1. Introduction

Tooth extraction whether due to caries, trauma or advanced periodontal disease causes commonly the alveolar ridge to decrease in volume, including reduced height and width and a change in morphology (Atwood 1971, Lekovic et al. 1997, Lekovic et al. 1998, Camargo et al. 2000, Iasella et al. 2003, Schropp et al. 2003, Botticelli et al. 2004). The restoration of a lost tooth to re-establish its function and esthetics is accomplished by either using conventional prosthetics such as a bridge and partial denture or by placement of an implant supported prosthesis (Taylor et al. 2000, Zitzmann and Marinello 2002). However, changes of the alveolar ridge after tooth extraction are usually clinically significant, with ridge width reduction of up to 50 % in the first year as well as vertical loss of 2-3 mm (Camargo et al. 2000, Iasella et al. 2003, Schropp et al. 2003). The severity of the healing pattern and shrinkage of the hard and soft tissue poses a problem for the clinician. Firstly, it may make the placement of the implant extremely challenging because of lack of bone with the need of bone augmentation (Dies et al. 1996, Bartee 2001, Buch et al. 2005, Irinakis 2007). Secondly, it could create an esthetic problem in the fabrication of an implant-supported restoration or a conventional prosthesis (Sheibert and Salama 1996, Buch et al. 2005, Shi et al. 2007). Bone loss in the vertical and horizontal plane will also affect the implant position (John et al. 2007, Keith and Salama 2007), the peri-implant soft tissues esthetic (pink esthetics) including height of the gingival margin, presence of a dental papilla (soft tissue in the interproximal areas) and concavity of the alveolar crest (Buser et al. 2004, Kois 2004, Keith and Salama 2007) and finally the shape and length of the restoration (white esthetics) (Kois 2004). Preservation of the alveolar ridge bone after tooth extraction would minimize these problems.

An understanding of extraction site wound healing and subsequently the histology of the healing socket with regard to bone formation and remodeling of the healing socket is necessary to determine the ideal concept or treatment protocol to preserve the alveolar ridge and minimize the loss of bone height and width (Bartee 2001, Winkler 2002, Schropp et al. 2003, Buser 2004, Irinakis 2007).

1.1. Anatomical preview

1.1.1. Alveolar bone

The alveolar bone is that part of the maxilla (upper jaw) or mandible (lower jaw) which supports and protects the teeth. This hard, calcified tissue with all the components of bone tissue surrounds the roots of the teeth and is also called alveolar process, or alveolar ridge. Together with the cementum of the tooth's root and the periodontal ligament (PDL), the alveolar bone constitutes the periodontium (attachment apparatus) for the tooth (Figure 1), the main function of which is to distribute and resorb forces generated by, for example, mastication (Lindhe et al. 1998).

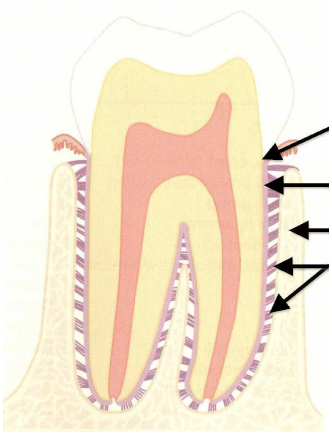

Figure 1: The alveolar bone supports and protects the teeth. It contains the roots of teeth and is attached to the cementum of teeth by the PDL.

The alveolar bone is divided into the alveolar bone proper and the supporting alveolar bone. The alveolar bone proper consists of plates of compact bone lining the tooth socket or alveolus that surrounds the tooth (Figure 2). Alveolar bone proper is also called bundle bone because Sharpey's fibers, a portion of the fibers of the PDL, insert here (Bath-Balogh 2006, Newman et al. 2006).

The alveolar crest is the most cervical rim of the bundle bone. In a healthy situation, the alveolar crest is slightly apical to cementum–enamel junction (CEJ) approximately 1 to 2 mm. The supporting alveolar bone consists of cortical and trabecular bone. The cortical bone, or cortical plates, consists of plates of compact bone on the facial and lingual surfaces of the alveolar bone. The trabecular bone consists of cancellous bone that is located between the bundle bone and the plates of cortical bone. The alveolar bone

between two neighboring teeth is called the interdental septum, or interdental bone. The alveolar bone between the roots of the same tooth is called the interradicular septum, or interradicular bone (Figure 3) (Misch 1999, Bath-Balogh 2006, Newman et al. 2006).

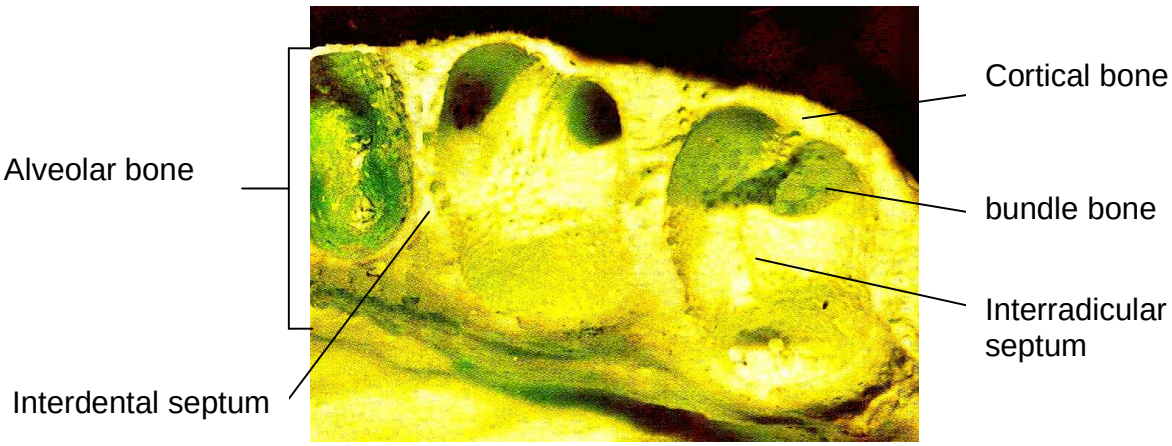

Figure 2: Part of the maxilla with the teeth removed.

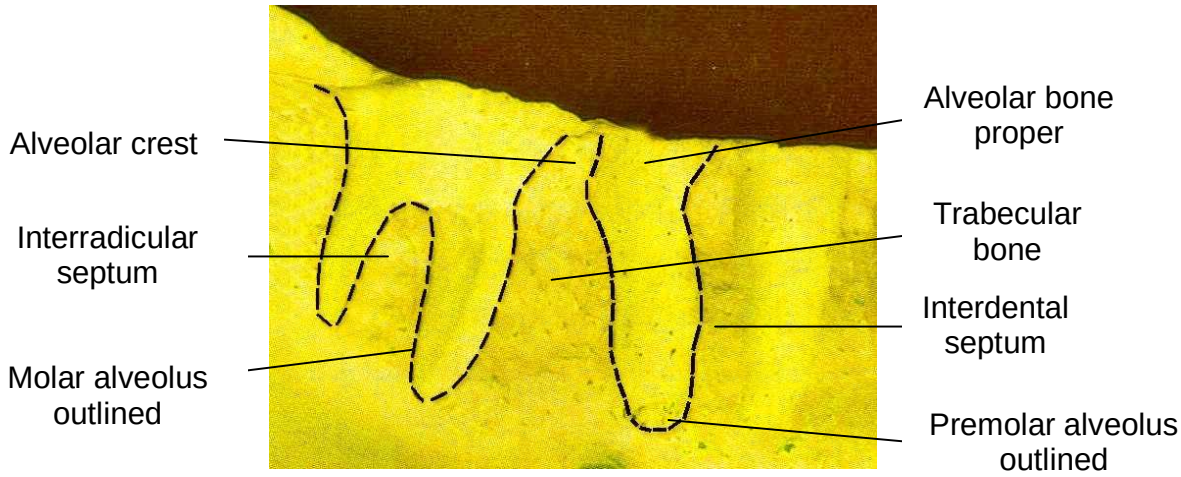

Figure 3: Cross section of alveolar bone of the mandible.

The alveolar process is considerably thicker at the palatal than at the buccal aspect of the maxilla and the margin of facial surface is thin, knife edged and frail. The alveolar process of the mandible varies in thickness from one region to another. In the incisor and premolar regions, the cortical bone plate at the buccal aspects of the teeth is considerably thinner than at the lingual aspects. In the molar region, the bone is thicker at the buccal than at the lingual surfaces (Ash 1984).

1.1.2. Periodontal ligament (PDL)

The periodontal ligament is the part of the periodontium which joins the root cementum with the alveolar bone. The PDL is a richly vascularized cellular fibrous connective tissue. It contains cells such as fibroblasts, cementoblasts, osteoblasts and osteoclasts, collagen fiber bundles and vascular and nerve supply. The presence of PDL makes it possible to transmit occlusal forces from the tooth to the bone. It participates in the formation and resorption of hard tissues of the periodontium and provides nutrition for the surrounding tissues (Lindhe et al. 1998, Rose et al. 2004, Newman et al. 2006).

1.1.3. Extraction sockets (Alveoli)

The tooth socket (alveolus) is the cavity formed by the facial and lingual plate and the interdental septum of the alveolar bone. The form and depth of each alveolus are determined by the form and length of the root it supports. After tooth extraction, one wall or more of the socket can be partially or totally missing or present fissures due to previous tooth trauma, traumatic extraction, deep periodontal pocket or previous apical surgery. Bone loss at the coronal part of facial or lingual wall (dehiscence) or at the apical part (fenestration) can be observed (Lindhe et al. 1998, Newmann et al. 2006). According to number of the intact remaining bone walls of the socket after tooth removal, the extraction site could be classified as 4-, 3-, 2- or 1-wall defect (Misch 1999). The socket is considered intact or 4-walled when the socket is surrounded by bone with the absence of fissures, dehiscence and fenestration (Figure 4).

Figure 4: 4-walled extraction socket with no wall missing.

1.2. Indication of tooth removal

Teeth are removed for many reasons. The indications of tooth extraction are listed in Table 1.

Teeth are usually extracted when:
- teeth are severely carious and non-restorable.
-presence of pulp necrosis and tooth is untreatable by standard endodontics.
- irreversible tooth mobility and excessive bone loss (periodontal disease).
- teeth have cracked or fractured roots (trauma).
- teeth are involved in pathologic lesions.
- teeth are involved in jaw fracture.

Table 1: Indications of tooth removal (Peterson et al.1997).

1.3. Dimensional changes of extraction sockets

The alveolar process is tooth-dependent tissue that develops in conjunction with the eruption of the teeth. Further, the volume as well as the shape of the alveolar process is determined by the form of the teeth, their axis of eruption and inclination (Schroeder 1986, Araujo and Lindhe 2005). The early clinical and cephalometric studies have shown that subsequent to the removal of all teeth, the alveolar process will undergo atrophy with dimensional reduction in both the horizontal and vertical axes (Calsson et al. 1967, Atwood and Coy 1971, Tallgren 1972). The greatest reduction occurs in the first 6-12 months after extraction and then bone resorption continues at a slower rate (Calsson et al. 1967,

Tallgren 1972). However, the rate of residual ridge resorption varies considerably between different individuals (Calsson et al. 1967, Atwood and Coy 1971, Tallgren 1972) and is four times greater on the mandible than on the maxilla (Atwood and Coy 1971, Tallgren 1972). The amount of tissue resorption was found to be greater in the edentulous molar region than in the incisor and premolar region of both jaws (Pietrokovski and Massler 1967). These changes of shape of the alveolar process of both jaws follow a predictable pattern and vary with sites (Tallgren 1972, Cawood and Howell 1988). Bone loss occurs in the anterior mandible and the maxilla mainly at the labial site (Cawood and Howell 1988, Winkler 2002).

Recent studies have examined the resorption pattern and assessed changes of the alveolar process following single and multiple tooth extractions by means of subtraction radiography (Schropp et al. 2003), study cast measurements (Lekovic et al. 1997, Lekovic et al. 1998, Camargo et al. 2000, Schropp et al. 2003), and direct measurements of the ridge following surgical re-entry procedures (Lekovic et al. 1997, Lekovic et al. 1998, Camargo et al. 2000, Iasella et al. 2003, Schropp et al. 2003, Botticelli et al. 2004).

The width of the alveolar ridge was found to be reduced by 50 % during the first 12 months after tooth extraction (about 5 to 7 mm), and two thirds of this bone loss occurs in the first three months after tooth extraction (Schropp et al. 2003), suggesting that most of the dimensional alteration of the alveolar ridge takes place within the first 3 months of healing (Figure 5) with a corresponding vertical bone loss of 0.9 to 3.25 mm (Lekovic et al. 1997, Lekovic et al. 1998, Camargo et al. 2000, Iasella et al. 2003, Schropp et al. 2003). Alveolar bone resorption is more pronounced at the buccal than the lingual aspect of the socket walls (Pietrokovski and Massler 1967, Iasella et al. 2003, Botticelli et al. 2004). Studies in canines have shown that this resorption is based on bundle bone resorption which loses its function after tooth removal and the alveolar bone is resorbed due to osteoclastic activity (Araujo and Lindhe 2005).

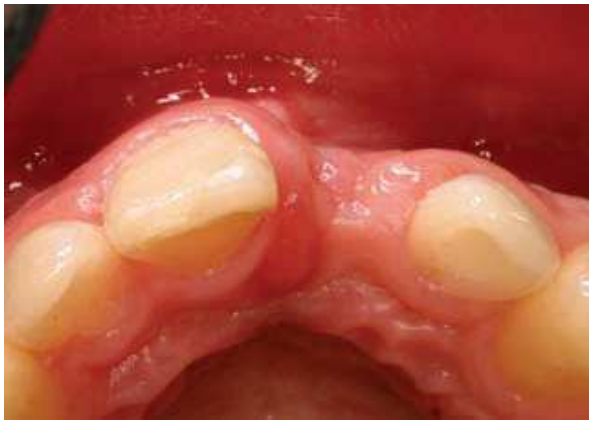

Figure 5: Horizontal alveolar ridge defect two months after tooth extraction.

Loss of alveolar bone may occur prior to tooth extraction because of advanced periodontal disease, periapical pathology, or trauma to teeth and bone. Damage of the bone tissue during tooth extraction procedure may also result in bone loss (O'Brien et al. 1994, Jahangiri et al. 1998, Chen et al. 2004, Irinakis 2007).

A variety of factors may influence the dimensional changes of the alveolar ridge following tooth removal, and it is clear hat current knowledge is limited in many areas. The rate and pattern of bone resorption may be influenced by the traumatic extraction of the tooth including flap elevation, damaging 1 or more of the bony walls of socket (Wood et al. 1972, O'Brien et al. 1994, Chen et al. 2004, Brkovic et al. 2008). Some others local factors such as the width of the labial cortical plate, periodontal health of the neighboring teeth and periodontal biotype (shape and thickness of soft tissue) may also affect the bone resorption process. The resorption of the bone of the facial plate after tooth extraction has been found to be greater when the residual facial plate thickness was thin or less than 2 mm (O'Brien et al. 1994, Lekovic et al. 1997, Spray et al. 2000, Botticelli et al 2006, Fickl et al. 2008). The thin, high scalloped periodontium, characterized by thin underlying alveolar bone might exhibit more post-surgical hard tissue resorption and soft tissue recession than the thick flat periodontal biotype (Block and Kent 1990, Sclar 2004, Fugazzotto 2005, Fickl et al. 2008). Systemic conditions such as osteoporosis, renal disease and endocrine disorders may accelerate bone loss by altering normal bone physiology and metabolism (Atwood 1962, Hirai et al. 1993, Bartee 2001). Patient habits including smoking and functional forces like bruxism have been implicated as contributing factors in accelerated bone loss (Bartee 2001, Chen et al. 2004).

1.4. Healing of extraction sockets (Histologic Events)

The healing events and new bone formation that occur in the extraction socket following tooth removal have been studied in different animal models (Claflin 1963, Cardaropoli et al. 2003) and in biopsies obtained from humans (Mangos 1941, Amler et al. 1960, Claflin 1963, Boyne 1966, Carlsson et al. 1966, Amler 1969, Evian et al. 1980, Ahn and Shin 2008, Trombelli et al. 2008). The early studies (Mangos 1941, Amler et al. 1960, Boyne 1966, Carlsson et al. 1966, Amler 1969) have shown that during the process of healing of the extraction socket the following events occur: firstly, a blood clot (mainly blood cells and network of fibrin) forms and fills the empty socket. Then, the blood clot matures and is organized by the formation of granulation tissue (rich in newly formed vascular structure, abundance of inflammatory cells such as neutrophils, macrophages and lymphocytes and fibroblasts infiltrate). The granulation tissue arises two to three days after tooth extraction and replaces the blood clot completely by the seventh day (Amler et al. 1960, Carlsson et al. 1966, Amler 1969). Thereafter, a gradual replacement of the granulation tissue by provisional connective tissue takes place. This provisional connective tissue is made up of densely packed mesenchymal cells, osteoblasts (cells produce bone), collagen fibers and vessels. In the next stage, formation of osteoid (non-mineralized bone matrix) by the osteoblasts begins and gradually converts to new mineralized bone filling of the socket from the base and periphery of the socket (Amler et al. 1960, Amler 1969). An epithelialization of the wound surface occurs parallel with the reparative processes in the socket. However, the available human studies on extraction wound healing, cited above, are affected by significant limitations. These included tissue samples from cadavers (Mangos 1941) and evaluated only a few samples and/or short observation intervals (Mangos 1941, Amler et al.1960, Boyne 1966). Quantitative analysis of the tissue has not been performed so that the amount of new bone formation could not be evaluated. Furthermore, most of the studies cited above were of comparatively short duration and, thus, have no information related to the later phase of socket healing including the process of remodeling of newly formed bone tissue and connective tissue.

In recent studies using a canine model, the process of healing in mandibular extraction sockets during 6-month interval shows that within the first 4 weeks there is an initial phase

of progressive bone formation so that the socket is mainly dominated by newly formed bone by the 30th day. After that, the onset of resorption and remodeling of this newly formed bone begins and gradual placement of bone marrow occurs and by 6 months after tooth extraction, mineralized bone occupies only 15 % of the extraction socket (Cardaropoli et al. 2003). Since the extraction wound healing and bone regeneration is slower in humans than in dogs (Claflin 1963, Al Pearce et al. 2007), there will be some variation of the rate of bone formation and remodeling between humans and dogs at certain periods. Little is known about the peak of the initial phase of bone formation and the time in which bone turnover in human begins. Such information could be of great value to determine the ideal time of implant placement after tooth extraction.

1.5. Implant placement and bone resorption

The reduction of alveolar bone volume following tooth extraction may interfere with the placement of implants and influence the treatment success with regard to function and esthetics (Irinakis 1997, John et al. 2007). Many concepts and treatment protocols have been suggested to preserve the alveolar ridge and minimize the loss of bone height and bucco-lingual width or to facilitate bone formation in extraction sockets.

Immediate implant placement (inserting implants into the fresh extraction socket) was claimed to prevent bone resorption and hence preserve the osseous structures while maintaining the original shape of the ridge (Garber et al. 2001, Paolantonio et al. 2001, Wagenberg and Ginsburg 2001). The advantages of immediate implant placement have been reported to include reduction in the number of surgical interventions and in the treatment time required, ideal orientation of the implant and optimal soft tissue esthetics (Webitt and Goldberg 1992, Missika et al. 1997). Findings from clinical studies and animal experiments, however, failed to support this hypothesis (Araujo et al. 2005, Araujo et al. 2006 a, Araujo et al. 2006 b, Botticelli et al. 2006, Covani et al. 2007). The buccal as well as the lingual bony walls of the socket were found to undergo marked resorption and remodeling in spite of immediate implant placement following tooth extraction. The resorption of the buccal bone wall was three times greater than that observed at the buccal aspect of the surgically involved control teeth with obvious vertical bone reduction (more

than 2 mm) and loss of bone-to-implant contact at the marginal portion of the implant during the first 6 months of healing (Araujo et al. 2005, Araujo et al. 2006 a, Araujo et al. 2006 b, Botticelli et al. 2006, Covani et al. 2007). The horizontal buccal and lingual bone resorption amounted to about 56 % and 30 %, respectively during the first 4 months of healing (Botticelli et al. 2004). Immediate implant placement shows no evident decrease of the resorption rate or pattern of the alveolar bone. Some authors reported that a favorable implant success rate can be achieved with immediately placed implant regardless of the dimension of bone reduction (Kan et al. 2003, Schrop et al. 2003 b, Covani et al. 2007). However, when a site is compromised by infection or a thin labial bone plate is present with the presence of thin, high scalloped periodontal biotype, immediate implant placement would carry a significant esthetic risk (Kao et al. 2007, Evan and Chen 2008, Ferrus et al. 2010, Tomasi et al. 2010), and exhibit serious gingival recession (Chen et al. 2007).

Delayed implant placement (6-12 weeks after extraction) might be a more appropriate treatment approach (Scalr 2004). Delayed implant placement might prevent the problems associated with immediate placement (Grunder et al. 1999, Nemcovsky et al. 2002, Chen et al. 2004, Sclar 2004). However, a delay of 3 months or more could result in a marked alveolar ridge reduction (Schropp et al. 2003). Hence, a procedure to minimize bone resorption would be favorable.

1.6. Socket preservation and grafting materials

Various methods, based on implanting grafting material with or without a barrier membrane in the residual alveolar socket at the time of tooth removal, have been described to maintain the alveolar ridge dimension and to protect the contours of the ridge bone and the soft tissue after tooth extraction or (Bartee 2001, Buch et al. 2005, Fugazzotto 2005, Irinakis 2007). The aim of procedures to preserve the socket dimension (socket preservation) is to prevent or minimize jaw bone atrophy and maintain adequate height and width of bone and the volume of soft tissue and to allow bone formation within the socket (Bartee 2001, Sclar. 2004).

The concept of guided tissue regeneration (GTR) using a membrane barrier to prevent the migration of epithelial cells into the wound to allow time for bone formation has gained wide

acceptance in healing periodontal defects (Laurel and Gottlow 1998, Needleman et al. 2001). Membranes known from GTR were utilized to preserve the alveolar ridge after tooth removal using non-absorbable (e.g. ePTFE) or bioabsorbable (e.g. collagen) membranes (Lekovic et al. 1997, Lekovic et al. 1998, Iasella et al. 2003). The original ridge contour was not preserved since a horizontal buccal-oral tissue loss up to 2 mm was found (Lekovic et al. 1997, Lekovic et al. 1998, Iasella et al. 2003).

Heterologous grafting materials, covered with a barrier membrane or not, have been widely utilized in augmentation procedures (reconstruction of the vertical and horizontal bone deficiency of alveolar ridge) and in sinus floor elevation (McAllister and Haghighat 2007). The biologic principles that support the use of heterologous grafting materials are the osteoconductive and osteoinductive properties which they might possess (Lindhe et al. 1998, Misch 1999, Newman et al. 2006). Osteoinduction means that the grafting material stimulates pluripotential mesenchymal cells to differentiate into osteoblasts (Misch 1999, Newman et al. 2006). On the other hand, osteoconductivity describes the enhancement of osteoblasts from the wound margins to infiltrate the defect and migrate across the grafting material to produce new bone (Lindhe et al. 1998, Misch 1999). These materials have also been used to stabilize the blood clot in bone defects and hence avoid volume reduction and surface invagination of overlying tissue, acting as a space-maintaining device (Friedmann et al. 2002, Cardaropoli et al. 2005).

Heterologous grafting material used in the oral cavity could be divided into: xenograft and alloplastic material (Lindhe et al. 1998, Newman et al. 2006, Irinakis 2007). Xenograft bone is derived from a donor of another species such as bovine, ovine and porcine bone. Alloplastic materials are synthetic products used as substitutes for bone graft such as hydroxyapatite, beta-tricalcium phosphate (TCP), poly-lactide sponge (Lindhe et al. 1998, Newman et al. 2006).

Heterologous grafting material was first used for socket preservation after tooth extraction in the late 1970s and the early 1980s, when hydroxyapatite (HA) in the form of root shaped cones and particles was placed into the extraction socket to prevent ridge resorption (Denissen et al. 1979, Quinn and Kent 1984). However, the use of these cones has not seen wide-spread acceptance related to problems of maintaining adequate soft tissue closure over the grafts (Kwon et al. 1986). Since then, several studies have proposed the

use of autogenous grafts (Becker et al. 1994, Becker et al. 1998), allograft bone (Brugnami et al. 1996, Zubillga et al. 2003), alloplastic material (Camargo et al. 2000, Serino et al. 2003, Luczyszyn et al. 2005, Rothmal et al. 2007), and xenografts (Artzi and Nemcovsky 1998, Becker et al. 1998, Arzi et al. 2000, Carmagnola et al. 2003, Fugazzotto et al. 2003, Norton et al. 2003, Nevins et al. 2006) as a socket preservation technique during bone healing following tooth extraction. However, no data is available on the course of healing in relation to time.

1.6.1. Deproteinized bovine bone mineral (Bio-Oss)

Bio-Oss bovine bone is a xenogenic grafting material obtained from calf bone that has been chemically treated to remove its organic components, leaving a trabecular and porous architecture similar to human bone. Inorganic bovine bone is similar to human cancellous bone in its hydroxyapatite crystalline, morphological structures and porosity (Berglundh and Lindhe 1997, Tapety et al. 2004). In addition, the physical properties of Bio-Oss approximates the value of human bone tissue (Yildirim et al. 2000). The Structure of Bio-Oss consists of a wide interconnecting pore system (75 % pores) that may enable this material to serve as physical scaffold for osteogenic cells and vascularization (Yildirim et al. 2000, Weibrich et al. 2000, Tapety et al. 2004), this allows osteoconductive properties with an effective bone graft matrix for bone formation (Valintini et al. 1998, Arzi et al. 2000, Proussaefs and Lozada 2003, Cardaropoli 2005, Araujo et al. 2008). Furthermore, Bio-Oss was seen to promote osteoblastic differentiation in artificial bone defects in rat femurs (Tapety et al. 2004).

Bovine bone mineral is a biocompatible grafting material and no inflammatory responses have been reported with the use of its particles (Piattelli et al. 1999, Jensen et al. 1996, Arzi et al. 2000, Arauio et al. 2001, Norton et al. 2003, Proussaefs and Lozada 2003, Araujo et al. 2008).

The degradation of Bio-Oss particles seems to be a slow process and the resorption of the material may require more than one year (Becker et al. 1998, Piattelli et al. 1999, Artzi et al. 2000, Fugazzotto 2003, Proussaefs and Lozada 2003).

Inorganic bovine bone has been widely used in sinus augmentation, repairing intra bony defects and in socket preservation procedures (Berglundh and Lindhe 1997, Artzi and

Nemcovsky 1998, Fugazzootto et al. 2003, Froum et al. 2008). The efficiency of bovine bone mineral placed in extraction sockets has been evaluated in experimental and clinical studies regarding new bone formation patterns after a prolonged healing period (Arzi et al. 2000, Carmagnola et al. 2003, Norton et al. 2003, Fugazzotto et al. 2004, Vance et al. 2004, Nevins et al. 2006). Bovine bone was found to be effective in limiting both horizontal and vertical bone resorption compared with non-grafted sockets 7 months after tooth removal (Nevins et al. 2006, Chen et al. 2007, Barone et al. 2008). Moreover, total incorporation of the bovine bone particles into newly generated osseous tissue was reported after this prolonged healing time (Artzi et al. 2000, Proussaefs and Lozada 2003, Barone et al. 2008). However, there are no reports of assessment of bone formation and incorporation of bovine bone particles into the grafted socket within the early healing period.

1.6.2. Bio-Oss Collagen

Bio-Oss Collagen consists of Bio-Oss granules (Ø 0.25 - 1 mm) with the addition of 10 % highly purified porcine collagen to ensure cohesion of bovine bone particles; it enables convenient handling and simple application. The consistency of this material provides stability to the Bio-Oss particles when placed within a defect (Jung et al. 2004).

Recent data indicate that the application of Bio-Oss Collagen minimizes the resorption pattern of original alveolar bone, allows adequate new bone formation within the extraction socket and is ideal for socket preservation in animal models (Araujo et al. 2008, Fickl et al. 2008). Moreover, it was observed that Bio-Oss collagen might act as space holder for epithelialization, minimizing the soft tissue collapse of the alveolar ridge (Fickl et al. 2008). Information about Bio-Oss Collagen used for socket preservation in humans is lacking.

2. Aim of the study

An estimated 13 million teeth are extracted in Germany each year (KZBV yearbook 2008). The replacement of the lost tooth using dental implants has become more popular. In the period from 2001 to 2008 the annual number of implants which were placed has increased from 200,000 to 500,000 implants yearly (KZBV yearbook 2001-2008). For a predictable esthetic and functional outcome of implant-retained restorations after tooth removal, the biology of the healing of extraction sockets needs to be considered.
Animal experiments and clinical studies revealed that the alveolar ridge undergoes dimensional alterations in both horizontal and vertical directions and morphologic changes after tooth extraction. The highest resorption of the alveolar ridge occurs during the first 3 months of healing. Many studies have stated the necessity of socket preservation using bone grafting material to minimize alveolar ridge alteration, while allowing adequate bone formation within the socket and preservation of normal soft tissue contour, thus avoiding the need for complex alveolar ridge reconstruction for esthetic implant restoration. However, the clinical and histologic performance of heterologous grafting material such as hydroxyapatite, beta-tricalcium phosphate (ß-TCP) and bovine bone mineral has been evaluated only after a prolonged healing period extending from 4 months up to 12 months.

The effectiveness of Bio-Oss Collagen placed in extraction sockets has been evaluated in animal studies regarding bone formation in healing periods of 3 or more months (Araujo et al. 2008, Fickl et al. 2008). No information is available on the rate of bone formation in extraction sockets after a shortened healing period of 6 or 12 weeks. No histological-histometric analysis has been performed to evaluate the tissue distribution in extraction sites grafted with Bio-Oss Collagen within these healing times. The objective of the present study was to assess the amount of new bone formation in the human extraction socket after 6 and 12 weeks as well as the amount and mode of incorporation of Bio-Oss particles at this time point.

3. Materials and Method

The study protocol was approved by the Ethics Committee of the Charité University Medicine in Berlin, Germany (Ethicsnumber: EA/142/00).

3.1. Patient and site selection

Twenty-nine patients (14 females and 15 males) with 34 extraction sites participated in this prospective study.

The patients were referred for the removal of teeth for endodontic reasons, caries or root fracture. Patients with active periodontal lesions or advanced periodontal disease comprising severe tooth mobility with deep pockets, chronic periodontal abscess, and radiographically showing no lamina dura and destruction in the interseptal bone of molars were excluded from this study. Teeth with evident periapical radiolucency and/or periapical abscess were not included. Only 4-walled extraction sockets with no fissures, dehiscences and fenestration were selected to take part in the study. Severely resorbed sockets with a remaining height of less than 5 mm were not included.

All patients were healthy and did not have any systemic disease such as endocrine disturbances and nutritional insufficiencies. Patients taking regular medications or having previous history of radiotherapy or chemotherapy were excluded. Only non-smokers were allowed to participate in this study.

Two groups of patients were randomly formed based on the time between tooth extraction and biopsy retrieval. In Group 1 the biopsy was taken 6 weeks after tooth extraction, and in Group 2 after 12 weeks. The patient distribution is presented in Tables 2 and 3. An implant placement was planned for all of the extraction sites.

Patient (initials)	Gender M=male F=female	Age (years)	Tooth socket (region)	Time between extraction and biopsy (weeks)
6-1	M	46	26	6
6-2	F	46	16	6
6-3a [1]	F	53	26	6
6-3b [1]	F	53	27	6
6-4	M	53	16	6
6-5	F	28	37	6
6-6	M	49	16	6
6-7	F	59	16	6
6-8	F	52	16	6
6-9	F	31	46	6
6-10	F	65	16	6
6-11	M	68	16	6
6-12	M	44	36	6
6-13	F	52	15	6
6-14	F	58	14	6
6-15	F	69	13	6
6-16	M	35	16	6

Table 2: Patient and site distribution in Group 1.

[1] In this patient, biopsy was retrieved from more than one extraction site.

Patient (initials)	Gender M=male F=female	Age (years)	Tooth socket (region)	Time between extraction and biopsy (weeks)
12-1	M	52	24	12
12-2	M	49	24	12
12-3	M	36	11	12
12-4	F	59	15	12

12-5a [1]	M	72	11	12
12-5b[1]	M	72	14	12
12-5c[1]	M	72	15	12
12-5d [1]	M	72	26	12
12-6	F	36	46	12
12-7	M	72	32	12
12-8	M	51	47	12
12-9	M	50	46	12
12-10	M	56	24	12
12-11	F	55	14	12
12-12	M	55	47	12
12-13a [1]	F	49	21	12
12-13b [1]	F	49	25	12

Table 3: Patient and site distribution in Group 2.

[1] In this patient, biopsy was retrieved from more than one extraction site.

3.2. Surgical procedure

3.2.1. Extraction of teeth

The extraction procedure was performed under local anesthesia (Ultracain D-S forte, Sanofi-Aventis GmbH, Bad Soden am Taunus, Deutschland) without the elevation of a mucoperiosteal flap; therefore, no primary wound closure was performed. Meticulous care was taken to avoid surgical trauma of the surrounding tissue by using a periotome and the appropriate dental forceps (Aesculap AG & Co KG, Tuttlingen, Germany) (Figure 6).

For consideration in the study, all extraction sockets had to be intact (4-wall defect), with no alveolar wall loss (Figure 7). A thorough curettage of all soft tissue debris in the alveolus was performed using a periapical curette (Aesculap AG & Co KG, Tuttlingen, Germany) to ensure the removal of all granulation tissue and periodontal ligament PDL and to stimulate bleeding from the osseous base.

3.2.2. Grafting procedure

Thereafter, Bio-Oss Collagen (Geistlich Pharma AG, Wohlhusen, Switzerland) was applied, not exceeding the height of the alveolar crest, into the extraction site without pressure to allow a passive fit and to maintain equal proportions of particle quantities in the socket per volume unit (Figure 9). Care was taken to ensure that the collagen was saturated with blood. The Bio-Oss Collagen was cut to the appropriate dimensions of the alveolar socket to enable uncondensed placement with dental forceps (Figure 8) (Aesculap AG & Co KG, Tuttlingen, Germany). No primary wound closure was performed.

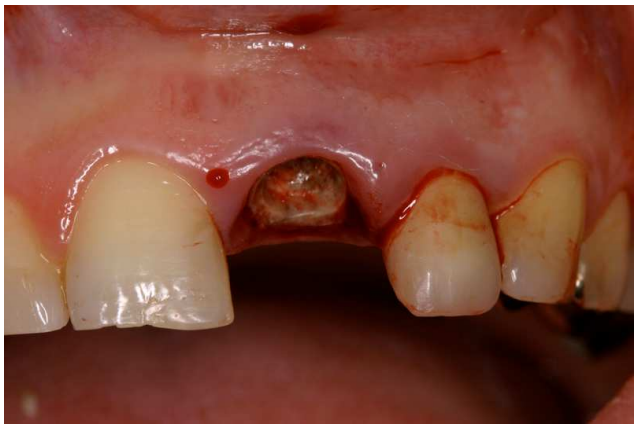

Figure 6: The site before extraction and after using a periotome.

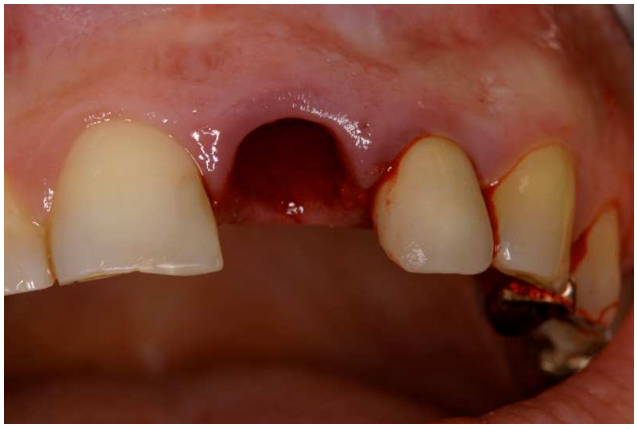

Figure 7: The socket showed no bony defect after the extraction.

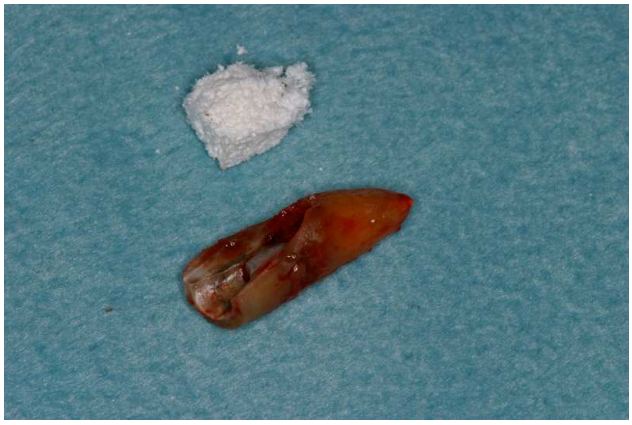

Figure 8: The extracted root and the root-shaped Bio-Oss Collagen.

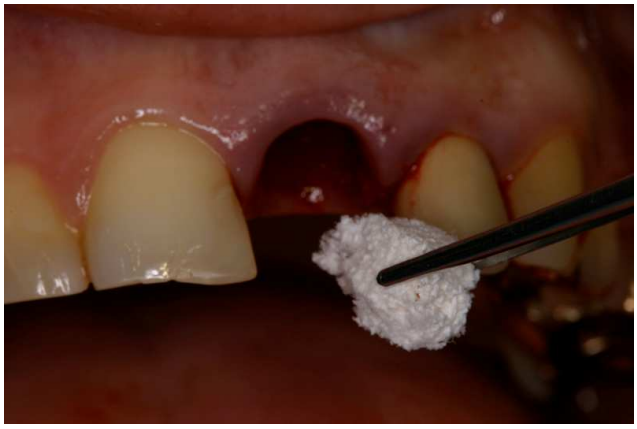

Figure 9: Application of Bio-Oss Collagen into the socket.

3.2.3. Post-operative Follow-up

The patients were clinically evaluated at post-operative days 1, 7, and 30 for the assessment of complications such as inflammation, mucosal erythema, wound dehiscences, or loss of grafting material (Figure 10).

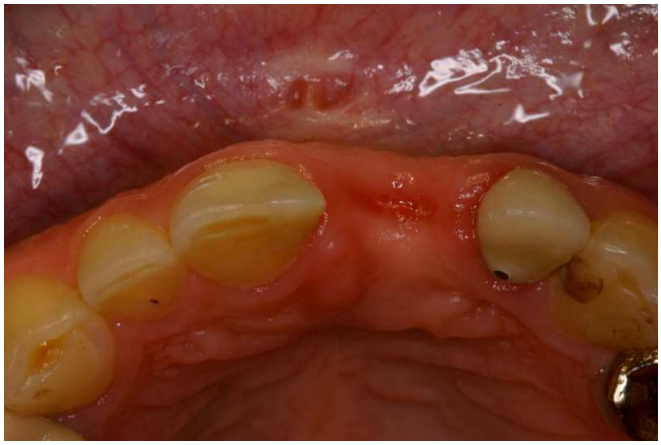

Figure 10: 30 days post operation, epithelial closure over the Bio-Oss Collagen grafted extraction site.

3.2.4. Biopsy retrieval procedure

At the time of implant placement, 6 weeks post operation in the first group and 12 weeks post operation in the second group, a mucoperiosteal flap was raised, the site of extraction clearly identified and a core biopsy taken from the center of the extraction site with a minimum depth of 8 mm. For this, a trephine bur (ø 2 mm) (Straumann AG, Basel, Switzerland) was used for the retrieval of the bone biopsy for histologic evaluation (Figure 11). This was followed by dental implant placement according to the manufacturer's surgical protocol. For implant placement, Camlog RootLine implants (Camlog Biotechnologies, Wimsheim, Germany) or Straumann ITI (Straumann AG, Basel, Switzerland) implants were utilized. The mucoperiosteal flaps were closed with interrupted sutures (5-0 Monocryl, Ethicon, Hamburg, Germany).

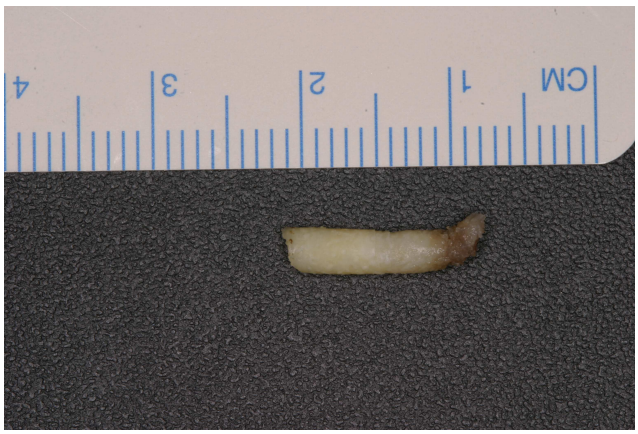

Figure 11: Cylindrical bone sample was retrieved from each extraction socket 6 or 12 weeks after extraction.

3.3. Histologic evaluation

Before histological preparation, the tissue samples were marked with blue or red ink (Marker II/Superfrost, Precision Dynamics Corp., San Fernando, CA, U.S.A.) at the coronal side to differentiate the coronal and apical regions.

3.3.1. Paraffin Embedding

Bone biopsy specimens (length 8 – 10 mm) obtained from the grafted areas were fixed in 4 % formalin for 2 days. Then they were decalcified in 17 % nitric acid for 12 hours (Callis 2002). After routine tissue processing, including dehydration with a series of alcohol and cleaning with xylene in a tissue processor (Citadel, Shandon, Frankfurt a.M., Germany), tissues were embedded directly in a paraffin block.

3.3.2. Sectioning

5 µm thick serial sections were prepared using a rotary microtome (Leica Mikrosysteme HandelsgesmbH, Vienna, Austria). Once sections were cut, they floated in a warm water bath (50℃) to remove wrinkles and allow flattening of the slice. Then, they were picked up and placed on a glass microscopic slide (75X25X1 mm) to dry at room temperature for 12 hours.

3.3.3. Staining

Before staining, the slides were deparaffinized by running them through xylenes, a series of alcohols and water as described in Tables 4, 5 and 6. Then, sections were stained with Hematoxylin-Eosin, Masson´s trichrome and Toluidine blue stain.

3.3.3.1. Haematoxylin and eosin (H&E) staining- for paraffin

Osteoid, calcified bone and connective tissue stained by H&E appear in different shades of pink. Nuclei stain blue. Hematoxylin-Eosin staining was performed as described in Table 4.

Reagent		Incubation time
Xylene	2X	10 min.
100% alcohol	2X	02 min.
96% alcohol	1X	02 min.
80% alcohol	1X	02 min.
70% alcohol	1X	02 min.
Aqua dest (distilled water)	wash	
Harris' haematoxylin		07 min
Aqua dest	wash	
HCL 0,25%	1X	Immerse for short time
Wash in running tap water 10 min.		
Eosin	5X immerse	
Aqua dest	wash	
80% alcohol (dehydration)	wash	
90% alcohol	wash	
100% alcohol	wash	
Xylene (cleaning)	2X wash	5 min.
Vitroclud (mounting medium) ([4]Langenbrink, Emmendingen, Deutschland)		

Table 4: Steps for H&E staining from deparaffinizing to coverslipping (covering the portion of the slide containing tissue using a thin glass coverslip and a clear synthetic mounting medium as a bond).

3.3.3.2. Masson’s trichrome staining

The osteoid stains orange/red, whereas mineralized bone appears in green color. Connective tissue stains green and nuclei brown/black. The Steps of staining are listed in Table 5.

Reagent		Incubation time
Xylene	2X	10 min.
100% alcohol	2X	02 min.
96% alcohol	1X	02 min.
80% alcohol	1X	02 min.
70% alcohol	1X	02 min.
Aqua dest	wash	
Weigert's iron hematoxylin 1+2	stain	20 min.
Wash in running tap water		10 min.
Acid fuchsin-Ponceau		05 min.
1% acetic acid	wash	15 min.
Phosphomolybdic acid/Orange G	stain	
1% acetic acid	wash	
Light green	stain	8-10 min
1% acetic acid	wash and 1X immerse	
100% alcohol (dehydration)	3X wash	
Xylene (cleaning)	wash	2X5 min.
Vitroclud (mounting medium)		

Table 5: Steps for Masson´s trichrome staining from deparaffinizing to coverslipping.

3.3.3.3. Toluidine staining

Bone stained by toluidine is colorless or pale blue/purple. Soft tissue present in varying shades of blue. Staining steps are described in Table 6.

Reagent		Incubation time
Xylene	2X	10 min.
100% alcohol	2X	02 min.
96% alcohol	1X	02 min.
80% alcohol	1X	02 min.
70% alcohol	1X	02 min.
Aqua dest	wash	
Toluidine blue		15 min.
80% alcohol (dehydration)	wash	
90% alcohol	wash	
100% alcohol	wash	3 min.
Xylene (cleaning)	wash	
Vitroclud (mounting medium)		

Table 6: Steps for toluidine staining from deparaffinizing to coverslipping.

3.4. Histologic and quantitative histomorphometric analysis of samples

The two most central sections were obtained from each specimen. The sections were line-scanned using ScanScope T3 (Aperio Technologies Inc, Vista, USA) with a resolution of 0.25 µm/Pixel and a 40x objective.

For the histologic qualitative analysis of the remodeling process, the stained preparations were examined under a light microscope (AxioPhot I, Zeiss, Jena, Germany) at a magnification of up to 40x. The histologic appearance of the total specimens was described.

A digital imaging system AXIO VISION 4.6 (Zeiss, Jena, Germany) was used to do the histomorphometric assessment. Two regions of interest ("ROI"), measured 0.5 mm^2 each, were determined within each specimen, located within the same proximity in the specimens

in the apical and coronal portion (see Figure 12). In each ROI the following parameters were assessed:

- The whole area of ROI
- The area of viable bone.
- The area of Bio-Oss particles.
- The area of connective tissue.

The parameters mentioned were marked interactively at the screen (Figure 13) and measured by the program. The relative volume of new bone, Bio-Oss particles as well as connective tissue were then estimated in each ROI in the coronal and apical region of every section using the same program as following:

- The volume of newly formed bone = area of viable bone/area of whole ROI (%).
- The volume of Bio-Oss particles = area of Bio-Oss particles/area of whole ROI (%).
- The volume of connective tissue = area of connective tissue/area of whole ROI (%).

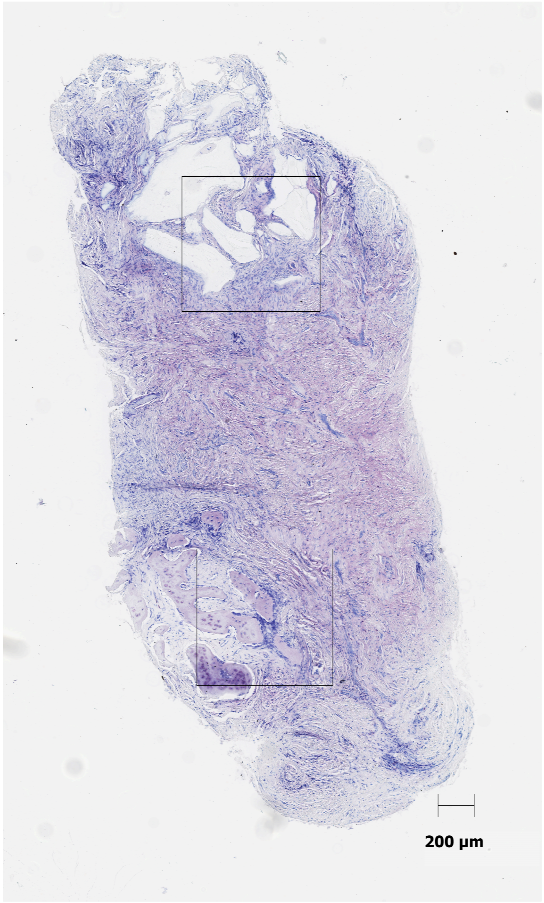

Figure 12: The first ROI was determined in the coronal portion of the sample, and the second one in the apical portion. Each ROI measured 0.5 mm^2.

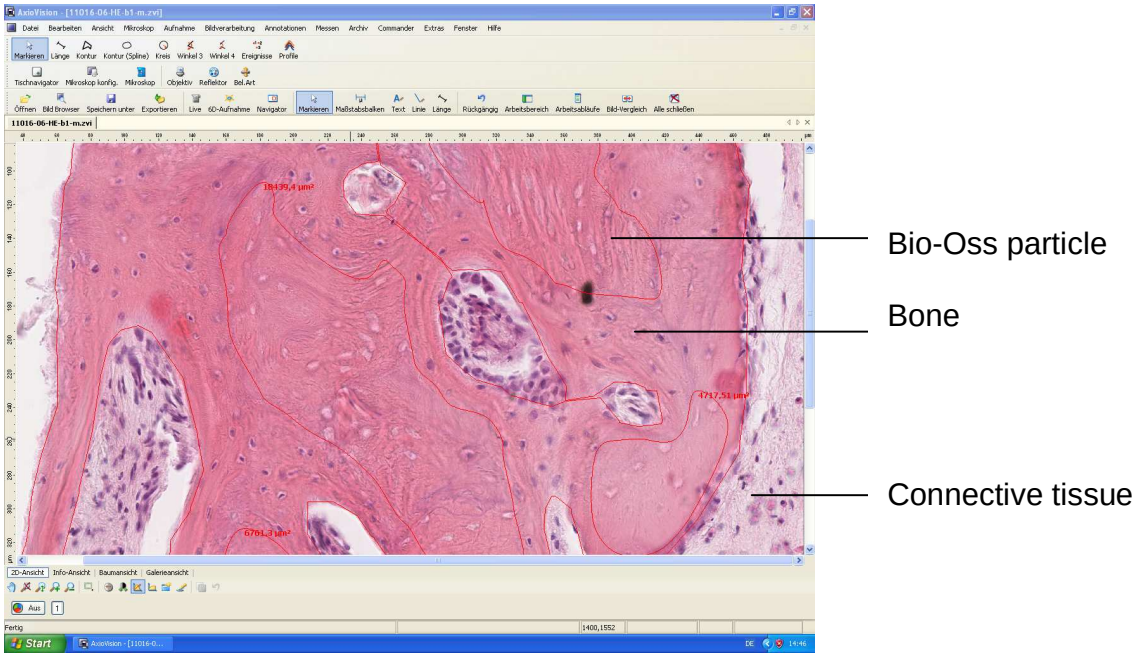

Figure 13: AXIO VISION imaging system program was used to measure the volume of bone, Bio-Oss Collagen particles and connective tissue.

The newly formed bone was distinguishable from Bio-Oss particles depending on this criteria: the osseous lamellae of healthy leaving bone tissue contained osteocytes, located in the bone lacunae, which send out branched protoplasmic processes into the small bone canal. Bio-Oss particles resembled the feature of necrotic bone material, characterized by a lack of osteocyte nuclei. The osteocyte lacunae were empty and the lamellar layer was indistinct. The shape and size of the Bio-Oss particles also showed clear differences in comparison to natural bone. While natural bone lamellae appeared long and thin, the particles of the bone substitutes were short, thick and had sharp boundaries.

To ensure high intra-observer reliability of the quantitative histomorphometric assessment of the samples, the morphometric analysis was based on recording the assessed data of each slide at three different time points.

The calibration of the section image was checked manually by placing a stage micrometer 25+50/10 mm (Zeiss, Göttingen, Germany) diagonally across the image to confirm the automatic calibration of the program.

3.5. Materials and devices overview

Materials	
Formalin 4% buffered	Herbeta-Arzneimittel[1]
Nitric acid-decalcifying agent	Herbeta-Arzneimittel[1]
Xylene	J.T.Baker[2]
Ethanol	J.T.Baker[2]
Methanol	J.T.baker[2]
Paraffin	Merck[3]
Vitro-Clud, Xylene based mounting medium	R.Langenbrink[4]
Haematoxylin and eosin (H&E) staining	
Eosin	Sigma[5]
Harris alum haematoxylin	Chroma[6]
Masson's trichrome staining	
Weigert's iron hematoxylin 1+2	Chroma[6]
Acid fuchsin-Ponceau-Fuchsin	Chroma[6]
Light green	Merck[3]
Orange G	Merck[3]
Toluidine staining	
Na-Tetraborat	Merck [3]
Toluidine blue	Chroma[6]
Pyronin-G	Merck[3]

Table 7: Reagent.

Devices	
Citadel- Tissue Processor	Shandon[7]
EG 1160-paraffin embedding station	Leica[8]
Microtome 2065	Leica-Reichert-Jung[8]

Table 8: Devices.

[1]Herbeta-Arzneimittel, Berlin, Deutschland. [2]J.T.Baker, Deventer, Holland. [3]Merck KgaA, Darmstadt, Deutschland. [4]Langenbrink, Emmendingen, Deutschland. [5]Sigma Diagnostics, St.Louis, Missouri, USA. [6]Chroma-Gessellschaft Schmid GmbH & Co, Münster, Deutschland. [7]shandon, Thermo Fisher Scientific, Waltham, USA. [8]Leica Mikrosysteme HandelsgesmbH, Vienna, Austria.

4. Statistics

The intraclass correlation coefficient (ICC) was used to determine the intra-observer reliability for the histomorphometric measurements (SPSS 13.0, SPSS Inc., Chicago, IL, USA).

The histological and histomorphometrical data were descriptively analyzed using the software SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and Excel 2002 (Microsoft, Seattle, USA).

Comparative statistical analysis between the apical and coronal region of the specimens was performed using the Wilcoxon signed-rank test with the software version SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Comparison between the 6-week patients group and the12-week patients group was statistically analyzed using Wilcoxon-Mann-Whitney test with the software SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

5. Results

The patients (n=29) with 34 extraction sites were divided into two groups. In Group 1 (n=16) with 17 extraction sites, the time of implant placement was 6 weeks after the grafting procedure. In Group 2 (n=13) with 17 extraction sites, the implants were installed after 12 weeks. The mean age of the patients in both groups was 50.5 years (ranging from 28 - 72 years). The age and sex distribution for 6-week and 12-week group is presented in Table 9.

Age (years)		20-29	30-39	40-49	50-59	60-69	70-79	**Total**
Male	Group 1 (6-week)	-	1	3	1	1	-	6
	Group 2 (12-week)	-	1	1	5	-	2	9
Female	Group 1 (6-week)	1	1	1	5	2	-	10
	Group 2 (12-week)	-	1	1	2	-	-	4
Total		1	4	6	13	3	2	29

Table 9: Age and sex distribution for the two groups.

All extraction sites, except one, healed uneventfully and showed no signs of inflammation. In one patient of group 1, one surgical site infection occurred after the extraction and grafting procedure and a re-entry was performed to remove all material from the socket. This site was excluded from further analysis.

The distribution of the sites within the jaws is given in Table 10.

Location of site		Anterior Tooth	Premolar	Molar	Total
Maxilla	Group 1 (6-week)	1	2	11	14
	Group 2 (12-week)	3	8	1	12
Mandible	Group 1 (6-week)	-	-	3	3
	Group 2 (12-week)	1	-	4	5
Total		5	10	19	34

Table 10: The location of sites within the jaw.

The clinical examination of the augmented area at time of implant insertion, 6 weeks after extraction in group 1 and 12 weeks in group 2, showed soft tissue closure in all cases without signs of inflammation. After elevation of the mucoperiosteal flap prior to implant placement, all extraction sites of group 1 could be clearly differential from the alveolar crest. Extraction sites of group 2 were either visible or identified using a surgical splint. This ensured the retrieval of samples from the center of the extraction socket. Successful implant placement was performed in all sites.

To ensure reliability of the observer, the intraclass correlation coefficient (ICC) was determined for histomorphometric technique used. The ICC was 0.919 with a 95 % CI of 0.843–0.972, indicating an excellent reliability of the measurements.

A total of 17 surgical sites of 6-week group and 17 sites of 12-week group were quantitatively analyzed in the 29 patients.

5.1. Histological observation

The overall characteristics of the healing sockets were examined in an all samples of both groups. The histologic examination of the samples revealed that the shape and size of the Bio-Oss particles showed clear differences in comparison to natural bone. While natural bone lamellae appeared long and thin, the particles of bone substitute were short, thick and had sharp boundaries. Bio-Oss particles also resembled the feature of necrotic bone material, characterized by a lack of osteocyte nuclei. The osteocyte lacunae were empty and the lamellar layer was indistinct (see Figures 14 and 15). The particles of Bio-Oss identified in the histologic analysis of the current study appeared to be in tight contact within newly formed bone and were well integrated and surrounded by newly formed woven or lamellar bone particularly in the apical region of most samples retrieved after 6 and 12 weeks of healing period (see Figures 18 and 21). Blood vessels were seen to infiltrate some Bio-Oss particles. In the coronal fraction of one half of the 6-week samples and one third of the samples of 12-week samples and in few samples from both healing periods, Bio-Oss remnants were found to be surrounded by provisional connective tissue (see Figures16 and 26).

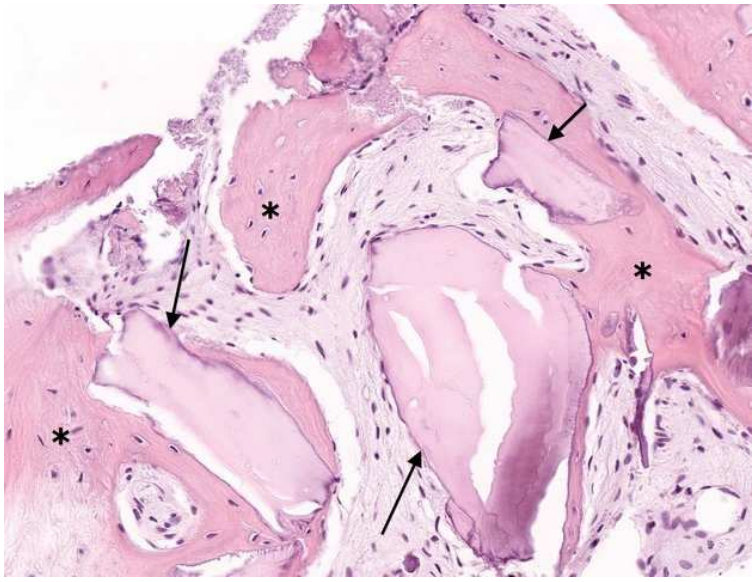

Figure 14: Bio-Oss particles (arrows) show a distinctive shape compared to new bone (asterisk). Osteocyte located in the bone lacunae in newly formed bone. Lack of osteocyte nuclei and empty lacunae in Bio-Oss particle. (H&E staining, 40x).

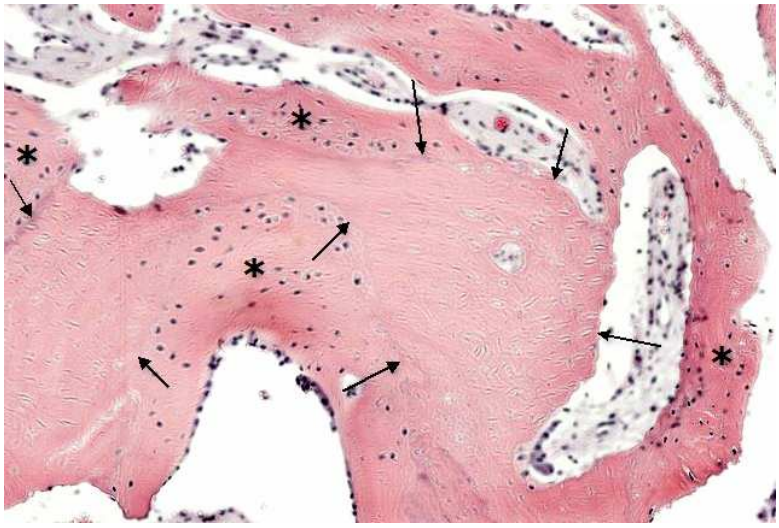

Figure 15: Bovine bone (arrows) appeared with empty lacunae compared to surrounding new bone (asterisk). (H&E staining, 10x).

5.1.1. The gross morphological characteristics of the 6-week tissue samples

Early phase matrix with red blood cells and neutrophil granulocytes embedded in a network of fibrin were not visible in any of the 6-week specimens, rather a maturing provisional matrix, oriented collagen fibers and a developing vasculature was present.

Three main characteristic histological appearances of sites derived after 6-week healing time have been observed. According to these characteristic histological appearances, the extraction sites could be divided into three types (see Table 11).

Type I	Presence of provisional connective tissue dominating the coronal region of the samples is characteristic of this type.
Type II	Presence of mature bone and bone marrow is characteristic of this type.
Type III	Presence of provisional connective tissue predominantly in both the coronal and apical region of the samples is characteristic of this type.

Table 11: Types of extraction sockets of 6-week samples.

5.1.1.1. Samples Type I

The tissue of the extraction sites of Type I contained a large amount of trabeculae of mineralized bone made of woven bone in the apical region surrounded by provisional connective tissue. The Bio-Oss particles were found to be in direct contact with newly formed bone as well as with provisional connective tissue. The tissue of the coronal region was dominated by mature provisional connective tissue rich in fibrocytes and a dense network of collagen fibers and Bio-Oss particles embedded in this provisional matrix. Small newly woven bone trabeculae could be observed (see Figures 16, 17 and 18). This histologic appearance was observed in the majority of the specimens obtained from extraction site after a 6-week healing period (n=10) In fact, all specimens were free of inflammatory cells except one. This specimen showed focal lymphocytic inflammatory infiltrate in the coronal region with bone formation adjacent to the area. The samples displayed an average of 33 % (1 – 57 %) of new bone formation and 12 % (3 - 31 %) of Bio-Oss remnants as well as 54 % (31 - 84 %) of connective tissue.

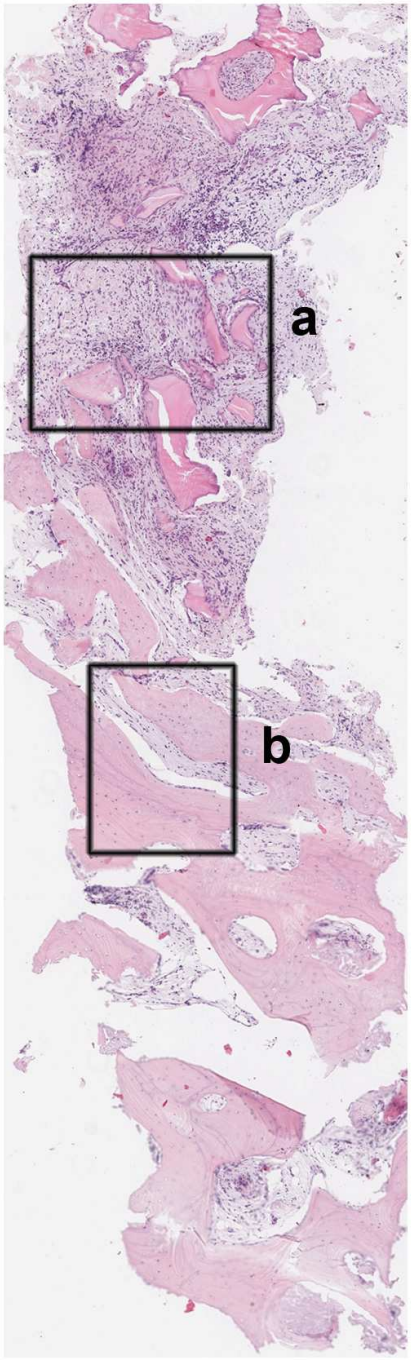

Figure 16: This histologic picture resembles the appearance of Type I specimens obtained from extraction sockets after a 6-week healing period. It shows bone formation in the apical region (b) and mature provisional matrix surrounding Bio-Oss particles in the coronal region (a) with a minor focus of lymphocytes at the surface of the specimen. (H&E staining, 2x).

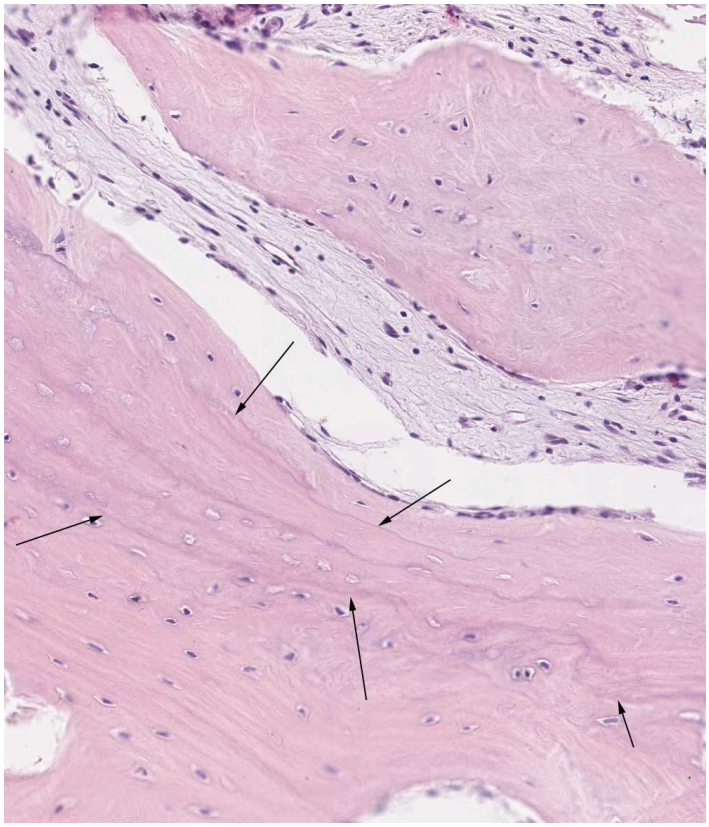

Figure 17: Woven bone surrounding and streaking the barely visible Bio-Oss particle (arrows), with provisional matrix in between. (H&E staining, 40x).

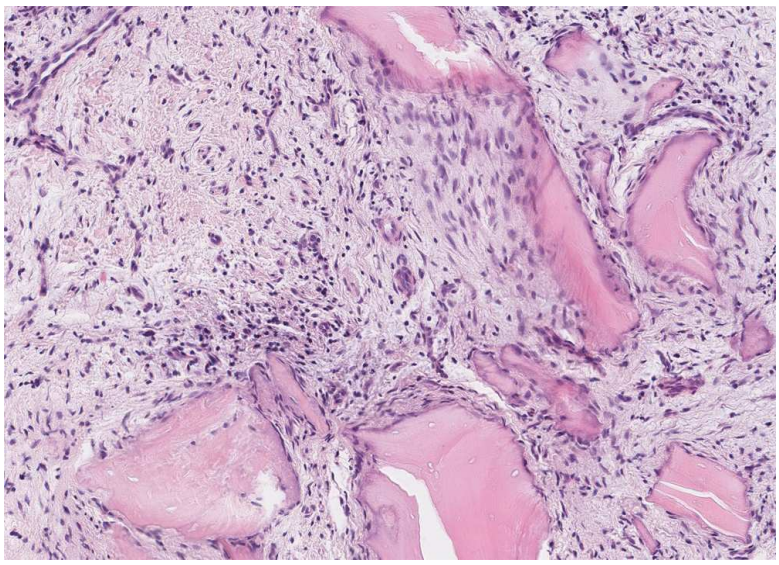

Figure 18: Bone formation around the Bio-Oss remnants next to an island of chronic inflammatory cells (lymphocytes) and provisional matrix with a slit-like blood vessel in the upper left corner.
(H&E staining, 40x).

5.1.1.2. Samples Type 2

The tissue of the extraction sockets of Type II was dominated by newly formed mineralized bone (Figure 19). The apical region showed a large amount of newly formed bone and mature fibroadipose bone marrow made up of densely packed adipocytes. The bone compartment was composed of woven bone and mature lamellar bone and appeared to be in direct contact with relatively large Bio-Oss particles (Figure 20). Large trabeculae of newly mineralized bone occurred also in the coronal portion of the sample with connective tissue containing a few adipocytes and was associated with graft particles with a varying degree of contact. This was observed in 3 samples in which the amount of lamellar and woven bone averaged 46 %, varying between 43 and 49 %. Remaining Bio-Oss particles were estimated at 6, 18 and 24 %. The connective tissue comprised fibroadipose tissue showing an average of 38 % (33 – 45 %).

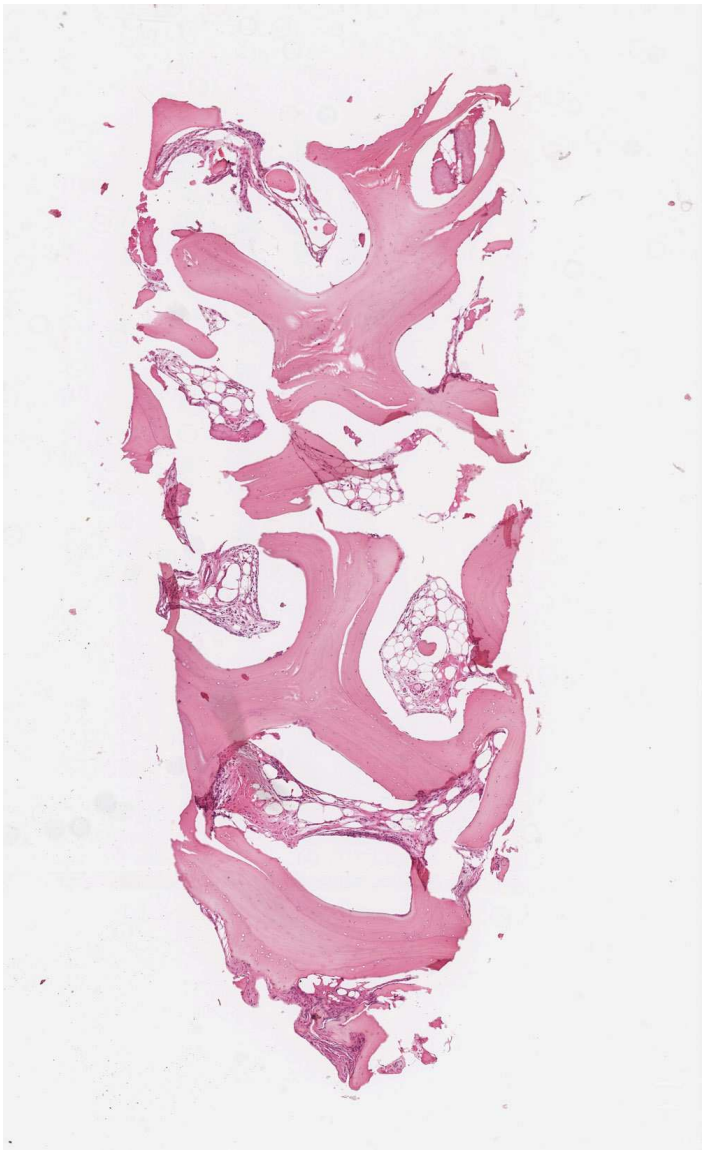

Figure 19: A specimen with > 40 % of new bone formation and mature bone marrow. (H&E staining, magnification 2x).

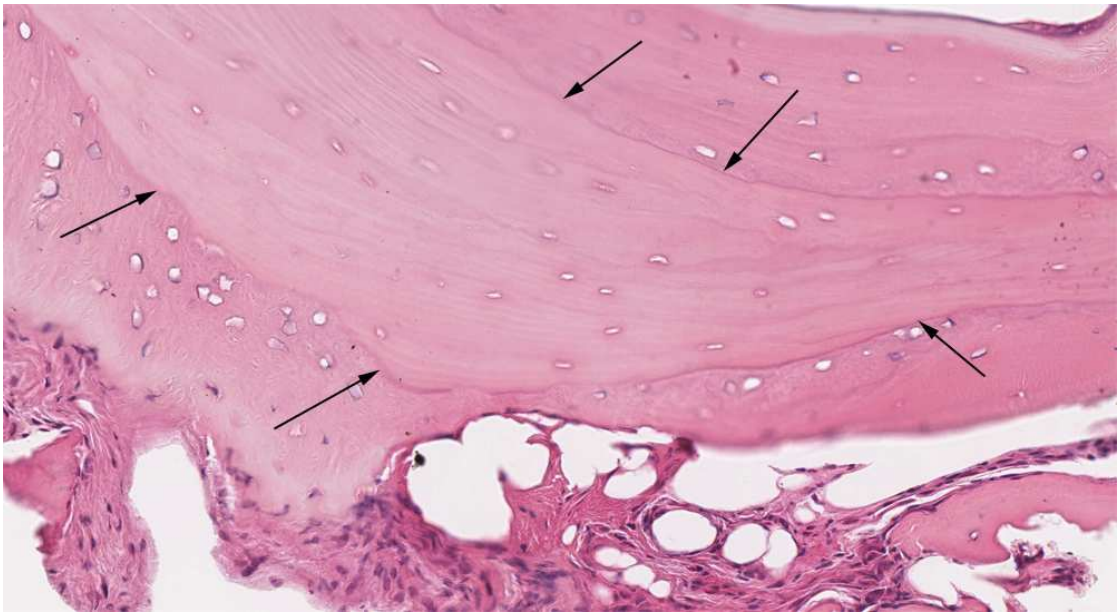

Figure 20: Bio-Oss particle (arrows) surrounded by lamellar bone (top) and woven bone (bottom). (H&E staining, 40x).

5.1.1.3. Samples Type 3

The histological examination of Type III sites revealed an abundance of provisional connective tissue which occupied an average of 71 % (58 – 83 %) of the whole sample (Figure 21). This provisional connective tissue consisted of mature oriented collagen fibers and mesenchymal cells with active fibroblasts (Figures 22 and 23). Bio-Oss particles in the coronal fraction were surrounded mainly by a provisional matrix. Small parts of newly formed bone were visible in close proximity to remnant Bio-Oss particles in this region. The apical region showed a small amount of trabeculae of newly woven bone and ongoing bone formation embedded in oriented collagen fibers. This histological appearance was found in 4 samples of extraction sockets with a 6-week healing period, in which small amounts of newly formed bone ranging from 1 to 19 % and Bio-Oss particles from 13 to 28 % were analysed.

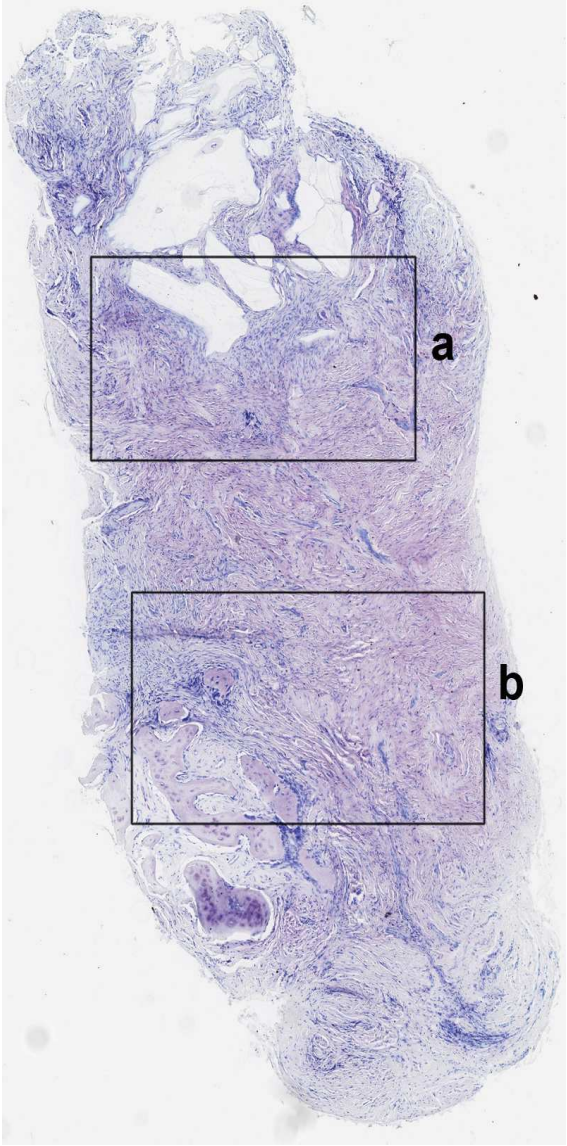

Figure 21: Biopsy predominantly displaying a provisional matrix. Bio-Oss remnants are visible in the coronal region (a) and islands of beginning bone formation located throughout the central and apical portion (b). (Toluidine blue staining, 2x).

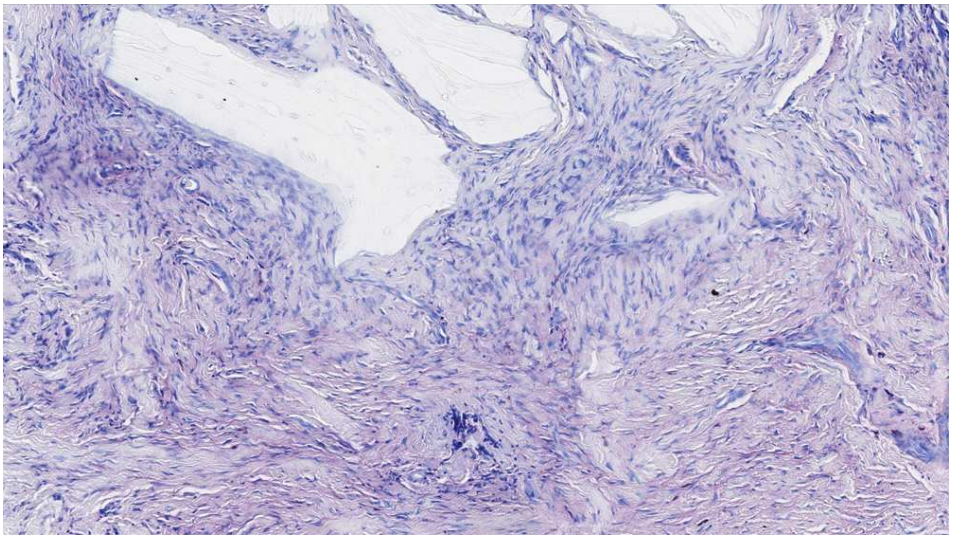

Figure 22: Mature, oriented collagen fibers visible with active fibroblasts and beginning bone formation. (Toluidine blue staining, 20x).

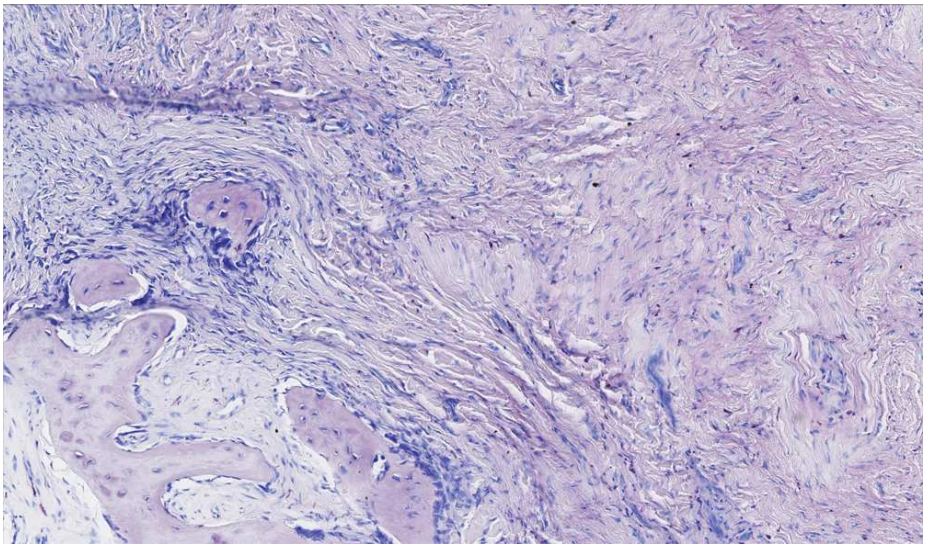

Figure 23: Newly woven bone with osteoblasts and ongoing bone formation embedded in oriented collagen fibers. (Toluidine blue staining, 20x).

5.1.2. The gross morphological characteristics of the 12-week tissue samples

The histologic examination of the 12-week extraction samples revealed an amount of new bone formation comparable to that observed at 6 weeks, but a more mature bone morphology is evident.

Three appearances of tissue were distinguished and extraction sites were also divided into three types (see Table 12).

Type I	Presence of provisional connective tissue dominating the coronal region of the samples with evidence of mature lamellar bone in the apical region characterizes this type.
Type II	Presence of mature lamellar bone and mature bone marrow characterizes this type.
Type III	Presence of provisional connective tissue predominantly in both the coronal and apical region of the samples characterizes this type.

Table 12: Types of extraction sockets of 12-week samples.

5.1.2.1. Samples Type 1

The histologic appearance of tissue of Type I showed many small trabeculae of parallel-fibered und woven bone distributed in connective tissue made up of provisional matrix in the coronal portion. A large amount of Bio-Oss particles surrounded mainly by provisional connective tissue was seen in this type. The apical portion comprised a large area of bone compartment made up of woven and mature lamellar bone, connective tissue, and a few Bio-Oss particles. This histological appearance, was observed in six specimens. The samples displayed an average of 24 % (8 – 37 %) of new bone formation, 18 % (11 - 22 %) of Bio-Oss remnants and 58 % (50 - 74 %) of connective tissue.

5.1.2.2. Samples Type 2

The extraction sockets of Type II were characterized by the presence of trabeculae consisting of mature lamellar bone, mature bone marrow in the apical region and a few Bio-Oss particles (Figures 24 and 25). In the coronal portion of the samples, islands of woven bone enclosed in the provisional connective tissue and associated with graft particles with a varying degree of contact were observed. The connective tissue observed in this part comprised more or less adipocytes. This histological observation was found in 6 samples. The amount of lamellar and woven bone in these samples averaged 31 %, varying between 25 and 41%. The mean of remaining Bio-Oss was 8 % (5 – 12 %). The connective tissue comprised of fibroadipose tissue accounted for an average of 62 % (51 – 65 %) of the samples.

Figure 24: This histologic picture has the appearance of Type II specimens obtained from extraction sockets after a 12-week healing period. Bone marrow and mature lamellar bone are seen in the lower portion. (H&E staining, magnification 2x).

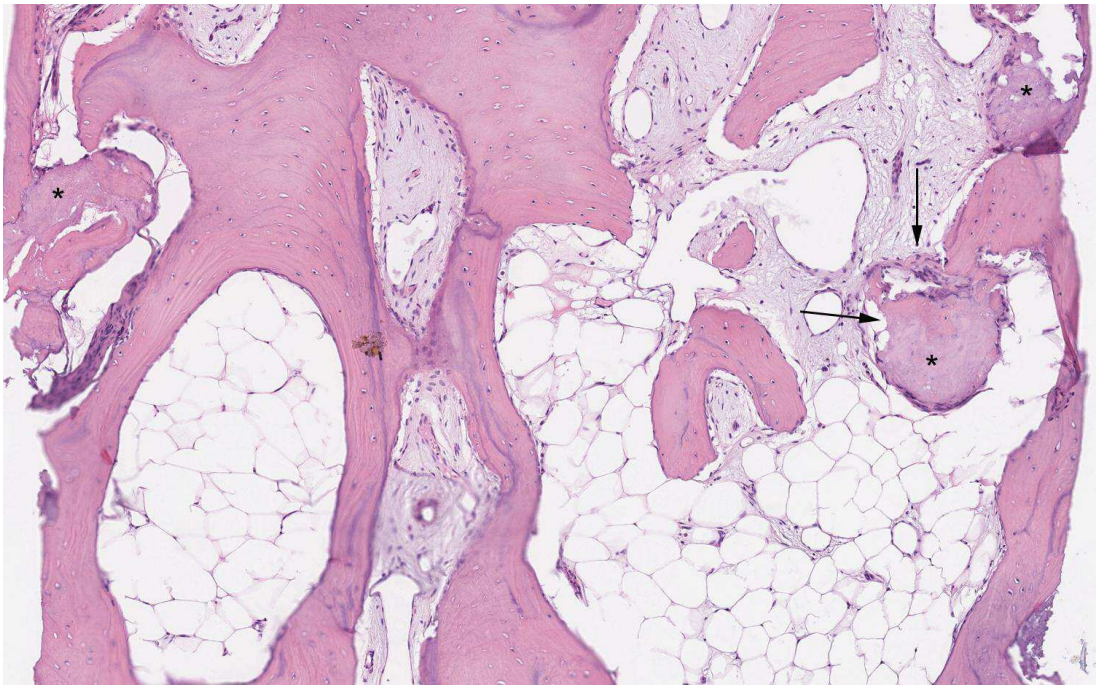

Figure 25: Bio-Oss particles (asterisk) with formation of woven bone and beginning resorption of the xenograft material (arrows). In the lower half mature bone marrow is visible. (H&E staining, 20x).

5.1.2.3. Samples Type 3

The third type of tissue comprised predominantly provisional connective tissue. Bio-Oss particles in the coronal fraction were surrounded mainly by the provisional matrix, where the newly formed bone was composed of woven bone. Trabeculae of varying dimensions were formed in the apical fraction and were composed of woven and less lamellar bone. Connective tissue was made up of collagen fibers and mesenchymal cells (Figure 26). Five specimens showed these histologic characteristics. The provisional connective tissue made up an average of 70 % (61 – 91 %), newly formed bone 8 % (3 – 17 %) and Bio-Oss particles 22 % (17 – 35 %) of the samples.

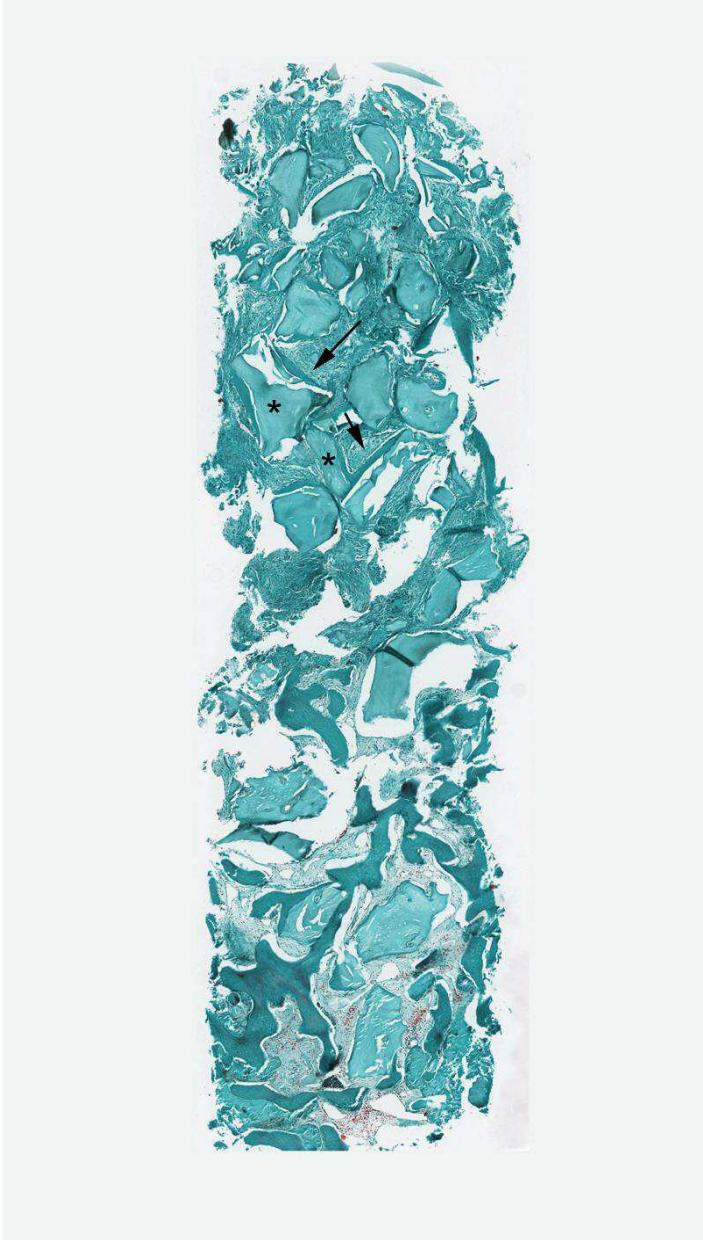

Figure 26: Biopsy displaying beginning bone formation and provisional matrix. Bio-Oss remnants (asterisk) are visible throughout the specimen with islands of beginning bone formation (arrows). The coronal region shows predominantly mature provisional matrix and the apical region (lower portion of the specimen) shows beginning formation of bone marrow. (Masson Trichrome, 2x)

5.2. Histomorphometric measurements

5.2.1. Overall histomorphometric assessments

5.2.1.1. 6-week samples

The histomorphometry revealed that the mean overall new bone formation in 6-week healing period grafted extraction sites was 28 % of the area (range 1 – 57 %) while the amount of Bio-Oss remnants was 11 % (range 3 – 31 %). Connective tissue consisting of collagen and fibroblasts was present in the grafted sites, comprising 54 % (range 31 - 87 %) of the tissue (Figure 27).

5.2.1.2. 12-week samples

After a 12-week healing period, the morphometric assessments showed that the mean overall new bone formation in the grafted extraction sockets was 25 % of the area (range 3 – 41 %). The amount of Bio-Oss remnants was 15 % (range 5 – 35 %). Connective tissue consisting of collagen, fibroblasts, or bone marrow was present in the grafted sites, comprising 60 % (range 50 - 91 %) of the tissue (Figure 28).

5.3. Statistical comparison between 6-week and 12–week samples

There is no significant difference between the quantity of newly formed bone after a 6-week and a 12-week healing period (P=0.090). No significant difference between the rate of Bio-Oss remnants (P=0.983) or the rate of connective tissue (P=0.69) was found in either group.

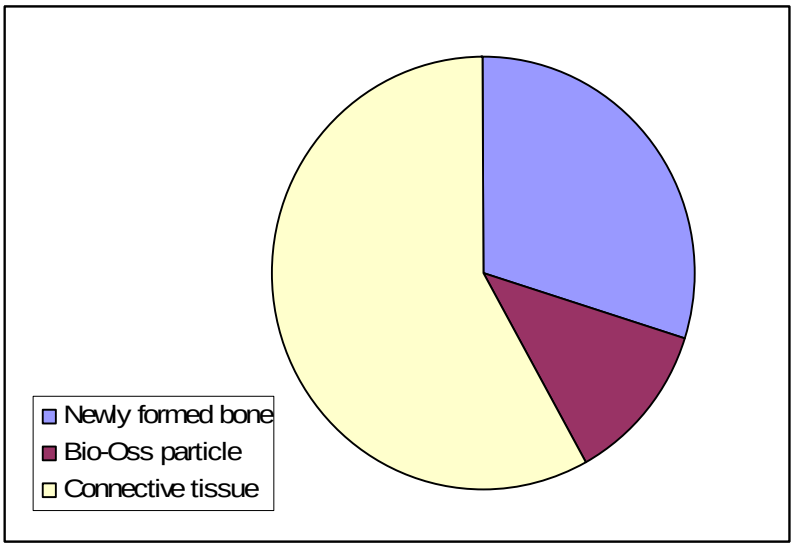

Figure 27: Histomorphometric findings for overall mean value of 6-week samples.

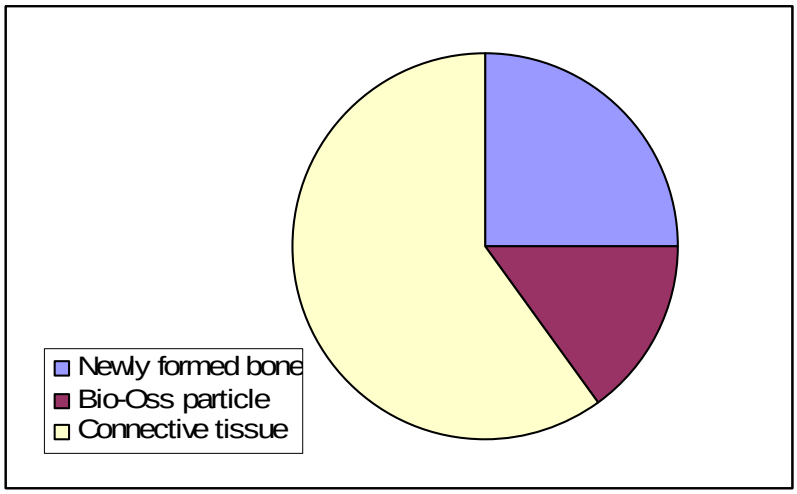

Figure 28: Histomorphometric findings for overall mean value of 12-week samples.

5.2.2. Individual histomorphometric assessments

5.2.2.1. 6-week samples

The histomorphometric evaluation of each extraction site of the 6-week samples is shown in Figure 29.

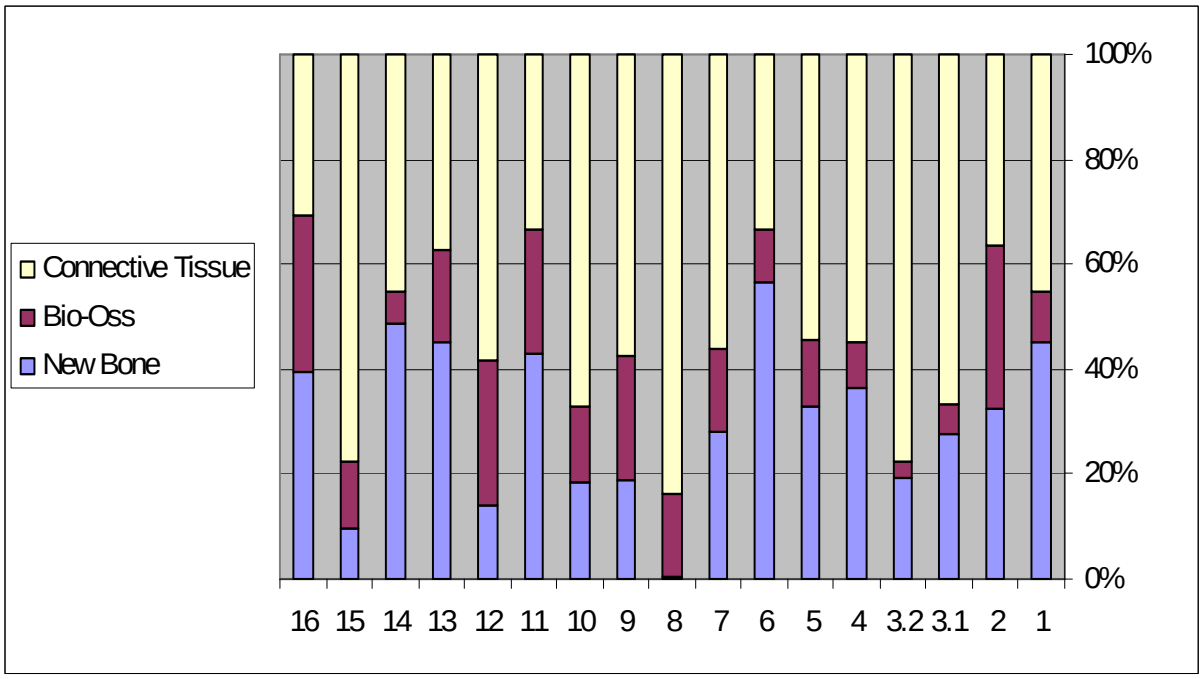

Figure 29: Mean % area of bone, Bio-Oss particles and connective tissue of 6-week grafted sockets for individual patient.

In five of the 6-week specimens, the amount of newly formed bone was more than 40 % of the tissue and showed an average of 47 % (range from 43 – 57 %), whereas the remaining Bio-Oss particles varied from 6 % to 24 % and the connective tissue from 33 % to 45 %. In six samples, newly formed bone accounted for less than 20 % of the area, connective tissue for 70 % (range 58 – 84 %) and Bio-Oss particles for 16 % (range 3 - 27%). The remaining samples (n=6) showed new bone formation representing 20 – 40 % of the area with an average of 33 % and 17 % (range 5 – 31 %) of Bio-Oss remnants and 50 % (range 37 – 67 %) of connective tissue.

5.2.2.2. 12-week samples

The histomorphometric evaluation of each extraction site of the12-week samples is shown in Figure 30.

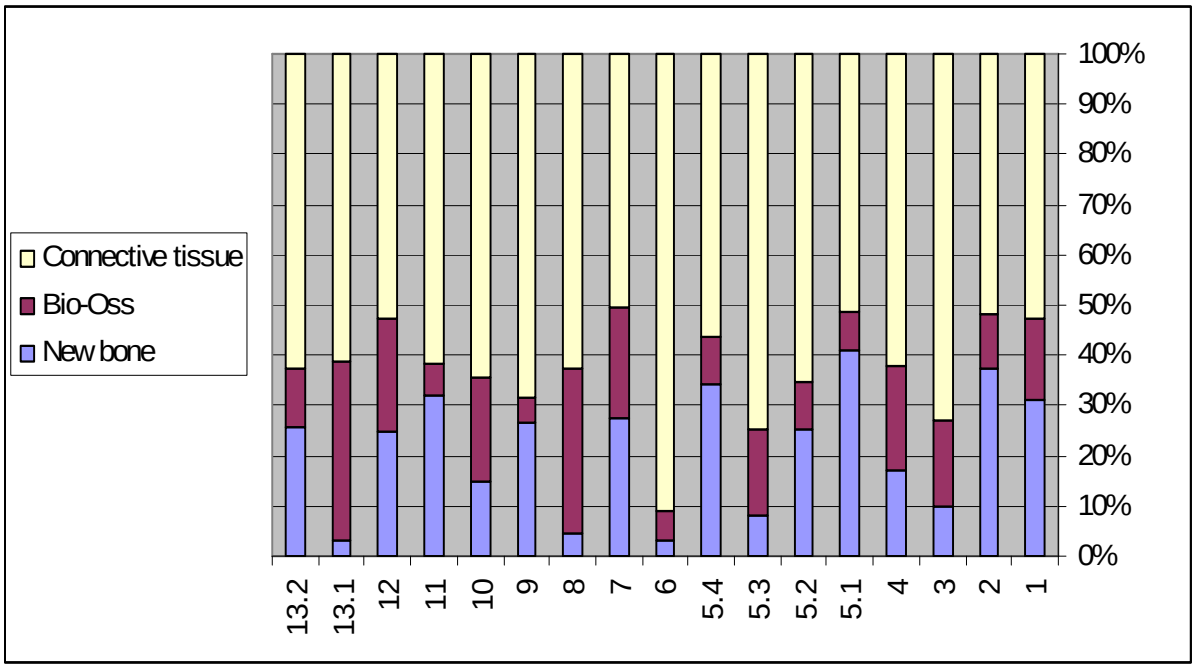

Figure 30: Mean % area of bone, Bio-Oss particles and connective tissue of 12-week grafted sockets for individual patient.

Only one sample of the 12-week healing period extraction sites showed a mean of newly formed bone of more than 40 %. The amount of bone in this sample was 41 % whereas Bio-Oss particles and connective tissue made up 8 % and 51 %, respectively, of the tissue. In seven samples, newly formed bone accounted for less than 20 % of the area, connective tissue for 70 % (range 61 – 91 %) and Bio-Oss particles for 20 % (range 5 – 35 %). The majority of samples (n=9) showed new bone formation between 20 – 40 % with an average of 27 %, and 11 %(range 5 – 22 %) of Bio-Oss remnants as 58 % (range 52 – 68 %) of connective tissue.

5.2.3. Morphometric evaluation with regard to region

5.2.3.1. 6-week samples

The specimens collected from the molar region in the 6-week healing period group (n=14) showed a mean of newly formed bone of 30 % (range 9 – 57 %), 15 % (range 3 – 31 %) of Bio-Oss particles, and 55 % (range 31 – 77 %) of connective tissue. The sample retrieved from the extraction sockets of the molar region (n=11) of the maxilla showed a mean of newly formed bone of 33 % (range 9 – 57 %), 54 % (range 31 – 77 %) of connective tissue, and 4 % (range 3 – 31 %) of Bio-Oss particles. The Bio-Oss Collagen-grafted areas in the molar region (n=3) of the mandible displayed 33, 19 and 14 % of newly formed bone and 13, 23 and 18 % of Bio-Oss remnants in the biopsies obtained from these regions respectively. The connective tissue represented 54, 58 and 58 % of the areas, respectively. The Bio-Oss Collagen-grafted areas in the canine of the maxilla displayed 13 % of Bio-Oss particles, 10 % of newly formed bone and 77 % of provisional connective tissue. The specimens obtained from the premolar region (n=2) of the maxilla showed 45 % and 49 % of newly formed bone, 18 % and 6 % of Bio-Oss particles and 37 % and 45 % of connective tissue, respectively (Figure 31).

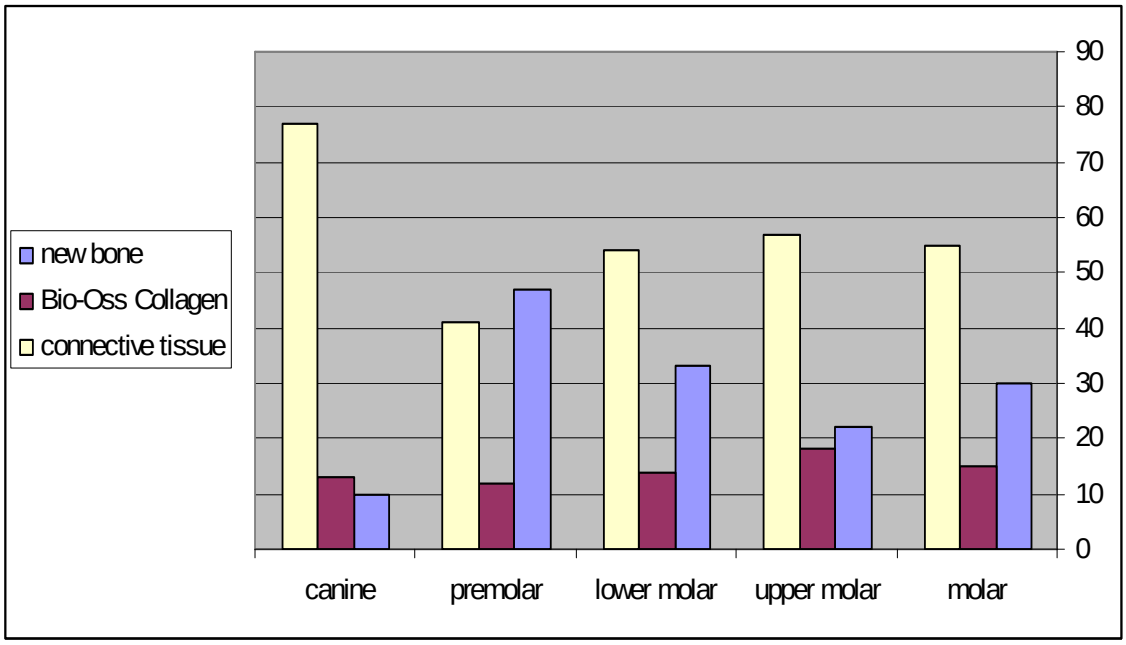

Figure 31: Histomorphometric findings for different regions of teeth in 6-week group.

5.2.3.2. 12-week Samples

The specimens collected from the molar region (n=5) in the 12-week healing period group, mainly from the mandible (n=4), showed a mean of newly formed bone of 13 % (range 3 – 27 %), 17 % (range 5 – 33 %) of Bio-Oss particles, and 70 % (range 52 – 91 %) of connective tissue. The Bio-Oss Collagen-grafted area in the molar region (n=1) of the maxilla displayed 8 % of newly formed bone, 17 % of Bio-Oss and 75 % of connective. The sample retrieved from the extraction sockets of the molar region (n=4) in the mandible showed a mean of newly formed bone of 15 % (range 3 – 27 %), 68 % (range 52- 91 %) of connective tissue, and 17 % (range 5 – 33 %) of Bio-Oss particles.

The Bio-Oss Collagen grafted areas in the premolar region (n=8) of the maxilla displayed 29 % (range 15 – 41 %) of newly formed bone and 13 % (range 6 – 21 %) of Bio-Oss remnants in the biopsies obtained from these regions, respectively. The connective tissue occupied 58 % (range 57 – 64 %) of these regions. The sample retrieved from the extraction socket of the anterior teeth region (n=4) showed a mean of newly formed bone of 18 % (range 3 – 27 %), 62 % (range 61 – 72 %) of connective tissue, and 20 % (range 10 – 35 %) of Bio-Oss particles (Figure 32).

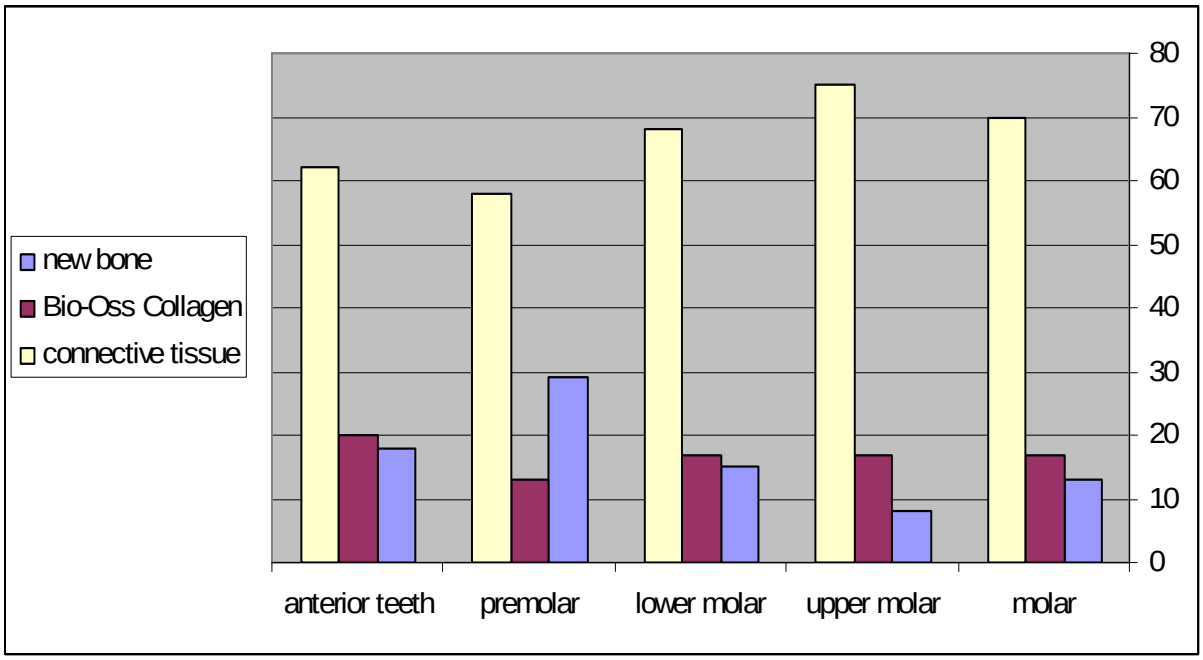

Figure 32: Histomorphometric findings for different regions of teeth in 12-week group.

5.2.4. Morphometric evaluation with regard to localization

5.2.4.1. 6-week Samples

The amounts of newly formed bone, Bio-Oss remnants and connective tissue in the coronal and apical region of the 6-week specimens are summarized in Table 13.

Patient Number	Region within jaw (FDI)	Localization within Specimen	New bone (%)	Bio-Oss Particles (%)	Connective Tissue (%)
1	26	coronal	37	17	46
		apical	53	3	44
2	16	coronal	27	35	38
		apical	38	27	35
3.1	26	coronal	27	3	70
		apical	28	8	63
3.2	27	coronal	15	3	82
		apical	23	4	73
4	16	coronal	20	18	62
		apical	52	1	47
5	37	coronal	24	20	56
		apical	42	5	53
6	16	coronal	53	17	30
		apical	60	4	36
7	16	coronal	7	21	72
		apical	50	10	40
8	16	coronal	1	22	77
		apical	1	10	89
9	46	coronal	12	31	57

		apical	26	16	58
10	16	coronal	16	17	66
		apical	21	11	68
11	16	coronal	33	36	30
		apical	52	11	37
12	36	coronal	1	55	44
		apical	27	1	72
13	15	coronal	45	10	45
		apical	45	25	30
14	14	coronal	34	7	59
		apical	63	6	31
15	13	coronal	1	23	76
		apical	19	3	78
16	16	coronal	29	28	43
		apical	50	32	18

Table 13: Mean percentage of the tissues found in the histologic specimens of 6-week extraction sockets with regard to localization.

There was a variation of the amount of tissues in the apical compared to the coronal portion of the biopsies retrieved from 6-week grafted extraction sockets. The apical portion of the specimens consisted of a mean of 40 % of new bone formation within a range of 1 to 63 %. Up to 10 % of remnant Bio-Oss particles were found in this region ranging from 1 to 32 %, and the connective tissue consisted of 50 % of the specimen with a range of 18 to 89 %. The coronal region had a mean of 20 % new bone formation (1 – 53 %) and 20 % of remaining Bio-Oss particles (3 – 55 %) with 60 % of provisional matrix (30 – 83 %) visible. The rate of newly formed bone was significantly different between the apical and the coronal regions (P=0.002). The amount of connective tissue and Bio-Oss remnants did not show a significant difference between the apical and the coronal regions within the specimens (P=0.40, P=0.10).

5.2.4.2. 12-week Samples

The amounts of newly formed bone, Bio-Oss remnants and connective tissue in the coronal and apical region of the 12-week specimens are summarized in Table 14.

Patient's Number	Region within jaw (FDI)	Localization within Specimen	New bone (%)	Bio-Oss Particles (%)	Connective Tissue (%)
1	24	coronal	26	6	68
		apical	37	26	37
2	24	coronal	47	5	48
		apical	28	16	56
3	11	coronal	3	19	78
		apical	17	15	68
4	15	coronal	18	18	64
		apical	17	23	60
5.1	14	coronal	31	9	60
		apical	50	7	43
5.2	11	coronal	18	15	67
		apical	32	4	64
5.3	26	coronal	3	14	83
		apical	14	19	67
5.4	15	coronal	24	10	66
		apical	46	9	45
6	46	coronal	2	10	88
		apical	7	1	92
7	32	coronal	10	8	82
		apical	45	37	18
8	47	coronal	5	33	62

		apical	5	32	63
9	46	coronal	19	8	73
		apical	35	2	63
10	24	coronal	10	30	60
		apical	23	11	66
11	14	coronal	24	9	67
		apical	42	4	54
12	47	coronal	16	30	54
		apical	33	16	51
13.1	21	coronal	4	38	58
		apical	3	33	64
13.2	25	coronal	15	18	67
		apical	36	7	58

Table 14: Mean percentage of the tissues found in the histologic specimens of 12-week extraction sockets with regard to localization.

A variation of the amount of tissues in the apical compared to the coronal portion of the 12-week sample existed. The apical portion of the specimens consisted of a mean of 28 % of new bone formation within a range of 3 to 50 %. Up to 15 % of remnant Bio-Oss particles were found in this region ranging from 1 to 37 %, and the connective tissue made up 57 % of the specimen with a range of 19 to 92 %. The coronal region had a mean of 16 % new bone formation (2 – 47 %) and 17 % of remaining Bio-Oss particles (5 – 38 %) with 67 % of fibrous tissue (48 – 88 %) visible. The rate of new bone formation was significantly different between the apical and the coronal regions (P=0.01). The amount of connective tissue and Bio-Oss remnants did not show a significant difference between the apical and the coronal regions within the specimens (P=0.7, P=0.4).

6. Discussion

The healing of the extraction socket includes a series of events, from the stabilization of the blood coagulum to bone formation, maturation and remodeling (Amler et al. 1960, Cardaropoli et al. 2003). Simultaneously, morphological and dimensional changes of the alveolar ridge take place and a reduction of 50 % of the original volume occurs within the first 12 months after extraction. Most of this ridge tissue collapse appears within the first 3 months (Schropp et al. 2003). Such changes often result in esthetic compromises in the area of tooth extraction or inadequate bone for implant placement or ideal implant positioning (Fugazzotto 2005, Irinakis 2007). Current animal studies revealed that Bio-Oss Collagen minimizes the shrinkage of the alveolar ridge after a 12-week healing period (Araujo et al. 2008, Fickl et al. 2008). In the present study, the histologic observation of the Bio-Oss Collagen filled socket and the histomorphometric measurement of newly formed bone, Bio-Oss collagen and connective tissue volume was performed in a relatively early stage of extraction socket healing after 6 and 12 weeks.

6.1. Discussion of patient selection and method

Several systemic and local factors as well as patient habits could influence the process of healing and the outcome of therapy procedure. These factors may affect ridge alteration and bone formation and remodeling after tooth extraction.

All patients participating in this study were healthy, with the influence of some systemic diseases altering normal bone physiology and metabolism being excluded. Diabetes is known to alter tissue integrity, impair wound healing and increase susceptibility to infection (Klokkevold and Thomas 2007). The formation of the collagenous framework in the tooth extraction socket was found to be inhibited in diabetes patients, resulting in delayed healing and increased alveolar destruction (Grandini 1978, Devlin et al. 1996). Osteoporosis was found to be associated with reduced and irregular bone formation during healing of extraction socket and delayed healing time (Iizuka et al. 1992, Erdogan et al. 2007). Smoking is known to affect hard and soft tissue healing in many ways, including arteriolar vasoconstriction which alters the blood flow to tissue and diminishes oxygen

perfusion causing cellular hypoxia. Some study showed that nicotine reproducibly inhibits osteogenesis, delays revascularization and may postpone post extraction socket healing. (Rosen et al. 1996, Hoogendoorn et al. 2002, Saldanha et al. 2006, Ziran et al. 2007, Klokkevold and Thomas 2007, Glowacki et al. 2008).

Absence of infection is mandatory for the success of the grafting material. The invasion of bacteria into the grafted site causes inflammation with a resultant decrease in bone formation, and bone and grafting material rapidly resorbs in the condition of a low pH (Smion et al. 1994, Misch 1999). Osseous regeneration in extraction socket with previously advanced periodontal disease was found to develop more slowly than disease-free sockets (Ahn and Shin 2008). To eliminate the influence of these local factors, patients with severe periodontitis or active periodontal lesions comprising periapical abscesses and teeth with evident periapical radiolucency were excluded from this study.

The tooth extraction procedure and the condition of socket wall are important factors in the healing pattern of alveolar ridge. More alveolar ridge shrinkage and bone loss was related to surgical trauma, including flap elevation in canines (Fickle et al. 2008 a) and humans (Atwood 1963). Although one experimental study in canines showed that flap elevation did not influence the long-term dimensional alterations of the extraction sites (Araujo and Lindhe 2009). Surgical trauma often results in loss or fracture of the bone plate which affects bone formation within the socket, as fibrous tissue could grow into the socket and less blood supply is provided due to the loss of bony walls (Misch and Dietsh 1993). By elevating the periosteum, the blood supply of the denuded bone surface will be compromised leading to osteoclastic activity and bone loss (Wood et al. 1972, Fickle et al. 2008 a). In the current study, care was taken to ensure atraumatic tooth extraction without mucoperiosteal flap elevation using a periotome and appropriate forceps and avoiding buccolingual forces. Only intact 4-wall sockets were included to ensure a better hold of the bone graft and a revascularization from the surrounding tissue.

The curettage of the alveolar walls was performed not only to remove granulation tissue and periodontal ligament but also to stimulate bleeding from the osseous structure which encloses the grafting material. This stimulation helps to supply the graft with blood in order to provide osteoprogenitor cells and associated growth factors essential for bone formation (Misch 1999).

With regard to the biopsy retrieval procedure, surgical splints were fabricated and used to identify the extraction sites to ensure that the biopsy cores were taken from the center of extraction sites.

To ensure high accuracy of the quantitative histomorphometric assessment of the samples, the observer had to repeat the histomorphometric analysis of each slide three times at different time points. Repeated assessment is used to make the histomorphometric measurements as accurate as possible (Revell 1983) besides determining the intra-observer reliability. There was no considerable variation with respect to the assessed data at each time point, suggesting an excellent reliability of the measurements as the ICC indicated. To eliminate the influence of microscopic and line-scanning resolution on the results of the histomorphometry (Revell 1983), only a 40x objective was used for the line-scanning of the sections and for the data analysis. Furthermore, the calibration of the image of each sample was checked manually prior to the histologic evaluation to confirm the automatic calibration of the program in order to ensure reliable measurements.

6.2. Discussion of results

The present histomorphometric investigation of Bio-Oss Collagen-filled extraction socket demonstrated pronounced bone formation after healing periods of 6 and 12 weeks. Data also showed that there were large variations in bone formation within extraction sockets of both healing period groups. Sockets were filled with mature or immature bone in the lower half or two thirds in most specimens retrieved 6 or 12 weeks after extraction time. Bone fill was only restricted to the lower one third in 4 samples of the 6-week group and in 5 samples in the 12-week group. No significant difference between the quantity of newly formed bone after 6 and 12 week healing periods was found.

Apart from the individual variation, the picture of bone formation in 6-week sockets is largely consistent with early human studies (Amler et al. 1960, Carlsson et al. 1966) showing equivalent time points for the formation of bone in unfilled sockets. Amler had examined, in his frequently cited study, human extraction socket biopsies at time intervals up to 50 days. He stated that after about 6 weeks of healing, two-thirds of the socket were filled with mineralized bone (Amler et al. 1960). However, a comparison with the findings of

present study is limited, as the study by Amler does not document the rate of bone formation, nor histomorphometric assessment.
Consistent with the present study, Calsson et al. reported that large variation of bone formation within human extraction sockets occurred 40 days and 3 months after extraction. They found that most sockets after a 40-day healing time displayed moderate bone formation towards the centre of the socket and only 25 % of sockets showed two thirds bone fill, whereas 50 % of 3 months extraction socket were largely filled with osseous tissue and the rest showed moderate or restricted bone fill (Calsson et al. 1966). Yet, the study lacks a histomorphometric analysis of the tissue which could be compared to data provided by the current study (Heberer et al. 2008).
Serino et al. reported a slightly higher rate of overall bone formation in polylactide grafted extraction sites after 3 months compared to those observed in the present study with an equal pattern of healing (Serino et al. 2008).

The present study indicates that bone compartments compromising the majority of the 6-week grafted extraction sockets showed mainly immature mineralized woven bone with an average rate of 33 %. In 20 % of the 6-week defects evaluated, over 40 % of mature lamellar bone with bone marrow was seen, suggesting an advanced stage of remodeling. Only 2 sockets displayed a bone formation lower than 10 % surrounded by mature provisional matrix with bone formation beginning. Osseous structures composing the 12-week grafted extraction sockets exhibited higher maturity as trabeculae consisting of mature lamellar bone with secondary osteons surrounded by bone marrow and were markedly observed in more than one third of samples and composed up to 40 % of the tissue volume. The average bone formation rate was 31 %. On the other hand, bone formation in 3 defects of the 12-week group was less than 10 % and mature provisional matrix was predominated in these defects. In conclusion, the amount of new bone at 12 weeks was comparable to that found at 6 weeks, but on histologic evaluation of the biopsy specimens a more mature bone morphology is evident.

Two studies of ungrafted extraction sockets are available to give comparative histologic and histomorphometric data about human wound healing (Trombelli et al. 2008, Ahn and Shin 2008).

A study on unfilled extraction sockets in humans found that the rate of de novo bone formation varied markedly between subjects and was slightly higher in the 6 - 8 week healing period group (34 %) compared with that of 12 - 24 week interval group (32 %) (Trombelli et al. 2008). This finding is largely consistent with the finding of the current study with a little higher bone formation rate. In a previous study done by Trombelli et al., 6 sites were evaluated in the 6 - 8 week healing interval group and 11 sites in the 12 to 24 weeks healing interval group and the exact time point of biopsy retrieval in each group was not mentioned. Thus, the amount of bone formation at a specific time point of healing could not be concluded from their study. Ahn and Shin reported about 50 % new bone formation in ungrafted human extraction socket after a 8-week healing period (Ahn and Shin 2008). This result is only in concordance to the finding of 20 % of 6-week extraction sockets made in the current study, where a similar rate of newly formed bone was estimated. Nevertheless, only 3 specimens were evaluated in the study done by Ahn and Shin for the corresponding time point, hence, information on possible variation of the rate of de novo bone formation between subjects can not be derived from the study.

Studies in canine models have shown a bone formation rate of up to 80 % after 30 days and up to 23 % after 60 days which is equivalent to a 40 and 90 day healing period, respectively, in humans as the physiologic bone turn-over in dogs is 1.5 x that of humans (Cardaropoli et al. 2003, Pearce et al. 2007). The average bone formation rate found in the specimens obtained in the current investigation showed an average bone formation rate that is lower than that found in the extraction sockets in the mandible of dogs. In the study performed on dogs, 2 defect sites where evaluated for each time point (1, 3, 7, 14, 30, 60, 90, 120 and 180 days) and the specimens retrieved at 30 days show a high rate of bone formation and low rate of provisional connective tissue, these sites might therefore represent the extraction sites with a high bone formation rate. The existence of bone marrow and lamellar bone as seen in samples in this study of human biopsies has been described at 90 days in unfilled extraction sockets in the canine model. Bone marrow

formation appears to be concomitant with the remodeling of the woven bone into lamellar bone with a synchronistic reduction of the amount of bone by osteoclastic activity (Cardaropoli et al. 2003) and a reduction of osteoblasts (Evian et al. 1981).
The findings of this study corroborate the assumption that bone formation follows the mechanism described by Amler et al. in man and Cardaropoli et al. in canine models, that after an initial boost of bone formation there is a maturation of the bone.
An early study on human extraction socket by Evian described a similar pattern of bone healing over a 16 weeks healing period. In that study 10 sites were examined at 6 time points (4, 6, 8, 10, 12, and 16 weeks). He concluded that a progressive osteogenic phase of bone healing is apparent from the 4^{th} week to the 8^{th} week after extraction and after that the bone formation rate slows down and bone components mature gradually and show signs of remodeling. The extraction socket was found to be completely filled with bone at the 10^{th} week. Evian does not provide detailed information on how many specimens were examined for each time point and whether or not there was primary wound closure after the extraction procedure, therefore information on a possible variation in the stage of maturation can not be derived.

Taken together, the data acquired in the present study, compared to previous studies, which were from ungrafted extraction sites, indicate a possible retardation of new bone formation and maturation in sockets filled with a xenograft material. Findings from experiments in canine models that investigated surgically created defects and extraction sockets filled with Bio-Oss Collagen corroborate this hypothesis (Cardaropoli et al. 2005, Araujo et al. 2008, Araujo et al. 2009). The placement of Bio-Oss Collagen in fresh extraction socket of canine mandible was found to delay the socket healing after two weeks of extraction since the newly formed bone made up 48 % of non-grafted socket compared with only 14 % of bone and 18 % of xenograft remnants in the grafted site (Araujo et al. 2009). Furthermore, it was observed that a xenograft placed in the extraction socket and defect in canine after a 3-month period of healing appeared to interfere with the process of remodeling and maturation taking place in the socket. Thus, non-grafted defects showed higher rate of bone marrow and mature lamellar bone formation but lower rate of overall bone formation when compared with grafted defects (Cardaropoli et al. 2005, Araujo et al.

2008). Heterologous grafting material is known to interfere with bone formation in wound sites and decelerate bone formation (Dies et al. 1996, Buser et al. 1998, Becker et al. 1998, Santos et al. 2008) without the existence of a foreign body reaction (Tapety et al. 2004, Carinci et al. 2006).

The specimens analyzed after 6-week and 12-week healing time in the current study showed a low bone formation rate in the apical region in comparison with the coronal region. The rate of newly formed bone was found to be significantly different between the two regions in the 6-week group as well as in the 12-week group. Thus, it would appear that bone formation in the extraction socket, when primary wound closure is not performed, is initiated from the apical region, as this showed a higher rate of bone formation compared to the coronal region. This new bone formation is also not enhanced from the coronal direction after epithelial closure of the extraction site.

As evidenced in previous studies in humans, bone formation in extraction socket takes place as early as 7-10 days after extraction and is found first at the base and lateral aspects of the socket (Amler et al. 1960, Boyne 1966, Carlsson et al. 1966). Trabeculae of new bone were found to form and fill gradually the apical 1/3 of the socket after 19 days (Boyne 1966) and the apical 2/3 after 38 days. Thus, bone formation would be initiated from the apical or lateral region of the extraction socket.

The biopsies obtained in this study demonstrate a partial area of the healing socket allowing the assessment of the healing process of the apical and coronal region. Whether bone formation was also initialized from the sides of the socket can not be determined from the data of this study.

The formation of new bone in extraction sockets in rodents and canines has been correlated to the existence of cells from the periodontal ligament (PDL) in the early phase of healing (Lin et al. 1994, Cardaropoli et al. 2003). Lin et al. used a cell labeling technique to follow the fate of PDL fibroblasts in extraction sockets of rat. They concluded that PDL fibroblasts proliferated and migrated into the center of the extraction socket, where they differentiated into osteoblasts that became involved in the formation of new bone. A similar observation was made by Cardaropoli et al. 2003 who demonstrated that vital PDL cells

adjacent to socket walls apparently migrated into the provisional connective tissue residing in the socket during the first week of healing following tooth extraction and contributed to hard tissue formation within the healing socket. On the other hand, an experimental study in canines has shown no correlation between successful new bone formation and the presence of PDL cells, as sockets that had their PDL removed exhibited similar wound healing characteristics to those that had retained the PDL (Cardaropoli et al. 2005). Hence, PDL cells, if they exist in the extraction socket, may contribute to bone regeneration in addition to osteoblasts originate from bone marrow of alveolar bone walls (Devlin and Sloan 2002).
Within this study, all PDL was removed from the extraction sockets as they where instrumented thoroughly after root removal, minimizing the importance of the PDL for the formation of bone in human extraction sockets.

A hard tissue bridge at the marginal entrance of extraction socket was described in experimental animal studies (Cardaropoli et al. 2003, Rothamel et al. 2007, Araujo et al. 2008, Fickl et al. 2008). In these studies, the extraction sockets of canines were examined after three to four months of primary wound healing. The histologic observation showed that a bridge of mineralized bone seals the entrance of the socket. In the current study, this hard tissue bridge was not seen in either 6-week or 12-week samples, and this is consistent with the data from human studies (Amler et al. 1960, Arzi et al. 2000, Carmagnola et al. 2003, Norton et al. 2003, Trombelli et al. 2008). Amler et al. did not describe a hard tissue bridge at the marginal entrance of extraction sockets in humans after 38 days, where primary closure was not performed. Nor was the bone bridge observed in human studies, when primary tissue closure was carried out and extraction sockets were evaluated in a time period extended from 2 to 12 months after extraction (Arzi et al. 2000, Carmagnola et al. 2003, Norton et al. 2003, Trombelli et al. 2008). However, coronal corticalization at the crestal margin of non-grafted human extraction sockets after a 10-week healing period was only reported by one study (Ahn and Shin 2008).

Within this study the extraction sockets filled with the Bio-Oss Collagen were left open to heal and wound closure was achieved by gradual lateral epithelial overgrowth. Epithelial

proliferation over the surface of the extraction wound in the unfilled extraction socket in humans was described in early studies (Amler et al. 1960, Carlsson et al. 1966, Amler 1969). The epithelium was found to grow from the alveolar border and to invaginate into the extraction socket with incomplete wound closure after 24 days and a not yet complete fusion of the touching adjacent epithelium after 35 days (Amler et al. 1960). Experimental animal studies showed that the degree of epithelium invagination with Bio-Oss Collagen-filled mandibular defects seems to be decreased in comparison to unfilled defects (Cardaropoli et al. 2005) and suggest that the mechanical stability provided by the graft filled in canine extraction socket could act as placeholder or a scaffold for the epithelialization (Fickl et al. 2008, Araujo et al. 2008). Within this study, wound closure was seen after 40 days in all patients with varying degrees of thickness of the overlying mucosa. Soft tissue collapse into the socket was not observed which supports the scaffolding function of the heterologous material suggested by animal studies. A quantification of the thickness and the degree of invagination of the epithelium cannot be concluded from this study, as it was not evaluated.

In comparison to the findings of this study, human extraction sockets filled with Bio-Oss revealed higher rates of Bio-Oss particles after a healing period of 8 to 9 months (Artzi et al. 2000, Carmagnola et al. 2003, Norton et al. 2003), whereas an equivalent rate of remnants was found in extraction sockets filled with Bio-Oss Collagen in canine with primary wound closure after 3 months of healing (Araujo et al. 2008).
Bio-Oss Collagen consists of Bio-Oss spongiosa granules embedded in a 10 % highly purified porcine Collagen Type I in a block form to ensure the cohesion of the spongiosa particles and when not compressed into the empty alveolus as performed in the current study, the Bio-Oss does not completely occupy the alveolar space. Whereas after application of pure Bio-Oss granules, the granules populate more space in the socket, resulting in a higher quantity of graft particles found after the same observation period. The extraction sockets in this study were filled without condensed placement of the grafting material and were left open to heal, thus not preventing minor displacement of the Bio-Oss particles into the oral cavity, which could contribute to a diminished amount of Bio-Oss particles within the biopsy specimens compared with following primary wound healing

grafted sockets in humans (Artzi et al. 2000, Carmagnola et al. 2003, Norton et al. 2003). There was no significant difference in the amount of particles found between the apical and coronal portion of the biopsies in the current study suggesting, only minimal, if any, dislodgement of the granules from the extraction site, as a uniform distribution of the particles was observed. The findings of the canine study would support these assumptions, as a similar rate of Bio-Oss Collagen remnants were found after 3 months' healing time, although the extraction sockets underwent primary closure by repositioned flaps (Araujo et al. 2008).

Primary soft tissue closure of grafted site was proposed to improve the predictability of grafting procedure as it prevents the dislodgment of graft particles, protects them from contamination and hence, minimizes the incidence of infection (Misch 1999). The exposure of Bio-Oss Collagen to the oral environment in the current study did not make the area susceptible to infection and minimal graft dislodgement was demonstrated, suggesting that secondary wound healing over Bio-Oss Collagen placed in extraction sockets is a safe and simple method.

The findings of the present study indicated that Bio-Oss particles showed minimal resorption and turn over in the healing period between 6 and 12 weeks after extraction socket grafting, as no significant differences between the amount of remnants in samples retrieved after 6-week and 12-week healing period were found. This observation is in agreement with data of previous studies, which demonstrated that the elimination of Bio-Oss is a slow process that may require a long time (Dies et al. 1996, Becker et al. 1998, Piattelli et al. 1999, Artzi et al. 2000, Carmagnola et al. 2003, Fugazzotto 2003, Norton et al. 2003, Proussaefs and Lozada 2003). Fugazzotto reported that there was nearly complete elimination of bovine bone particles grafted in extraction socket after 12 months of healing time as these particles was seen to occupy just 0.13 % of the sockets' volume. Yet, in one histologic human study, Bio-Oss particles were evident in situ 4 years after the augmentation procedure (Piattelli et al. 1999). Therefore, the exact duration in which bovine bone substitute material could be completely resorbed is not clear.

The finding within this study is consistent with the observation made in human and experimental studies showing that an intimate contact frequently is established between Bio-Oss particles and newly formed bone suggests the osteoconductivity of bovine bone particles (Arzi et al. 2000, Proussaefs and Lozada 2003, Cardaropoli 2005, Araujo et al. 2008, Fickl et al. 2008 a). The osteoconduction of Bio-Oss particles as a scaffold for new bone formation may depend on their physical and chemical properties similar to those of human cancellous bone and the porosity of the material. The large interconnecting pore system of the material increases its inner surface and permeability which tends to positively influence the penetration of osteogenic cells, vessels and ingrowth of new bone (Klinge et al. 1992, Yildirim et al. 2000, Weibrich et al. 2000).
Bio-Oss particles have been shown to be biocompatible, as no inflammatory infiltrate was found at the interface or around the Bio-Oss particles. No foreign body reaction around bovine bone remnants was observed. These findings support the data from human and animals studies which stated that bovine bone is a biocompatible bone grafting material (Jensen et al. 1996, Piattelli et al. 1999, Arzi et al. 2000, Araujo et al. 2001, Proussaefs and Lozada 2003, Norton et al. 2003, Araujo et al. 2008).

The presence of bovine bone particles used in maxillary sinus augmentation or treatment of defects around implants did not jeopardize the osseointegration of inserted dental implants if not enhancing it, as reported by histologic studies in humans (Valentini et al. 1998, Scarano et al. 2004, Iezzi et al. 2008) and animals (Berglundh and Lindhe 1997, Hürzeler et al. 1997, Haas et al. 1998, Hämmerle et al. 1998, Polyzois et al. 2007). A high intimate bone-to-implant contact percentage without an interposition of the grafting material particles was observed. No bovine bone particle was found to be in direct contact with the implant surface. Moreover, implants placed in Bio-Oss required significantly higher pull-out force than the control implants in sheep (Haas et al. 1998). Human data of long-term clinical outcome and survival rate of implants inserted into grafted extraction sockets are lacking and may be beneficial to elucidate whether the presence of the grafted particles would eventually interfere with the longevity of functional implants in such grafted sockets.

Human extraction sockets filled with bovine bone mineral evaluated after a healing period of 4 months showed equivalent or only slightly higher rates of new bone formation compared to the current study with 6 and 12 weeks healing period (Norton et al. 2003, Vance et al. 2004), whereas sockets investigated after 7-10 months revealed either equivalent or somewhat lower quantity of bone (Artzi et al. 2000, Carmagnola et al. 2003, Norton et al. 2003, Barone et al. 2008).

Existing animal studies suggest that there might be a boost of bone formation within the first few weeks after extraction and that after a prolonged period of missing mechanical load the onset of resorption begins (Cardaropoli et al. 2003, Skerry 2008). The process of osseointegration of the implant is known to increase bone density as a result of the stimulation of the remodeling process in the bone. This has been described as the regional acceleratory phenomenon (RAP) (Frost 1994). Mechanical loading is known to activate several cellular processes in osteocytes, promoting bone formation and remodeling of woven bone into lamellar bone carried out by basic multicellular units (BMU) of osteoclasts and osteoblasts. In the absence of mechanical loading the osteoclasts' activity is increased and osteoblasts' activity is reduced leading to bone resorption not followed by compensating bone formation (Skerry 2008, Van Oers et al. 2008). Therefore, it is necessary to acquire data on human extraction sockets over various time points to determine the optimal time point for the placement of implants.

The bone formation rate in the extraction socket found after 6 and 12 weeks is high when compared to augmentation procedures with bovine bone mineral in the sinus. This might be due to the fact that there is a more favorable blood supply as the surrounding walls are in close proximity resulting in a smaller distance to the center (Yildirim et al. 2000, Froum et al. 2006, Froum et al. 2008).

7. Summary

After tooth extraction, the alveolar ridge will frequently decrease in volume and change morphologically. Existing animal studies suggest that after a prolonged absence of mechanical loading, the onset of resorption begins. Post extraction maintenance of the alveolar ridge might minimize the residual ridge resorption and, thus, allow placement of an implant that satisfies esthetic and functional criteria. As the process of osseointegration is known to enhance bone density (regional acceleratory phenomenon (RAP)), it is necessary to acquire data on human extraction sockets over various periods of time to determine the optimal time point for the placement of implants after tooth extraction. Immediate implant placement has not proven to prevent resorption. Several studies have proposed the use of heterologous grafting material such as hydroxyapatite, ß-tricalciumphosphate and bovine bone mineral for ridge preservation during bone healing. The efficiency of heterologous bone substitute placed in extraction sockets has been evaluated after prolonged healing periods in experimental and clinical studies on bone formation and the substitute influence on the resorption pattern. No information is available on the rate of bone formation in human extraction sockets filled with Bio-Oss Collagen after shortened healing periods of less than 3 months. This prospective study focuses on the bone formation of human extraction sockets augmented with Bio-Oss Collagen after a healing period of 6 and 12 weeks.

Twenty-nine patients, 15 male and 14 female, with an average age of 50.5 years (range 28 - 72 years), were included in this study. All patients were healthy, none had any systemic disease and none were nicotine users. In all patients, the extraction sockets showed no defect and were instrumented to eliminate all remnants of periodontal ligament tissue. Each socket was grafted with Bio-Oss Collagen (Geistlich, Switzerland) without flap management or application of a membrane. After a 6-week (16 patients with 17 extraction sites) and 12-week (13 patients with 17 extraction sites) healing period, at the time of implant placement, bone biopsy specimens were taken using a trephine bur. The bone biopsy specimens were fixed, decalcified and paraffin-embedded. Serial sections 5 µm thick were prepared and stained with Hematoxylin eosin, Masson´s trichrome and Toluidine staining. A mean of 2 sections were obtained from each specimen. The sections were line-scanned using ScanScope T3 (Aperio Technologies Inc., Vista, USA) with a 40x objective.

For the qualitative and morphological analysis of the remodeling process, the stained preparations were examined under a light microscope (AxioPhot I) at a magnification of up to 40x. Two regions of interest (ROI) were determined within each specimen, one in the apical portion and the other in the coronal part. In each ROI, the quantification of new bone formation, Bio-Oss remnants and fibrous tissue or bone marrow was performed by a single experienced observer using the digital imaging system AXIO VISION (Zeiss, Jena, Germany) and analysed statistically using Wilcoxon-Mann-Whitney test and Wilcoxon signed-rank rest.

After a 6-week healing period, the biopsy specimens showed a mean of 28 % (range 1 – 57 %) of newly formed bone, 11 % (range 3 – 31 %) of remaining Bio-Oss particles and 54 % (range 31 – 87 %) of connective tissue. After a 12-week healing period, the rate of new bone formation was 25 % (range 3 - 41 %) while the amount of remaining Bio-Oss particles was 15 % (range 5 – 35 %) and of connective tissue 60 % (range 50 – 91 %). There was no significant difference between the quantity of new bone after 6 and 12 weeks healing periods (P=0.09). The amount of bone tissue in the apical portion of the biopsies was significantly higher than that in the coronal portion in the 6-week and 12-week samples (P=0.002, P=0.01).

This descriptive study provides data demonstrating that a great variability exists with respect to hard tissue formation in human extraction sockets filled with Bio-Oss Collagen after a healing period of 6 and 12 weeks. These variations in bone formation and maturation within the socket evaluated might be due to unidentified factors contributing to the individual healing pattern.

The findings of the current study demonstrate that the rates of bone formation after 6 weeks of healing are similar to those found after a 12-week healing period, with more mature bone morphology being evident in 12-week samples. The placement of biomaterial might delay but not prevent overall bone formation with the advantage of providing scaffolding for the overlying epithelium. Bone formation in extraction sockets with secondary wound healing is initiated from the apical region and is not enhanced from the coronal direction after epithelial closure of the extraction site.

These results encourage an early implantation after healing period of 6 weeks, as the bone formation rate after 6 weeks is similar to that after 12 weeks.

8. Zusammenfassung

Bereits unmittelbar nach der Zahnextraktion kommt es zu einer physiologischen Atrophie des Kieferkamms. Diese führt zu einer Dimensionsveränderung und zum Knochenverlust in horizontaler und vertikaler Richtung. In tierexperimentellen Studien wurde nachgewiesen, dass die Knochenresorptionsvorgänge das Resultat eines dauernd fehlenden mechanischen Belastungsreizes sind. Es wird vermutet, dass durch die Erhaltung des Alveolarkamms nach Zahnextrakion der Knochenverlust minimiert werden könnte. Somit wären besser planbare Implantatinserationen möglich und deren Ergebnisse würden ästhetischen und funktionellen Kriterien entsprechen. Über den Prozess der Osseointegration ist bekannt, dass sich die Knochendichte erhöht (regional acceleratory Phänomen (RAP)). Deshalb es ist notwendig, Daten auf die menschlichen Extraktionsalveolen über verschiedene Zeiträume zu gewinnen, um den optimalen Zeitpunkt für die Implantatinseration nach Zahnextraktion zu bestimmen. Durch die Sofortimplantation können die Resorptionsvorgänge offensichtlich nicht kompensiert werden. Mehrere Studien stellen die Verwendung von heterologen Knochenersatzsmaterial wie Hydroxylapatit, ß-trikalziumphosphat und bovines Knochenmineral für Alveolarkammprävention (socket preservation) während der Knochenheilung dar. Der Einfluss von Knochenersatzmaterialen auf die Resorptionsmuster und die Knochenbildung in den Extraktionsalvolen wurden in tierexperimentellen und klinischen Studien nach Heilungszeiten von mehr als 3 Monaten untersucht. Es liegen keine Informationen über die Rate der Knochenbildung in mit Bio-Oss Collagen augmentierten menschlichen Extraktionsalveolen nach verkürzten Heilungszeiten von weniger als 3 Monaten vor. Ziel der vorliegende Prospektivstudie ist die Knochenneuformation der frischen menschlichen Extraxtionsalveole, nach Augmentation mit Bio-Oss Kollagen und einer Heilungszeit von 6 und 12 Wochen histomorphometrisch zu analysieren

In dieser Studie sind Neunundzwanzig Patienten, 15 männliche und 14 weibliche, mit einem Durchschnittsalter von 50.5 Jahren (Bereich 28 bis 72 Jahre) enthalten. Alle Patienten waren gesund, ohne systemische Erkrankung und keiner war Raucher. Die Extraktionsalveolen bei allen Patienten waren intakt und wurden gesäubert um alle Reste desmodontalen Gewebes zu beseitigen. In jede Extraktionsalveole wurde Bio-Oss Collagen (Geistlich, Schweiz) ohne primäre Deckung oder die Anwendung einer Membran

appliziert. Nach einer Heilungszeit von 6-Wochen (16 Patienten mit 17 Extraktionswunde) und 12-Wochen (13 Patienten mit 17 Extraktionswunde) wurden die Implantate inseriert. Dabei wurde eine Knochenbiopsie mit einem Trepanbohrer entnommen. Die Knochenproben wurden fixiert, entkalkt und in Paraffin eingebettet. Nach der Anfertigung eines Dünnschliffes auf eine Stärke von 5 µm erfolgte die Färbung nach Masson-Goldner, Hämatoxylin-Eosin und Toluidin blau. Bei einer 40-fachen Vergrößerung wurde ein digitales Foto von jedem Präparat mittels ScanScope T3 (Aperio Technologies Inc., Vista, USA) aufgenommen. Die histologische Auswertung der gefärbten Präparate erfolgte im Durchlichtmikroskop (Axiophot I) bei einer 40-fachen Vergrößerung. Es wurde zwei Bereiche von Interesse pro Präparat definiert. Ein Bereich liegt in der apikalen und ein anderer in der koronalen Region des Präparates. In jeder Region wurde die Knochenneuformation, der Anteil der Bio-Oss Kollagen Partikel sowie Bindegewebe und Knochenmark von einem erfahrenen Untersucher mittels des Bildanalyseprogramms AXIO VISION (Zeiss, Jena, Deutschland) quantitativ erfasst. Zur statistischen Auswertung wurden der Wilcoxon-Mann-Whitney test und der Wilcoxon signed-rank rest herangezogen
Der mittlere Anteil des neu gebildeten Knochens nach sechswöchiger Heilungszeit betrug 28 % (1 – 57 %), der Bio-Oss Partikeln 11 % (3 – 31 %) und der des Bindegewebes 54 % (31 – 87 %). Nach zwölfwöchiger Heilungszeit betrug der mittlere Anteil.des neu gebildeten Knochens 25 % (3 – 41 %), der Bio-Oss Partikeln 15 % (5 – 35 %) und des Bindegewebes 60% (50 – 91 %). Die Ergebnisse zeigten keinen signifikanten Unterschied bezüglich des Anteils der Knochenneubildung zwischen den 6 - und 12 Wochen Proben (P=0.09). Der Anteil des Knochengewebes im apikalen Bereich der Biopsien war signifikant höher als im koronalen Bereich in den 6- und 12-Wochen Proben (P=0.002, P=0.01).
Die in dieser Deskriptivstudie erhobenen Daten deutet auf eine große Variabilität bezuglich der Knochenneubildung mit Bio-Oss Collagen augmentierten menschlichen Extraktionsalveolen nach sechswöchiger und zwölfwöchiger Heilungzeit hin. Die Unterschiede bei der Knochenneubildung und Knochenreifung deuten auf nicht definierte Faktoren hin, welche zu unterschiedlichen individuellen Heilungsmustern geführt haben.
Die Ergebnisse der vorliegenden Studie zeigen, dass kein signifikanter Unterschied bezüglich der Knochenneubildung zwischen 6-Wochen Heilungzeit Proben und 12-Wochen Heilungszeit Proben ermittelt werden konnte, wobei mehr reife Knochenmorphologie in den

12-Wochen Proben nachwiesbar ist.

Die Einlage des Biomaterials in die Extraktionswunden könnte insgesamt die Knochenbildung verzögern, aber nicht verhindern, mit dem Vorteil dass es als Gerüst für das sich darüber bildende Epithel dient. Die Knochenbildung in Extraktionsalveolen mit sekundärer Wundheilung wird von der apikalen Region ausgelöst und wird nicht vom koronalen Bereich nach epithelialen Verschluss der Extraktionwunde unterstützt.

Diese Ergebnisse fördern die Auffassung zu einer frühen Implantation nach einer Heilungszeit von 6 Wochen, da die knöcherne Auffüllung der Extraktinalveole nach sechswöchiger Heilungszeit vergleichbar mit der nach 12 Wochen ist.

9. References

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10. Curriculum vitae (Lebenslauf)

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

11. Selbsständigkeitserklärung

„Ich, Baem Al Chawaf, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: „Healing of human extraction sockets augmented with Bio-Oss Collagen after 6 and 12 weeks“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

17.08.2010

Basem Al Chawaf