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Hydroxyapatite paste Ostim®, without elevation of full-thickness flaps, improves alveolar healing stimulating BMP- and VEGF-mediated signal pathways: an experimental study in humans

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Running title: Ostim® improves alveolar healing without full-thickness flap elevation

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ABSTRACT

Objective: Tooth extraction is considered as the starting point of jaw atrophy, via osteoclast activity stimulation. The maintenance of dental alveolar bone depends on surgery procedure and use of materials to maintain prior space favouring bone regeneration. Among substitutes used in dentistry to fill bone defects, Ostim-Pastes (Ostim®, Osartis, Obernburg, Germany) is a nanocrystalline paste tested for treatment of severe clinical conditions. This research firstly investigated the effect of Ostim on alveolar healing comparing in the same healthy subjects an Ostim®-filled socket with a not-filled one. Moreover, it also proposed a new surgical protocol for the post-extractive socket treatment using the graft materials without elevation of full-thickness flaps.

Material and Methods: Fourteen patients were enrolled to bilateral maxillary or mandibular extraction, that was performed without elevation of full-thickness flaps. In each patient one sockets was filled using Ostim®, the other one was allowed to undergo natural healing. No suture was carried out. Clinical and biological parameters were screened at 1, 7, 14 and 21 days.

Results: Obtained results evidenced that nanocrystalline hydroxyapatite supports bone regeneration increasing the synthesis of the synthesis of pro-osteogenic factors, as BMP-4, BMP-7, alkaline phosphatase, osteocalcin. Moreover, filling post-extractive socket with nanocrystalline hydroxyapatite paste leads to a complete epithelialization already at 7 days after extraction, despite the teeth were extracted without elevation of full-thickness flaps. The improved epithelialization is mediated by increased VEGF expression. No significant change was observed in inflammatory parameters, with exception of a early and transient IL-1 β induction, that could trigger and improve alveolar healing.

Conclusions: Clinical and biomolecular observations evidenced that nanocrystalline hydroxyapatite improves alveolar socket healing increasing angiogenesis, epithelialization, and osteogenesis, also in absence of elevation of full-thickness flaps.

INTRODUCTION

Alveolar healing following tooth extraction is a complex repair process involving different types of tissues, including bone. Among tissue regeneration, osteogenesis occurs later than those of other tissues. In healthy subjects, epithelial cells start to migrate early during the first day of post-extraction period and their proliferation is already marked at fourth day; bone production begins at 10 days after extraction (Boyne 1966) and is not more evident after 20 weeks (Ahn & Shin 2008).

Post-extractive osteogenesis is due to the osteoprogenitor cells present in the socket, passing through various maturational stages. Runx2 is a specific marker of osteogenic differentiation and is strongly expressed by osteoprogenitors and by a subpopulation of mature endosteal osteoblasts in the extraction socket (Devlin & Sloan 2002). This recently discovered protein regulates osteoblast function and differentiation because it induces the expression of osteoblast-specific genes (Karsenty 2000).

The maintenance of the dental alveolar bone after extraction depends on the attentive surgery procedure and the use of materials capable to maintain the prior space and be helpful in bone tissue healing.

Natural or synthetic bone fillers are frequently used in dentistry to augment bone tissue or to fill bone defects, but they could also be helpful in accelerating bone repair in extraction socket. Among synthetic substances, various types of hydroxylapatite and synthetic glasses have been tested also in dentistry. Ostim-Pastes (Ostim®, Osartis, Obernburg, Germany), a rather new material, is an injectable nanocrystalline paste [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$], and consists of a suspension of pure HA in water (Tadic & Epple 2004). It is characterized by a large bioactive specific surface area, resembles human bone, is fully reabsorbed and quickly replaced with bone tissue (Chiapasco et al. 2009; Huber et al. 2006). It has been tested for treatment of severe clinical conditions, such as tooth perforations and jaw cysts

(Bezrukov et al. 1998; Grigor'ian et al. 2000), and in addition to tricalcium phosphate granules for acetabular bone impaction grafting procedures (Arts 2006).

At the present little is known about the molecular mechanisms underlying osteogenic properties of hydroxyapatite nanocrystalline paste. Using the technique of intravital fluorescence microscopy, Laschke and coll. (2007) demonstrated that the implantation of Ostim® into the dorsal skinfold chamber of Syrian golden did not induce inflammation process, and stimulated angiogenesis. It has been suggested that this guided vascularization may accelerate the formation of new bone, because osteoblasts are facilitated to migrate into these vascularized areas; moreover, endothelial cells from new formed vessels could stimulate the differentiation of preosteoblasts to osteoblasts by the expression of osteotropic growth factors, such as endothelin-1 and IGF-1 (Rubanyi & Polokoff 2007).

This research firstly investigated the effect of hydroxyapatite nanocrystalline paste on healing process after tooth extraction in healthy subjects, comparing in the same patient an Ostim®-filled socket with a not-filled one. Both clinical and biological parameters have been evaluated. In particular, in each socket biological parameters were determined in both mucosa and filling material; moreover, attention has been paid to the signalling transduction pathways that could be modulated by hydroxyapatite nanocrystalline paste leading to bone production.

The study also proposed a new surgical protocol for the postextractive socket treatment using the graft materials without elevation of full-thickness flaps to protect the postextractive socket.

MATERIALS AND METHODS

Fourteen patients (3 females and 11 males), ranging in age from 45 to 60 years have been enrolled to bilateral maxillary or bilateral mandibular extraction.

Each patient had 2 monoradicular teeth, both maxillary or mandibular, to be extracted.

Indications for tooth extraction included root or crown fractures, nonrestorable caries and residual roots. The exclusion criteria were the following: teeth with acute infection, smokers with >10 cigarettes/day, patients medical illness, irradiation to the head or neck region within 12 months before surgery, pregnancy or breastfeeding, poor oral hygiene.

Before entering the study, all patients were informed about the nature of this clinical trial and they signed an informed consent form. The study was approved by the Ethics Committee of the University of Turin, Italy.

Patients received initial periodontal preparation 1 week before extractions and exhibited good oral hygiene at the time of extractions.

Extractions of the 2 monoradicular teeth were performed in the same surgical time (Figure 1A). Following local anesthesia (mepivacaine 2% with adrenaline 1:100000) the teeth were extracted in an atraumatic way without elevation of full-thickness flaps. The sockets were thoroughly debrided (Figure 1B). In each patient one of the sockets was filled using an injectable nanocrystalline paste (Ostim®, Osartis, Obernburg, Germany) (T sites)(Figure 1C). Natural healing by clot formation was allowed at the other socket (C sites) (Figure 1D). The areas were assigned randomly. The sockets, both T sites and C sites, were not sutured. Instead both Ostim® and clot remains *in situ* with no attempt to achieve primary closure of the surgical wound. No antibiotic and analgesic therapy was given.

Patients were screened clinically at 1, 7, 14 and 21 days. Samples of soft tissues around the tooth were removed at the following times: before extraction, after 1 day and 7 days or before extraction, after 1 day and 14 days.

Clinical parameters

For the clinical examination, the patients were asked to score his/her feeling of pain, for both postextraction sites, on a 10 cm visual analog scale (VAS), with 0 cm reflecting no pain and 10 cm

reflecting worst possible pain. The pain was evaluated each day at the same time starting at 2 hours after extraction (T1) until day 7 (T7) in the postoperative period.

In addition to any clinical control the degree of epithelialization of the postextractive sockets was assessed clinically (both in T and C sites).

Biological analyses

Molecular parameters were evaluated immediately before the extraction, after 1 and 7, or immediately after the extraction, after 1 and 14 day.

All specimens were placed in RNA Later solution (Qiagen, Milan, Italy) to prevent RNA degradation, and maintained at -80°C until use. Mucosae and material present in the corresponding alveolar socket filled or not with Ostim (about 30 mg) were mechanically homogenized using a rotor-stator homogenizer (Ultra Turrax T25, IKA®-Werke, Staufen, Germany); obtained homogenates were used for RNA extraction using NucleoSpin® RNA II kit (MACHEREY-NAGEL, Düren, Germany).

The expression of factors involved in osteogenesis was evaluated by using Real-time polymerase chain reaction (PCR). Interleukin (IL)-1 β , IL-6, transforming growth factor (TGF)- β 2, bone morphogenetics protein (BMP)-4 and BMP-7, alkaline phosphatase, (ALP), osteocalcin (OCN), peroxisome proliferators activating receptor (PPAR)- β , collagene type I and type III were examined.

Total RNA was extracted using the NucleoSpin RNA II Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Real-time PCR was performed with single-stranded cDNA prepared from total RNA (1 μg) using a High-Capacity cDNA Archive kit (Applied Bio Systems, Foster City, CA).

Forward (FW) and reverse (RV) primers were designed using the Beacon Designer® program (Bio-Rad, Hercules, CA) as reported in Table 1.

Twenty-five microliters of a PCR mixture containing cDNA template equivalent 40 ng of total RNA, 5 pmoles each of forward and reverse primers, and 2X IQ SYBR Green SuperMix (Bio-Rad,

Hercules, CA) were amplified using an iCycler PCR instrument (Bio-Rad, Hercules, CA) with an initial melt at 95°C for 10 min, followed by 35-40 cycles at 95°C for 40 s, annealing temperature for each primer set for 40 s, and 72°C for 40 s.

Each sample was tested in duplicate, and threshold cycle (Ct) values were averaged from each reaction. The fold change was defined as the relative expression in all samples compared with that in non-filled alveolar socket at 1 day, calculated as $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct_{\text{sample}} - Ct_{\text{GAPDH}}$ and $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{non-filled alveolar socket}}$.

Ostim® samples at day 1 and both control and Ostim® samples at 7 or 14 days were compared with the values of controls taken as 1.

Statistical analysis

Statistical analyses were performed using InStat3 software. All data were expressed as means \pm SD. The significance of differences between control and hydroxyapatite nanocrystalline means was assessed by Wilcoxon Matched Pair Test ($p < 0.05$).

RESULTS

Clinical parameters

28 extractions, 16 in the mandible and 12 in maxilla of teeth irreparably compromised, were carried out on fourteen patients (3 females and 11 males) enrolled in this study. The reasons of extraction are reported in Table 1.

No patients dropped out of the study and no postsurgical complications were observed in all extraction sites; in particular, nanocrystalline hydroxyapatite paste did not result in adverse or infective reactions, although it was not covered from full-thickness flaps and was exposed to oral bacteria. Although not

covered with a flap, nanocrystalline hydroxyapatite paste was well stabilized in alveolar sockets and at 7 days a complete epithelialization. The fast timing of epithelialization allowed to have no case of dry sockets in T sites. In Figure 1, a case report: protocol regarding the extraction of 1.3 and 2.3 teeth is showed. Both extractions were performed at the same surgical time (panel A) and were performed in an atraumatic way without elevation of full-thickness flaps (panel B). One of the sockets was filled using an injectable nanocrystalline paste (Ostim®, Osartis, Obernburg, Germany) (panel C, T site); natural healing by clot formation was allowed at the other socket (panel D, C site). Both T and C sites were not sutured (panel D). Clinical control at 7 days evidenced in C sites a complete epithelialization in comparison with T site (panel F). Moreover, Ostim did not result in adverse or infective reactions, although it was not covered from full-thickness flaps.

The score of VAS pain was greater for Ostim® site respect to the Control site when measured in the second day, while in the other times it was lower. The difference between the two treatments was statistically significant only at day 2 and 7 ($4,21 \pm 2,27$, P value= 0,004) (Figure 2).

Biological parameters

For each alveolar socket, the analyses were carried out on both filling material and mucosa. Real-time PCR analysis was performed to determine the expression of several genes. With regard to inflammation process, the expression of pro-inflammatory (IL-1 β , IL-6) and anti-inflammatory cytokines (IL-10) were evaluated; with regard to wound healing TGF β 2 and PPAR β were evaluated; with regard to bone synthesis BMP-4, BMP-7, ALP, and OCN were evaluated. At each experimental time, the values of non-filled alveolar sockets were taken as 1, and values of Ostim®-filled ones expressed as variation with respect to corresponding non-filled socket.

With regard to inflammation process, in Ostim®-filled alveolar sockets no significant increase in IL-1 β mRNA was present at 1 and 7 days in comparison with corresponding non-filled alveolar sockets; on

the contrary, mRNA level was decreased at 14 days (Fig. 3, panel A). In mucosa no significant increase of IL-1 β was present at 7 days in Ostim $^{\circledR}$ -filled alveolar sockets; whereas a significant decreased content was observed at 14 days (Fig. 3, panel B).

IL-6 mRNA was unchanged in Ostim $^{\circledR}$ -filled alveolar sockets in comparison with corresponding non-filled ones at all experimental times (Fig. 3, panel C); on the contrary, in the mucosae a significant increase was evidenced at 1 day. No change was present at 7 and 14 days (Fig. 3, panel D).

The anti-inflammatory cytokine IL-10 was increased in Ostim $^{\circledR}$ -filled alveolar sockets at 1 and 14 days, being the increase significant at 1 day (Fig. 3, panel E); differently, no significant change was observed in mucosae at all experimental times (Fig. 3, panel F).

With regard to parameters involved in wound healing, a significant increase was observed at 1 day in VEGF in Ostim $^{\circledR}$ -filled sockets and a not significant trend to increase was also evidenced in corresponding mucosae at the same experimental time (Fig. 4, panel A). On the contrary, TGF β 2 and PPAR β , did not change in both tooth socket and mucosa filled or not with Ostim $^{\circledR}$ at all times examined (Fig. 4, panel B, C, D, E).

With regard to the analysis of parameters involved in osteogenesis, the mRNA content of BMP-4 and BMP-7 is reported in Figure 7. No significant change in BMP-4 was observed in Ostim $^{\circledR}$ -filled alveolar sockets at all times (Fig. 5, panel A) and in mucosae at 7 and 14 days (Fig. 5, panel B); on the contrary, a significant increase was present in mucosae at 1 day.

A significant increase in BMP-7 mRNA was present in Ostim $^{\circledR}$ -filled alveolar sockets at 1 and 7 day (Fig. 5, panel C), and in mucosae at 1 day ((Fig. 5, panel D). No significant change was observed in socket and mucosae at the other experimental times.

Figure 6 reports the mRNA content of ALP (panels A and B) and OCN (panels C and D). ALP was significant increased in both Ostim $^{\circledR}$ -filled alveolar sockets and in mucosae at 1 day, but was

unchanged at the other experimental times; OCN was increased at 1 and 7 days in socket, and at 1 day in mucosae.

DISCUSSION

The literature is unanimous in identifying tooth extraction as the starting point of atrophy of the jaw. In fact, it seems to stimulate osteoclast activity during the first 8 weeks with vertical and horizontal bone resorption. Every tooth extraction determines an average volume loss in thickness of about 50% of the alveolar bone in 12 months (Schropp et al. 2003).

As we need to preserve the alveolar process we must perform bone graft within the first week to address the issue of reconstitute the normal healing process. Indeed the physiological process includes: bone deposition between 3 and 6 months and, new bone remodeling with further resorption after 6 months. Accordingly during this last stage the graft should be reworked and replaced by newly formed bone to induce the transformation of the grafted site into bone tissue.

Since the time period from 0 to 3 months is experiencing a loss of bone volume increased, immediately after extraction could be the ideal time to implement the methods of preservation of the alveolar process (Trombelli et al. 2008).

Darby and coll. (Darby et al. 2009) reported that to correct the atrophy, the graft is ineffective if it is not aimed at implant-prosthetic rehabilitation and becomes essential whether dental implant is delayed (after 6-8 weeks). Quinn and Kent argued that the use of a graft material gives the best results when placed immediately after tooth avulsion, thus guaranteeing as a bone regeneration than that obtained with regenerative techniques performed later to correct the atrophy. The immediate placement exploits the spontaneously occurring healing process, improving the postextractive regenerative potential of the socket and reducing the postoperative resorption (Quinn & Kent 1984).

Using different materials, such as biomaterials xenogenic origin (Norton et al. 2003; Carmagnola et al. 2003), ceramics based on CaP (Weiss et al. 2007) and polyglycolic or polylactic acid (Serino et al. 2008)), some authors suggested that whether a graft is run after extraction, coverage with elevation of full-thickness flaps allows healing and aesthetic better than the spontaneous recovery. Coverage through full-thickness flap also seems to preserve, stabilize and protect the grafted (Landsberg & Bichacho 1994).

The present study firstly shows that nanocrystalline hydroxyapatite is able to function as a support for bone regeneration, probably preventing the collapse of the surrounding soft tissue inside socket during the healing process also in absence of protection by full-thickness flap. In fact, biomolecular evaluations carried out in this research evidenced that nanocrystalline hydroxyapatite significantly improves the synthesis of pro-osteogenic factors, as BMP-4, -7, ALP, and OCN. To be noted that the stimulation occurs very early, being mainly evident at day 1 in both OSTIM-filled tooth socket and corresponding mucosa. The market anticipation of osteogenesis process induced by nanocrystalline hydroxyapatite filling could be decisive in preventing the atrophy of the jaw.

Another important observation from this study is that filling postextractive socket with nanocrystalline hydroxyapatite paste leads to a complete epithelialization already at 7 days after the tooth extraction, which has not been reported with other graft material. This improvement of epithelium repair happened despite the teeth were extracted without elevation of full-thickness flaps. This occurs due to stabilization of the graft material in to the alveolar sockets. With the aim of investigating the molecular mechanisms underlying the ability of nanocrystalline hydroxyapatite in favouring epithelium and vessel repair, its effect on VEGF expression was determined in tooth socket and mucosa. Results from biomolecular analysis confirmed the ability of this material in inducing angiogenesis process via increased expression of VEGF in Ostim-filled socket. Certainly, a major blood provision plays a pivotal role in improving and accelerating tissue healing, and in this case epithelialization.

With regard to pain, the higher value of VAS score registered in Ostim® site at day 1 respect to the Control site can be explained by the increased IL-1 β mRNA observed in tooth socket. In fact, it has been observed that during inflammatory process hyperalgesia is associated with an upregulation of IL-1 β and other inflammatory cytokines. Increased IL-1 β production is known to reduce mechanical nociceptive thresholds, acting via a complex signalling cascades leading to the release and/or activation of nociceptive molecules, such as prostaglandin, IL-6, substance-P, and MMP9 (Inoue et al. 1999; Samad et al. 2001; Ren 2009). The reduction of pain referred in the following days by patients in nanocrystalline hydroxyapatite filled socket agrees with the progressive decrease of IL-1 β mRNA evidenced in this site. The decrease of inflammatory process could also be due to the increase of the anti-inflammatory cytokine IL-10 present after 14 days.

The increased pain at day 1 is the only negative side effect observed in nanocrystalline hydroxyapatite-filled sites; anyway, it can be suggested that this very transient induction of a proinflammatory molecule could act as factor able to trigger an early tissue repair regarding bone, vessels, and epithelium.

CONCLUSIONS

The nanocrystalline hydroxyapatite, a graft material of last generation, for the first time was applied in postextractive socket, without elevation of full-thickness flaps. Both clinical observations and biomolecular analyses evidenced that this material improves alveolar socket healing increasing angiogenesis, epithelialization, and osteogenesis, also in absence of elevation of full-thickness flaps.

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FIGURE CAPTIONS

FIGURE 1. Case report: protocol of extraction of 1.3 and 2.3 teeth

A: both extractions were performed at the same surgical time.

B: the extractions were performed in an atraumatic way without elevation of full-thickness flaps.

C: one of the sockets was filled using an injectable nanocrystalline paste (Ostim®, Osartis, Obernburg, Germany) (T site). Natural healing by clot formation was allowed at the other socket (C site)

D: both T and C sites were not sutured. Instead both Ostim and clot remains in situ with no attempt to achieve primary closure of the surgical wound.

E: Clinical control at 7 days: In C sites a complete epithelialization is evident. Ostim did not result in adverse or infective reactions, although it was not covered from full-thickness flaps.

FIGURE 2. Pain measurement using Visual Analog Scale (VAS) in OSTIM® and control sites.

Values are means \pm SD of 14 patients. The significance of differences between control and OSTIM means was assessed by Wilcoxon Matched Pair Test ($p < 0.05$).

FIGURE 3. mRNA content of IL-1 β , IL-6 and IL-10 in tooth socket filled or not with Ostim and in corresponding mucosa.

For day 1 data are means \pm S.D. of 14 patients; for other experimental time data are means \pm S.D. of 7 patients. Means with different letters are significantly different from one another ($p < 0.05$) as determined by Wilcoxon Matched Pair Test ($p < 0.05$).

FIGURE 4. mRNA content of VEGF, TGF- β 2 and PPAR β in tooth socket filled or not with Ostim and in corresponding mucosa.

For day 1 data are means \pm S.D. of 14 patients; for other experimental time data are means \pm S.D. of 7 patients. Means with different letters are significantly different from one another ($p < 0.05$) as determined by Wilcoxon Matched Pair Test ($p < 0.05$).

FIGURE 5. mRNA content of BMP-4 and BMP-7 in tooth socket filled or not with Ostim and in corresponding mucosa.

For day 1 data are means \pm S.D. of 14 patients; for other experimental time data are means \pm S.D. of 7 patients. Means with different letters are significantly different from one another ($p < 0.05$) as determined by Wilcoxon Matched Pair Test ($p < 0.05$).

FIGURE 6. mRNA content of ALP and OCN β in tooth socket filled or not with Ostim and in corresponding mucosa.

For day 1 data are means \pm S.D. of 14 patients; for other experimental time data are means \pm S.D. of 7 patients. Means with different letters are significantly different from one another ($p < 0.05$) as determined by Wilcoxon Matched Pair Test ($p < 0.05$).

FIGURE 1



A



B



C



D



E

FIGURE 2

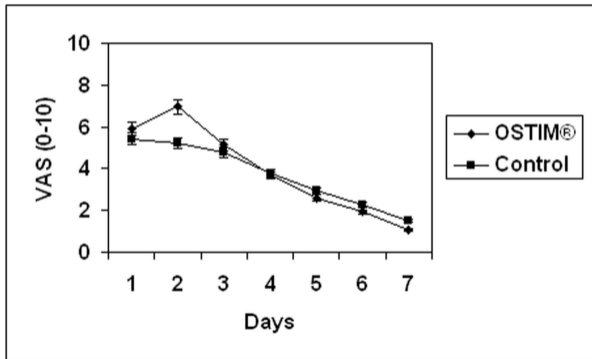


FIGURE 3

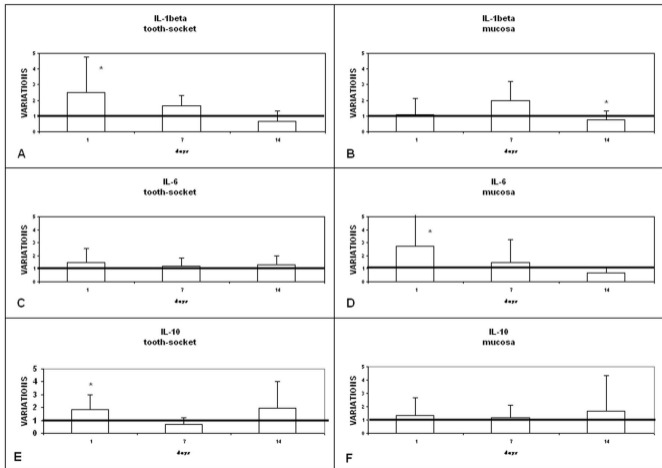


FIGURE 4

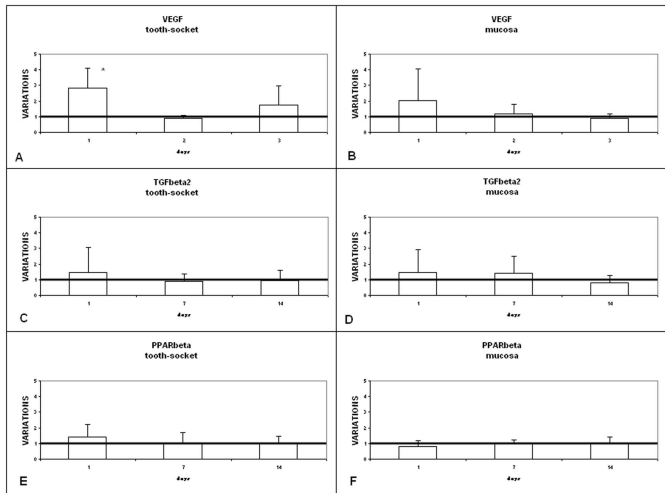


FIGURE 5

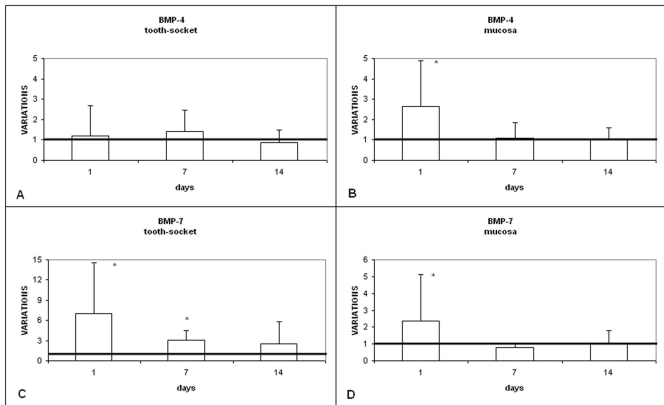


FIGURE 6

