Principles of preclinical biomedical studies for biomaterial-based scaffolds intended for bone defects replacement

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Abstract. Biomedical studies of materials precede their clinical trials. Based on our own experience and literature data, an in vitro and in vivo screening type testing algorithm for bioresorbable materials is presented, which allows one to describe cytotoxicity, cytocompatibility, cell matrix properties, biocompatibility and osteoplastic potential of the studied biomaterials.

1. Introduction

Conducting biomedical studies of materials intended to replace bone defects is an obligatory step prior to obtaining permission for limited clinical trials. This stage must give clear answers to several questions: are these materials toxic, are they cyto- and biocompatible, and will implants consisted of studied material sustain osteogenesis, providing an adequate scenario for organotypic tissue repair in the defect zone. The last thesis relates, to a large extent, to bioresorbable materials.

The literature describes a wide range of in vitro approaches for assessing the cytocompatibility of materials, all of them include different research protocols and registration methods [1-3]. Different models are also used to assess biocompatibility and osteoplastic properties in vivo [4-6].

As a result of the comprehensive literature analysis and our own empiric experience, we formed an algorithm for preclinical biomedical testing of materials designed to fill bone defects, which is usually conducted in a “funnel” manner - “from wide to narrow”. This approach is due to the presence, as a rule, a line of materials with smoothly changing properties at the start of testing. In accordance with the design of the study, materials that successfully “withstand” the previous stage pass to each subsequent stage of testing.

2. Results

2.1. The study of cytotoxicity, cytocompatibility, and adhesive properties of biomaterials.

This step is carried out in vitro. To prepare the “extracts” from materials that are used for testing according to ISO 10993, samples of materials (for example, in the form of granules) are usually kept in a culture medium for 24-48 hours. Keeping in mind the possibility of the material passive resorption, it is additionally recommended to evaluate the weight of the samples before and after incubation in the medium. The extracts of biomaterials are then added to cell cultures in order to assess their cytocompatibility by investigation of the cell population increase / decrease dynamics. In general, the material can be classified as non-cytotoxic if after 24-72 hours of incubation with cells the extract causes...
death of no more than 30% of the initial amount. Additionally, the dynamics of the cell growth and the doubling time duration as well as cellular morphology and motility should be evaluated in the presence of extracts. Altogether it provides the full estimation of the biomaterial cytocompatibility. One of the common causes of potential cytotoxicity may be a change in the medium pH by the adding of the material that is also advisable to control.

The next step of in vitro biomaterials “casting” is evaluation of their matrix properties for cell adhesion, which depend both on the chemical composition of the materials and the architectonics of their surface, surface charge, and a number of other physicochemical properties. To assess this characteristic of materials, they are usually saturated with cell suspension and the dynamics of the cell growth is estimated during the cultivation period up to 14-21 days. Finally, the light or fluorescence microscopy is carried out for visualisation of the surface colonisation with proliferating cells. Figure 1 shows the variants in the growth kinetic of the cell population seeded on calcium phosphate granules with pronounced (A) or moderate (B) matrix properties, and in case of cytotoxicity (C).

![Figure 1](image.png)

**Figure 1.** Variants of the dynamics of the human fibroblasts cell population during the cultivation on calcium phosphate granules with various matrix surface properties.

Figure 2 demonstrates the prominent matrix properties of 3D constructs based on β-tricalcium phosphate resulted in the active expansion of their surface by human fibroblasts in the process of cultivation.

![Figure 2](image.png)

**Figure 2.** Growth of human fibroblasts on the surface of βTCP- based 3D-printed construct, MTT assay
At its simplest, this system uses the line of immortalized human fibroblasts or the highly differentiated human osteosarcoma line MG63, which reflects the behavior of osteoblasts. Since some materials can be inducers/promoters of cell differentiation, this step could be repeated for the most promising of them using primary cultures of multipotent mesenchymal stromal cells (MMSCs) with evaluation of the osteogenic differentiation genes expression. Thus, figure 3 shows the in vitro stimulation of the osteogenic differentiation genes in the MMSCs from human adipose tissue on the granules of the natural *Acropora cervicornis* coral skeleton.

![Figure 3. Expression of osteogenic genes in human adipose derived-MMSC after 14 days of cultivation on the granules of A. cervicornis coral skeleton](image)

2.2 *The classic model for studying the biocompatibility of materials* is the “subcutaneous test” in mice: fragments of biomaterials are placed under the skin of mice and, after removing of 2 animals from the experiment every 2 weeks (up to 14-16 weeks), all bordering tissues from the implantation zone are cut out for histological preparations. Before cutting, the implantation zone is always visually assessed for analysis of the capsule formation, vascularization, and the presence/absence of an inflammatory reactions.

Even Alexis Carrel (1872-1944) wrote that the body’s reaction to the “foreign” material is universal and leads to its rejection, accompanied by oedema, inflammation, and necrosis. So, he introduced the concept of biocompatibility, thus laying the foundations of transplantology and implantology. In histological studies of implanted materials the presence of these signs, which are manifested in the accumulation of lymphoid cells in the implantation zone, diffuse/granulomatous inflammation, oedema, interstitial hematomas, necrosis, and cell detritus should be assessed first of all.

In the absence of signs of rejection, a histological examination is detailed to make a conclusion about the degree of "biocompatibility" of the material and the adequacy of the biological response from the organism of laboratory animals. To this end, the thickness and structural features of the connective tissue formed the capsule around the implant, the degree of its vascularization, the presence of foreign body giant cell, and the dynamics of filling the inner space of resorbable implant with connective tissue should be evaluated.

Figure 4 shows the view of an implant made from granules from *A. cervicornis* coral three weeks after surgery. The macroscopic observation revealed a well-vascularized capsule around the implant (figure 4A), while the histological preparation shown that the granules (visualized as voids after decalcification) were surrounded by vascularized connective tissue rich in multinuclear foreign body giant cell (figure 4B).
Figure 4. Formation of vascularized connective tissue capsule around the implant from *A. cervicornis*; A - macrograph, B - micrograph; 3 weeks after surgery; HE staining.

2.3. The next (final) stage of *in vivo* research is aimed at studying the osteoplastic potencies of the biomaterial. This stage can be performed on larger laboratory animals, for example, rats or rabbits, the bone size of which allows you to create a fenestrated (u-shaped) defect of the tibia bone with a volume of ~ 24-30 mm³ and depth to the lower cortical layer.

A “good” osteoplastic biomaterial intended to replace bone defects must be bioactive, that is, create conditions in the defect zone that facilitate the transition of tissue from a state of inflammation (developing due to trauma) to a state of organotypic regeneration. Such a scenario in the defect zone requires consistency in the rate of biomaterial resorption (which has an active and passive component) with the coordinated activity of various bone cells types (in particular, osteoblasts and osteoclasts).

To evaluate the events described above, osteogenesis in the defect zone is studied in dynamics for at least 16 weeks, with deducing from the experiment 2 animals every 3-4 weeks. Specimens of tissue for the preparation of histological preparations must contain not only the implant, but also the surrounding host bone structures.

Several types of reparative osteogenesis are well known: periosteal - from the inner layer of the periosteum containing MMSC; direct - with bone marrow MMSC participation; and indirect - enchondral – also with MMSC but through the formation of cartilage tissue, gradually replaced by new bone (so-called, embryonic type of osteogenesis). Accordingly, it is advisable to take into account the presence, intensity and correlation of these types of osteogenesis on histological sections. In addition, it is necessary to analyze the degree of implant consolidation with the recipient bed and the timing of the formation of compact and spongy bone tissue at the implantation site, correlating them with the dynamics of the implanted material bioresorption.

Figure 5A demonstrates the formation of bone trabeculae adjacent to granules of carbonate hydroxyapatite (CHA) in the model defect of the rat tibia (marginal resection) 9 weeks after surgery, which indicates the osteoconductive properties of such biomaterial. Obviously, the spaces between the granules are populated by bone marrow cells, i.e. hematopoiesis was restored in the defect zone. Similar processes were also revealed when using a whole porous 3D-printed implant from βTCP (figure 5B): after 9 weeks, this implant was also populated with bone marrow cells without signs of inflammation, while the formation of cancellous bone tissue in the area of contact between the implant and bone marrow was observed (figures 5C,D).
Figure 5. Different implants in rat tibia model defect: CHA (A) granules and porous scaffold 3D-printed from βTCP (B, C, D). 9 weeks. HE staining.

3. Conclusion.
The described above biomedical studies for the developed materials are a necessary minimum for selecting the most perspective types for further implantation into bone defects. When testing composite or tailored biomaterials with specific substitutions in their structure, as well as during creation of tissue-engineering constructs with cells, genetic vehicles, bioactive or pharmaceutical substances etc., the presented research algorithm can be expanded by immunohistochemical and molecular genetic tests corresponding to certain translational tasks.

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