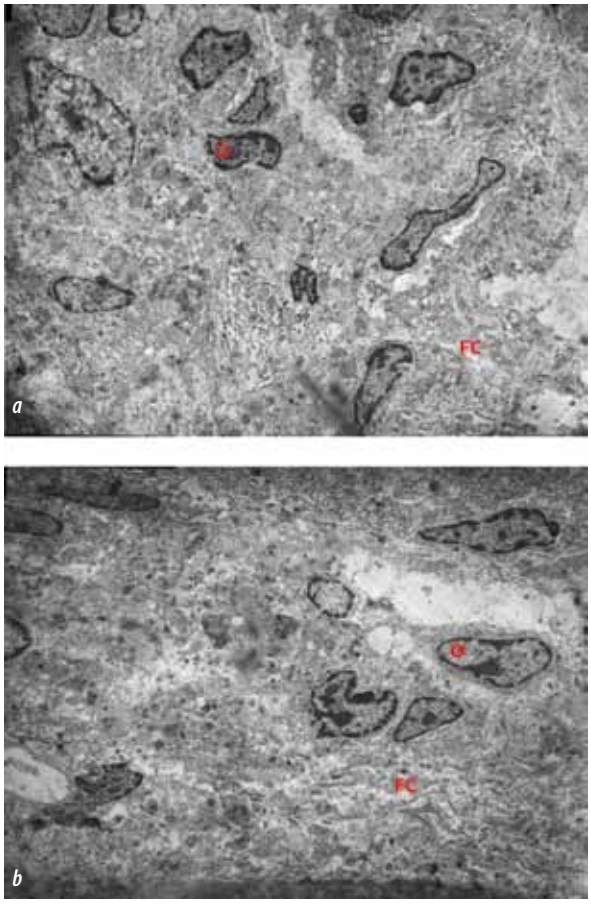

Fig. 2. *(a, b) Section of cellular reorganisation around the site of application of the BG composite. Accumulation of osteocytes (O) and single fibroreticular cells (FC). Electrophotogram, ×2400*

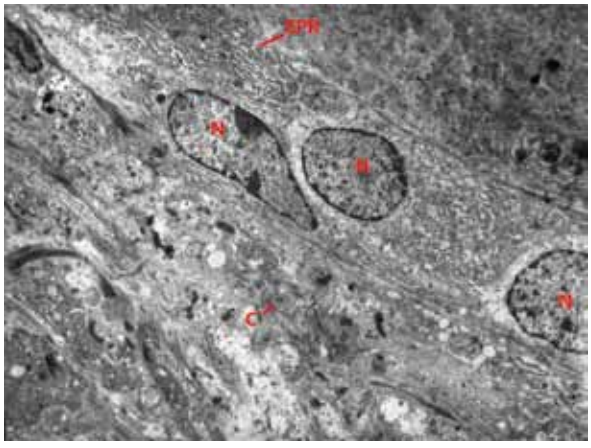

Fig. 3. *Accumulation of osteocytes with a developed endoplasmic reticulum (EPR) and a dominance of euchromatin in the nuclei (N). Bundles of collagen fibres (C) are present in the extracellular matrix. Electrophotogram, ×2400*

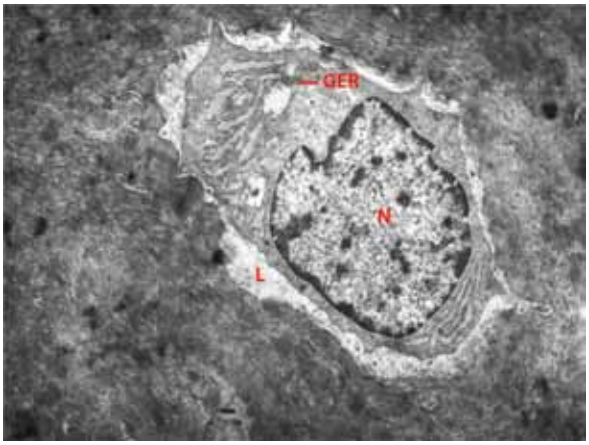

Fig. 4. *Osteocyte in the lacuna (L) of the bone tissue after application of the bioactive glass-based composite. A developed granular endoplasmic reticulum (GER) and nucleus (N) with euchromatin dominance indicates an active functional (synthetic) state. The bone matrix (BM) contains fibrous structural elements. Electrophotogram, ×4000*

Table 1. *Levels of osteocalcin and the dynamics of osteocalcin at the control points of the experiment*

Group / Deadline	Osteocalcin, ng/ml			
	day 1	day 22	Δ abs	Δ %
Control	61,4	67,9	+6,5	+10,6%
Bioglass	66,2	74,6	+8,4	+12,7%
Laser 660	61,5	67,1	+5,6	+9,1%
Laser 810	63,4	69,9	+6,5	+10,3%
Bioglass + Laser 660	62,2	79,9	+17,7	+28,5%
Bioglass + Laser 810	61,9	84,4	+22,5	+36,3%

Table 2. *Level of alkaline phosphatase and its dynamics at the control points of the experiment*

Group / Deadline	Alkaline phosphatase, units/l			
	day 1	day 22	Δ abs	Δ %
Control	97,7	102,2	+4,5	+4,6%
Bioglass	100,2	114	+13,8	+13,8%
Laser 660	96,6	109,3	+12,7	+13,1%
Laser 810	100,7	122,2	+21,5	+21,4%
Bioglass + Laser 660	99,3	116	+16,7	+16,8%
Bioglass + Laser 810	95,5	144,4	+48,9	+51,2%

The results of the analysis of AP expression indicate that both the bioactive glass and laser irradiation demonstrated a stimulating effect on bone

regeneration in comparison with the control group of animals which were treated only with the bioactive glass, where alkaline phosphatase expression index was 114.0 units/l. The effect of λ660 nm laser stimulated alkaline phosphatase level to 109.3 units/l, while at λ810 nm laser alkaline phosphatase reached 122.2 units/l (see Fig. 6).

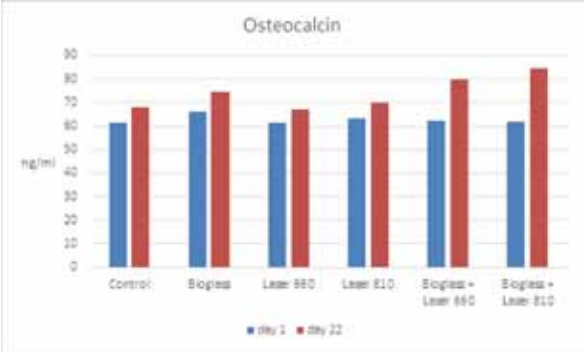

Fig. 5. *Level of osteocalcin (OC) in different groups at day 1 and day 22 of the experiment*

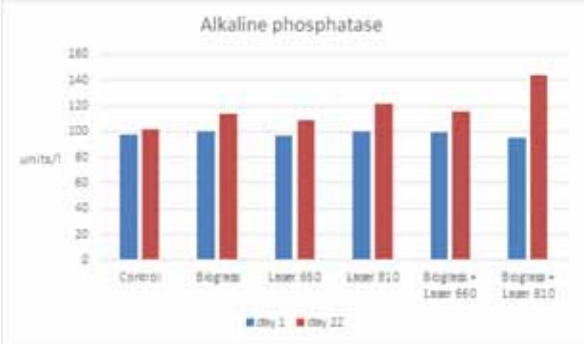

Fig. 6. *Level of alkaline phosphatase (AP) in the test groups at day 1 and day 22 of the experiment*

Similar to the increase in OC level, the highest LF expression rate on the day 22 of the observation was recorded when the bioactive glass was combined with the photobiomodulatory effect of 810 nm wavelength light: 144.4 units/l, while in the Bioglass+Laser 660 group its level was 116.0 ng/ml.

Similar data have also been observed by other researchers [4-10], since the infrared light has greater penetrating power and results in increased biochemical activity of cells, particularly during proliferation period, which, at the same time, is safer.

CONCLUSION

1. The experimental data show that the use of the bioactive glass-based composite stimulates bone regeneration and creates conditions for bone regeneration and repair.

2. Our results confirm the use of low level laser therapy with a wavelength of 810 nm as an effective photobiomodulatory factor, which in the presence of the bioactive glass significantly increases serum markers, namely OC and AF, indicating a positive effect on bone regeneration.

3. To ensure timely introduction of this method into clinical practice, the study of LLLT on bone regeneration needs to be continued in new experimental models and according to the principles of translational medicine.

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