Bone repair of implants surfaces in rats: histomorphometric analysis, scanning electron microscopy and energy dispersion X-ray spectroscopy. A Randomized study

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Article

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Abstract

This study aimed to characterize the newly formed bone tissue adjacent to implant surfaces histologically. The implants were previously analyzed by scanning electron microscopy and energy dispersion X-ray spectroscopy and installed in the right tibia of 75 rats divided into the: polished group, machined group and treated group. The animals were euthanized at 3, 7, 15, 21, and 40 postoperative days, and the tibias were histologically analyzed for bone/implant contact. Scanning electron microscopy of the surfaces showed topographic differences, with the smoothest and most regular surfaces in the polish group, followed by the surface of the machine group, while the treated group showed surfaces with the greatest roughness. The energy dispersion X-ray spectroscopy revealed surfaces without contamination and showed similar Titanium, Aluminum, and Vanadium contents in all groups. The implant osseointegration process showed healthy and vital bone with similar qualitative patterns in all groups. Bone cortilization occurred in contact with the implants' titanium surfaces, even in the areas close to the tibial bone marrow. The peri-implant repair pattern was similar between all the surface types at all other timelines, with cortical bone formation in all the titanium surface of the three implant groups.

Introduction

It has been proposed that the topographic and physical-chemical modifications of the implant's surface increase the contact between the bone and implant. These modifications create a roughness that, not only increase the area of contact with the bone, but also favors retention of the clot on the surface and adsorption of proteins, and increase cell migration and proliferation, which is essential for bone formation closer to the implant, guaranteeing higher levels of osseointegration and long-term success[1,2,3]. The most important factors affecting the quality and speed of osseointegration are the chemical nature and physical nature of the implant's surface[4], whereas implants with rough surfaces requires a higher torque for their removal as compared to those with smooth surfaces[3,5,6].

Different methods have been used for surface treatment, including addition and subtraction techniques, wich can also be associated with titanium oxide (TiO_2) or aluminum oxide (Al_2O_3) blasting, laser irradiation, and surface oxidation[2,7]. The formation of TiO_2 on the implant surface shows a significant improvement in the blood response in the osseointegration process[8].

Osseointegration between the implant surface and bone tissue is a pre-requisite for the long-term success of the implant supported fixed prosthesis. Hence, changes in the implant surfaces have gained an important and decisive place in implantology research in recent years[16]. The surface roughness of the implants affects the biomechanical quality of the bone embedded in these surfaces, the bone integrated with rough surfaces is harder and more rigid as compared to bone that integrated with smooth surfaces[14].

This study analyzes the polished surfaces, machined surfaces, and acid treated, as well as the histological characterization of the newly formed bone tissue.

Materials And Methods

A total of 75 male rats (Rattus Norvegicus Albinus, Wistar), aged between 70–90 days and weighing approximately 250–300 mg, were randomly divided into three groups (n = 25) according to the type of implant surface: polished group (PG), animals received implants with a polished surface; machined group (MG), animals received implants with a machined surface; and treated group (TG), animals received implants with a treated surface.

The randomization of the rats into the three groups, was conducted by drawing lots in an envelope containing one of the three group names.

This study follows ARRIVE guidelines and all methods were carried out in accordance with relevant guidelines and regulations, approved by the Ethics Committee on Use of Animals of the Faculty of Dentistry of Araçatuba (UNESP) (process n° 00253–2012).

This study used 75 mini-implants with internal insertion manufactured by Implalife Biotechnology (Jales, São Paulo, Brazil), for the tibia region of the rat, with dimensions of 2 mm in diameter and, 7 mm in length having machined, polished, and treated types of surfaces. The subtraction method was used on the surfaces of the treated implants, where acid solutions baths of nitric acid, hydrochloric and phosphoric acid were used. Surface treatment was carried out entirely by the implant manufacturing company.

Study samples were anesthetized with 0,2 ml Ketamine Hydrochloride (Ketamina Agener®, União Química Farmacêutica Nacional SA, São Paulo, SP, Brazil) and 0.1 ml Xylazine (Dopaser®, Laboratório Calier do Brasil, Osasco, SP, Brazil), for 250 mg of body weight. After trichotomy on the anterior surface of the right posterior limb and antisepsis with polyvinylpyrrolidone iodine degermante (10%, Riodeine Degermante®, Rioquímica, São José do Rio Preto, SP, Brazil), the superior third of the tibia was infiltraded using 0.1 ml of Lidocaine Hydrochloride with 1:100,000 epinephrine (Alphacaíne lidocaine 2% epinephrine 1:100,000 DFL®, Rio de Janeiro, RJ, Brazil), to ensure local hemostasis. The incisions were made by planes and the flap was detached, and the bone tissue was exposed to perform the bicortical osteotomy.

The milling implants installation was performed with a 2.0 mm diameter drill, electric motor (BLM 600®; Driller, São Paulo, SP, Brazil) at a speed of 1500 rpm and a speed reducer handpiece (1:16; (KaVo®, Kaltenbach & VoigtGmbH & Co., Biberach, Germany), under constant irrigation of sterile 0.9% sodium chloride solution. The implants were installed according to the group to which the animal belonged. The skin and muscular flaps were repositioned and closed with a tight suture in layers.

All the animals were injected intramuscularly with 1 mg/kg/day of sodium dipyrone (Ariston Indústrias Químicas e Farmacêuticas Ltda, São Paulo, Brazil) and a 20,000 I.U. of penicillin G benzathine (Fontoura Wyeth S.A. Industrias Farmacêuticas, São Bernardo do Campo, SP, Brazil) per animal for immediate postoperative period. The animals were euthanized by anesthetic overdose (about three times the anesthetic dose) with Ketamine Hydrochloride (Ketamina Agener®, União Química Farmacêutica

Nacional SA, São Paulo, SP, Brazil) and Xylazine (Dopaser®, Laboratório Calier do Brasil, Osasco, SP, Brazil) on days 3, 7, 15, 21 and 40 postoperatively.

Analysis by scanning electron imcroscopy and X-Ray spectroscopy by energy dispersion

The implants in each group were analyzed for surface topography using Scanning Electron Microscopy (SEM) with an energy dispersion X-Ray Spectroscopy (EDS) system (model EVO LS15, Zeiss, Germany), to analyze the chemical composition and elementary mapping of the surfaces[17].

Histological and histometric analysis

The tissue samples from the bone/implant interface were obtained and fixed in 10% formaldehyde solution for 48 hours, washed in running water for 24 hours, decalcified in 20% ethylenediamine tetraacetic acid for 5 weeks, and then dehydrated using alcohols and diaphanized. The pieces obtained were placed in separete paraffin blocks and 6 µm thick, cuts were obtained, which were later stained using hematoxylin and eosin and mounted on histology slides.

The images obtained were analyzed using an optical microscope (Jenamed 2 Histology, Carl Zeiss, Germany), and captured using a photographic camera (AxioCamICc1, Carl Zeiss Microimaging system) connected to the microscope and the computer. The images were captured using the application Axio Vision (Release 4.8.2 SP1 12-2011, Carl Zeiss, Germany).

Histometric analyses were performed using the ImageJ image analysis software, version 1.47. The linear extent of contact between the newly formed tissues and the implant surface (LECOI) was calculated as a percentage, from the initial organization of the clot (3 days) to the bone deposition over the entire implant (40 days).

Statistical analysis

The data obtained in each type of comparison were subjected to statistical analysis using the statistical analysis program in experimental work BioEstat 5.3.

Results

Scanning electron microscopy associated with X-ray spectroscopy by energy dispersion

The EDS analysis of the surfaces revealeds the exact composition of: titanium, aluminum, and vanadium (Table 1).

Table 1
Chemical composition of the surface of the implants of the polished group (PG), machined group (MG) and treated group (TG).

Group	Element Weight (%)			
	Aluminum	Titanium	Vanadium	Total
PG	6.44	89.81	3.75	100.000
MG	7.08	89.09	3.83	100.000
TG	7.21	89.00	3.79	100.000

Histological analysis

The representative histologic images of the evolution of bone formation in groups PG, MG and TG is illustrated in Figs. 2 and 3, captured in 250x and 400x magnification respectively.

On postoperative day 3, the presence of mature connective tissue with adult fibroblastic cells and well-arranged collagen deposition areas was observed. In areas with the most contact with the spirals and close to the areas of corticalized bone tissue, bone deposition was observed. This aspect of the connective tissue was noted in all three groups, with variations due to the area of analysis, depending on the medullary or cortical regions of the tibiae. In a short postoperative period, the clot was organized in the connective tissue and granulation tissue, with no inflammatory or foreign body reactions.

At 7 days, there was neoformed bone tissue in contact with the implant spirals and bone neoformation at some distance. There was no qualitative difference between the groups. The newly formed bone presenteds a thin trabeculate with wide medullary spaces, but intense osteoblastic activity. There was no granulation tissue or inflammatory reaction.

On Day 15, we noticed a different behavior between the regions of the bone marrow and cortical tibia. In both locations, in direct contact with the surface of the implants, we noticed corticalized bone tissue with few medullary spaces. When we analyzed the regions of the implants that were in contact with the tibial bone marrow, the spaces were filled with tissue in medullary differentiation, with numerous white undifferentiated cells and adipocyte vessels. In the tibial cortical areas, deposition of mature bone occured with thick trabeculae and few medullary spaces filling the entire space between the spirals of the implants. This qualitative factor probably indicates the completion of the process of differentiation of reparative tissue.

At 21 days, a better differentiatied tissue was formed in the bone marrow that presented a cellular infiltrate characteristic of white cells and vessels. The surface of the implants showed a prevalence of lamellar bone tissue at their contact area.

At 40 days, the remodeling process was more specifc with respecto to the disposition of the newly formed tissue, showing remodeled corticalized bone, with lamellar bone in contact with the surface and thickness diversification according to the tibial region. The bone marrow occupieds most of the spaces in the central areas of the tibia, while the lamellar bone prevailed next to the cortical bone.

Statistical analysis

The statistical and histomorphometric analyses were performed for the different surfaces of the studied groups at 3, 7, 15, 21, and 40 postoperative days. The averages of the bone-to-implant contact (BIC) values of the samples (measuring bone in contact with both the cortical and medullary region of the tibia) represented the percentage of bone in contact with the spirals of the implants in each of the three groups, as well in the five analyzed periods (Fig. 1, Table 2).

Table 2
Percentage of bone in contact with the implant for different periods and different surfaces.

Days	Polished Group	Machined Group	Treated Group
3	10.42 ± 12.2%	6.25 ± 6.85%	14.58 ± 14.61%
7	70 ± 16.95%	87.5 ± 11.18%	94.16 ± 6.45%
15	77.08 ± 16.61%	81.25 ± 15.31%	83.33 ± 6.45%
21	91.66 ± 6.45%	90 ± 5%	92.08 ± 6.21%
40	91.66 ± 6.45%	95.83 ± 6.45%	91.67 ± 6.45%

Comparing the values of the three groups in the same period, there was a statistically significant difference ($p < 0.05$) between PG and TG on postoperative day 7 $p = 0.0189$ with BIC values 70 ± 16.95% and 94.16 ± 6.45%, respectively. In general, the BIC percentages of the PG, MG, and TG groups were 68.16 ± 33.62%, 72.17 ± 37.22% and 75.16 ± 34.12%, respectively.

Discussion

The topographic analysis of the surfaces showed that TG presented morphology with more roughness as compared to MG and PG. This difference was probably responsible for the higher percentage of BIC in the TG than that of PG in the initial 7 days period, with a statistically significant difference (± 6.45% vs. 70 ± 16.95%). The osteogenesis that occurs at the bone/implant interface is directly influenced by the physicochemical and morphological properties of the implant surface that further influence a series of events such as protein adsorption, cell migration and proliferation, cell differentiation and bone matrix deposition[18].

The stabilization of implants machined in bone tissue occurs by the growth of bone on the titanium surface resulting in a biomechanical union. An unfavorable aspect of surfaces with less roughness is

that this interaction with bone tissue is time-dependent[19,20]. Therefore, to favor the osseointegration process and decrease the time for implant-supported rehabilitation, the surfaces of the implants can be modified by different treatment methods, and this is mainly indicated in cases of bones with deficient quality and quantity.

The process of treating the implant surface with an acid can increase the oxide layer and roughness by immersing it in an acid solution that corrodes the surface and forms micropores[21]. However, there is still no consensus about defining a surface as being smooth or rough. What is considered a smooth surface in one study may be considered rough in another[3,23]. The acid conditioning provides a homogeneous rough surface, increasing the active contact area that favors a better adhesion and cell proliferation, thus enabling faster osseointegration as observed in this study in the 7-day period for TG[24].

Butz et al[14] in 2006 conducted a study on rats where they found that the bone surrounding the acid-treated titanium surfaces was significantly harder than that found around the machined surfaces. In a systematic review conducted by Wennerberg and Albrektsson[3] in 2009, a positive correlation was found between surface topography and osseointegration. It was demonstrated that surfaces treated with acid and those with roughness between 0.6–0.9 μm were found to be more resistant in the removal torque tests, and showed a larger amount of BIC when compared to implants with machined surfaces. These results corroborated with our study.

Some authors concluded in their studies that a very rough surface seems to interfere with the bone response, and may not be beneficial for adequate osseointegration[1,25,26]. According to our study results, we were able to observe a higher percentage of BIC in the initial 7 days TG. There was statistically significant difference ($p < 0.05$) only in this period when comparing the PG and TG groups. Similar data were found in some studies where there was a statistically significant difference in the initial period of osseointegration, but not in the later period. This suggests that the chemical modification of the implants surface may interfere with bone apposition properties in early periods of osseointegration[27,28].

Some authors have described that in addition to the changes made to the implant's surface to modify the macro, micro or nano roughness, some chemical changes also occur on this surface. Therefore, identifying the most important factor responsible for the results obtained when comparing implants with different types of surface treatments may be difficult[3,29]. Despite the large number of studies evaluating the surfaces of the implants, is the ideal microtexture and biomechanical properties promoting the optimization of the BIC interface are yet unknown[1].

It is of fundamental importance to carry out studies on the biological behavior of the modified surfaces, since the topographic and physical-chemical properties obtained favor osseointegration. The implant osseointegration process in this study followed the same qualitative template in the PG, MG, and TG. The bone differentiation process occurred early in the TG at 7 days; however, all groups showed similar results in the end.

Conclusion

The cortical bone formation occurred in contact with the titanium surface of the three implant groups, even in areas close to the tibial bone marrow.

Declarations

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Author contributions statement

GBF: designed the study, collected the data, analysed the data, writing. LCCC: led the writing, analysed the data. KRT: analysed the data. IS: analysed the data. DP: led the writing. IRGJ: collected the data, analysed the data, led the writing.

Data availability statement

All data generated or analysed during this study are included in this published article, and the raw data are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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Figures

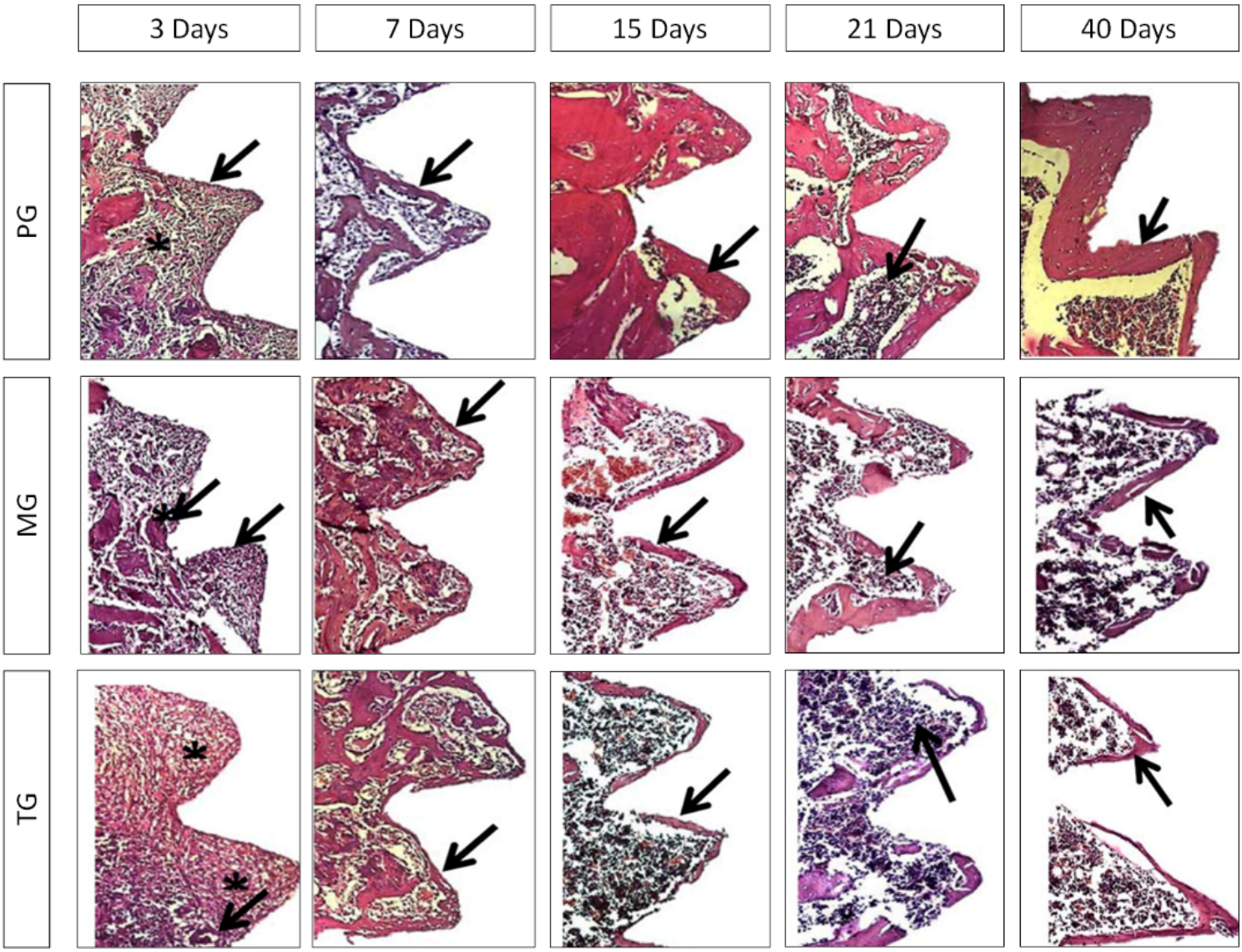

Figure 1

Graph illustrating the means (in percentage) of the BIC (Bone-implant contact) values for the 3 groups (PG, MG and TG) in the 5 periods analyzed

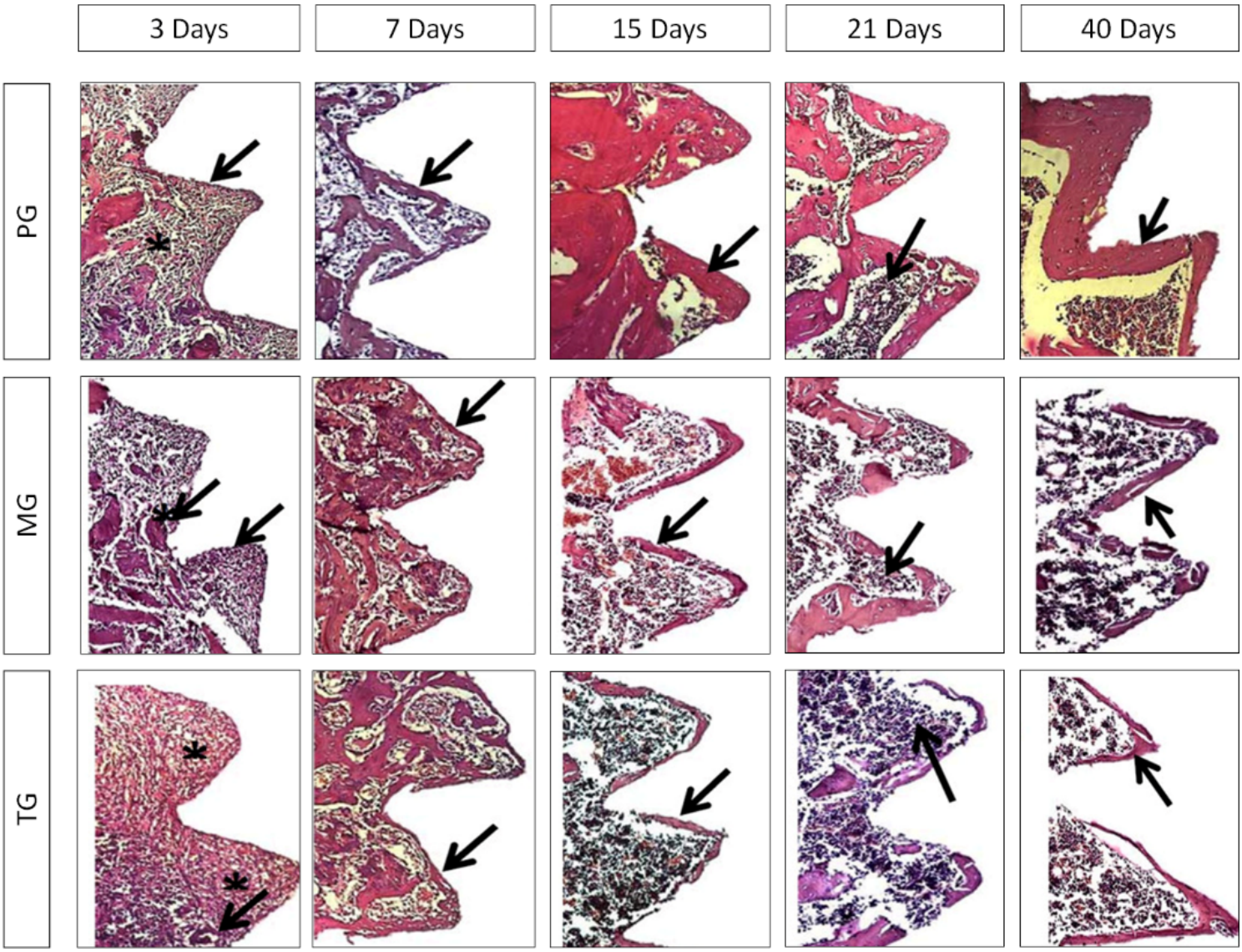

Figure 2

Graph illustrating the means (in percentage) of the BIC (Bone-implant contact) values for the 3 groups (PG, MG and TG) in the 5 periods analyzed

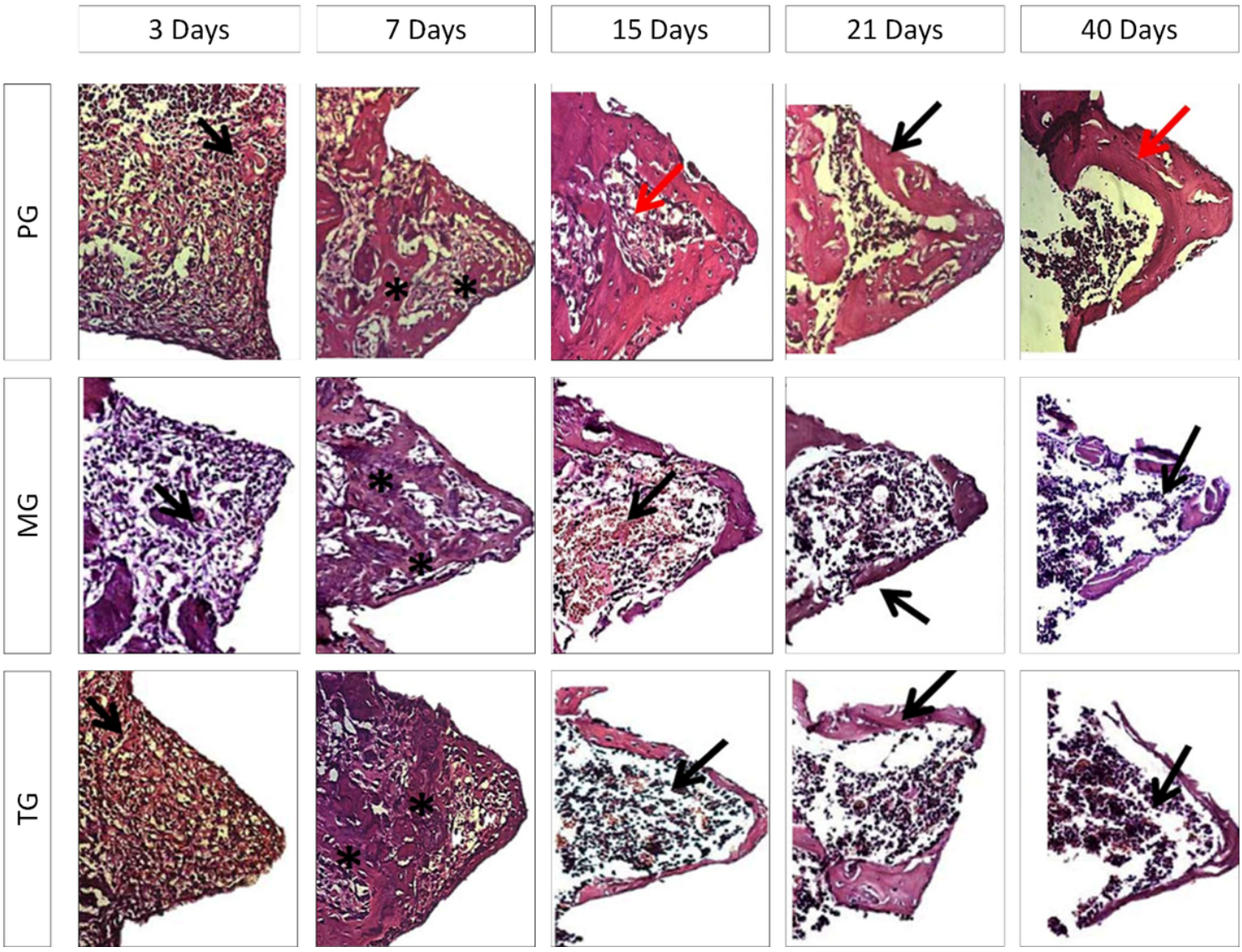

Figure 3

Representative histologic images of the evolution of bone formation (arrows) filling the valleys of the implants (*) in groups PG, MG and TG. All pictures were captured in 400x magnification.