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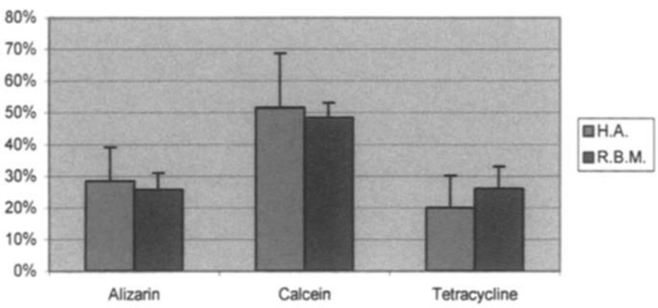
Table 2. Results and descriptive statistic of the Polyfluorochrome Sequential Labeling

Implant	Alizarin		Calcein		Tetracycline	
	H.A.	R.B.M.	H.A.	R.B.M.	H.A.	R.B.M.
1	24.1%	41.1%	66.9%	50.2%	9.0%	8.7%
2	20.3%	37.5%	70.2%	41.9%	9.5%	20.6%
3	8.3%	22.5%	88.3%	38.4%	3.5%	39.1%
4	16.1%	19.4%	28.8%	57.9%	55.1%	22.6%
5	45.9%	26.1%	27.0%	48.0%	27.1%	25.9%
6	23.1%	16.6%	54.9%	45.1%	22.0%	38.3%
7	62.7%	31.5%	0.7%	55.2%	36.6%	13.3%
8	10.0%	20.5%	81.5%	36.9%	8.4%	42.6%
9	33.7%	25.2%	59.5%	51.0%	6.8%	23.8%
10	40.2%	16.0%	37.7%	58.5%	22.0%	25.5%
average (μ)	28.4%	25.6%	51.6%	48.3%	20.0%	26.0%
standard deviation (ρ)	17.2%	8.6%	27.5%	7.7%	16.3%	11.1%
standard error (ρ/?n)	5.4%	2.7%	8.7%	2.4%	5.1%	3.5%
Confidence	95%					
C.I.	10.6%	5.3%	17.0%	4.8%	10.1%	6.9%
Max.	39.1%	31.0%	68.6%	53.1%	30.1%	32.9%
Min.	17.8%	20.3%	34.5%	43.5%	9.9%	19.2%



Graphic 1 Mean Values and Standard Error for the Polyfluorochrome Sequential Labeling.

and HA-coated surfaces. After an 8-week healing period, active remodelling with secondary osteons and lamellae was seen in the bone close to the implant in both rough (RBM) and HA surfaces. Newly formed bone was seen at the sub-periosteal and endosteal surfaces as well as inside the medullar cavity. The amount of new bone formed in the marrow cavity was irregular as described by Jansen et al. (1991). These authors reported that bone remodeling was very active after eight weeks of healing and it presented diverse degrees of bone maturation and also an irregular new bone formation in the marrow cavity. The polyfluorochrome sequential labeling is in accordance with Haider et al. (1993) who have described the same color pattern of bone labeling where the correspondence of colors was detected. Haider et al. (1993) also described woven bone formation showing a diffuse pattern after tetracycline and calcein labelling and formation which started directly at the wall of the drilled hole without preceding resorption. The results in the present study describe the remodelling at the sub-periosteal and endosteal surfaces. It was noticed that the structural anatomy of the tibia changed in order to anchor the implant during the bone modelling and remodelling. The results of this work are also compared with those presented by König et al. (1998) where they found three different patterns of bone deposition and Rahn (1976) where the method of fluorescent bone labelling was used. The histomorphometry confirmed the findings using the fluorescent microscopy technique. Calcein had greater mean values of deposition compared to alizarin and tetracycline for both surfaces. It was due to the fact that calcein was deposited between 28 and 35 days, indicating the higher bone metabolism at this period. Alizarin was the first label injected at the time that there was bone resorption due to the drilling preparation. Tetracycline was the last label and represented the final stage of the bone remodeling process, osteoconduction and maturation.

It is concluded that polyfluorochrome sequential labeling is an important tool in the identification of bone remodeling after the insertion of titanium implants with various surfaces inside rabbit tibias.

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of alizarin was: rough surface 25.6% ± 2.7%, HA coated surface 28.4% ± 5.4%, calcein: rough surface 48.3% ± 2.4%, HA coated 51.6% ± 8.7%, and tetracycline: rough surface 26% ± 3.5%, and HA coated 20% ± 5.1% (Table 2 and Graphic 1). ANOVA test showed that no statistical difference was found in polyfluorochrome sequential labelling deposition between the rough and HA coated implants ($p = 0.999$). The Tukey test verified that calcein was deposited in a statistically higher amount compared to alizarin and tetracycline on both surfaces ($p < 0.05$). Nonetheless, there was no statistical difference between the deposition of alizarin and tetracycline inside the threads of rough (RBM) and HA coated implants.

Discussion

Analysis under the fluorescent microscope demonstrated bone remodeling at the bone/implant interface with rough

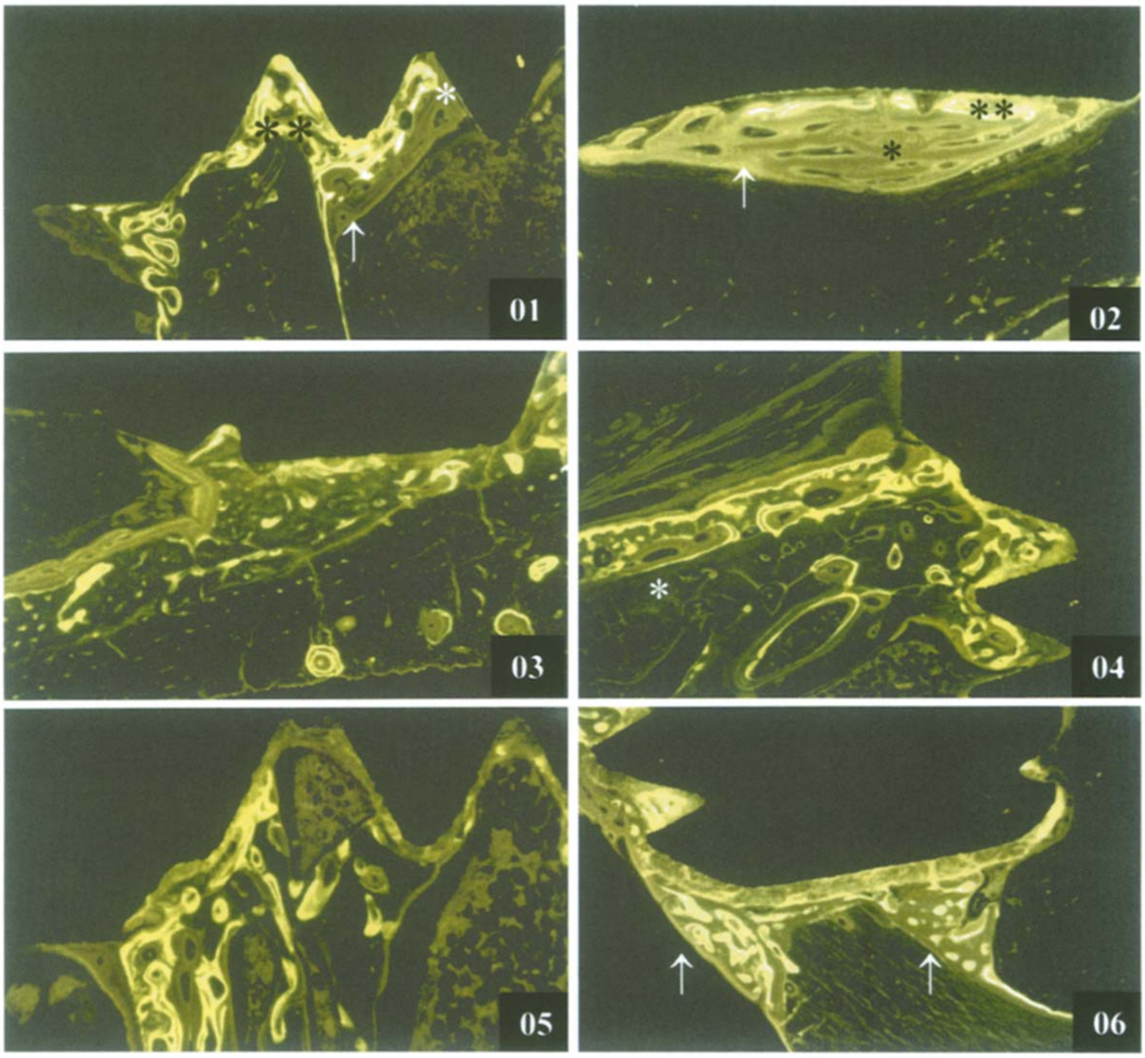

Fig. 1. Photomicrograph under fluorescent microcopy. Head of the rough surface implant. Remodeling at the sub-periosteum and endosteum related to the implant surface. Alizarin – tile red (*), calcein – green (**), and tetracycline – yellow (↑). ×35.

Fig. 2. Photomicrograph under fluorescent microcopy. Apical region of the rough surface implant. Newly formed bone in direct contact with the implant surface due to osteoconduction. Alizarin – tile red (*), calcein – green (**), and tetracycline – yellow (↑). ×57.

Fig. 3. Photomicrograph under fluorescent microcopy. Apical region of the rough surface implant anchored in the cortical bone opposite the surgical bed of the tibia. Bone remodeling through osteoconduction. ×57.

Fig. 4. Photomicrograph under fluorescent microcopy. Head of the HA-coated implant. Remodeling below the periosteum and the old cortical bone in dark green (*). Direct apposition of polyfluorochrome sequential label inside the implant thread. ×35.

Fig. 5. Photomicrograph under fluorescent microcopy. Head of the HA-coated implant at a higher magnification. Newly formed bone between the periosteum and cortical bone. ×57.

Fig. 6. Photomicrograph under fluorescent microcopy. Apical region of the HA-coated implant anchored in the cortical bone of the tibia. Note that the tibial bone fitted (*) the implant in order to anchor it. HA-coating (↑). Alizarin – tile red (*), calcein – green (**), and tetracycline – yellow (↑). ×35.

Materials and methods

Operative Procedures. Commercially pure titanium implants with a rough surface and HA-coated implants were inserted in rabbit tibias where they remained for eight weeks. This study followed the Rules of the Ethics Committee in Animal and Research, in accordance with the surgical protocol (Protocol 096/2000). After a period of housing, 8 male rabbits (*Oryctolagus cuniculus*) with an average weight of 3 kg received 15 commercially pure titanium implants with rough "resorbable blast media" surface (Restore®, Lifecore Biomedical, Inc., Chaska, MN, USA) and 15 HA-coated (Sustain®, Lifecore Biomedical, Inc., Chaska, MN, USA). The implants were thread shaped, measuring 7 mm in length and 3.75 mm in diameter. According to the surgical plan two implants were inserted near the proximal margin of each tibia under general anesthesia and antibiotic protection. The rough implants were inserted in the right and the HA-coated in the left tibia. The surgical protocol was the same for all the animals under general anesthesia (intramuscular injection of ketamine 20 mg/kg). The skin of both legs was shaved, antisepsis was performed, and local anesthesia was applied with 1:100,000-epinephrine vasoconstrictor. A full flap was made in the area near the surgical site and the implants were inserted in the proximal area of the epiphysis of the tibias under saline irrigation. After the insertion, the flaps were carefully closed with a silk thread suture (Ethicon– Johnson's & Johnson's). For the evaluation of bone remodeling during the healing period, the polyfluorochrome sequential labeling introduced by Rahn (1976) as described in Table 1 was followed.

Section Preparation. After a healing period of eight weeks (56 days) the animals were sacrificed by an overdose of pentobarbital. The tibias were dissected and bone blocks containing the implants were removed and maintained in a 4% neutral formalin buffered solution. After fixation, the samples were washed in running water for 12 h and dehydrated in graded solutions of ethanol from 70% to 99% for 24 hours in each solution. The samples were then embedded in a metacrylate solution (Technovit VCL, Sigma) and after polymerization they were processed according to the cutting-grinding technique (Donath and Breuiner 1982). The sections remained unstained. All sections obtained were analyzed under the fluorescent microscope (Leitz, Aristoplan) with a blue filter (I3, BP 450–490nm).

Histomorphometry. The amount of alizarin, calcein, and tetracycline that were deposited during the healing period were measured for the three best consecutive threads of both sides of the rough and HA-coated implants using the fluorescent microscope

Table 1. Polyfluorochrome sequential labeling and days of application

Postop.	Substance	Doses	Injection (mg/kg)
14 days	Alizarin	30	3 g/100 ml + 2 g $NaHCO_3$
21 days	Alizarin	30	3 g/100 ml + 2 g $NaHCO_3$
28 days	Calcein	10	3 g/100 ml + 2 g $NaHCO_3$
35 days	Calcein	10	3 g/100 ml + 2 g $NaHCO_3$
42 days	Tetracycline	60	3 g/100 ml + 2 g $NaHCO_3$
49 days	Tetracycline	60	3 g/100 ml + 2 g $NaHCO_3$
56 days	Exitus		

(Eclipse 1000 M. Nikon, and double F–R green filter) connected to a computer equipped with a digital camera and an image analysis system (Image-Pro Plus, Media Cybernetics, U.S.A.). All measurements were statistically evaluated using the analysis of variance, ANOVA, with two factors (surface and label) and Tukey test for multiple comparisons among the means. The confidence level was $p < 0.05$.

Results

Fluorescent microscopy. The analysis under fluorescent microscopy showed an intense bone remodeling and osteoconduction for both rough and HA coated implants. Newly formed bone was characterized by polyfluorochrome sequential labeling (Figs. 1, 3–6). The old bone was characterized by a dark green without label. In some areas secondary osteons were demonstrated by the deposition of the labels in a concentric arrangement (Figs. 1, 4). The tight adaptation of the bone/implant favored new bone growth immediately adjacent to the implant, especially in the vicinity of the cortical bone of the tibia. Cortical as well as newly formed bone could also be seen in the medullary cavity, promoted by osteoconduction but in a small quantity (Fig. 1). In some samples, newly formed bone was seen in the medullary cavity in direct contact with the implant surface (Fig. 2). It is interesting that the newly formed bone was completely marked by the polyfluorochrome sequential labeling. It was observed that the fluorescent analysis showed a special contrast of the material in different layers related to the amount of newly formed bone (Fig. 3). Alizarin had a tile red color in a diffuse pattern; calcein was represented by green bands, and tetracycline by thin yellow lines. Polyfluorochrome sequential labeling was observed in the well-organized osteons as well as in the vascular channels and in the lamellar bone. The cortical layer of the respective tibiae also revealed the labels due to a physiological bone remodeling process. In some samples, the labels were deposited in sheets or layers (Figs. 1–5). Other samples represented three types of bone tissue, e. g. newly formed bone related to the periosteum, old cortical bone, and remodeling due to the endosteum and conductive properties of the implant. At the apical part of the implant, tetracycline was related to the endosteum; alizarin was present in the middle layer and calcein in remodeling bone representing primary osteons (Fig. 3). At the top of the implant, tetracycline was present at the sub-periosteal surface, alizarin was below the tetracycline, and calcein as well as alizarin marked osteons (Figs. 1, 5). It was interesting to observe that below those layers there was old cortical bone that was marked by the labels due to the remodeling process. The HA coated implants showed that the labels were deposited directly in the ceramic coating (Figs. 4, 5).

Histomorphometry. Table 2 shows the result of the measured percentage of the labels inside the three best implant threads. After an 8-week healing period the average

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Bone remodeling analysis of various dental implant surfaces using polyfluorochrome sequential labeling in rabbit tibias

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Summary. The purpose of this study is to analyze the bone remodeling process after the placement of threaded implants with rough (RBM) and hydroxyapatite coated surfaces (HA) in rabbit tibias using polyfluorochrome sequential labeling. Histomorphometry was performed in order to quantify the amount of each label deposited during the healing period. This work demonstrates the possibility of periodic identification of apatite deposition during bone remodeling around titanium and ceramic implants. It has been concluded that the polyfluorochrome sequential labeling is an important tool for identification of bone remodeling after the insertion of titanium and ceramic implants inside rabbit tibias.

Key words: Osseointegration – Hydroxyapatite – Rough Surface – Histomorphometry – Fluorescent microscopy – Polyfluorochrome Sequential Labeling

Introduction

The research conducted by Brånemark et al. (1969), Schroeder et al. (1976), and clinically demonstrated in 1981 by Adell et al., has allowed for the creation of implants with new designs and different surfaces in the hope of improving clinical results. The development of a dynamic functioning attachment of implants to bone is imperative for the long-term success of implant-supported dental prostheses. The most successful material in long-term clinical studies of osseointegrated oral implants is commercially pure titanium (König et al. 1999; Sul et al. 2002). Special surfaces have been studied in order to be used in more complex surgical situations such as: immediate implant placement, expansion of the residual ridge, or maxillary sinus floor elevation. The HA-coated implants should have the advantage of providing an osteoconductive surface for enhanced bone growth (Kay 1992; Reddy 1995). More recently, novel types of implant systems have been developed with rough surfaces using different methods such as: plasma spraying, blasting, etching, beading or sintering (Schroeder et al. 1981; Deporter et al. 1986; Buser et al. 1991; Dominici et al. 1994; Wennerberg et al. 1995, 1998; Cochran et al. 2001) in order to increase the bone/implant contact surface. The use of cellular bone labels allows for the verification of temporal bone remodeling using a fluorescent microscopic technique demonstrating the reaction with the newly deposited apatite (Rahn 1976; Roberts et al. 1984; Haider et al. 1993; Beck 1997; König and Lopes 2002).

The objective of this study is to analyze the efficiency of bone tissue labeling using polyfluorochrome sequential labeling during bone healing at the interface between titanium and ceramic implants. Histomorphometry was performed in order to quantify the amount of each label after the healing period.

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