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Biotechnological aspects of the working-out and manufacturing of living bone equivalent

D. O. Zubov [1, 2, 3], Y. V. Poliachenko [1], O. O. Kostrub [1], R. I. Blonskyi [1], O. M. Magomedov [1], O. V. Dolgopolov [1], I. A. Zasadnyuk [1]

[1] State Institute «Traumatology and Orthopaedics NAMS of Ukraine», Kyiv

[2] SI «NSC «M. D. Strazhesko Institute for Cardiology, Clinical & Regenerative Medicine NAMS of Ukraine», Kyiv

[3] «Medical & Diagnostic Centre «Profimed» LLC, ADONIS Medical Group of Companies, Kyiv. Ukraine

Objective. To handle biotechnological aspects in manufacturing processes of three-dimensional living bone equivalent for restoration of critical sized bone defects for innovative treatment of combat-related casualties. Methods. To fabricate living bone equivalent we used devitalized xenogeneic bone scaffolds (DBM chips) and autologous fibrin hydrogel seeded with autologous cultured bone marrow-derived multipotent mesenchymal stem/stromal cells (BM-MSCs). Quality/identity control of cell cultures was assured by donor and cell culture infection screening (IFA, PCR), flow cytometry (cell phenotype), karyotyping (GTG banding), functional assays (CFU assay, multilineage differentiation assay). Results. The BM-MSC cultures had a normal karyotype and appropriate phenotype, multilinear differentiation potential and functional properties, appropriate CFU frequency and hadn't any signs of cell senescence. The FDA/PI combined staining showed the demineralized bone chips' regular seeding with viable cells. Conclusions. An actual regenerative medicine approach to organ-saving transplantation of the three-dimensional living bone equivalent for combat-related casualties requires further preclinical and clinical approbation for thorough studies on the bone integrity restoration, forming new bone tissue in a site of bone defect, and duration of rehabilitation period compared to the gold standard of the conventional bone defect cure.

Мета. Вивчити біотехнологічні аспекти процесу виробництва тривимірного живого еквівалента кістки для відновлення кісткових дефектів критичного розміру. Методи. Для його виготовлення живого еквівалента кістки використовували девіталізовані ксеногенні кісткові каркаси (чіпси з демінералізованого кісткового матриксу) й аутологічний фібриновий гідрогель, попередньо засіяні аутологічними культивованими мультипотентними мезенхімальними стовбуровими/стромальними клітинами кісткового мозку (МСК-КМ). Контроль якості/ідентичності клітинних культур для еквіваленту включав: інфекційний скринінг донора та саме культури клітин (ІФА, ПЛР), проточну цитометрію (фенотип клітин), каріотипування (G-забарвлення), функціональні тести (КУОф-аналіз, аналіз диференціювання на мультипотентність). Результати. Культури МСК-КМ мали нормальний каріотип і відповідний для МСК фенотип, потенціал до відповідного мультипотентного диференціювання, доцільну частоту КУОф і не мали ознак старіння клітинної культури. Комбіноване фарбування FDA/PI показало рівномірне розподілення життєздатних клітин на демінералізованих кісткових чипсах. Висновки. Сучасний підхід із використанням методів регенеративної медицини до органозбережної трансплантації тривимірного живого еквівалента кістки потребує подальшої доклінічної та клінічної апробації, включаючи проведення ретельних досліджень щодо ефективності відновлення цілісності кістки, формування нової кісткової тканини в місці кісткового дефекту та порівняння тривалості реабілітаційного періоду з «золотим стандартом» традиційного лікування дефектів кісток.

Ключові слова. Регенеративна медицина, дефекти кісток, живий еквівалент кістки, лікарські засоби на основі клітин людини, мультипотентні мезенхімальні стромальні/стовбурові клітини.

Keywords. Regenerative medicine, bone defects, living bone equivalent, human cell-based medicinal products, multipotent mesenchymal stromal/stem cells

Introduction

In the current wartime realities in our country, restoring the integrity of bone tissue using the approaches and methods of regenerative medicine and bone tissue engineering in some cases can become an organ-saving option and therapy of despair for the patients with high-energy combat-related trauma and bone defects of critical size. The actual reconstructive surgery methods for bone integrity restoring are mostly based on the use of autografts (the gold standard), allografts and xenografts or artificial scaffolds made of osteoplastic materials. The first option has their well-known limitations, in particular, the limited availability of such grafts and the morbidity of the bone donor site during the procedure of the autologous bone harvesting, while the use of osteoplastic materials usually leads to their poor integration into the bone, which leads to an unbalanced distribution of loads, bone formation disorders, increased pain and the risk of fractures, which ultimately leads to repeated surgical interventions. In the last years, studying efforts have been focused on the development of innovative bone substitutes that not only provide immediate mechanical support, but also ensure proper fixation of the graft, for example, by promoting bone formation (e. g., so called 'cell-guided bone regeneration'). There are some clinically successful cases of such bone devices development, including biological glues, stem cell-seeded scaffolds, and gene-therapy bone substitutes. An important aspect of the clinical effectiveness of this regenerative medicine approach is the search for optimal ways of proper introducing innovative biomedical products based on human cells to restore bone integrity.

This bone integrity restoration alternative approach is becoming in demand in our wartime. The problems of high energy traumatic injuries of skeleton bones and the methods of their reconstruction in correlation with reparative osteogenesis are relevant for actual clinical and experimental traumatology and orthopedics. Temporary and economic costs for treating patients with alterations of reparative regeneration processes, especially in the high-energy mechanism of trauma, the complexity of their social adaptation, justify the need to search for innovative organ-saving technologies of regenerative medicine for bone integrity restoration. Particularly, it is actual the problem of treatment of combat high energy bullet, shrapnel and blast injuries faced Ukrainian traumatologists, seeing the Ukraine-Russia war.

The gold standard for extended bone defects' treatment is autologous osteoplastic surgery. Then there are methods of the defect filling with use of different osteoplastic materials, such as allogeneic and xenogeneic bone (e. g., DBM); synthetic and polymer materials; and using the method of compression-distraction osteosynthesis by Ilizarov. The choice of the method of plastic of the bone defect depends on its location and size.

The newest alternative to the conventional methods of bone plastic surgery is bone restoration methods based on the approaches of regenerative medicine. Currently, there are two approaches to bone regeneration. The first one is based on the delivery of high doses of bone morphogenetic and other factors to the defect zone, such as autologous platelet concentrate or recombinant bone morphogenetic proteins (BMPs). Marx et al. first evaluated the use of platelet concentrate for bone healing in 1998, and his studies led to the development of an entire industry for its use in traumatology and orthopedics using platelet concentrator devices [1]. The BMPs were first proposed as bone morphogenes by Urist in 1965. In the late 1980s his team performed the first transplantation with their use [2, 3].

The second approach in bone regeneration is based on the delivery to the defect area of the critical mass of osteoprogenitor cells, which realize their trophic and integrative potencies in a wound for the formation of bone regenerate and new bone at final. For the first time Phemister in the 1940s used injections of bone marrow aspirate into the fracture non-unions [4]. In the 1960s Burwell proposed as an autograft of the iliac crest bone containing the bone marrow [5]. Subsequently, as a source of osteoprogenitor cells, a concentrated bone marrow-derived mononuclear fraction was used. Connolly first showed in 1998 that four-fold concentration of the mononuclear fraction of bone marrow significantly increases the degree of fusion of non-consolidated fractures in humans [6]. Muschler et al. in 2003 proposed the technique of saturation of allogeneic bone chips and crumbs with a bone marrow clot, both in animal models and in clinical practice of fracture consolidation [7, 8]. However, in some cases, neither the autograft nor Muschler's technique led to the formation of a dense bone in non-consolidated fractures revealed by the X-ray examination. Such dysfunction may be related to the individual osteogenic insufficiency of bone marrow cells, as well as to the compromised immune status of the patient. Thereby, the next step in bone regeneration was the use of immunomodulating allogeneic cultured osteoprogenitor bone marrow-derived multipotent mesenchymal stromal/stem cells (BM-MSCs) [9–12]. Of note, the organ-saving bone regeneration

technology Trinity Evolution™ (Orthofix, USA) based on Mushler's approach has been proposed to restore defects in casualties with combat related trauma obtained in Iraq and Afghanistan war conflicts. As an osteoinductive scaffold the authors from Walter Reed National Military Medical Center (Bethesda, USA) have used the allogeneic demineralized bone matrix' (DBM) chips pre-saturated with allogeneic cadaver mononuclear fraction of bone marrow containing uncultured and non-purified multipotent mesenchymal stromal/stem cells (MSCs) [13]. Furthermore, in other studies for bone fracture consolidation, it was applied an alternative cell component — the adipose-derived MSCs, or ADSCs [14, 15]. It is known also an incomplete pilot study on a complex approach which was developed earlier in Ukraine by Zubov et al. with use of a cocktail of expanded autologous BM-MSCs, periosteal progenitor cells and endothelial progenitor cells on a fibrin hydrogel/DBM composite, to restore critical-sized bone defects of 47 casualties with complicated gunshot bone wound. The X-ray examination determined that within 4–6 months post-operatory, 90.4 % of the treated defects with such a composite regained native integrity [16, 17].

In this biotechnological study we are considering the manufacturing aspects, as well as the further possibility of using the three-dimensional living bone equivalent (3D LBE) as an innovative medicinal product based on DBM seeded with autologous cultured cells for prospective restoration of the critical-sized bone defects in combat-related casualties. In its last modification the advanced 3D LBE consists of decellularised xenogeneic bone chips as a scaffold. Such an a scaffold was used as osteoinductive and osteoconductive carrier for cultured autologous osteoprogenitor BM-MSCs for formation of the critical cell mass enhancing the vascularization and trophics of the manufactured 3D LBE for the new bone formation *in situ*.

Materials and methods

All procedures for the harvest, isolation, culturing, biotesting, cryopreservation, banking and release of human cell cultures, as well as the manufacturing of 3D LBE, were carried out in the clean rooms of the biotechnology laboratory of Bank of human cord blood, other tissues & cells, «Medical & Diagnostic Centre «Profimed» LLC (License of Ministry of Health of Ukraine under No. 1706 of September 20, 2018) in accordance with the requirements of actual Ukrainian legislation.

Cell cultures

Culture of the BM-MSCs. Heparinized human bone marrow aspirate in a volume not more 5 ml was obtained with an aspirating needle from the iliac crest. Then, 1 ml of aspirate was spread over culture flasks with an area of 175 cm^2 (SPL, Korea). The primary culture was seeded into multi-flasks with an area of 875 cm^2 (Corning, USA) for large scale cell expansion. The therapeutic dose of BM-MSCs ($50–300 \times 10^6$ cells) was available over 30–40 days.

The growth medium for BM-MSCs culturing consists of α-MEM modified (BioWest, France), 10 % FBS (Sigma-Aldrich, USA), 1 ng/ml bFGF (Sigma-Aldrich, USA), 2 U/ml heparin sodium (Indar, Ukraine), 100 U/ ml antibiotic-antimycotic solution (BioWest, France). Cell subculturing was carried out with a mixture of trypsin and EDTA solutions in a ratio of 0.05 % : 0.02 % in DPBS (Sigma-Aldrich, USA). The seeding density was $1–3 \times 10^3$ cells per 1 cm^2.

All of the above cell types were grown in a multigas incubator (Binder CB 210, Germany) in an atmosphere of 5 % CO_2 and a saturating humidity of 97 %.

All autologous donors (peripheral blood — by IFA, PCR) and finalized cell cultures (by PCR) were screened for absence of HIV½, HBV, HCV, HSV½, CMV, EBV, Treponema pallidum and Mycoplasma ssp.

Functional assays for QC

A set of quality control (QC) procedures were established to release personalized 3D LBE as the human cell-based medicinal product. To determine the cell plating efficiency, colony forming units (CFU) analysis was performed according to the conventional protocol. For CFU staining, the cell colonies were fixed with cold ethanol and stained with azure-eosin by Romanowsky-Giemsa (Makrokhem, Ukraine) for 20 min [18]. Multilineage differentiation into the osteogenic and adipogenic directions was carried out according to the standard protocols [19]. The fluorescein diacetate (FDA) / propidium iodide (PI) (Sigma-Aldrich, USA) combined fluorescent staining assay was applied for checkup of the cell seeding regularity over the bone chips. The normal karyotype of cultured cells was confirmed by GTG banding method [18].

Directed multilineage differentiation assays

Adipogenic differentiation was performed in the following medium: DMEM-HG (4.5 g/L) supplemented with 10 % FBS, 1μM dexamethasone, 200 μM indomethacine, 500 μM isobutylmethylxanthine, 5 μg/ml insulin (all ex Sigma-Aldrich, USA) and 5 % donor horse serum (BioWest, France). After 14 days the cells were fixed and stained. To detect the adipogenic differentiation, the cells were stained with 0.5 % solution of Oil Red O dye for neutral intracellular lipids (Sigma-Aldrich, USA) [19].

Osteogenic differentiation was performed in the following medium: DMEM low glucose (1.0 g/L) with 10 % FBS, 100 nM dexamethasone, 10 mM ß-glycerophosphate and 50 µg/ml ascorbate-2-phosphate (all ex Sigma-Aldrich, USA). After 21 days the cells were fixed and stained. To detect osteogenic differentiation, the cells were stained with 2 % solution of Alizarin Red S dye for calcified extracellular matrix deposition (Sigma-Aldrich, USA) [19].

Flow cytometry

The number of positive and negative cells for corresponding markers (conventional, but not all-sufficient set) [12, 19] was measured for each accession of human cultured BM-MSCs (at P2) with use of flow cytometer BD FACSAria (BD Biosciences, USA). Staining with the monoclonal antibodies (PerCP-Cy5.5 mouse anti-human CD105, PE mouse anti-human CD73, FITC mouse anti-human CD90, APC-Cy7 mouse anti-human HLA-DR, APC mouse anti-human CD34, FITC mouse anti-human CD45, DAPI mouse anti-human CD14) was performed according to the manufacturer's instructions (BD Pharmingen, BD Horizon, USA). The cell viability was determined by a fluorescent dye PerCP-Cy5.5 7AAD (BD Pharmingen, BD Horizon, USA).

Microscopy

Inverted fluorescent microscope ECLIPSE Ti2-U equipped with CMOS USB 3.0 10MP digital camera and NIS-Elements analysis software (Nikon Instruments Inc., USA) were applied.

Statistics

The data are presented as mean and SEM (x ± m). Statistical significance was estimated using the Student's t-criterion.

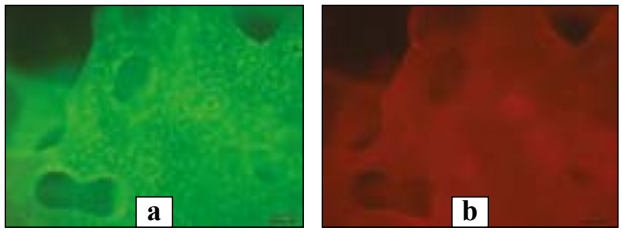

Fig. 1. Functional assay for 3D LBE quality control during its manufacturing: the fluorescein diacetate (FDA) / propidium iodide (PI) combined fluorescent staining assay showed the demineralized bone chips' regular seeding with viable cells: a) FDA green staining for viable seeded cells (fluorescent microscopy); b) PI orange staining for dead seeded cells (fluorescent microscopy)

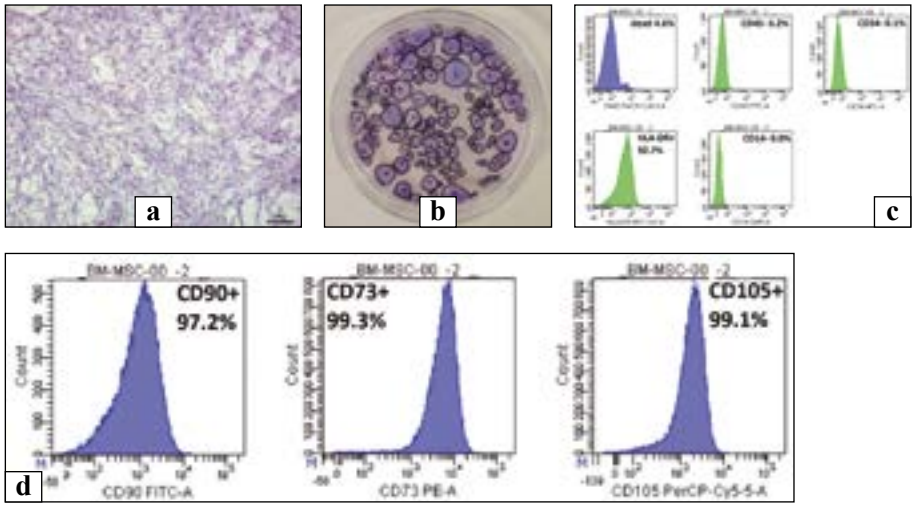

Fig. 2. Cell cultures' functional assays for quality control of human cultured BM-MSCs: a) cultured cell morphology at passage 1, P1 (Romanowsky-Giemsa staining, bright-field microscopy); b) colony forming unit assay for human BM-MSCs (ø100 mm Petri dish seeded in triples for 14 days with 5.7×10^6 nucleated cells isolated from heparinised aspirate of red bone marrow; Romanowsky-Giemsa staining); the efficiency with which MSCs form colonies is an important functional assay for the quality of medicinal products based on human cells; c) immunophenotyping data on negative and viability human cultured BM-MSC markers; d) immunophenotyping data on positive human cultured BM-MSC markers

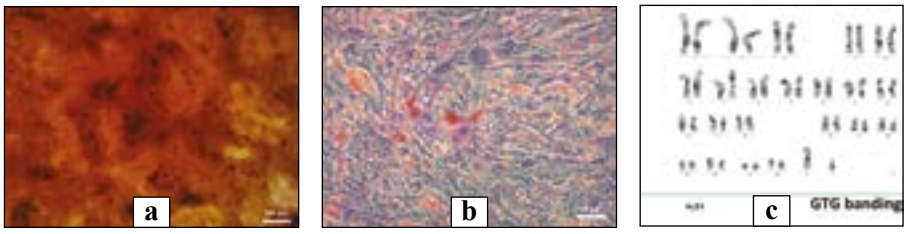

Fig. 3. Cell cultures' functional assays for quality control for medicinal product based on human cultured BM-MSCs: a) directed multilineage differentiation assay for osteogenic differentiation (Alizarin Red S staining for mineralized matrix deposition); b) directed multilineage differentiation assay for adipogenic differentiation (Oil Red O staining for intracellular lipids, Romanowsky-Giemsa counterstaining); c) karyotyping, a standard metaphase normal male karyotype, 500–550 bands (GTG banding method)

Results and their discussion

The gold standard for critical sized bone defect restoration is autologous bone graft transplantation. Although this surgery possesses its drawbacks, such as morbidity, risk of donor wound contamination and non-healing, risk of lysis of transplanted bone autograft. As a substantial alternative for autologous bone grafting we developed the regenerative medicine organ-saving biotechnology of the 3D LBE transplantation.

The manufactured bone equivalent was homogenously seeded with cells after incubation on a scaffold as proved by fluorescein diacetate (FDA)/ propidium iodide (PI) combined staining procedure. The cell cultures were appropriately characterized for their identity, purity, potency, viability and suitability for the intended use (fig. 1–3) [12, 19, 20]. All cell cultures had a normal karyotype and phenotype, differentiation potential and functional properties, CFU frequency (range: 42–316 per 5.7×10^6 nucleated cells, n = 10; fig. 2, b) and hadn't any signs of cell senescence.

Cultured BM-MSCs were positive for conventional (but not unique) stromal markers CD105, CD90, CD73, and were negative (< 2 %) by hematopoietic markers CD34, CD45, CD14, although they were positive in some donors for MHC class II molecules — HLA-DR. Cell viability by 7AAD marker was at least 90 % (fig. 2, c, d).

Three dimensional living bone equivalent manufacturing

As a scaffold for cells, a treated, cell-, DNA-free, xenogeneic and non-immunogenic bone chips were used (e. g., DBM of local Ukrainian manufacturers or a commercial dental product – spongious bone substitute «Large Geistlich Bio-Oss® granules», 1–2 mm, Geistlich Pharma AG, Germany). In a first step the carrier was seeded directly with cells. Initially, bone chips were evenly seeded in the biotechnology laboratory (clean rooms facility) with autologous cultured BM-MSCs (P2–P5) and incubated in a CO_2-incubator for 7–14 days, depending on the volume of the bone defect and the uniform overgrowth of the chips with seeded cells. The homogenous cell distribution on the surface of the bone chips at the end of incubation period was confirmed by the combined staining with FDA/PI fluorescent dyes (fig. 1). After incubation, as a second step the cell-seeded carrier was placed into the autologous fibrin hydrogel containing an additional portion of autologous cultured BM-MSCs (P2–P5); it was used to shape chips with pre-seeded cells to the required bone defect form. Cells were seeded over a carrier and into the fibrin hydrogel at a ratio of 5×10^6 cells per 1 cm^3 scaffold. In this final pre-incubated form (bone chips, fibrin hydrogel, cultured cells), the living bone equivalent is ready for transplantation into the site of the bone defect. The freshly prepared LBE can be stored in the operating room in the cold (+4°C…+8°C) in a firmly closed thermo-box with cold accumulators (pre-cooled to –20°C) for 6–8 hours.

The developed medicinal product based on the human cells can be attributed, depending on the main regulatory systems (European Medicines Agency-Committee for Advanced Therapies, EMEA-CAT, EU vs Food and Drug Administration, FDA, USA) either to the (1) «Human Cell-Based Medicinal Products (CBMPs)» – in terms of the European Medicines Agency (EMEA) [21], or to the (2) «Human cells, tissues, and cellular and tissue-based products (HCT/Ps)», those in terms of the FDA [22]. Seeing the signing of the Association agreement between Ukraine and the European Union in 2014, our legislation and regulatory on the transition of medicinal product based on human cells into the clinical practice should be gradually harmonized with the EU legislation.

Thereof, the developed three-dimensional living bone equivalent requires further preclinical and clinical approbation for thorough studies on restoring the bone integrity, forming new bone tissue in a site of bone defect, and duration of rehabilitation period compared to the gold standard of the conventional bone defect cure.

Conclusions

The following preclinical and clinical tasks remain to hurdle towards the implementation of the 3D LBE: to determine the effectiveness of the equivalent in an animal model of a critical bone defect, to begin clinical testing of the 3D LBE, to study its effectiveness, to determine indications and contraindications for its use, to evaluate the histo-morphological processes of reparative osteogenesis and remodeling of the equivalent in the bone wound that occur at the site of equivalent transplantation (e. g., 3, 6 and 12 months, long term), and also consider further possibility of recommending this medicinal product as an alternative to autologous bone transplantation for certain clinical cases. In the future, we can hope that innovative bone engineering technologies with use of different type of bioreactors and bone 3D bioprinting will be able to save the patients from amputation and subsequent prosthetics of a limb.

Conflict of interest. The authors declare no conflict of interest.

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БІОТЕХНОЛОГІЧНІ АСПЕКТИ РОЗРОБКИ ТА ВИГОТОВЛЕННЯ ЖИВОГО ЕКВІВАЛЕНТА КІСТКИ

Д. О. Зубов [1, 2, 3], Ю. В. Поляченко [1], О. О. Коструб [1], Р. І. Блонський [1],
О. М. Магомедов [1], О. В. Долгополов [1], І. А. Засаднюк [1]

[1] ДУ «Інститут травматології та ортопедії НАМН України», Київ
[2] ДУ «ННЦ «Інститут кардіології, клінічної та регенеративної медицини ім. акад. М. Д. Стражеска НАМН України», Київ
[3] ТОВ «Лікувально-діагностичний центр «Профімед» Медичної Групи Компаній ADONIS, Київ. Україна

✉ Dmytro Zubov, PhD in Biol. Sci.: zoubov77@yahoo.com
✉ Iurii Poliachenko, MD, Dr. Sci. in Medicine, Prof.: info@ito.gov.ua
✉ Oleksandr Kostrub, MD, Dr. Sci. in Medicine, Prof.: akostrub@ukr.net
✉ Roman Blonskyi, MD, Dr. Sci. in Medicine: drblonskiy@ukr.net
✉ Oleksandr Magomedov, MD, Dr. Sci. in Medicine, Prof.: alexandr@magomedov.kiev.ua
✉ Oleksii Dolgopolov, MD, Dr. Sci. in Medicine: ortotravma.kiev@gmail.com
✉ Ivan Zasadnyuk, MD, PhD in Medicine: zasadnyuk@ukr.net