Histological evidence of novel ceramic implant: evaluation of tolerability in rabbit femur

Cristian Adrian Rațiu(1), Simona Daniela Cavalu(2), Viorel Miclăuş(3), Vasile Rus(3), Grigore Ion Lăzărescu(4)

1) Department of Dentistry, Faculty of Medicine and Pharmacy, University of Oradea, Romania
2) Department of Preclinical Sciences, Faculty of Medicine and Pharmacy, University of Oradea, Romania
3) Department of Histology, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania
4) Faculty of Dental Medicine, “Titu Maiorescu” University, Bucharest, Romania

Abstract

The investigation of desired optimal interface between bone and dental or orthopedic implants, and whether a newly developed material conforms to the requirements of biocompatibility and mechanical stability, are important and rigorous procedures as an essential step prior to clinical trials. The present study proposes the investigation of a novel ceramic implant in terms of biocompatibility, osseo integration and bone response, by an experimental study using a small animal model (rabbit). Radiological examination after six weeks post-surgery highlighted the stability and well integration of the implant, without fibrous tissue or other undesirable complications. The histological analyses highlighted the stability and well integration of the implant, without fibrous tissue or other undesirable complications. The histological analyses ensured not only the coverage of the implant hole, but also the continuity with the adjacent bone. The chemical stability is suggested by the XRD (X-rays diffraction) pattern, as the implant did not showed erosion marks at the surface, not even discrete ones. Moreover, the XRD pattern recorded on the surface of femoral bone showed the fingerprints of hydroxyapatite indicating that the new bone covered the surface of the implanted area. The qualitative and quantitative aspects of the new bone were highlighted through light microscopy and SEM/EDX (scanning electron microscopy/energy dispersive X-rays), especially the lamellar architecture of the new bone at the contact area with the implant, six weeks after insertion. The Ca/P ratio was evaluated, which is a valuable indicator in qualitative assessment of the osseous tissue.

Keywords: osseointegration, rabbit femur, ceramic implant.

Introduction

The challenge to provide safe and efficacious biomaterials materials for osseous tissue, particularly to overcome the problems of implant rejection and related infections, is part of the most innovative therapeutic strategy in tissue engineering. The actual tendency is to replace, where possible, the metal material and devices, not only for esthetic consideration, but also because of the inconveniences related to the limitation of NMR (nuclear magnetic resonance) investigations [1, 2]. In order to be embedded in the bone, the implant has to have a particular structure, which provides mechanical resistance and biocompatibility to the bone and surrounding tissues [3, 4]. Depending on the final destination of the implant, its testing can be made in either the femoral condyle, long bone diaphysis, maxillary or mandibular bones [5]. Dense ceramic materials, alumina/zirconate composite-like, are very tempting for applying in oral and orthopedic implantology, including hip and knee prostheses, dental implants [6–8]. Structural analysis and mechanical properties of different composites with variable alumina/zirconate content were investigated and recently presented in specialty articles, highlighting some advantages in comparison to other composites [9, 10]. They are considered to be bioinert materials, having a minimal interaction with the adjacent tissue. For this reason precisely, the study of the biological fixation of the implant and also its adaptation to the biological site it was conceived for, is a very important topic. Thus, after determination of the structural properties and mechanical performances of these composites, in vivo testing on an animal model adequate to the type of the implant is imposed [11–13]. Within the chosen model for implant material testing, they are inserted in experimental animals, in cortical or trabecular bone. The bone response to the implant can be assessed at the end of the experimental period through different techniques: light microscopy, transmission electron microscopy and scanning electron microscopy. Considering the fact that electron microscopy offers information on a very small area, while the light microscopy covers the whole tissue containing the implant, some researchers consider light microscopy as being the most adequate method to gather up, as complete as possible, the information regarding the impact of the implant toward the tissue [4].

The aim of our study is to investigate a novel ceramic implant in terms of biocompatibility, osseo integration and bone response, by an experimental study using a small animal model (rabbit). The chemical composition of the ceramic implant used in our study consist of Al₂O₃ and ZrO₂ (stabilized with yttria), being processed by a novel technique, namely spark plasma sintering (SPS). Both the composition and processing techniques of the ceramic material represent a novelty in this domain and belongs to a more complex study, which implies the investigation of physicochemical and mechanical properties.
of some ceramics with variable composition depending on the Al$_2$O$_3$/ZrO$_2$ ratio. In the same time, a comparison with some other studies related to biological behavior of synthetic materials for orthopedic and dental applications was made, in the context of biomaterials evolution and their clinical availability, as well as the different approaches used to fulfill the challenges faced by this medical field.

Materials and Methods

For the purpose of in vivo testing, alumina–zirconia ceramic with the composition 80% Al$_2$O$_3$–20% YSZ was selected, based on previous studies regarding the processing, structural and mechanical characterization [8–10]. With this purpose in view, the ceramic specimens were manufactured in a shape of a truncated cone with the following sizes: H=8 mm, D=3 mm, d=2 mm. As for the animal model, four clinically healthy young male rabbits were selected, with a 4.2 kg weight. The management of animal husbandry, pre and postoperative care in the vivarium, were standardized in-house, as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments, according to the European Legislation and Ethics, and approved by the Ethics Committee of the Faculty of Medicine, University of Oradea, Romania (No. 09/17.11.2012). Anesthesia was performed with an intramuscular injection containing xylazine and ketamine.

An incision was then made in order to have access to the femur bone. An osseous defect was made by using dental drills and titan implant insertion (original TBR), with an increasing diameter. The size of the osseous defect was strictly calculated so that the implant matches exactly, in order to ensure its immobilization throughout the osseointegration process. The basic rules for implant therapy were followed in order to achieve osseointegration: the implant was sterilized prior to surgery, the implant site preparation was performed under sterile condition and completed with non-traumatic surgical technique that avoided overheating of the bone during preparation of the recipient site and the implant was placed with good initial stability. While using the drills, the irrigation with normal saline solution was practiced, in order to avoid tissue overheating and necrosis [14–17]. The implant was fixed so that it would totally be buried in the osseous defect, after which the soft tissues, muscle and skin, were sutured in layers. Radiographic examination was performed after six weeks in order to assess the position, stability of the implant and the aspect of the surrounding proliferated tissues; then, the animals were sacrificed and the femur was extracted. The clean, dry femoral bone pieces (without any connective tissue) were analyzed by XRD (X-rays diffraction) spectroscopy, by focusing the X-ray beam in the implanted area, using a Shimadzu XRD-600 diffractometer equipped with Cu-K$_\alpha$ radiation ($\lambda=1.5418$ Å) and Ni-filter. For the histological and SEM (scanning electron microscopy) examination, the bones were transversely sectioned with a dental drilling machine, at 1 cm distance from each side of the implant. Half of the resulted pieces were fixed in 10% buffered formalin for seven days and decalcified in 7% trichloroacetic acid solution during 20 days, the solution being replaced every four days [18]. We considered the decalcification process to be completed when we were able to section a small fragment from the sample, using a blade [18]. The following protocol was used to determine the point at which decalcification was complete: 5 mL of used decalcification fluid from the tissue processing vessel was removed and ammonium hydroxide dropwise added until the pH of the solution turned to neutral. Next, 5 mL of saturated ammonium oxalate was added, well shaken and allowed to stand at room temperature for 30 minutes. Formation of a precipitate (calcium hydroxide) after the addition of ammonium hydroxide indicates the presence of substantial amounts of calcium in the spent fluid, so the fluid should be changed and the tissue further decalcified. Formation of a precipitate after ammonium oxalate addition shows less calcium. If the solution remains clear for 30 minutes after addition of the oxalate, the tissue is essentially calcium free. After decalcification was completed, we proceeded to the final trimming of the samples and we also extracted the implant in this stage. The samples were embedded in paraffin, and 5 µm thick sections were stained with Goldner’s trichrome method [19]. The rest of the pieces were prepared for electronic microscopy investigation, sputter-coated with gold before examination. The new-formed bone at the surface of the implant area, but also the bone-implant contact area was analyzed through SEM/EDX (energy dispersive X-rays) electron microscopy (JSM 7000F, JEOL, Tokyo, Japan) and the Ca/P ratio resulted from the elemental analysis was assessed.

Results

The surgical procedure during in vivo tests on rabbit femur is presented in photographic images in Figure 1 (a–c).
The image shows several stages of the surgery performed in order to insert the ceramic implant in rabbit femur. Radiological examination after six weeks highlighted the fact that the implant was stable, well integrated and without fibrous tissue or other undesirable post-surgery complications (Figure 2).

The implant proved to be stable both from mechanically and chemically point of view. Thus, it did not suffer noticeable changes throughout the experiment, finally presenting the same aspect as at the implantation moment, without fissures or other aspects that would suggest mechanical decay. The chemical stability is suggested by the fact that the implant did not present erosion marks at the surface, not even discrete ones. Moreover, the XRD pattern of femoral bone presented in Figure 3 shows the fingerprints of hydroxyapatite at 2θ=31.8° and 39.7° indicating that the new bone covered the surface of the implanted area, as the marker bands are visible along the characteristic patterns of Al2O3/ZrO2 ceramic recorded before the implantation procedure.

Over the defect where the implant was inserted, new bone formed, ensuring not only the coverage of the implant, but also the continuity with the adjacent bone, as can be seen in the histological details of Figure 5.

On the lateral sides of the implant, the newly formed bone is deployed as elongated trabeculae emerging from two directions with an obvious tendency to meet each other. The newly formed bone is well vascularized throughout its thickness, including the edge facing the implant, even if in this area the vessels shows a small caliber (Figure 6).

Regarding its organization, the newly formed bone is relatively different from one area to another. In some areas, it resembles like primary bone, in other areas, it contains more or less oriented trabeculae and even haversian systems in development stage. The osseous lamellae prevail in the case of trabeculae progressing on the lateral side...
of the implant, being disposed along the trabeculae (Figure 7). The large number of osteoblasts on the edge of these trabeculae suggests that the osteosynthesis processes are still unwinding.

The electron microscopy details of the newly formed bone are presented in Figure 8 (a and b) corresponding to the contact area with the connective tissue, as well as the cross-section, after the detachment of the implant (Figure 8, d and e). As an interesting detail, in Figure 8b we can observe the structure of a haversian canal, as an evidence of a good vascularization of the new bone. Another detail presented in Figure 8e demonstrates the lamellar architecture of the new bone at the contact area with the implant, six weeks after insertion. The EDX spectra in both situations, according to Figure 8 (c and f) confirms the quality of the newly formed bone, because the quantitative composition of the tissue indicates a 1.72 respectively 1.77 Ca/P ratio, values that are within normal limits.

![Figure 6](image1.png)  
**Figure 6** – New bone formation in apposition with the ceramic implant – six weeks (Goldner’s trichrome). Black arrows – young osteons; blue arrow – primary bone.

![Figure 7](image2.png)  
**Figure 7** – The architecture of the new bone – six weeks (Goldner’s trichrome). Black arrows – osseous trabeculae; blue arrow – bone marrow.

![Figure 8](image3.png)  
**Figure 8** – Scanning electron microscopy (SEM) images recorded on the new bone surface as well as the corresponding energy-dispersive X-rays (EDX) spectrum: (a–c) Area of the newly formed bone – surface view at the contact with the connective tissue area; (d–f) Implant–bone interface area, cross-section.

## Discussion

Zirconia toughened alumina (ZTA) composite is a relatively new ceramic consisting of an alumina matrix in which there are embedded different percent of zirconia particles, either unstabilized or stabilized. The resulted material shows improvements in mechanical properties and makes it a candidate material for prosthetic surgery and tissue regeneration. Many studies in literature presents the important efforts made to improve the material quality by modifying the production process and design requirements [20–23]. These non-metallic inorganic materials have limited range of formulations because their micro-
structure is highly dependent on the applied manufacturing process including temperature, purity of the starting oxides powder, size and grains distribution and porosity. These parameters have direct effect on both the mechanical and biological properties. In order to evaluate if the new biomaterial conforms to the requirement of biocompatibility, mechanical stability and safety, rigorous testing procedure must be performed both in vitro and in vivo. Animal models allow the evaluation of materials in loaded or unloaded situations over potentially long time durations and different tissue qualities [24–26]. Guidelines were provided from the previous studies in literature for the dimensions of the implants according to the animal size and bone chosen but also the implant design, in order to avoid pathological fracture of the site. The rabbit is one of the most commonly used animals for medical research, being used in approximately 45% of musculoskeletal research studies [24, 27]. The literature presents only few papers regarding the differences between human and rabbit bone composition and density, but some similarities in the bone mineral density were reported [27, 28]. On the other hand, the rabbit has faster skeletal change and bone turnover [24].

Assessing the osseointegration process in our rabbit model, we firstly observed that the optimum conditions were created. Thus, we can say that the osseous defect was correctly made, with the adequate shape and sizes for the implant fixation, so that it does not shift at all during the investigation period. Under these conditions, the proliferated bone surrounded the implant shape, in direct contact with the surface. The organism tolerability towards the implant chemical composition proved to be very good, illustrated by the fact that the organism did not trigger a rejection reaction. No clinical signs of inflammation or mobility were present and moreover, the reparative process around the implant was noticed in terms of bone apposition, proliferation and consolidation of osseous tissue to intimately cover the implant, including the penetration area of the experimental bone defect. The osseous tissue covering the implant all over ensures a very good consolidation, conferring high mechanical resistance. The bone around the implant has a relatively good density, with an aspect of a young bone, with differences from one area to another. This fact proves that the consolidation and remodeling degrees are not identical with respect to direction. Some areas emphasize primary bone, while in others, the bone gradually acquires an aspect of a secondary bone (lamellar). Of course, there are numerous other intermediary stages. Even in the areas where the bone has a lamellar aspect, there is a high polymorphism regarding the number and density of the lamellae, but also their disposition (plexiform or ordered). Even where the osseous lamellae are well represented or dominant, their disposition is very different from one area to another, the majority being oriented in a certain direction, probably on the direction of the applied force. There are situations in which they seem to dispose circularly around some spaces that contain blood vessels, gradually forming young haversian systems. All these aspects clearly suggest that the osseous proliferation processes around the implant are in a relatively advanced stage, the remodeling process to a haversian bone is very active, but far from being over.

These observations are also supported by the XRD pattern, emphasized by the presence of hydroxyapatite in the XRD spectrum.

These aspects were also stated by previous studies in the literature, even if some clinicians consider that only long-term results can be helpful to evaluate the real biological response of each given surface [4, 29, 30–33]. In order to qualitatively and quantitatively assess the quality of the newly formed bone, images were recorded through scanning electron microscopy and EDX spectrum, which highlighted both ultrastructural details and the composition of the new bone. The fact that the Ca/P ratio is an indicator of bone quality is well known, assuming that pure hydroxyapatite has a 1.67 Ca/P ratio, in the ideal case [29, 30]. This suggests that the specificity of the Ca/P ratio is better than that of Ca and P concentrations and may be more reliable for the diagnosis of bone disorders. Of course, as mentioned by others authors [31–33], mean values for Ca/P ratios between different bone sites and different animals are highly significant, demonstrating a dependence on lifestyle and bone use of these species.

Conclusions

We can state that the ceramic implant used in our experiment has an appropriate mechanical and chemical resistance, being well tolerated by the body as demonstrated by the absence of inflammatory process or mobility. In this context, exclusively osseous tissue proliferated around the implant, with an obvious tendency of remodeling and consolidation. The newly formed bone was also well vascularized throughout its thickness, and, from the qualitative point of view, the Ca/P ratio indicated a good value. Considering the aspects observed by light microscopy and SEM/EDX, we can state that the new composite based on alumina/zirconia meets the necessary requirements to be used in the medical prosthetics. Our results aligned to the general efforts related to the research of upgraded or new biomaterials for orthopedic and dental applications, to fulfill the challenges faced by these medical fields.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

Authors Cristian Adrian Rațiu and Simona Daniela Cavalu acknowledge the support from UEFISCDI, PN II-ID-PCE-2011-3-0441, contract 237/2011.

References


