Histol Histopathol (2010) 25: 463-471

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

TGF-ß1 and VEGF after fresh frozen bone allograft insertion in oral-maxillo-facial surgery

L.F. Rodella[1]*, G. Favero[1]*, R. Boninsegna[1,2], A. Borgonovo[2], R. Rezzani[1] and F. Santoro[2]

[1]Department of Biomedical Science Biotechnology Section of Human Anatomy, University of Brescia, Brescia, Italy, [2]Unit of Oral Surgery, Department of Dentistry and Stomatology, ICP Hospital, University of Milan, Milan, Italy

*These authors equally contribuited to this work

Summary. Bone regeneration technique using allografts is widely used in oral surgery to repair alveolar defects and to increase alveolar volume for endosseous implant insertions. Bone allografts promote the reabsorption and neo-synthesis of bone tissue, which are regulated by numerous cytokines, proteins and growth factors. In this study, six patients with insufficient alveolar volume for endosseous implant insertions, were treated with bone regeneration technique using Fresh Frozen Bone (FFB) allografts collected from the femoral head or the hip.

Samples of bone graft collected during graft insertion surgery and biopsies collected six months later during implantology were fixed, decalcified and analyzed histomorphologically and morphometrically by haematoxylin-eosin staining. In addition, TGF-ß1 and VEGF were analyzed by immunohistochemistry.

The histological analysis of FFBs showed wide areas of calcified bone organized in osteons intermingled with areas of non-calcified matrix containing osteoblasts. However, the regenerated alveolar bone, collected six months after the graft insertion surgery, showed wide areas of non-calcified matrix. TGF-ß1 and VEGF were less expressed in FFB than in regenerated alveolar bone.

Key words: Fresh Frozen Bone (FFB), Bone regeneration, TGF-ß1, VEGF

Introduction

Bone regeneration using bone grafts is widely used in oral surgery to repair small and medium size defects and represents an auxiliary medical therapy for endosseous implant insertion (Sanz Casado, 2002). Bone graft substitutes induce or increase bone formation and healing process (Gulaldi et al., 1998; De Biase et al., 2005) promoting both osteogenesis and bone remodeling (Hatano et al., 2004). Different bone graft materials are used to correct alveolar defects: intra- or extra-oral autologous bone, allografts, xenografts and alloplastic grafts (Listgarten and Rosenberg, 1979; Simion and Fontana, 2004; De Biase et al., 2005; Aguirre Zorzano et al., 2007; Kao and Scott, 2007; de la Piedra et al., 2008, Labanca et al., 2008).

Bone graft materials can also be classified according to their biological properties into: osteogenetic (Kao and Scott, 2007; de la Piedra et al., 2008), osteoinductive (Burchardt, 1983; Ripamonti and Duneas, 1998; Simion and Fontana, 2004; Kao and Scott, 2007) and osteoconductive biomaterials (De Biase et al., 2005;). Only the autografts have these three properties, in fact they contain both osteogenic cells and osteoinductive growth factors; in addition they are an osteoconductive scaffold (Adell et al., 1981; Hardesty and Marsh, 1990; Giannoudis et al., 2005). However, the use of autografts for bone regeneration therapy is limited by insufficient sources and by the increase in patient morbidity (Aguirre Zorzano et al., 2007).

Many clinical studies support allogenic bone use and its potential to induce correct bone repair and remodeling (Weyts et al., 2003; Faiella, 2007). Allogenic bone is available in different forms, e.g., Freeze-Dried Bone Allograft (FDBA), Decalcified Freeze-Dried Bone Allograft (DFDBA), Fresh Bone (FB) and Fresh-Frozen Bone (FFB) (Burchardt, 1983; Weyts et al., 2003; Kao and Scott, 2007).

FFB is collected from cadaver or live donors from different anatomical areas in the first 12 hours after donor death, or more frequently during total hip replacement in patients, according to the standards of the Musculoskeletal Council of the American Association of Tissue Banks (AATB) and the European Association of

Offprint requests to: Luigi F. Rodella, Department of Biomedical Sciences and Biotechnology, Viale Europa 11, 25124 Brescia, Italy. e-mail: rodella@med.unibs.it

Musculo Skeletal Transplantation (EAMST) (American Association of Tissue Banks 2008; European Association of Musculo Skeletal Transplantation 2008).

FFB is immediately frozen at -80°C and stored for a minimum of six months (Perrott et al., 1992; D'Aloja et al., 2008; Stacchi et al., 2008).

FFB is frequently used by orthopaedic surgeons to treat bone loss (Perrott et al., 1992; van Biezen et al., 2000; Buma et al., 2001). This clinical succes has led to FFB use in oral surgery and in dental-regenerative techniques to increase maxillary volume and so support loaded endosseous implants (Heyligers and Klein-Nulend, 2005; Stacchi et al., 2008).

The FFB obtained from the Lombardia Tissue Bank is a mineralized, non-irradiated, disinfected and frozen allograft, but in many papers the allografts were treated with 25 KGY of X-ray as reported (Rawashdeh et al., 2008).

The main advantages of the use of this material are low morbidity, shorter surgical times, greater availability and good osteoconductive properties (D'Aloja et al., 2008; Stacchi et al., 2008). Even if there are not many works regarding FFB, this allograft seem to have osteoinductive properties, due to the presence of Bone Morphogenetic Proteins (BMPs) in non-calcified matrix (Lalani et al., 2003; Board et al., 2008).

The disadvantages of FFB are the potential risk of disease transmission (Perrott et al., 1992), due to viral, bacterial or oncogenetic contaminants (Stacchi et al., 2008) and of immunological reaction (Köndell et al., 1996). These risks are minimized using the freezing technique, inducing cell death through the disruption of the cell membranes as a result of ice crystal formation (Stacchi et al., 2008). In this way, the graft becomes inert and non viable, and well accepted by the recipient host without immunological reaction (Köndell et al., 1996). Nevertheless, some authors showed viable cells in the graft after freezing treatment (Simpson et al., 2007). In addition, some authors suggest that fast freezing using the cryoprotective substance dimethyl sulfoxide (DMSO) should be a promising mean of improving immune tolerance to allograft bone and enhance biological function by maintaining viable cells and promoting cell growth (Heyligers and Klein-Nulend, 2005; Egli et al., 2006; de la Piedra et al., 2008). There are few studies reporting the clinical effect of FFB grafts in oral maxillo-facial surgery (D'Aloja et al., 2008).

Several cytokines, proteins, proteases, growth factors and angiogenetic factors play a role in bone regeneration after allograft insertion. However, their exact activity, signaling pathways and regulating mechanisms are not completely understood.

Transforming Growth Factor-beta 1 (TGF-ß1) and Vascular Endothelial Growth Factor (VEGF) appear to play a pivotal role in new bone formation.

Mammalian bone cells synthesize TGF-ß and store it in an inactive form into non-calcified matrix (Hauschka et al., 1988; Centrella et al., 1991; Zimmermann et al., 2005). As a consequence of bone injury, the inactive form is released and converted to an active form (Centrella et al., 1991) that induces the expression of BMPs 1-8, Growth Differentiation Factor -1, -5, -8, -10 and TGF-ß1, -ß2 and -ß3, by binding its own receptors. In the end the effects influence cell growth and differentiation.

TGF-ß1 is a multi-functional growth factor member of the TGF-ß superfamily (Joyce et al., 1990a,b; Bostrom, 1998; Ozkan et al., 2007); it is involved in the proliferation of osteoblasts, osteoclasts, chondrocytes and mesenchymal bone-precursor cells (Joyce et al., 1990b; Zimmermann et al., 2005). It stimulates the synthesis of bone matrix proteins, such as collagen I, II, III and IV (Campbell and Katawa, 1997; Dubois et al., 2001), fibronectin, matrix-metalloproteinases, angiogenetic factors (VEGF and Fibroblasts Growth Factor) (Takeuchi and Shidou, 1993; Chua et al., 2000; Lalani et al., 2003; Chen et al., 2004) and BMPs (Sun et al., 1997; Ozkan et al., 2007; Board et al., 2008) and accelerates fracture healing by chemotaxis and osteoblast stimulation (Lind, 1998). All these potential effects have an important role in the early phases of bone regeneration.

VEGF is a polypeptide that stimulates new blood vessel formation (Tanaka et al., 2007) through the induction of endothelial cell proliferation (Kleinheinz et al., 2005), differentiation and migration (Dagtekin et al., 2003), playing a role in angiogenesis (Lalani et al., 2005).

During osteogenesis, VEGF and its receptors have been found in endothelial cells, osteoblasts, osteoclasts and chondrocytes (Huebsch and Hansen, 1969; Orgill and Demling, 1988; Hollinger et al., 1996; Wong et al., 1996). The direct roles of VEGF during bone formation have been demostrated by several *in vitro* and *in vivo* studies (Chen et al., 2004; Lalani et al., 2005), suggesting that VEGF enhances osteoblast differentiation, migration and activity (Wong et al., 1996) and osteoclast recruitment (Lalani et al., 2005). So it is able to enhance capillary density (Geiger et al., 2005) playing an important role in angiogenesis associated with osteogenesis (Dagtekin et al., 2003; Chen et al., 2004; Mori et al., 2006). In addition, osteoblasts produce VEGF that enhances differentiation of the osteoblasts themselves (Deckers et al., 2000). TGF-ß1, TGF-α and Endothelial Growth Factor are also involved in regulating osteoblast VEGF expression (Chua et al., 2000) through the up-regulation of BMPs.

On the basis of these considerations, TGF-ß1 and VEGF are fundamental in bone regeneration following graft insertion surgery.

There are few histological studies about FFB applications in oral-maxillo-facial surgery and no data are available about TGF-ß1 and VEGF immunohisto-chemical analyses, so the aims of this study were to evaluate: 1) bone regeneration after FFB insertion and 2) spatial localization and histomorphometric levels of TGF-ß1 and VEGF both in the grafts before the insertion and in alveolar bone six months after graft insertion.

Materials and methods

Six patients (3 females and 3 males, aged between 35-60 years) were studied. The patients were treated over a 3-year period (2005-2008) in the Unit of Oral Surgery, Department of Dentistry and Stomatology, ICP Hospital, University of Milan, Italy. All patients needed endosseous implant insertions and had insufficient alveolar bone volume. All patients were systemically healthy at the time of surgery.

Surgical protocol

All patients were operated on graft and implant insertions by the same surgical team (three surgeons). Five patients were treated with maxillary sinus augmentation procedures and with onlay-grafts to increase alveolar volume using FFB, collected from the cancellous block of femoral heads (2 patients) or cortical-cancellous hip bone (3 patients); one patient was treated to repair bone defect after cistectomy with FFB collected from femoral head. All FFB grafts were obtained from the muscular-skeletal tissue bank: Orthopaedical Insitute "Gaetano Pini", Milan, Italy.

After the elevation of a mucoperiosteal flap, FFBs were inserted and fixed using a rigid fixation method (Fig. 1A,B). The grafts were collected from a single donor and were then assigned randomly to the patients.

In all cases non-reasorbable sutures were used and removed after 7-14 days. All patients received

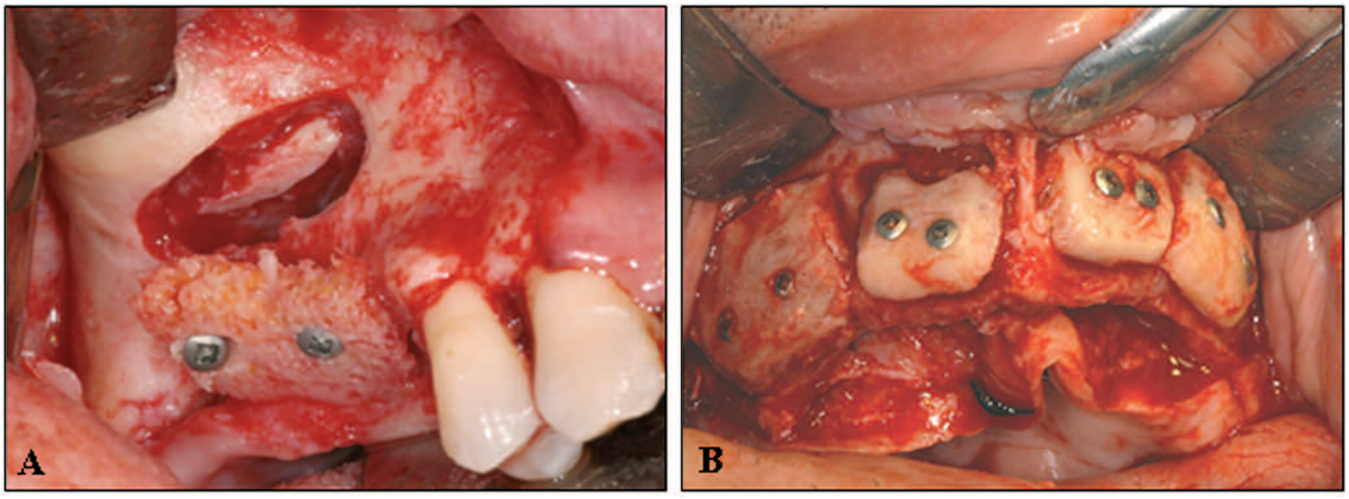

Fig. 1. FFB grafts as on-lay augumentation fixed to the maxilla during regenerative surgery. **A.** Femoral head graft; **B.** Hip bone graft (iliac crest).

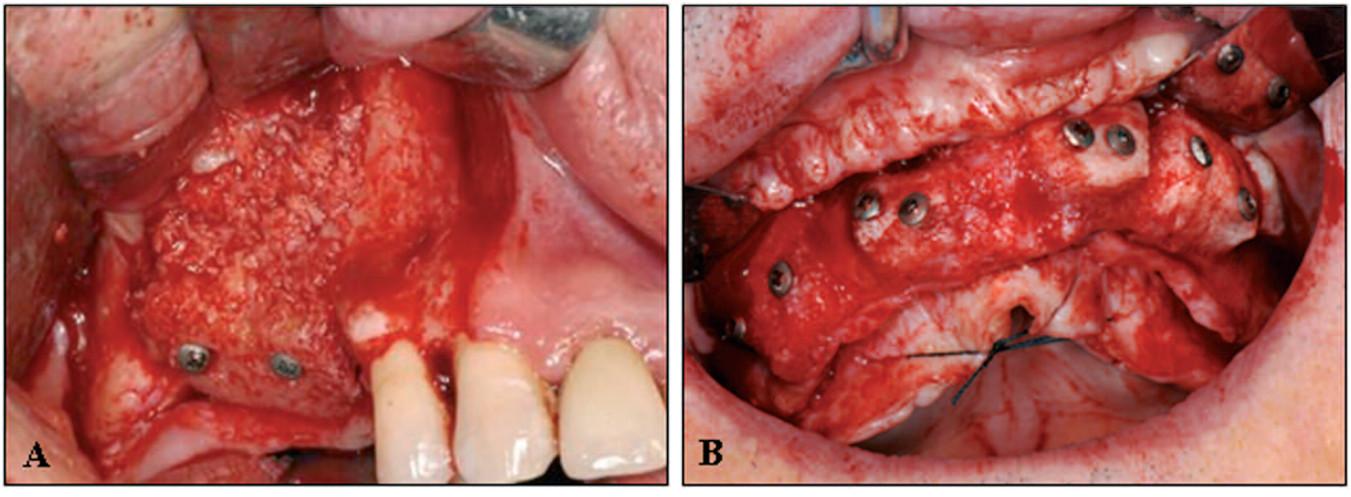

Fig. 2. Surgical reopening six months after implant placement. **A.** Femoral head graft; **B.** Hip bone graft (iliac crest).

Ampicillin (2 g) intravenously at the time of induction and Amoxicillin with Clavulanic acid (1 g) every 8 hours for 7 days.

After six months, the rigid fixations were removed and the patients were submitted to implant surgical therapy using a trephine device (Komet, Milan, Italy) to prepare the implant site, so were collected the alveolar regenerated bone biopsies.

During this surgery, a clinical evaluation of regenerating bone was made by estimating the bone resorption level with respect to the head of the screw, used for fixing grafts (Fig. 2A,B). A value of 1 mm or less indicates that the grafted alveolar bone covers the screw apex, while a value higher than 1 mm indicates that the screw apex is completely covered by the grafted alveolar bone.

In addition, the reabsorption of elevated sinus floor six months after implant insertion (1 year after graft insertion surgery) was evaluated by analyzing radiographically the level of elevated sinus floor with respect to the apex of the implant according to Hatano et al. (2004).

All observations were made independently by the three surgeons. We assumed that the observations were correct if there was complete concordance among all surgeons.

Biopsy

In this study we analyzed the FFB grafts (G), collected during graft insertion surgery, and alveolar biopsies (A), collected six months after the FFB insertion. FFBs were taken from femoral heads (GF) or from hip bone (iliac crest) (GH). The grafts were then cut into a cube of 10 mm^3 using a low-speed handsaw. The cubes were then cut in line with the axis of the femoral neck or in the lateral side of the iliac bone. Six months after graft insertions, the alveolar regenerated bone samples were collected near the midline on the top of the bone graft, during implantology. The biopsies of 2 mm diameter and 5 mm length was made from the external surface of the graft to native bone. All biopsies were fixed in 10% neutral formalin for 48 h and then decalcified in Osteosoft (Merk, Darmstadt, Germany).

The decalcified specimens were dehydrated by multiple graded alcohol solutions followed by xylene and then embedded in paraffin wax using a routine protocol. Twenty serial sections (7 μm thick) of each biopsy were cut using a microtome (Microm HM 325).

Histological analysis

Alternate sections were stained with haematoxylin-eosin (five sections for each biopsy) and with Van Giesson staining (five sections for each biopsy). The heamatoxylin-eosin staining showed bone tissue morphology, while collagen fibres in bone sections were detected using Van Giesson staining. Before both stainings the sections were hydrated in graded alcohol solutions. In particular, they were stained in heamatoxylin for 10 minutes, washed in tap water and then stained in eosin for 1 minute. For Van Giesson staining, the hydrated sections were immersed in iron-heamatoxilyn for 10 minutes, rinsed with tap water and then stained in Van Giesson solution (picric acid and acid fuchsin 1%, 1:10) for 5 minutes.

Immunohistochemical analysis

Alternate sections (five sections for all biopsies) were immersed in 3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity. The sections were then incubated with normal goat serum (Vector anti-rabbit) for 60 minutes and successively with polyclonal anti-TGF-ß1 (TB21: sc-52893, Santa Cruz Biotechnology) and anti-VEGF (A-20: sc-152, Santa Cruz Biotechnology) diluited 1:50 in PBS containing 3% normal goat serum and 0,1% Triton X-100 for 1 hour at room temperature and overnight at 4°C. After incubation in the primary antiserum, the sections were sequentially incubated with biotinylated anti-rabbit immunoglobulins (Vector anti-rabbit) and avidin-biotin peroxidase complex (Vector Labs, Burlingame, CA) and then stained in a solution of 0,05% 3-3-diaminobenzidine tetrahydrochloride (DAB 5 mg/10 ml PBS) (Sigma). Negative control of immunohistochemistry was performed by omitting the primary antibody solution and incubating the sections with non-immune goat serum.

Histomorphometric analyses

Ten fields randomly selected from each section, five in the apical area and five in the crestal area, were analyzed and the percentange of calcified bone and non-calcified matrix were evaluated in haematoxylin-eosin staining; the same method was used to measure TGF-ß1 and VEGF percentage of stained area in immunohistochemical analyses.

Digital pictures were taken using a light microscope (Olympus, Germany) and then the percentage of area was calculated using an image analyzer (Image-Pro Plus 4.5.1, Immagini e Computer, Milan, Italy) that quantizes the levels of positive immuno-label in each field, as previously described (Chen et al., 2004; Lalani et al., 2005). The analyses were made by two blinded investigators. We assumed that the evaluations were correct if there were no statistically different values between the investigators.

Statistical analysis

The results were expressed as mean ± standard deviation (SD). All data were analyzed by ANOVA and Bonferroni's test. A p-value less than 0,05 was considered statistically significant.

Results

Clinical results

The clinical results showed an evident restoration of alveolar bone morphology and volume. There were no signs of complications such as infection, edema, soft tissue laceration or abscess in any patients. We observed only inflammation and swelling processes, mainly due to the surgical procedure.

Bone regeneration was evaluated, during implant placement, by the same surgeons that made graft insertion and it was observed both in patients treated with femoral head and in patients treated with hip bone. The regenerated alveolar bone induced by femoral grafts appeared more vascularized and it showed a well evident bleeding. On the contrary, in the patients receiving hip graft (iliac crest), bleeding was less evident.

Femoral FFB allografts were completely integrated and it was not possible to distinguish the residual FFB from the preexisting bone; on the contrary, in patients with hip allograft were observed residual cortical FFB. Only two patients showed a partial reabsorption of the onlay mandible graft and no significant reabsorption was detected in sinus elevation.

Histological results and histomorphometric results

The analysis of femoral heads and hip bones showed wide areas of calcified bone, containing numerous osteocytes, organized into osteon systems. In addition, we found some areas of non-calcified matrix containing

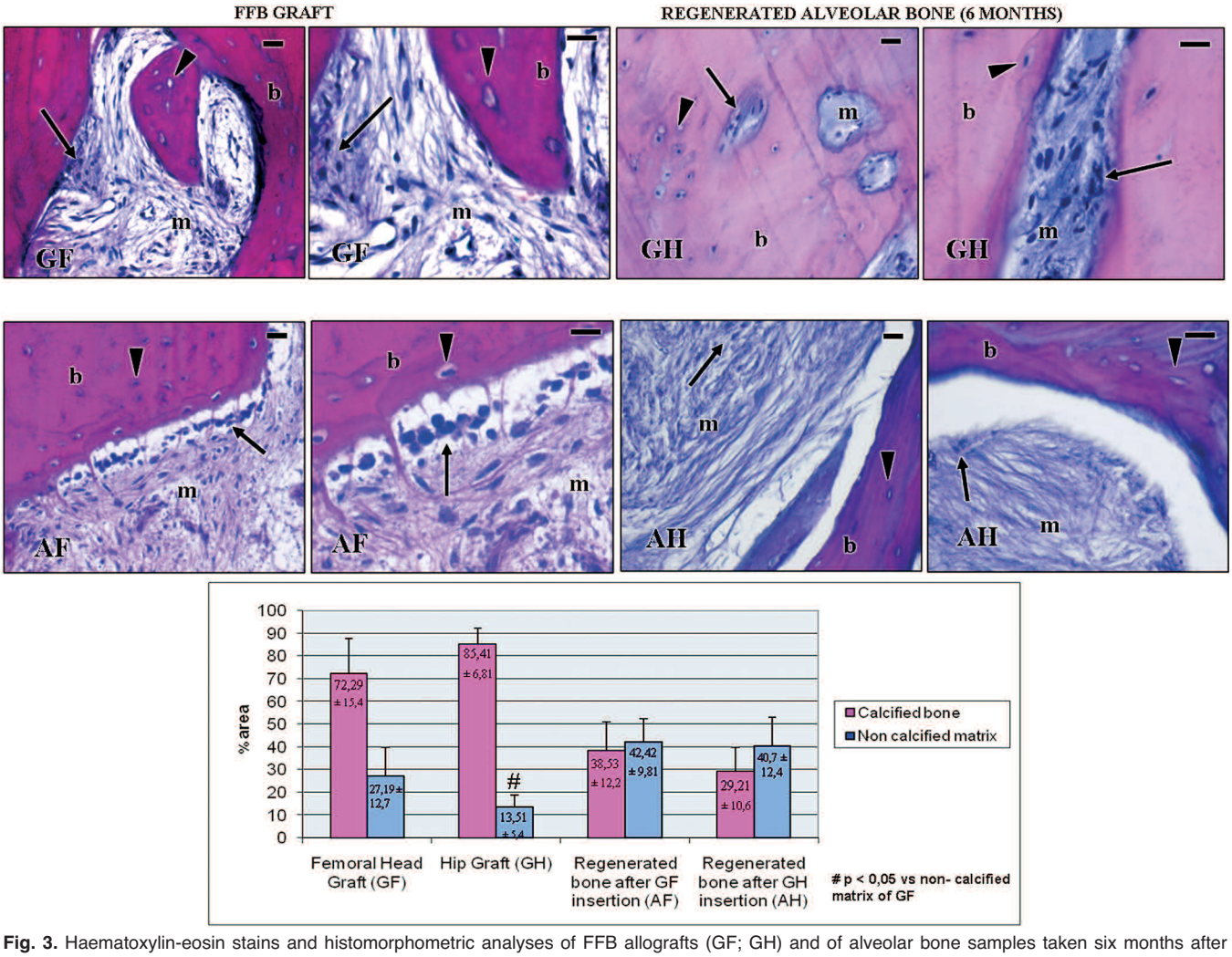

Fig. 3. Haematoxylin-eosin stains and histomorphometric analyses of FFB allografts (GF; GH) and of alveolar bone samples taken six months after graft insertions (AF; AH). The arrows indicate the osteoblast secreted osteoid, organic bone matrix (m); while the arrowheads indicate osteocytes in the regenerated calcified bone (b). The graph shows the comparison between the percentange of area of non-calcified matrix and of calcified bone both in graft and alveolar samples. Bar: 20 μm.

osteoblasts. In particular, hip grafts showed less non-calcified matrix than femoral bones (GF).

Six months after graft insertions, the regenerated alveolar bone showed good bone quality and wide areas of bone regeneration both in patients receiving femoral bone and hip bone. These areas of matrix are more evident in femoral bone (Fig. 3). In particular, haematoxylin-eosin staining showed calcified bone areas stained in pink/fuchsia and non-calcified matrix stained in blue/violet.

We did not find inflammatory cells in any samples. All bone defects were partially healed by bone formation.

Immunohistochemical and histomorphometric results

We did not find any significant differences between apical and crestal area. TGF-ß1 and VEGF were present in all studied bone samples. The immunohistochemical analysis showed that both growth factors are localized only in the bone non-calcified matrix in FFB grafts and regenerated alveolar bone. In particular, TGF-ß1 and VEGF have a high immunoreactivity in the regenerated alveolar bone compared with FFB grafts (Figs. 4, 5) without significant difference between patients treated with femoral bone or hip allograft.

The quantitative data of TGF-ß1 and VEGF stained area are reported in Figs. 4, 5.

Discussion

Bone regeneration using allografts is characterized by a simultaneous process of reabsorption and neo-synthesis of bone tissue. Since there are no data regarding alveolar bone regeneration after FFB insertion in human, in this study we showed that FFB allografts

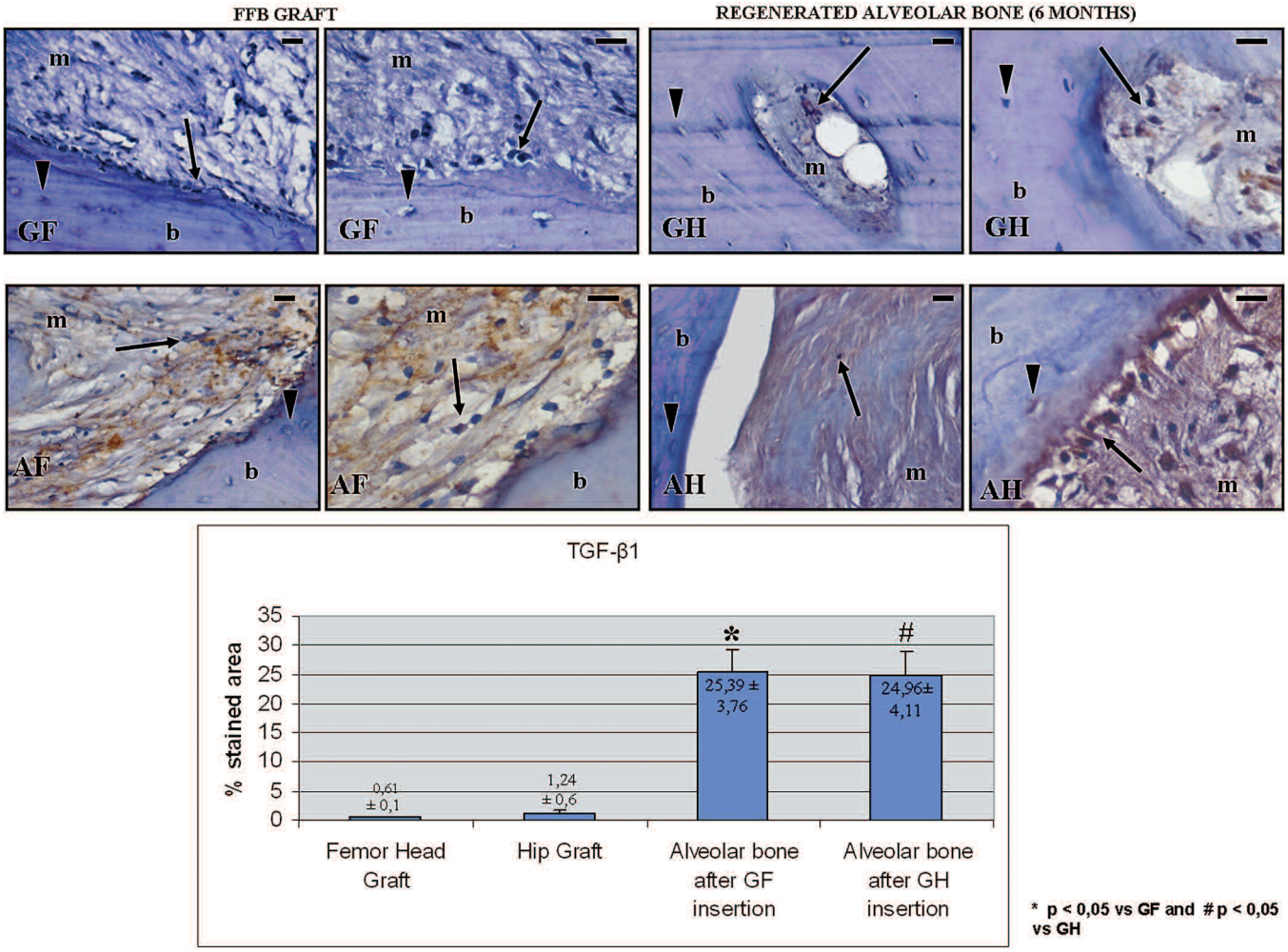

Fig. 4. TGF-ß1 immunohistochemistry and histomorphometric analyses of FFB allografts (GF; GH) and of alveolar bone samples taken 6 months after graft insertions (AF; AH). The arrows indicate the osteoblast secreted osteoid, organic bone matrix (m); while the arrowheads indicate osteocytes in the regenerated calcified bone (b). The graph shows the percentange of area of TGF-ß1 both in graft and alveolar samples. Bar: 20 μm.

are useful to increase alveolar volume for six months after femoral head or hip allograft insertion.

Hip bone grafts do not seem to be completely substituted by neo-synthetized bone at the external cortical bone level, probably because cortical bone of hip graft was more compact than cancellous bone of the femoral head (Dragoo and Sullivan, 1973a,b).

Nevertheless, with both grafts we observed a good bone consistence that permits a correct implant insertion.

Morphometrical analysis showed that FFB grafts contain less non-calcified matrix than alveolar bone; this could relate to the regeneration process, induced from the FFB graft during alveolar bone regeneration.

We also analyzed the localization and the histomorphometric levels of two growth factors, TGF-ß1 and VEGF, which stimulate the neo-synthesis and regeneration of bone tissue, influencing osteoconduction and osteoinduction properties of grafts.

These factors are localized in the non-calcified matrix, which provides a scaffold for the mineralization process and plays a fundamental role in the subsequent incorporation of grafts and in bone regeneration process. This finding is in agreement with that observed in animal studies (Lalani et al., 2003, 2005).

We showed for the first time that both TGF-ß1 and VEGF are present in the non-calcified matrix of both FFB grafts, even if in a small amount. These data are in agreement with the results of Lalani et al. (2003, 2005) and suggest that the grafts possess an intrinsic growh factor pattern that could act on resident osteoblasts to induce new bone formation.

In addition, it is evident that both femoral head and hip grafts induce the synthesis of TGF-1ß and VEGF from osteoblasts of alveolar bone, without significant

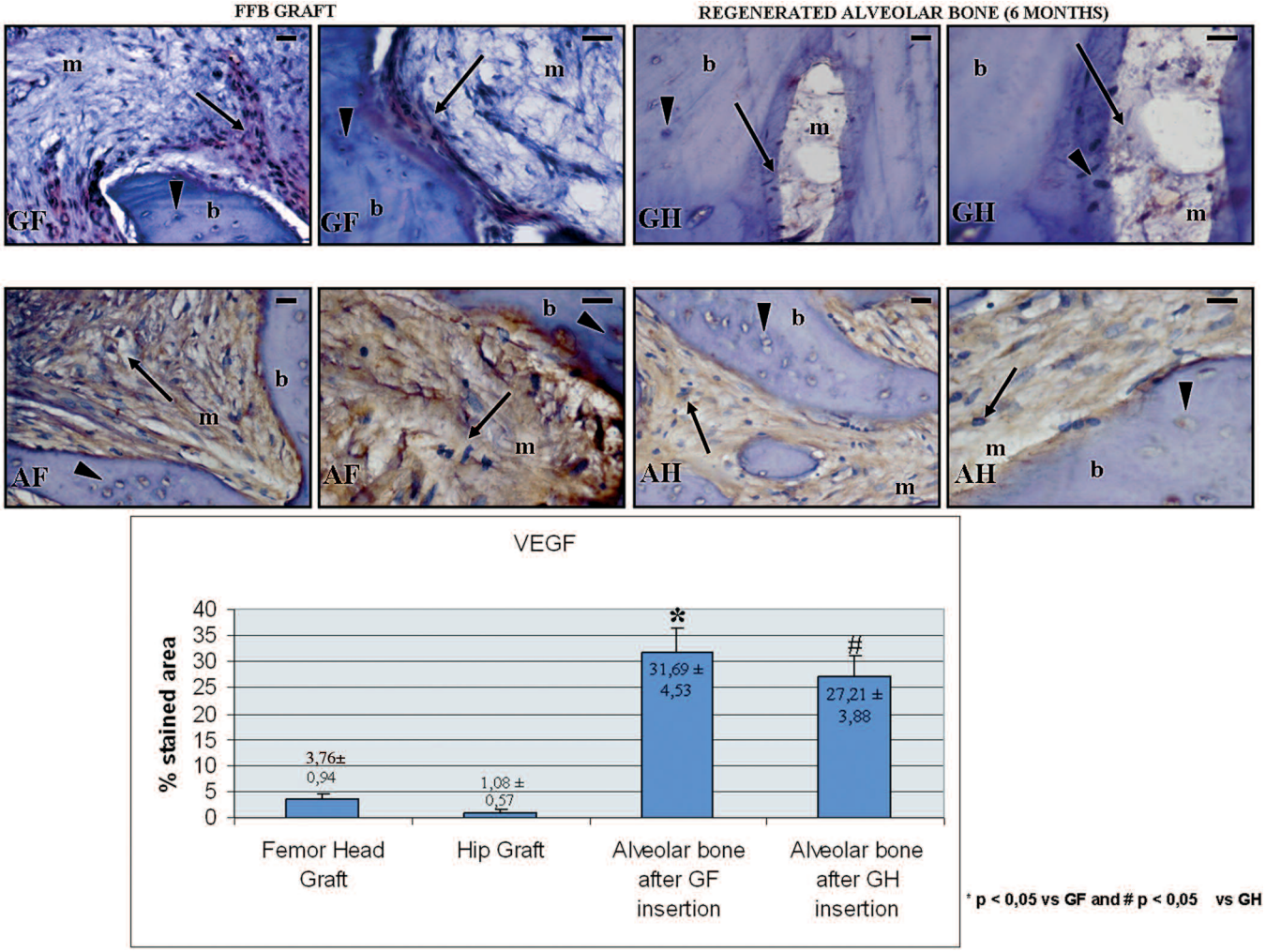

Fig. 5. VEGF immunoistochemistry and histomorphometric analyses of FFB allografts (GF; GH) and of alveolar bone samples taken 6 months after graft insertions (AF; AH). The arrows indicate the osteoblast secreted osteoid, organic bone matrix (m); while the arrowheads indicate osteocytes in the regenerated calcified bone (b). The graph shows the percentange of area of VEGF both in graft and alveolar samples. Bar: 20 μm.

differences between femoral and hip bone.

In conclusion, our study suggests that FFBs, from femoral head and hip, are useful in oral maxillo-facial surgery to increase or reconstruct the athropic jaw and to support loaded endosseous implants, as they are bioactive materials that promote new bone formation involving TGF-ß1 and VEGF.

The main advantages of FFBs are low morbidity, short surgical time and greater availability (D'Aloja et al., 2008). It will therefore partially replace autogenous bone, used as gold standard in dentristry (Faiella, 2007; de la Piedra et al., 2008). The preparation tecnique of FFBs decreases antigenicity and the risk of disease transmission (Perrot et al., 1992), so allogenic bone has a very minimal risk of disease transmission. However, further experimental studies involving many patients are required to confirm our hypothesis and to evaluate long term maintenance of implant success after FFB insertion.

Acknowledgements. The authors thank Dr. R. Coates for English revision of the manuscript and Sig.ra Giugno Lorena for her technical contribution to this work. This study was supported by the grant (ex-60%) of the University of Brescia.

References

Adell R., Lekholm U., Rockler B. and Branemark P.I. (1981). A 15-year study of osseointegrated implants in the treatment of edentulous jaw. Int. J. Oral. Surg. 10, 387-416.

Aguirre Zorzano L.A., Rodríguez Tojo M.J. and Aguirre Urizar J.M. (2007). Maxillary sinus lift with intraoral autologous bone and B--tricalcium phosphate: histological and histomorphometric clinical study. Med.Oral. Patol. Oral. Cir. Bucal. 12, E532-E536.

Board T.N., Rooney P. and Kay P.R. (2008). Strain imparted during impaction grafting may contribute to bony incorporation an in vitro study of the release of bmp-7 from allograft. J. Bone Joint. Surg. Br. 90, 821-824.

Bostrom M.P.(1998). Expression of bone morphogenetic proteins in fracture healing. Clin. Orthop. Relat. Res. 355 Suppl, S116-S123.

Buma P., Donk S., Slooff T.J. and Schreurs W. (2001). Bone graft incorporation after reconstruction of bony defects with impacted morselized bone graft. Histology of animals and patients. Ortop. Traumatol. Rehabil. 3, 41-47.

Burchardt H. (1983). The biology of bone graft repair. Clin. Orthop. Relat. Res. 174, 28-42.

Campbell S.E. and Katawa L. (1997). Angiotensin II stimulated expression of trasforming growth factor-beta1 in cardiac fibroblasts and myofibroblasts. J. Mol. Cell. Cardiol. 7, 1947-1958.

Centrella M., McCarthy T.L. and Canalis E. (1991). Trasforming growth factor-beta and remodeling of bone. J. Bone Joint. Surg. Am. 73, 1418-1428.

Chen Y-J., Wurtz T., Wang C-J., Kuo Y.R., Yang K.D., Huang H-C. and Wang F-S. (2004). Recruitment of mesenchymal stem cells and expression of TGF-,1 and VEGF in the early stage of shock wave-promoted bone regeneration of segmental defect in rats. J. Orthop. Res. 22, 526-534.

Chua C.C., Hamdy R.C. and Chua B.H. (2000). Mechanism of transforming growth factor-beta1-induced expression of vascular endothelial growth factor in murine osteoblastic MC3T3-E1 cells. Biochim. Biophys. Acta 1497, 69-76.

D'Aloja E., Santi E. and Aprili G. (2008). Fresh frozen homologous bone in oral surgery: case reports. Cell. Tissue Bank. 9, 41-46.

Dagtekin G., Schiffer R., Klein B., Jahnen-Dechent W. and Zwadlo-Klarwasser G. (2003). Modulation of angiogenic functions in human macrophages by biomaterials. Biomaterials 24, 3395-3401.

De Biase A., Guerra F., Cipriano L., Lamazza L. and Tucci E. (2005). Subantral filling by deantigenated heterologous bone and immediate fixture placement. Minerva Stomatol. 54, 99-108.

de la Piedra C., Vicario C., de Acuna L.R., Garcia-Moreno C., Traba M.L., Arlandis S., Marco F. and Lopez-Duran L. (2008). Osteoinductive effect of bone bank allografts on human osteoblasts in culture. J. Orthop. Res. Soc. 26, 200-207.

Deckers M.M., Karperien M., van der Bent C., Yamashita T., Papapoulos S.E. and Löwik C.W. (2000). Expression of vascular endothelial growth factors and their receptors during osteoblast differentiation. Endocrinology 141, 1667-1674.

Dragoo M.R. and Sullivan H.C. (1973a). A clinical and histological evaluation of autogenous iliaca bone grafts in humans II. External root resoption. J. Periodontol. 44, 614-625.

Dragoo M.R. and Sullivan H.C. (1973b). A clinical and histological evaluation of autogenous iliaca bone grafts in humans I. Wound healing 2 to 8 months. J. Periodontol. 44, 599-613.

Dubois C.M., Blanchette F., Laprise M.H., Leduc R., Grondin F. and Seidah N.G. (2001). Evidence that furin is an authentic trasforming growth factor-beta1-converting enzyme. Am. J. Pathol. 158, 305-316.

Egli R.J., Wingenfeld C., Hölzle M., Hempfing A., Fraitzl C.R., Ganz R. and Leunig M. (2006). Histopathology of cryopreserved bone allo- and isografts: pretreatment with dimethyl sulfoxide. J. Invest. Surg. 19, 87-96.

Faiella R.A. (2007). Is human bone allograft safe for the management of alveolar defects? A primer for patient consent. J. Mass. Dent. Soc. 56, 18-20.

Geiger F., Bertram H., Berger I., Lorenz H., Wall O., Eckhardt C., Simank H.G. and Richter W. (2005). Vascular endothelial growth factor gene-activated matrix (VEGF165-GAM) enhances osteogenesis and angiogenesis in large segmental bone defects. J. Bone Miner. Res. 20, 2028-2035.

Giannoudis P.V., Dinopoulos H. and Tsiridis E. (2005). Bone substitutes: an update. Injury 36 (Suppl 3), S20-S27.

Gulaldi N.C.M., Shahlafar J., Makhsoosi M., Caner B., Araz K. and Erbengi G. (1998). Scintigraphic evaluation of healing response after heterograft usage for alveolar extraction cavity. Oral. Surg. Oral. Med.Oral. Pathol. Oral. Radiol. Endod. 85, 520-525.

Hardesty R.A. and Marsh J.L. (1990). Craniofacial onlay bone grafting: a prospective evaluation of morphology, orientation and embryonic origin. Plast. Reconstr. Surg. 85, 4-14.

Hatano N., Shimizu Y. and Ooya K. (2004). A clinical long-term radiographic evaluation of graft height changes after maxillary sinus floor augmentation with a 2:1 autogenous bone/xenograft mixture and simultaneous placement of dental implants. Clin. Oral. Implants Res. 15, 339-345.

Hauschka P.V., Chen T.L. and Mavrakos A.E. (1988). Polypeptide growth factors in bone matrix. Ciba Found. Symp. 136, 207-225.

Heyligers I.C. and Klein-Nulend J. (2005). Detection of living cells in non-processed but deep-frozen bone allografts. Cell.Tissue Bank. 6,

25-31.

Hollinger J.O., Brekke J., Gruskin E. and Lee D. (1996). Role of bone substitutes. Clin. Orthop. Relat. Res. 324; 55-65. Erratum in: Clin. Orthop. 324, 55-65.

Huebsch R.F. and Hansen L.S. (1969). A histopathologic study of extraction wounds in dogs. Oral Surg. Oral. Med. Oral. Pathol. 28, 187-196.

Joyce M.E., Jingushi S. and Bolander M.E. (1990). Transforming growth factor-beta in the regulation of fracture repair. Orthop. Clin. North. Am. 21, 199-209.

Joyce M.E., Roberts A.B., Sporn M.B. and Bolander M.E. (1990). Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur. J. Cell Biol. 110, 2195-2207.

Kao S.T. and Scott D.D. (2007). A review of bone substitutes. Oral. Maxillofacial. Surg. Clin. N. Am. 19, 513-521.

Kleinheinz J., Stratmann U., Joos U. and Wiesmann H.P. (2005). VEGF-activated angiogenesis during bone regeneration. J. Oral. Maxillofac. Surg. 63, 1310-1316.

Köndell P.A., Mattsson T. and Astrand P. (1996). Immunological responses to maxillary on-lay allogeneic bone grafts. Clin. Oral. Implants Res. 7, 373-377.

Labanca M., Leonida A. and Rodella L.F. (2008). Biomateriali naturali di sintesi in odontoiatria: scienza ed etica come criteri per la loro scelta. Implantologia-La rivista per il clinico; 1/108 Anno 6, Quintessenza edizioni.

Lalani Z., Wong M., Brey E.M. and Mikos A.G. (2003). Spatial and temporal localization of transforming growth factor-,1, bone morphogenetic protein-2, and platelet-derived groth factor-A in healing tooth extraction sockets in a rabbit model. J. Oral. Maxillofac. Surg. 61, 1061-1072.

Lalani Z., Wong M., Brey E.M., Mikos A.G., Duke P.J., Miller J.M., Johnston C. and Montufar-Solis D. (2005). Spatial and temporal localization of FGF-2 and VEGF in healing tooth extraction sockets in rabbit model. J. Oral. Maxillofac. Surg. 63, 1500-1508.

Listgarten M.A. and Rosenberg M.M. (1979). Histological study of repair following new attachment procedures in human periodontal lesion. J. Periodontol. 50, 333-344.

Mori S., Akagi M., Kikuyama A., Yasuda Y. and Hamanishi C. (2006). Axial shortening during distraction osteogenesis leads to enhanced bone formation in rabbit model through the HIF-1·/vascular endothelial growth factor system. J. Orthop. Res. 24, 653-663.

Orgill D. and Demling R.H. (1988). Current concepts and approaches to wound healing. Crit. Care Med. 16, 899-908.

Ozkan K., Eralp L., Kocaoglu M., Ahishali B., Bilgic B., Mutlu Z., Turker M., Ozkan F.U., Sahin K. and Guven M. (2007). The effect of trasforming growth factor ß1 (TGF-ß1) on the regenerate bone in distraction osteogenesis. Growth Factors 25, 101-107.

Perrott D.H., Smith R.A. and Kaban L.B. (1992). The use of fresh frozen allogeneic bone for maxillary and mandibular reconstruction. Int. J. Oral. Maxillofac. Surg. 21, 260-265.

Rawashdeh M.A. and Telfah H. (2008). Secondary alveolar bone grafting: the dilemma of donor site selection and morbidity. Br. J. Oral. Maxillofac. Surg. 46, 665-670.

Ripamonti U. and Duneas N. (1998). Tissue morphogenesis and regeneration by bone morphogenetic proteins. Plast. Reconstr. Surg. 101, 227-239.

Sanz Casado J.V. (2002). Bone regeneration in oral surgery. An. R. Acad. Nac. Med. 119, 237-254.

Simion M. and Fontana F. (2004). Autogenous and xenogeneic bone grafts for the bone regeneration- A literature review. Minerva Stomatol. 53, 191-206.

Simpson D., Kakarala G., Hampson K., Steele N. and Ashton B. (2007). Viable cells survive in fresh frozen human bone allografts. Acta Orthop. 78, 26-30.

Stacchi C., Orsini G., Di Iorio D., Breschi L. and Di Lenarda R. (2008). Clinical, histologic, and histomorphometric analysis of regenerated bone in maxillary sinus augmentation using fresh frozen human bone allografts. J. Periodontol. 79, 1789-1796.

Sun Y., Zhang W., Ma F., Chen W. and Hou S. (1997). Evaluation of transforming growth factor beta and bone morphogenetic protein composite on healing of bone defects. Chin. Med. J. (Engl) 110, 927-931.

Takeuchi T. and Shidou T. (1993). Impairment of blood supply to the head of the femur after fracture of the neck. Int. Orthop. 17, 325-329.

Tanaka S., Matsuzaka K., Sato D. and Inoue T. (2007). Characteristics of newly formed bone during guided bone regeneration: analysis of cbfa-1, osteocalcin and VEGF expression. J. Oral. Implantol. 33, 321-326.

van Biezen F.C., ten Have B.L. and Verhaar J.A. (2000). Impaction bone-grafting of severely defective femora in revision total hip surgery: 21 hips followed for 41-85 months. Acta Orthop. Scand. 71, 135-142.

Weyts F.A., Bos P.K., Dinjens W.N., van Doorn W.J., van Biezen F.C., Weinans H. and Verhaar J.A. (2003). Living cells in 1 of 2 frozen femoral heads. Acta Orthop. Scand. 74, 661-664.

Wong M.E., Hollinger J.O. and Pinero G.J. (1996). Integrated processes responsible for soft tissue healing. Oral. Surg. Oral. Med. Oral. Pathol. Oral. Radiol. Endod. 82, 475-492.

Zimmermann G., Henle P., Kusswetter M., Moghaddam A., Wetzensen A., Richter W. and Weiss S. (2005). TGF-ß1 as a marker of delayed fracture healing. Bone 36, 779-785.

Accepted November 4, 2009