

Bone Reaction to a Newly Developed Fiber-reinforced Composite Material for Craniofacial Implants

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Abstract. *Although autologous bone graft is the gold standard in bone reconstruction, the limited volume, the morbidity associated with the donor site, the difficult modelling of complex forms and the unpredictable rate of resorption fuel the researches towards the development of alloplastic materials as bone substitutes. A new fiber reinforced composite (FRC) was developed using 35% combination of monomers bisphenol A glycidylmethacrylate [bis-GMA], urethane dimethacrylate [UDMA], triethylene glycol dimethacrylate [TEGDMA],) and 65% E-glass fibers (300 g/mp). Sixteen (n=16) male Wistar rats were used for the study. The experimental group (n=12) received intrafemoral implants of FRC. The control group (n=4) received intrafemoral titanium implants. After one month and three months respectively, tissues adjacent to implants were histologically evaluated. The intensity of the bone tissue inflammatory reaction, as well as the presence of the osteoblasts and the newly formed bone on the implant surface were the main criteria assessed. The FRC material determined a similar tissue reaction to Ti specimens, at one- and three-months follow-up. Both materials, inserted in the medullary canal, were surrounded by a fibrous connective tissue capsule, which, as time passed, underwent intramembranous ossification process. Fiber reinforced composite may be considered a promising alternative to titanium implants in critical size defects reconstruction.*

Keywords: *fiber-reinforced composite, biocompatibility assessment in vivo*

1. Introduction

Bone defects are associated with different medical conditions, from trauma or infections to congenital diseases or malignancies. Their reconstruction remains a surgical challenge. Although autologous bone graft is the gold standard, the limited volume, the morbidity associated with the donor site, the difficult modelling of complex forms and the unpredictable rate of resorption fuel the researches towards the development of alloplastic materials as bone substitutes [1].

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Metal implants have been successfully used for decades, even if they may have potential negative effects on the recipient bone due to stress-shielding or over-loading [2]. Metal implants may also induce cytotoxic reactions that arise from the liberation of heavy metal ions, corrosion products and nanoparticles [3, 4]. This is relevant especially for titanium, where Ti⁴⁺ ions may lead to soft tissue atrophy and potential exposure of the implant [5]. A new fiber reinforced composite was developed and previously characterized for chemical, physical and mechanical properties by our department [6]. Cytotoxicity and implantation tests (subcutaneous, intramuscular) were conducted and the material proved excellent biological behavior [7,8].

In the present work, the newly developed fiber-reinforced composite (FRC) was biologically tested using intramedullary bone implantation in rats. The bone reaction was recorded and characterized in comparison with titanium, material that already has a well-known behavior, to assess the possibility of replacing titanium for custom-made cranio-facial implants.

2. Materials and methods

2.1. Material preparation

To produce the fiber-reinforced composite (FRC) tested in the study, 2,2-bis[4-(2-hydroxy-3-methacryloxypropoxy)phenyl]propane (Bis-GMA), 1,6-bis(methacryloxy-2-ethoxy-carbonyl-amino)-2,4,4-trimethyl-hexane (UDMA), triethylene glycol dimethacrylate (TEGDMA), benzoyl peroxide (POB), 2,2-dihydroxyethyl-p-toluidine (DHEPT), butylatedhydroxy toluene (BHT) were used as matrix. All the above-mentioned materials were purchased from Sigma Aldrich Chemical Co. (Taufkirchen, Germany) and used without additional purification. Bidirectional woven E-glass 300g/mp was used as reinforcing material (Owens Corning, Brussels, Belgium). Two resins (base resin and catalyst resin) were prepared to produce the matrix material. The composition of the resins was UDMA 60 wt%, Bis-GMA 10 wt%, TEGDMA 30 wt%. POB (1 wt%) served as the initiator in the catalyst resins and DHEPT (1 wt%) was added as accelerator in the base resin. BHT was used as an antioxidant, dissolved in each resin in an amount of 0.65 wt%.

The bidirectional woven E-glass was silanated using 3-methacryloxypropyl-1-trimethoxy-silane (A-174).

2.2. Specimen preparation

Cylindrical specimens (1.5/10 mm) of glass fiber reinforced composites were fabricated by vacuum bag technology [9-12] for the study of bone tissue reaction to the implantation (fig. 1). Initially, the silanated E glass fibers were impregnated by hand lay-up technology on metallic plate mould. After this stage, the entire system (mould and FRC) was introduced in vacuum bag folia, closed and subjected to a vacuum pressure (-0.9 Barr). Thus, the resin excess and air bubbles were removed from composition, the entire surface being uniformly pressed, the fiber fraction ratio being constant throughout the material. The FRC was hardened by chemically initiated radical polymerization of the monomer mixture, followed by thermal treatment at 120⁰ C, for two hours, in an oven. Samples were cut along the fiber monofilament direction, using Water Jet technology, from the composite plate, obtained by synthesis. They were given the final dimensions by computer numerical control machining.

To determine the weight fraction ratio of the FRC obtained, the following formula was used,

$$W_f = \frac{w_f}{w_c} \times 100[\%] \quad (1)$$

where W_f is weight fiber fraction, w_f - weight of reinforced material and W_c - weight of composite materials. The FRC obtained contained 65% fiber-glass and 35% organic resins.

Following fabrication, FRC specimens were immersed in 70 degrees ethyl alcohol, for 48 hours, to remove the residual monomer.

Control specimens of the same dimensions were manufactured from pure titanium, by selective laser melting technology (Realizer II SLM 250, MCP, Borchon, Germany), using an especially gas-atomized

titanium (Ti) powder (99.5% Ti) (TILOP 45, OTC Osaka Titanium Technologies, Osaka, Japan). The powder had 45 μm mean particle diameter, the melting point temperature around 1670°C, 4.51 g/cm^3 density, being included in category of Titanium Grade I SLM process parameters were 120 W laser power, 500 mm/s scanning speed and 50 μm layer thickness. To obtain a proper surface roughness for osseointegration ($R_a \sim 4\mu\text{m}$) [13], the outer borders of parts were treated with 344 mm/s and 133 W laser power (Figure 1).

Prior to implantation, all specimens were sterilized using oxygen plasma (Sterrad, J&J, Irvine, CA, USA).

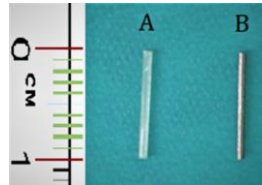


Figure 1. Specimens for intraosseous implantation:

A - fiber-reinforced composite (test);

B – pure Titanium (control)

2.3. Animals and housing

Sixteen ($n=16$) male Wistar rats with a mean weight of 323.1 ± 20.66 g were used for the study. The experimental group ($n=12$) received intrafemoral implants of FRC. The control group ($n=4$) received intrafemoral titanium implants. The groups were randomized in different cages.

Animals were housed in polysulfone type III-H open-top cages (Tecniplast, Buguggiate, Italy) and had access to filtered tap water in bottles and pelleted feed (Cantacuzino Institute, Bucharest, Romania) *ad libitum*. The bedding was standard wood chips aseptic bedding (Lignocel®; J. Rettenmaier & Söhne GmbH + Co. KG, Rosenberg, Germany). The rats were kept in the Laboratory Animal Facility of the "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania, at a standard room temperature of $22^\circ\text{C} \pm 2^\circ\text{C}$ and a relative humidity of $55\% \pm 10\%$, in a 12:12-hour light: dark cycle (lights on, 7 am to 7 pm), at a light intensity of 300 lx at 1 m above the floor. All experimental protocols were approved by the Ethics Committee of "Iuliu Hatieganu" University of Medicine and Pharmacy and were conducted in accordance with the EU Directive 63/2010 (203/20.04.2015). For environmental enrichment, autoclaved braided cotton dental rolls were used (Celluron®, Hartmann, Heidenhelm, Germany). All animal-handling procedures were performed according to the European and Romanian guidelines.

2.4. Experimental protocol

The rats were anesthetized by intraperitoneal injection of ketamine HCl (80 mg/kg) and xylazine (8 mg/kg). Anesthetized animals were restrained on an operation table in a lateral decubitus and the superior half of the left hind limb was shaved over a surface of approximately 40 mm \times 40 mm. Betadine solution was applied on the skin for antisepsis.

The left hind limb was flexed, and an incision of the suprapatellar skin was made. The patellar ligament was incised, exposing the distal epiphysis of the femur. Under constant saline cooling, with the help of a dental micromotor and a round bur, the medullary canal of the femur was trephined and the previously described specimens were implanted (Figure 2). The skin wound was sutured with a nonresorbable thread and then disinfected.



Figure 2. Insertion of the specimens into the femur medullary canal:

A- fiber-reinforced composite (test);

B- pure Titanium (control)

After the implantation of the specimens, the experimental animals were placed in tagged cages, assuring optimal vivarium conditions during the entire period of the study, normal diet (solid foods) and

water *ad libitum*. During the study, local reactions emerging near the implantation site were followed as well as their impact upon the general status of the animals.

After one month and three months respectively, six subjects having implanted FRC specimens and two subjects having titanium specimens were euthanized, with a pentobarbital overdose.

The implanted femoral bones were collected and fixed in 10% formalin solution for 5 days. Tissue samples have been decalcified, implants removed, and the remaining specimens processed to be embedded in paraffin. Sections of 5 μm thickness were prepared from the paraffin blocks, stained using the Hematoxylin Eosin for histologic interpretation.

Tissues adjacent to implants were evaluated and a first descriptive analysis of the identified histological modifications was made. The following histological parameters were recorded: the presence of inflammatory cells (polymorphonuclear neutrophils, macrophages, lymphocytes, and plasma cells), the structure of the fibrous capsule surrounding the implant, and the neovascularization: presence and aspect of blood vessels in the capsule. According to these parameters, the intensity of the bone tissue inflammatory reaction was scored as being:

0- absent: no signs of inflammation;

1- mild: 1-5 inflammatory cells of each type per enlargement field 400x; minimal, focal capillary proliferation, 1-3 vessels; thin fibrous capsule with few collagen fibers and fibroblasts;

2- moderate: 5-10 inflammatory cells of each type per enlargement field 400x; groups of 4-7 capillaries; moderate fibrosis: collagen fibers parallel to the implant surface and scattered fibroblasts;

3- severe: dense inflammatory infiltrate; multiple neoformation blood vessels; thick, fibrous connective tissue capsule with densely packed collagen fibers and numerous fibroblasts;

Regarding the osseointegration of the implants, the presence and the secretory activity of the osteoblasts (morphology and the presence of osteoid - the uncalcified bone matrix) and the newly formed bone (immature woven bone or mature lamellar bone) on the implant surface was assessed. Measurements of newly formed cortical bone around the intramedullary specimens were made and thus the scores were:

0- the absence of osteoblasts or newly formed bone on the implant surface;

1- woven bone spicules formed on the implant surface; few inactive, flattened osteoblasts arranged in a single discontinuous layer on the bone surface; absence of osteoid adjacent to the osteoblasts; few osteocytes in the bone matrix.

2- well consolidated trabeculae consisting of lamellar bone, with small outbreaks of immature bone, active cuboidal osteoblasts arranged in a continuous layer on the surface of the trabeculae; presence of the newly formed osteoid underlying the osteoblasts; multiple osteocytes embedded in the calcified bone matrix.

3- thick, well-consolidated bone trabeculae, consisting of mature lamellar bone; numerous active osteoblasts on the surface of the trabeculae; presence of osteoid; several rows of osteocytes in the lamellae composing the trabeculae.

The ANOVA test was used to compare the independent groups. Bonferroni method was used for the post-hoc test. The level of statistical significance was set at $p < 0.05$.

3. Results and discussions

The intraosseous implantation was well tolerated by all animals included in the study. After the implantation, no animal presented notable modifications of the general status. The skin healing was uneventful, and the convalescence period was short, with no clinical significance. There were no rejection phenomena.

Macroscopically, no differences were seen between the animal groups. None of the animals presented signs of necrosis, bleeding or granulation tissue around the implanted specimens.

3.1. Peri-implant bone reaction to titanium specimens, one-month follow-up

Titanium implant was completely surrounded by a fibrous capsule. Some spicules of immature, non-lamellar bone extended towards the medullary canal, without forming a continuous layer of bone tissue. The capsule covered the entire implant surface and contained collagen fibers, parallel oriented, fibroblasts and vascular elements. Thin and irregular bone spicules were noted on the internal surface of the capsule. They were composed of bone matrix and osteocytes in osteoblasts. On the external surface, adjacent to the medullary spaces, active osteoblasts were present. At the periphery, the spicules were in contiguity with the compact bone in which the implant was placed (Figure 3).

On the surface of bone trabeculae, adjacent to the implant, neither osteoclasts nor bone resorption phenomena were identified. There were no signs of inflammation or bone remodeling.

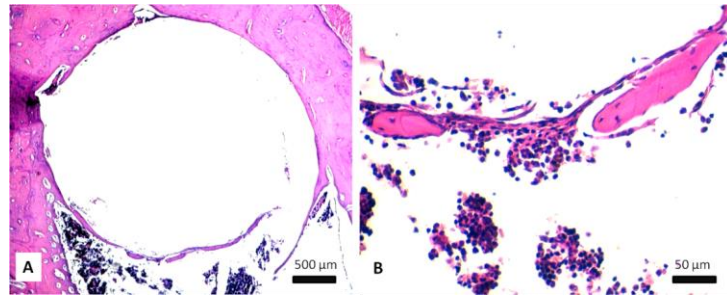


Figure 3. The histological aspect of the local tissue reaction in case of Titanium implant at one month: A - capsule and bone spicules on the implant surface, decalcified bone, H-E coloration, 40x; A - capsule with fibroblasts and capillaries, spicules with osteoblasts in a continuous layer on the external surface, decalcified bone, Hematoxylin- eosin (H-E) stain, 200x

3.2. Peri-implant bone reaction to FRC specimens, one-month follow-up

The aspects were similar to those of the titanium specimen, except the thicker capsule. The capsule contained parallel collagen fibers located adjacent to the implant, fibroblasts and capillaries. The spicules were composed of osteocytes included in the matrix and osteoblasts, on the surface, oriented towards the medullary canal (Figure 4).

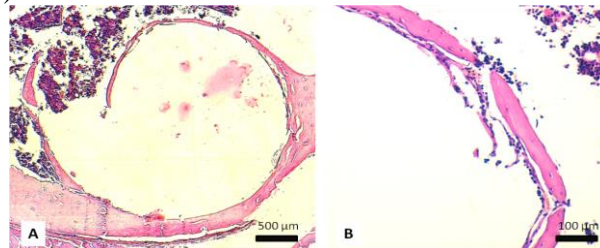


Figure 4. The histological aspect of the local tissue reaction in case of FRC implant at one month. A - Surface of the implant covered by the capsule and immature bone, decalcified bone, H-E coloration, 40x. B- Predominantly fibrillar capsule and bone spicules at the interface with the medullary canal, decalcified bone, H-E stain, 200x

3.3. Peri-implant bone reaction to titanium specimens, three months follow-up

Absence of the capsule was noticed, meanwhile the presence of some well consolidated bone trabeculae, made of mature bone, with bone lamellae oriented parallel to the implant surface. The internal surface of the trabecula, adjacent to the implant, was covered by an amorphous bone matrix. The external surface, adjacent to the medullary canal, was covered by a continuous layer of active osteoblasts. At the periphery, the contiguity relation of the trabecula with the compact bone of the diaphysis was maintained. Furthermore, the bone trabeculae of the adjacent spongy bone were joining with the newly formed trabecula (Figure 5). There were no signs of local inflammation or bone remodeling around the implant.

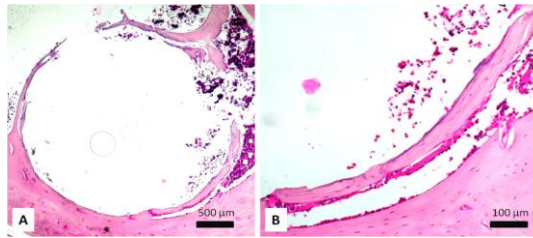


Figure 5. The histological aspect of the local tissue reaction in case of Titanium implant at 3 months. A - Thick trabecula on the implant surface, decalcified bone, H-E coloration, 40x. B - Bone blades and osteoblasts on the external surface of the trabecula, decalcified bone, H-E stain, 200x

3.4. Peri-implant bone reaction to FRC 3 specimens, three months follow-up

The persistence of a thin, discontinuous, capsule was observed on the internal surface of the trabecula, adjacent to the implant. Otherwise, the aspects were similar to those of the titanium specimen (Figure 6).

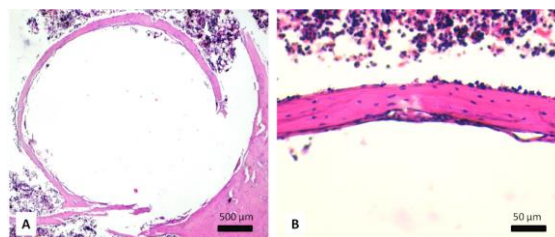


Figure 6. The histological aspect of the local tissue reaction in case of FRC implant at 3 months. A - The capsule and bone trabecula on the implant surface, decalcified bone, H-E coloration, 40x. B - Trabecula covered with thin fibrous tissue, on the internal surface and osteoblasts, on the external surface, decalcified bone, H-E stain, 200x

The histological parameters, following the descriptive analysis of the microscopical details observed, have been summarized in Table 1.

Table 1. Histological parameters of the biological reaction to the intramedullary bone implantation

Histological parameter	Titanium 1 month	FRC 1 month	Titanium 3 months	FRC 3 months
Presence of inflammation signs	0	0	0	0
Encapsulation	1	1.83±0.408	0	1.16±0.408
Neovascularization	1	2.16± 0.408	0	0.83± 0.408
Presence of osteoblasts	2	2.16±0.408	1	1.16±0.408
Presence of the bone on the implant surface	1	1.16±0.408	3	2.83±0.408
Average thickness of the bone on the implant surface	26.64 ± 3.28 μm	29.53±2.56 μm	53.77 ± 3.48 μm	52.715 ± 2.84μm

Although having significant advantages over some other metals, titanium, the most commonly used metal for implant production nowadays, pertains disadvantages that can lead to the biomaterial research in the rush of finding improved alternative solutions. Recently, a trend towards nonmetallic load-bearing implants in all fields of bone surgery was reported. Particularly, in cranial implantology, the urge for nonmetallic implants is justified by the requirements of medical imaging systems (CT, MRI) and radiation therapy, and also by the need to prevent the infections associated with the prosthetic pieces and the resorbing autologous bone flaps [2].

In this study, the biocompatibility of a newly developed FRC material was histologically assessed by its biological reaction when implanted in the bone. Control specimens were manufactured from highly pure Ti because rare cases of Ti hypersensitivity reactions or rejections were reported in the literature [14]. Moreover, the nanometric TiO₂ coated metal surface is linked to the corrosion resistance of the implants and their bio inert behavior in vivo, leading to an acceptable osseointegration. The changes that the material induced locally were analyzed thoroughly. Histological analysis was based on descriptive

data, depending on the scores attributed to different parameters (inflammation, healing) taken into account in the study.

Regarding the intraosseous implantation, the FRC material determined a tissue reaction similar to Ti specimens, at one- and three-months follow-up. Both materials, inserted in the medullary canal, were surrounded by a fibrous connective tissue capsule, which, as time passed, underwent intramembranous ossification process. However, for FRC specimens, 3 months after the implantation, there still were some persistent fine areas of a poorly vascularized connective tissue interface, which could not be observed in the case of the titanium specimens. Signs of inflammation were absent in case of FRC as well as titanium, certifying the bio acceptance of the materials, but also the strictness of respecting the asepsis and antisepsis, aspects that could have contributed to the faulty interpretation of the results. In the absence of inflammation, a viable cortical bone was formed on the surface of both materials, characterized by the presence of active osteoblasts and mature osteocytes, located in the osteoblasts. The thickness of the bone trabeculae for the two types of materials was almost equal, without any significant differences between the studied groups ($p > 0.05$), at both time intervals (1 month and 3 months, respectively). No osteoclasts were observed, as the bone formation process was in full anabolic phase.

A well organized, poorly vascularized connective tissue capsule with few inflammatory cells was formed around the FRC material. The capsule underwent intramembranous ossification process. By comparing the two materials (Ti and FRC), significant differences were recorded when assessing the formation of the peri-implant connective tissue capsule at 1 and 3 months, respectively. The capsule was thicker and contained more numerous neoformation vessels in the case of the composite material ($p < 0.05$). The formation of the connective tissue capsule near or around the implanted biomaterials is a very frequent behavior. *Thus, the macrophages and the gigantic multinuclear cells cannot remove the material through phagocytosis due to its volume and the best alternative for the host seems to be the isolation of this foreign body. This frequently involves the formation of a connective tissue capsule, which is almost avascular, with a layer of gigantic multinuclear cells that limits the potential host-implanted biomaterial interactions. The characteristics of the capsule depend on the cytokines released at the site [15], the intensity of trauma produced during implantation, the defect size and the quantity of temporary extracellular matrix formed locally [16].*

The fibrous capsule limits the inflammatory reaction but, on the other hand, if it is formed around an intraosseous implant, the osseointegration process might be compromised. The defective contact between the implant and the adjacent bone favors the initiation of the infectious process, as well. Supporting these findings, Santavirta *et al* have shown that this connective tissue capsule, also known as the interface membrane, forms around polymer implants (e.g. PMMA) and has the morphology of a synovial [17]. The same implants, surface conditioned and rigidly fixed, are superficially surrounded by a thin membrane, containing some macrophages aggregates [18]. The quantity and quality of the capsule formed around alloplastic materials depend on the implantation technique as well as on the material used [19-20]. Few lymphocytes and plasma cells occasionally present in the capsule cannot, by themselves, negatively influence the material bio acceptance. However, the presence of numerous macrophages, osteoclasts and gigantic multinuclear cells associated with an excessive thickness of the fibrous capsule, suggests an implant-host incompatibility reaction. The initial interaction between an implant and cells plays an important role in bone regeneration and osseointegration [21].

Intraosseous implantation tests depict the inflammatory reaction the new biocomposites produce on living tissues. The presence of a thin, well organized capsule around tested FRC allows their labeling as nontoxic and inert materials. This is even more underlined by the ossification process, which took place at the level of the surrounding capsule [22].

The favorable biological behavior induced by the FRC is due to its chemical and physical properties.

One of the most critical aspects of using polymer based composites for medical purposes is the release of residual monomer, which can induce cytotoxic, inflammatory, allergic and even mutagenic reactions. The gel effect which occurs during the dimethacrylate monomers polymerization, leads to the closing of macroradicals and unreacted monomers in the cross-linked three-dimensional polymer



network. Presence of residual monomer or unreacted double bonds in the harden matrix has also a plasticizing effect on the polymer and make the polymeric matrix prone to degradative reactions [23].

We have previously developed more formulations of FRCs meant for the fabrication of cranio-facial implants and selected the one used in this study. The polymeric matrix tested here is based on UDMA (60%), TEGDMA (30%) and Bis-GMA (10%) and has a degree of conversion of 85.58% (14.42% residual double bonds) [7]. The total percent of the extracted monomers, related to the weight of initial copolymer sample is 0.04% (0.001% Bis-GMA, 0.008% TEGDMA and 0.031 % UDMA), being lower than the data presented in the literature for the dental composites, which end up releasing, after 7 days of storing in ethyl alcohol up to 1.2% residual monomer [24].

This superior degree of conversion can be explained by the lower viscosity of UDMA compared with the Bis-GMA oligomers and a longer gel time which allows a greater number of methacrylic groups to react and convert to polymer.

The quantity of residual monomer released by the FRCs is influenced by the hydrophilic/hydrophobic character as well as by the amount, size and flexibility of the each monomer's specific molecules. UDMA, being a hydrophilic and flexible monomer, can be easily extracted in a hydrophilic medium (water, alcohol). TEGDMA has a smaller size and less hydrophilic character than UDMA, with relatively flexible etheric groups, whereas the Bis-GMA oligomers have large, rigid molecules, presenting a stronger hydrophobic character compared to the other monomers. This leads to a significantly smaller quantity of extracted Bis-GMA monomer in a hydrophilic medium [7].

Another aspect to be mentioned is that in case of FRC custom-made implants, the release of different monomers/activators will be even more limited, since the chemical polymerization and postcure thermic treatment are to be performed *ex vivo*. Due to this particularity of the fabrication process, the problem of toxicity related to clinical use of other materials such as glass-ionomer cements (that release polyacids and aluminum ions in contact with body fluids) is surmounted.

The reaction induced by a material at implantation may also be related not only to the chemical composition, but also to the trauma associated with roughness of the samples' surface. The morphological analysis of the external surface of FRC – assed by SEM- has previously shown that the reinforcing material was well incorporated in the matrix and monofilaments of fiber glass could not be observed on the material surface [6].

Other properties that recommend FRCs for being used to fabricate custom-made implants are mechanical properties that can be tailored by the degree of reinforcement [7] in order to achieve same flexural strength as the living bone. Compared with other nonmetal materials used (eg, bioactive glass), FRCs have the advantage of not being brittle. In case of a traumatic event, the glass-fiber reinforcement provides a better resistance and lowers risk of damaging internal organs and structures (as brain, eye, blood vessels, nerves) by sharp edges [25].

Moreover, FRCs lack thermal conduction, which can cause problems to the patient, and can be easily shaped in the operating room when necessary. FRCs do not produce artifacts during computed tomography or magnetic resonance imaging investigations of the operated area.

4. Conclusions

Fiber glass reinforced biocomposites could be used as reconstruction materials in bone defects, due not only to their chemical, physical and mechanical properties, but also to their favorable biological behavior.

A certain inflammatory reaction of the organism to the implantation of a biomaterial is tolerable and the fiber-reinforced composite implants can establish a close contact with the bone, comparable with the osseointegration of titanium implants.

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