On tissue reactions to dentin as a bone substitute material

Payam Farzad

Department of biomaterials
Institute of clinical sciences
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

Göteborg 2017

On tissue reactions to dentin as a bone substitute material

payam.farzad@gu.se
http://hdl.handle.net/2077/52410
ISBN: 978-91-629-0209-4 (TRYCK)
ISBN: 978-91-629-0210-0 (PDF)
Printed in Gothenburg, Sweden 2017
Ineko AB

To my father-you would have been proud.

Content

On tissue reactions to dentin as a bone substitute material

Click here to enter text.
4ff8
Department of biomaterials, Institute of clinical sciences
Sahlgrenska Academy at University of Gothenburg
Göteborg, Sweden

Abstract

Background Reconstruction of the jaws due to resorption of the alveolar crest may require bone augmentation prior to installation of endosseous implants. Active research on new bone graft materials with bone regeneration ability equivalent to autogenous bone but without the limitations of allogenic, xenogenic and synthetic bone are constantly ongoing. From clinical and experimental studies, it has been demonstrated that replanted teeth without a viable periodontal membrane will ankylose with the bone. The dentin of such teeth is fused with the bone, and will be gradually replaced by bone, also called replacement resorption or osseous replacement. In order to possibly modify treatment protocols and also exploring possible cost-benefit alternatives to commercially available bone replacement materials, there has been an increased interest to explore the use of human dentin as a source for graft material.

Aims The aim of the first study was to evaluate and compare the host tissue response to autogenous and xenogenic non-demineralized dentin blocks implanted in non-osteogenic areas, the abdominal connective

tissue and femoral muscle of rabbits. The objective of the second study was primarily to evaluate the healing pattern of xenogenic non-demineralized dentin granules and dentin blocks grafted to maxillary bone of rabbits and secondarily to study integration of titanium micro-implants installed in grafted areas. In paper III, we sought to evaluate the healing pattern of xenogenic demineralized dentin granules and dentin blocks grafted to cavities created in tibial bone of rabbits, secondarily to study integration of titanium micro-implants installed in grafted areas and thirdly to investigate the morphological appearances and differences between demineralized and non-demineralized dentin by means of Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX). Finally, the objective of study IV was to compare the host tissue response and remodelling of onlay grafts of demineralized dentin in comparison to onlay bone grafts transplanted to the native tibial cortical bone wall.

Material and methods In study I, fifteen 6-month old New Zealand male white rabbits were used. Dentin autografts taken from the same rabbit and dentin xenografts taken from human premolars were implanted in abdominal connective tissue and femoral muscles. All rabbits were sacrificed after 12 weeks for light microscopic analysis.

In study II, fifteen 6-months old New Zealand male rabbits were used. Dentin blocks and dentin granules from human premolars were implanted in cavities prepared on either side of the maxilla (n=15x2). After a healing period of 6 months, one micro implant (5 mm long, 2 mm in diameter) was installed in each surgical site. All rabbits were sacrificed 24 weeks after implant installation. The specimens were studied by light microscopic and histomorphometrical analysis. Study III included twelve 6-month old New Zealand male, white rabbits. Dentin blocks and dentin granules from human premolars were implanted in cavities prepared on

both tibial bones. Twelve hours prior to grafting the dentin grafts were rinsed in saline and demineralized on its surface by being placed in 24% EDTA neutral, pH7, for 12 hours. After a healing period of 24 weeks, one micro implant was installed in each surgical site.

To characterize the grafts, twelve additional dentin blocks were prepared in standardized sizes. All samples were conditioned in 24% EDTA neutral, pH7, for 12 hours followed by a second x-ray analysis. Four samples were chosen for conventional SEM and energy dispersive X-ray analysis (EDX), both image mode and element analysis mode. In study IV, we used eight 6-months old New Zealand male rabbits. Standardized sized dentin blocks from human premolars and similar autogenous bone blocks, harvested from tibia were grafted as onlay blocks on each tibia (n=8x2). All animals were sacrificed after a healing period of 12 weeks. Descriptive histology as well as histomorphometrical analysis of the remaining dentin, bone graft and soft tissue was determined using light microscopy.

Results Study I showed only minor signs of heterotopic bone formation. There were no significant differences between autografts and xenografts or grafts implanted in connective tissue or muscle with regards to tissue reactions except for a significant difference ($P = 0.018$) in findings of more local inflammatory cells in relation to grafts placed in connective tissue in the autograft group. In study II, no statistically significant difference could be observed in BIC and BA between dentin and native bone. Overall the BIC and percentage of new bone fill of the block specimens were higher than the same parameters for the particulate graft. Study III showed a tendency towards higher BIC and BA for the EDTA conditioned dentin in conjunction with installed implants, but the difference was not statistically significant. In addition, on the demineralized dentin surface the organic marker element C dominated, as

revealed by EDX image mode. The hydroxyapatite constituents Ca, P and O were close to devoid on the dentin surface. A similar pattern was discerned from the semi-quantitative data analysis where the organic markers C and N dominated. Study IV showed that in general, both the dentin and bone block grafts were fused to the bone, resorbed and replaced by bone and connective tissue to a varying degree. Resorption cavities could be seen in the dentin with bone formation. Zones of osseous replacement resorption of the dentin could be noted. In both graft types, higher rate of bone formation was seen at the interface between graft and recipient site.

Conclusion Non-demineralized dentin, whether autogenous or xenogenic did not have the potential to induce bone formation when implanted in non-osteogenic areas such as the abdominal wall and abdominal muscle of rabbits. Limited or no bone contact between micro-implants and xenogenic non-demineralized dentin grafts could be seen. Demineralized xenogenic dentin onlay grafts showed similar resorption characteristics as autogenous bone onlay grafts, being resorbed in a similar rate during 12 weeks. New bone formation occurred mainly in terms of replacement resorption in the interface between dentin/bone graft and native bone. The bone inductive capacity of the dentin material seemed limited although demineralization by means of EDTA indicated a higher BIC and BA value in conjunction with installed implants in the area.

Keywords Grafted dentin, tissue reaction, bone blocks, dental implants, experimental study

LIST OF PUBLICATIONS

This dissertation is based on the following papers, which will be referred to throughout by their Roman numerals I-IV:

I. Al-Asfour A*, Farzad P*, Andersson L, Joseph B, Dahlin C
Host tissue reactions of non-demineralized autogenic and xenogenic dentin blocks implanted in a non-osteogenic environment. An experimental study in rabbits. *Dent Traumatol. 2014;30:198-203*
**Equal contribution*

II. Farzad P, Al-Asfour A, Dahlin A, Andersson L, Dahlin C
Integration of dental implants in conjunction with grafted dentin. An experimental study in the rabbit maxilla. *Oral Health Dent Manag 2015;5:289-293*

III. Farzad P, Lundgren T, Al-Asfour A, Andersson L, Dahlin C
Integration and characterization of decalcified and non-decalcified dentin in conjunction with dental implants. An experimental study in rabbit tibia. *In Manuscript*

IV. Al-Asfour A*, Farzad P*, Al-Musawi A, Dahlin C, Andersson L.
Demineralized xenogenic dentin and autogenous bone as onlay grafts to rabbit tibia. *Implant Dent 2017;26:232-237.*
**Equal contribution*

Abbreviations

AB	Autogenous bone
ALP	Alkaline Phosphatase
BA	Bone to implant area
BCP	Biphasic calcium phosphate
BIC	Bone to implant contact
BMP	Bone morphogenetic protein
BMU	Basic multicellular unit
CaPs	Calcium phosphate ceramics
CSF	Colony stimulating factor
DFDB	Demineralized freeze-dried bone
EDS	Energy dispersive spectroscopy
EDTA	Ethylenediaminetetraacetic acid
FHA	Flourohydroxyapatite
FPD	Fixed partial denture (bridge)
HA	Hydroxyapatite
IGF	Insulin-like growth factor
IM	Intramembranous
MSC	Mesenchymal stem cell
MMP	Matrix metalloproteinase
OC	Osteocalcin

OP	Osteopontin
OPG	Osteoprotegrin
PDGF	Platelet-derived growth factor
PDL	Periodontal ligament
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear factor
RANKL	Receptor activator of nuclear factor-IB LIGAND
ROI	Region of interest
RPD	Removable partial denture
SEM	Scanning electron microscopy
TCP	Tricalcium phosphate
TGF	Transforming growth factor
TRAP	Tartrate-resistant acid phosphatase
TGF	Transforming growth factor
VEGF	Vascular endothelial growth factor

Introduction

Alveolar bone is a prerequisite for support of the teeth. Alveolar bone atrophy can be caused by systemic disorders, endocrine imbalance, age, mechanical forces or periodontal disease (Boyne 1982; Bays 1986) and is also seen after loss of teeth (Schropp 2003; Carlson 2004).

These factors may act independently or concordantly and lead to reduced chewing ability and morphological changes of the jaw bone. There are several different methods of restoring the chewing function. Conventional removable prostheses (RPD) retained by the remaining dentition and supported by the residual alveolar bone is one way of solving this functional problem. Another option is a fixed dental bridge (FPD), which is cemented to the remaining teeth anterior and posterior to the edentulous region. Both these options have drawbacks in that removable prostheses are not accepted by all patients and tooth-supported fixed bridges require a sufficient number of supporting teeth (Randow et al.1986; Jepson et al.1995). Since the concept of osseointegration was introduced back in 1969 by Brånemark and co-workers, endosseous implants have been used successfully as an alternative treatment to removable prosthesis and fixed dental bridges with good long-term clinical results (Brånemark et al. 1969). One major advantage of endosseous implants is that there is no need of engagement of remaining teeth. Sufficient bone height and bone width is however a prerequisite for achieving good results. If there is a bone deficiency in the maxilla, the problem may be solved by using narrow implants (Hallman 2001), short implants (Pohl 2017) or tilting the implants towards a new direction where bone can be found (Mattson et al. 1999; Krekmanov 2000; Aparicio et al. 2001). Other options include the use of specially designed long implants (zygomatic implants) which are placed through the

maxillary sinus into the zygoma (Higuchi 2000; Malevez et al. 2000; Farzad et al. 2006). In the mandible lateralization or transposition of the inferior alveolar nerve enables installation of implants posterior to the mental foramen when sufficient height bone superior to the nerve is not available, however this is a less suitable method since sensory disturbance might occur following this procedure (Hirsch and Brånemark 1995). Alveolar distraction osteogenesis is another method used to increase the height of the available bone above the mandibular canal in order to install dental implants. This technique avoids the sensory disturbance problems associated with lateralization or transposition of the inferior alveolar nerve (Felice et al. 2013).

In cases where atrophy of the alveolar bone is severe, there might be a need for augmentation procedures prior to implant treatment. Most often, a three dimensional lack of bone, *i.e.* lack of width and height, in the desired position can be solved by reconstruction using veneer grafts and allowing the graft to heal for a certain period prior to placement of dental implants (Bahat and Fontanessi 2001). However, this augmentation technique might not be applied in every bone deficiency situation. For instance, the technique most often used in the posterior part of the maxilla is augmenting vertically by grafting of the maxillary sinus floor (Boyne 1980; Wood and Moor 1988; Hallman et al. 2002; Hallman et al. 2002).

Autogenous bone grafts have been the gold standard to reconstruct bone deficiency situations for many years (Bloomquist 1980; Sakkas et al. 2017). Their range of advantages includes early revascularization, resistance to infections and evidence of immune activation (Burchardt 1983; Beirne 1986). Moreover, the autogenous bone graft possesses both osteoinductive and osteoconductive properties (Urist 1965; Urist 1980). However, a disadvantage is that this technique requires a second surgical site to harvest the bone graft. Moreover, there are drawbacks such as

donor site morbidity, limitations in the quantity of available bone, prolongation of surgery time and an increase of treatment cost (Dahlin et al. 1988; Raghoebar et al. 2001; Andersson 2008). Several studies have also shown that particularly onlay bone block grafts are prone to resorption and a large part of the bone graft can be lost during the healing period (Johansson et al. 2001; Nyström et al. 2002; Misch 2011). This has encouraged research to find an acceptable bone substitute. The ideal bone substitute should be readily available, well tolerated by the host, possess both osteoinductive and osteoconductive properties and be able to be resorbed gradually with the regeneration of new osseous tissue and healing of the bone defect (Jensen et al. 1996; Schilling et al. 2004). Available bone substitutes on the market are, either synthetic, inorganic or biologically organic and may be associated with additional cost for the patient. These materials are used solely to replace the bone grafting procedure or used in combination with a minor amount of autogenous bone to increase the volume of graft material. Since allogenic and xenogenic bone substitute have a potential risk of disease transmission, there has been an increasing demand for synthetic bone substitutes in recent years (Sogal and Tofe 1999; Kim et al. 2016).

Clinical implications of dentin as a bone substitute material

Dental trauma is one of the major causes for tooth loss. Alveolar bone resorption is an inevitable consequence of tooth loss and may be detrimental to long-term dental aesthetics and function. It is estimated that the prevalence of dental trauma is 17, 5% in a global perspective making it one of the most frequent traumas reported (Azami Aghdas et al. 2015). The tooth is physiologically connected to the alveolar bone via the periodontal ligament attaching into the “bundle bone” portion of the

socket-associated bone. This part of the alveolar bone is always resorbed following tooth loss as a normal physiological event (Araujo et al. 2006). This can, in particular be observed in younger patients suffering from a tooth loss. The change in dimensions is most pronounced in the anterior maxilla and during the initial 6 months following tooth loss (Rodd et al. 2007). This study also reports a difference between genders. Hence young women tend to have a more pronounced bone resorption compared to male persons. Recently differences in the pattern of resorption with regards to gingival biotype have also been described (Schappuis et al. 2013). It was demonstrated by means of CBCT analysis that a thin biotype is associated with more pronounced resorption along the axis of the socket while a thicker biotype tends to demonstrate more marginal bone loss (Schappuis et al. 2013). Since a final restoration supported by dental implants requires a completed skeletal growth, tooth loss in a relatively young age will create a need for a detailed treatment planning leading up to the final restoration. Based on these facts, researchers and clinicians have become interested in the use of human dentin from extracted teeth in the context of serving as graft material (Kim et al. 2010; Murata et al. 2011) since it is readily available, cheap and from biological origin. Dentin has inorganic and organic contents that are very similar to those of human bone. From clinical and experimental studies, it has been well documented that replanted teeth without a viable periodontal membrane will ankylose with the bone (Söder et al. 1977; Andreasen JO 1981; Blomlöf et al. 1983; Andersson et al. 1984; Andersson et al. 1989; Hammarström et al. 1989; Lindskog and Blomlöf 1992; Andreasen et al. 1995; Barrett and Kenny 1997; Trope 2011; Maslamani et al. 2016). The dentin of such teeth is fused with the bone (ankyloses), and will be gradually replaced by bone, also called replacement resorption or osseous replacement (Andreasen and Hjörting-

Hansen 1966; Andersson 1988; Andersson et al. 1989). This is considered to be mainly a bone remodelling process (Andreasen and Hjorting-Hansen 1966; Andersson et al. 1984; Andersson 1988).

Furthermore, it has been suggested that dentin possesses not only osteoinductive properties due to its content of bone morphogenic protein (BMP) but also osteoconductive properties. These facts might indicate that dentin might function as a bone substitute material (Pinholt et al. 1992; Ike and Urist 1998).

The increasing number of bone grafting procedures in the recent years and the subsequent introduction of different bone substitutes to the market require a better understanding of the bone biology and bone grafts.

Bone biology

Human bone is biologically active connective tissue, which has its own blood supply and consists of cells and extracellular matrix. This living tissue has several important functions for the organism; (i) gives mechanical support to the body; (ii) produces blood cells in the bone marrow; (iii) functions as a reservoir of Ca-ions; (iv) provides protection for internal organs and (v) serves as attachments for muscles, ligaments and tendons. The bones in the human body can be assorted to as long bones, short bones, irregular bones and flat bones. All bones are composed by an outer dense structure called the cortical bone and an inner layer of trabecular bone with lower density and a more porous structure. About 80% of the skeletal mass is composed of cortical bone. Mineralized bone appears in two forms, woven and lamellar. Woven bone is seen during early bone formation i.e. during growth and healing.

Lamellar bone is the form of mature bone and is formed during modelling and remodelling.

About 70% of the bone is composed of mineral, mainly hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$. The bone matrix consists of mainly type I collagen (up to 90%), proteins such as osteocalcin (OC), bone sialoprotein, osteopontin (OPN), osteonectin and a great number of growth factors, e.g. BMPs. The remaining content consists of 5% to 10% water and <3% of lipids (Buck and Dumanian 2012).

From an embryological standpoint, the craniofacial skeleton including maxilla and mandible is formed from the neural crest cells. There are two types of bone formation described: endochondral ossification (the most common mechanism of primary bone formation) and intramembranous ossification (Buck and Dumanian 2012; Makiewicz 2011).

In the regions of craniofacial skeleton, differentiation of mesenchymal cells directly into osteoblasts initiates production of a trabecular pattern of early bone matrix. Bone matrix matures through secretion of bone matrix components and cellular synthesis. At his stage, calcium phosphate, in the form of hydroxyapatite (HA) crystals are deposited at the bone matrix site. This procedure is called intramembranous (IM) bone formation and the flat bones of the skull, the clavicle and the mandible are formed in this way (Makiewicz 2011; Buck and Dumanian 2012).

The endochondral ossification occurs in the long bones, pelvis, skull base and vertebral column. In this type of ossification, mesenchymal cells differentiate into chondrocytes, which produce a hyaline cartilaginous framework. This cartilage is matured through hypertrophy of chondrocytes followed by matrix erosion. The remaining cartilage matrix mineralizes and the chondrocytes regress and die. Through invading blood vessels, mesenchymal cells enter the calcified cartilage model

which may be differentiated into osteoblasts and subsequently start bone formation (Zipfel et al.2003).

Bone remodelling refers to a continuous process throughout life where old bone is replaced by new bone and during normal conditions equal amount of bone is formed as the amount of bone resorbed keeping the total bone mass unchanged. This phenomenon aims at maintaining mechanical properties of the skeleton and support mineral homeostasis and for maintaining a constant serum level of calcium (Zipfel et al. 2003; Lerner 2006; Makiewicz 2011; Buck and Dumanian 2012). Bone remodelling begins before birth and continues until the organism's death. In adults about 25% of trabecular and 3% of cortical bone is replaced each year (Zipfel et al. 2003). The process of bone remodelling takes place in a basic multicellular unit (BMU), which consists of bone resorbing osteoclasts, the bone forming osteoblasts, osteocytes within the bone matrix, bone lining cells on the bone surface, and the capillary blood supply (Kular et al. 2012). The duration of the resorption process is 3 to 4 weeks and the subsequent bone formation takes about 3-4 months to be completed. The bone remodelling process is shorter in cortical bone than in cancellous bone where the length of the process is about 200 days in human iliac bone (Kular et al. 2012).

Bone cells

The osteoblast, the bone lining cell, the osteocyte and the osteoclast are the four cells types found in bone. In total, these cells make up around 10% of the total bone volume. The osteoclasts are formed by giant multinucleated cells whereas the other three types are derived from mesenchymal stem cells (Buck and Dumanian 2012).

-Osteoblast

Osteoblasts account for 4-6% of the bone cells and are estimated to have a lifespan of three months in human bone. Osteoblasts are the only cells with capability of bone formation through producing and secreting proteins, thus forming the bone matrix. They line the surface of bone, packed tightly against each other with a rounded, polyhedral form (Rochefort 2010; Capulli et al. 2014). Osteoblasts are derived from mesenchymal stem cells with a capability of differentiation into fibroblasts, chondrocytes, myoblasts and adipocytes (Ducy et al. 2000). Four maturational stages have been identified in osteoblast differentiation: pre-osteoblast, osteoblast, osteocyte and bone lining cells (non-active flattend osteoblast) (Kular et al. 2012). Several proteins such as collagen type I, osteocalcin (OC), alkaline phosphatase (ALP), osteonectin, osteopontin (OP), bone sioloprotein and a few other minor matrix proteins are produced by osteoblasts (Manolagas 2000).

Fibers of type I collagen, which is the major protein in the matrix, provide a structure on which mineral is deposited (Mackie 2003). At the end of a bone formation cycle, mature osteoblasts face one of three fates: approximately 50-70% undergoes apoptosis and the rest will either develop into bone lining cells or osteocytes (Manolagas 2000, Kular et al. 2012).

Osteoblasts are also responsible for regulating the differentiation of the bone resorbing osteoclasts by producing factors such as macrophage colony-stimulating factor (M-CSF), osteoprotegerin (OPG) and cytokine receptor activator of NF-κB ligand (RANKL). These factors play a major role in osteoclast formation, activation and resorption (Kular et al. 2012).

-Osteocytes

Osteocytes account for more than 95% of all the bone cells. They demonstrate a widely variable life expectancy, but a mean half-life time

of 25 years in human bone has been proposed, although it is probably less due to a constant bone turnover of approximately 10% (Rochefort et al. 2010). Osteocytes have been differentiated from osteoblasts and are entrapped in the bone matrix. The time span for a motile osteoblast to be an entrapped osteocyte in the bone matrix is about 3 days. Osteocytes demonstrate a size of 10 µm -20 µm in human bone, which is a reduction to 30% of the size of the osteoblast origin (Knoteh et al. 2003; Bonewald 2011). They lie in lacunae embedded in the bone matrix and once there, they start to extend projections through channels in the bone matrix called canaliculi (Bonewald 2011). These channels aid the osteocytes to communicate not only with each other but also with other bone cells on the bone surface such as bone lining cells and osteoblasts (Dudley and Spiro 1961; Tanaka-Kamioka et al. 1998; Bonewald 2011). Osteocytes serve as mechanosensors, having the ability to detect mechanical pressure and load through the interconnected network of fluid containing canaliculi (Aarden et al. 1994; Burger and Klein-Nulend 1999). This ability can induce bone repair following microdamage. Osteocytes are also responsible for maintaining the bone matrix (Aarden et al. 1994; Burger and Klein-Nulend 1999). By modulating secretion and expression of insulin-like growth factor (IGF), osteocalcin (OC) and sclerostin, the osteocytes are able to regulate skeletal homeostasis. Osteocytes also provide the majority of RANKL that controls osteoclast formation in cancellous bone (Robling 2008; Rochefort et al. 2010).

-Bone lining cells

The bone lining cells or surface osteoblasts are flattened, thin, differentiated cells, mainly derived from osteoblasts. These cells are located on top of a thin layer of unmineralized collagen matrix covering the bone surface (Miller et al. 1989). They connect to the osteocytes through gap junctions (Miller and Jee 1987). Lining cells can be activated

and differentiated into osteogenic cells and they also take part in the homeostasis of mineral through control of bone fluids and ions e.g. by immediate release of calcium from bone when the blood calcium level is low (Miller et al. 1989). When exposed to PTH, bone lining cells secrete collagenase to remove the collagen matrix so osteoclasts can attach to bone (Recker 1992).

-Osteoclasts

In an adult organism, osteoclasts are derived from hematopoetic stem cells and share precusrsors with macrophages and monocytes. They are the only cell type that can resorb bone and are formed by multiple cellular fusions of mononucleated cells (Vaananen and Laitala-Leinonen 2008). The osteoclast is found and formed in much smaller numbers compared to other bone cells on the surface of the bone. These cells are highly motile, but since they are only formed on the bone surfaces, they ate never encountered in the blood circulation (Lerner 2000). A differentiated human osteoclast contains about five to eight nuclei in each cell and has a diameter of 50-100 μm. Bone resorption takes place in a finger shaped extension of the ruffled border membrane. This is also the most characteristic feature of the osteoclast (Manolagas 2000; Vaananen and Laitala-Leinonen 2008). Osteoclast formation, activation and resoprtion are regulated by the ratio of receptor activator of NF-κβ ligand (RANKL, which binds to RANK and activates osteoclastogenesis) to osteoprotegerin (OPG, which inhibits osteoclastogenesis), IL-1 and IL-6, colony stimulating factor (CSF), parathyroid hormone, 1,25-dihydroxyvitamin D and calcitonin (Blair and Athanasou 2004). Resorbing osteoclasts have a unique ability to create an acidic environment in the resorption lacunae via secretion of hydrogen ions through proton pumps and chloride channels. Hydroxyapatite is

dissoluted when the pH within the bone-resorbing space is lowered to about 4,5. This is followed by secretion of tartrate-resistant acid phosphatase (TRAP), cathepsin K, matrix metalloproteinases (MMPs) and gelatinases from cytoplasmic lysosomes to digest the organic matrix. The result is formation of Howship's lacunae on the surface of trabecular bone and Haversian canals in cortical bone. Degradation products such as bicarbonate, calcium and phosphate ions are removed from the resoprtion lacunae by transportation through the cells for secretion (Reddy 2004). The resorption phase is completed by mononuclear cells after osteoclasts undergo apoptosis. Resoprtion is followed by osteoblast activation and formation of osteoid, which fills the cavities over a period of about three months (Deal 2009).

Physiology of bone healing

The use of a bone graft for purposes of achieving increased bone volume is affected by anatomical, histological, and biochemical principles. Additionally, several physiological properties of bone grafts directly affect the success or failure of graft incorporation. These properties are osteogenesis, osteoinduction and osteoconduction (Prolo 1990).

-Osteogenesis

Osteogenesis is the ability of the graft to produce new bone, and this process is dependent on the presence of live bone cells in the graft. Osteogenic graft materials contain viable cells with the ability to form bone (osteoprogenitor cells) or the potential to differentiate into bone-forming cells (inducible osteogenic precursor cells). These cells, which participate in the early stages of the healing process to unite the graft with the host bone, must be protected during the grafting procedure to ensure viability in order to produce osteoid. When new bone is formed by

osteoprogenitor cells within the wound defect, i.e. a bone fracture, it is called spontaneous osteogenesis. Transplanted osteogenesis is when new bone formation is related to presence of bone forming cells within the bone graft (Muschler et al. 1990).

The role of osteogenesis as a mechanism of new bone formation during nonvascularized bone graft healing, however, is thought to be of lesser significance than that of osteoconduction (Burchardt 1983).

-Osteoconduction

Non-vascularized bone grafts heal through a predictable sequence of events. In the first step, the graft will undergo partial necrosis, followed by an inflammatory stage. During this phase, the graft is invaded slowly by vessels, which in turn will deliver osteoclasts and osteoblasts to the region. Interaction between these 2 cell lines will lead to replacement of much of the grafted bone by new bone. The term creeping substitution is used to describe this slow vessel invasion and bony replacement, a process formally known as osteoconduction. The term refers to the process where bone grows on a surface. An osteoconductive surface is one that permits bone growth on its surface or down into pores, channels or pipes (Albrektsson and Johansson 2001). In the context of bone healing, the graft would serve as a scaffold on which new bone is deposited (Muschler et al. 1990).

The second step in the process of healing is the formation of a hematoma. Shortly after placement of the graft, a hematoma is formed around the graft, which is due to the surgical disruption of host soft tissues and the recipient bony bed. During this early stage, a small minority of cells on the graft's surface are able to survive, primarily as a result of plasmatic imbibitions (Heslop et al. 1960; Muliken et al. 1984). The third step is the start of an inflammatory reaction. The inflammatory reaction, which lasts for 5 to 7 days is focused around the graft and ensues after hematoma

formation. A dense fibrovascular stroma is formed around the graft and the onset of vascular invasion starts at 10 to 14 days (Gross et al. 1991). Vascular invasion brings additional cells with osteogenic potential into the graft, as the interstices of the old bone act as a directive matrix. As osteoblasts deposit new bone, osteoclasts resorb necrotic bone and pave the way for the graft to be penetrated by vascular tissue (Schmitz and Hollinger 1996; Gross et al. 1991).

-Osteoinduction

The principle of osteoinduction was described by Urist and the biochemical events by Reddi (Urist 1965; Bang and Urist 1967; Reddi and Wientroub 1987). They described the inductive process in rodents as ingrowth of vascular tissue and development of osteoprogenitor cells with subsequent new bone formation by enchondral ossification (Urist 1965; Bang and Urist 1967; Reddi and Wientroub 1987). Osteoinduction refers to the process by which active factors released from the grafted bone stimulate osteoprogenitor cells from the host to differentiate and form new bone. This process is highly dependent of a soluble protein called BMP. The BMP belongs to the family of transforming growth factors, (TGF)-β. Three phases of osteoconduction have been described: chemotaxis, mitosis, and differentiation. During chemotaxis, bone inductive factors direct the migration and activity of osteogenic cells via chemical gradients. The inductive factors then stimulate these osteoprogenitor cells to undergo intense mitogenic activity, followed by their differentiation into mature, osteoid-producing cellular elements (i.e., osteoblasts). Ultimately, the cells become revascularized by invading blood vessels and are incorporated as new bone. The ultrastructural character of the bone graft (i.e. cancellous versus cortical) determines the

ability of revascularization to take place and, therefore, significantly impacts the process of incorporation (Muschler et al. 1990).

Healing of bone grafts

A graft is transplantation of tissue or cells. The most commonly used materials for alveolar ridge augmentation purposes are:

-Autogenous bone (AB)

An autogenic graft is transplantation of tissue within the same individual and is considered to be the ‘’gold standard’’ in reconstruction of defects in the jaws. This is mainly due to its osteoinductive and osteoconductive properties as well as low cost and minimal risk for disease transmission (Burchart 1983). The healing of autogenous bone grafts is quite similar to that of fracture repair. An important similarity in bone graft healing is that a substantial portion of the biological activity originates from the host. This occurs because most viable osteocytes within the graft itself necrose shortly after transplantation. Nonetheless, substantial biological interactions still remain between graft and host. This important biological interplay contributes to the final outcome of graft take (Burchart 1983).

Most common donor sites in reconstruction of jaw defects prior to implant surgery are various areas of the mandible, tibia and the iliac crest. Cortical, cancellous or a combination of both can be obtained from these different sites (Buser et al. 1996; Sjöström et al. 2007). Cancellous bone is osteogenic providing vital osteoprogenitor cells, it is osteoinductive and is completely replaced in time by osteoconduction because the graft also is acting as a scaffold for bony ingrowth from the recipient site (Burchart 1983). Cortical bone may be osteogenic but heals mainly by osteoconduction. At the time of transplantation it provides more mechanical support than cancellous grafts but the later are revascularized more rapidly and completely than cortical grafts (Burchart 1983;

Sjöström et al. 2007). The graft consists partly of surviving cells (preosteoblasts and preosteoclasts), but also proteins capable of converting undifferentiated mesenchymal stem cells into bone producing cells (Burchart 1983; Sjöström et al. 2007). Since the blood supply to the bone graft is cut off at the time of harvest, revascularization needs to occur for incorporation of the bone graft and resorption of cortical bone is therefore a major part of bone graft healing (Urist 1980; Goldberg and Stevenson 1987). Differences in revascularization time and pattern are seen between trabecular and cortical bone. In trabecular bone, revascularization is re-established through micro-anastomosis with existing blood vessels. Since the cancellous bone is porous with marrow tissue between the trabeculae, vascular ingrowth occurs more rapidly and is completed after a few weeks. In contrast to cancellous bone grafts, cortical bone graft is densely packed and revascularization proceeds slowly and takes about almost two months to be completed (Albrektsson 1980).

The large spaces between trabeculae in cancellous grafts permit the unobstructed invasion of vascular tissue and the facile diffusion of nutrients from the host bed. This is thought to promote osteogenic cell survival, imparting increased osteogenesis when compared with cortical grafts. Osteoprogenitor cells, brought in by the invading vessels, differentiate into osteoblasts and deposit a layer of new bone around the necrotic trabeculae. An osteoclastic phase ensues, wherein the entrapped cores of dead bone are resorbed. Cancellous bone grafts are completely revascularized and ultimately replaced with new bone over several weeks to months (Stevenson et al. 1996; Pinholt et al. 1994).

Revascularization of cortical bone grafts proceeds with initial osteoclastic activity. Enlargement of the haversian and Volkmann's canals must occur before vessels are able to penetrate the graft. The dense lamellar structure

of cortical bone limits the vascular invasion, and the newly forming vasculature is constrained to invade the graft along these preexisting pathways. This process begins at the graft periphery and progress to the interior of the graft (Burchardt 1983). Revascularization in cortical bone grafts may also be restricted by the limited number of endosteal cells that remain viable after transplantation. These cells are thought to contribute to end-to-end vessel anastomosis during bone graft revascularization (Heiple et al. 1987). Studies have shown that cortical grafts in the onlay position show only superficial revascularization occurring in the first 10 to 21 days, and central revascularization by 8 to 16 weeks (Ozaki and Buchman 1998). Once a graft has been placed, mesenchymal cells recruited to the region will differentiate into fibroblasts, endothelial cells or osteoblasts depending on the stimuli. These cells form new connective tissue, vessels or osteoid respectively. One major factor of importance for graft survival is the stability of the graft. This will improve both revascularization and leads to a lower grade of resorption of the graft (Phillips and Rahn 1988; Phillips and Rahn 1990). In the competition between the soft and bone tissues, a cancellous bone graft may be more prone to soft tissue ingrowth and resorption than a cortical graft (Körloff et al. 1973; Gordh et al. 1998; Johansson et al. 2001).

Several different factors are considered important for the general bone metabolism and survival of autogenous bone grafts. These factors can either be systemic such as age, gender, physical activity, hormonal status and drugs or local factors such as graft orientation, fixation of the graft, recipient's site, mechanical stress and revascularization. Certain hormones such as calcitonin, insulin, vitamin D3 and parathyroid hormones are also essential.

A fresh autogenous bone graft contains osteoinductive proteins (BMPs) that stimulate the recruitment of mesenchymal stem cells and

osteogenesis and is therefore the golden standard in reconstructive surgery (Sampath & Reddi 1983). Bone morphogenic proteins (BMPs) are homodimeric proteins of approx. 30kD with two identical strands linked by a cysteine binding group. Nearly 20 modifications of BMPs with slightly different modifications in secondary structure elements have been identified so far (Miyazono 2000). BMP2-BMP9 belong to the TGF-β superfamily with a high degree of homology with the TGF-βs. TGF-β and BMP have a common scaffold with the cysteine knot motif and two double stranded beta sheets (Scheufler et al. 1999). BMP2, BMP4 and BMP7 are considered to be osteogenic and have been tested in experimental and clinical approaches. The content of BMPs in bone has been estimated to be 1µg/g bone tissue (Kubler 1997).

In humans, three different transforming growth factor β (TGF-β) have been identified and are primarily found in platelets. These growth factors have been proven to enhance bone formation around titanium implants (Clokie and Bell 2003). Other factors stimulating bone formation are insulin-like growth factors. Insulin-like growth factors (IGFs) are single chain peptides that exist in two isoforms (IGF-I and IGF-II). IGFs have approximately 40-50% homology between themselves and with insulin. Despite this significant homology between insulin and IGFs, all three have unique binding sites to their receptors (O'Connor 1998). IGF-I has been proven to be three times more efficient in bone cells than IGF-II (Schmid 1993).

Platelet-derived growth factor (PDGF) is another factor that might influence the speed of bone formation. PDGF is a highly basic dimeric glycoprotein of 30 kD consisting of two disulphide bonded polypeptides encoded by different genes (Cochran et al. 1993). There are three isoforms characterized by the combination of A- and B-chains featuring two homodimeric (PDGF-AA and PDGF-BB) and one heterodimeric

isoform (PDGF-AB) (Hock & Cannalis 1994). PDGF-BB and PDGF-AB are systemically circulating isoforms contained in alpha granules of platelets from where they are released after adhesion of platelets to injured sites of vessel walls, whereas PDGF-AA is secreted by unstimulated cells of the osteoblastic lineage (Cannalis 1992). Marx et al. (1998) used platelet rich plasma (PRP) which is rich in PDGF, TGF- β1 and β2, IGF and fibrin in treating large mandibular defects with autogenous bone as carrier and found increased bone maturation rate and bone density compared with defects augmented with autogenous bone only.

-Vascularized bone grafts

Free vascularized bone grafts are another option, widely used for postablative reconstruction in irradiated recipient beds, where standard bone grafts have been shown to be less viable. Bone flaps are in general used in defects larger than 6 cm or when composite tissues are required. Large bone segments from the fibula or iliac crest can be transplanted together with various amount of soft tissue to restore form and function. Instant blood circulation in the flap guaranties transfer of viable osteocytes, thereby bypassing the need for new bone formation apart from at the graft-host interfaces. Vascularized bone transfers are technically challenging though and donor site morbidity is an issue in some cases (Rohner et al. 2003; Jaquiery et al. 2004)

-Allogenic bone

An allogenic graft is obtained by transplanting tissue from one individual to a genetically non-identical individual of the same species and contains no viable cells (Urist 1965). For this reason allografts are considered to be mainly osteoconductive and have very little or no osteoinductive properties (Becker et al. 1995).

Different forms of allografts are available. Mineralized or demineralized bone, frozen or freeze-dried bone, demineralized dentin and antigen-extracted allogenic bone (AAA) are examples of allografts. All of the components in bone are potentially immunogenic but bone minerals and collagen are only weakly antigenic. For this reason, cortical bone is preferable due to its high content of collagen compared to cancellous bone, resulting in a weaker immunologic reaction (Dayi et al. 2002).

Transplantation of allogenic tissue initiates an immunological reaction in the recipient of the cell-mediated type (Burwell et al. 1985). It is believed that T-cell responses are the most significant in bone transplantation and that the cell-mediated mechanisms are the same as those in skin graft rejections. The immunologic responses result in impaired revascularization of the graft and subsequent necrosis. Allografts also carry the coincident risk of disease transmission. Extensive donor screening protocols have been implemented worldwide in order to reduce transmission of HIV and hepatitis B and C viruses (Buck et al. 1989). In order to sterilize and lower the antigenicity of the allogenic graft different processing methods have been tested. Freeze-drying, demineralization, deep freezing (<-70° C) chemo sterilization or radiation, have all been suggested (Chalmers 1959; Senn 1989; Lane & Sandhu 1987). The same factors that reduce immunogenicity, however, also deactivate the osteoinductive factors that are so critical to survival. In addition, deep freezing (<-70° C) and freeze drying- the two most common methods of preservation, may significantly alter the mechanical properties and strength of the graft (Voggenreiter et al. 1994).

It was shown in dogs that the acceptance of a frozen allograft was improved with histocompatibility matching or immunosuppression (Goldberg et al. 1985). However clinical trials revealed no clear relationship between the degree of histocompatibility of the donor and the

recipient and the incorporation of frozen bone allografts (Muscolo et al. 1987).

In maxillofacial surgery, frozen allogenic bank bone has mainly been used in combination with autogenic bone (Sailer 1983; Plotnicov and Nikitin 1985). The principles for incorporation of allografts follow the same principles as for autogenous bone grafts but probably proceed more slowly due to the absence of viable cells that are osteoinductive, although allografts might have some osteoinductive properties. Osteoblasts from the recipient generate bone as the transplanted bone is gradually resorbed. Clinical trials have revealed that the incorporation is a slow and incomplete process (Lane & Sandhu 1987). Pinholt et al. (1990) studied demineralized and lyophilized dentin and bone implants in rats, and demonstrated induction of new bone formation, however in two other studies in rats and goats respectively, no osteoinduction was found (Pinholt et al. 1991; Pinholt et al. 1992). Smiler et al. (1992) compared autogenous bone (AB), deep frozen demineralized bone (DFDB), and hydroxyapatite (HA) as grafting materials prior to implant placement, with equally good results. However the healing time for the various grafts differed significantly. In a human study by Boeck-Neto et al. (2002) bone formation was evaluated in 10 patients who underwent maxillary sinus floor augmentation using autogenous bone with DFDB or HA. They concluded that both materials were still present after 10 months.

In a study by Lohman et al. (2001), bone harvested from patients and processed by lyophilization, was divided into two portions, One of which was used directly while the other was demineralized. They concluded that the age of the patient played an important role in the osteoinductive capacity of the bon study using mineralized cancellous bone allograft for sinus augmentation a vital bone content of almost 26% was found after 9 months of graft healing (Froum et al. 2005). In a clinical study by Gapski

et al. (2006), human mineralized bone allografts were successfully used for sinus lift procedures before placement of implants.

The use of an ideal graft material should result in high formation of vital bone after graft maturation. The literature shows varying results for different grafting materials. Vital bone content of 14% to 44% has been reported in the literature.

-Xenogenic grafts

Tissue transplanted between individuals of different species is called a xenogenic graft. Examples are bone-like minerals derived from corals or algae, bovine bone and porcrine bone. One of the main tasks to overcome using xenogenic bone grafts has been the immunological response and to obtain safety of disease transmission (Enneking 1957; Nisbet 1977; Burwell et al. 1985). The antigenicity of the graft initiates a T-cell response and it is believed that the cell-mediated mechanisms are the same as those seen in skin graft rejections. In order to avoid an immunological rejection after implantation, the proteins have to be extracted using various procedures. In the process of eliminating the antigens the organic matrix is destroyed and thereby the osteoinductive properties and as the osteoinductive capacity disappears the graft can only act as an osteoconductive scaffold. Furthermore, the presence of minerals in the graft impedes the transformation of fibroblasts to osteoblasts (Urist 1971; Reddi and Huggins 1973). This leads to a formation of new bone at lower pace compared to autogenous bone grafts. Healing of xenografts follow the same principles as for allografts and today they are frequently used for bone augmentation procedures in implant dentistry due to their similarity to human bone.

Algipore®(Dentsply Friadent,Mannheim Germany) is a porous fluorohydroxyapatite (FHA) derived from calcifying algae (Corallina officinalis). Complete removal of organic components has been carried

out utilizing pyrolytical segmentation of native algae and hydrothermal conversion of calcium carbonate into FHA in the presence of ammonium phosphate at about 700°C (Thorwarth et al. 2007). Studies have shown that Algipore® is a suitable biomaterial for periodontal treatment and for sinus floor augmentation (Schopper et al. 2003; Roos-Jansaker et al. 2011, Scarano et al. 2012).

Deproteinized bovine bone (DPBB) is a natural bone mineral with extreme similarities in structural and chemical composition to human bone. DPBB consists of 100% deproteinized bovine hydroxyapatite. The most documented DPBB used for reconstruction in implant surgery is Bio-Oss®(Geistlich, Wolhausen, Switzerland). This material has been investigated in numerous clinical and experimental studies by several authors and since all proteins are claimed to be extracted, Bio-Oss® works only as a 3-dimensional scaffold for ingrowth of blood vessels and bone building cells (Maioranta et al. 2001; Hallman et al. 2002; Hallman et al. 2002; Froum et al. 2006; Esposito et al 2009; Felice et al. 2009; Esposito et al. 2010; Jensen et al. 2012; Lee et al. 2012; Lindgren et al. 2012). Bio-Oss® can either be used alone in various augmentation procedures or in combination with autogenous bone which would add on the osteoinductive properties of the autogenous bone to the transplant. In a systematic review assessing augmentation of the maxillary sinus floor with Bio-Oss® alone or Bio-Oss® combined with autogenous bone, it was concluded that the hypothesis of no differences between the two procedures could neither be confirmed nor rejected. They also concluded that the addition of AB to bio-Oss® did not influence the biodegradation of Bio-Oss® although long term studies were not available (Jensen et al. 2012).

There seem to be controversy in the literature whether DPBB is resorbable, slowly degraded, phagocytated or non resorbable. True

resorption by means of osteoclastic activity of Bio-Oss® is however, one topic where contradictory results have been found. In one study, maxillary sinus augmentation using DPBB and AB was performed in 20 patients. Signs of resorption lacunas and the presence of osteoclasts on the particle surface were observed in the specimens harvested four years after initial surgery (Piatelli et al. 1999). In another report, there were no signs of DPBB particles after 20 months of healing but it could not be determined whether this was dependent on the biopsy technique or was the result of a true resorption (Wallace et al. 1996). Other studies on maxillary sinus grafting with Bio-Oss show no signs of resorption of the material at all (Schlegel & Donatah 1998; Valentini et al. 1998, 2000). The fact that this material does not resorb when used for maxillary sinus grafting is highly important in comparisons to autogenous bone grafts only, where in some cases the resorption is more than 50% of the original volume (Körloff 1973; Johansson et al. 2001). The advantage of using a non-resorbable bone substitute due to, its low-grade resorption has been discussed in a paper by Cobb et al. (1990). They concluded that a 1:1 ratio is optimal and increased amounts of bone substitute result in more fibrous encapsulation. Nevertheless, in some studies 100% of DPBB has been used with results similar to those when it was used as an admixture (Hising et al 2001; Yildrim et al. 2001).

Some researcher have claimed that Bio-Oss® after all contains certain amount of both transforming growth factor β(TGF- β) and proteins, whilst others have questioned these findings (Honig et al. 1999; Schwartz et al. 2000; Benke et al. 2001). A systematic review by Kim and co-workers indicates that bovine derived bone substitutes may theoretically carry a risk of prion disease transmission to patients even though no clinical reports of this complication have been published yet (Kim et al. 2013, 2016).

-Alloplastic grafts

Alloplastic bone grafts are derived synthetically and are alternatives to autogenous, allogenous and xenogenic bone grafts in reconstructive surgery. The development of alloplastic bone substitutes evolved out of the operative morbidities of autografts and the limitation of allografts. Alloplastic grafts follow the same principles as for the allografts except that the materials contain no proteins and are only osteoconductive. Formation of new bone can start if the material is placed in close contact to bone, serving as a 3-dimensional scaffold for ingrowth of bone building cells and blood vessels.

Calcium phosphate ceramics (CaPs) including HA and tricalcium phosphate (TCP), calcium-sulphate, bioactive glasses and polymers are all examples of alloplastic bone grafts materials with different compositions. Furthermore, they exhibit different biological and mechanical properties.

Calcium phosphates resemble the inorganic matrix of bone more closely and therefore have greater utility as a bone substitute. Alloplastic bone graft substitutes usually contain hydroxyapatite (HA) and different calcium polymers such as, β-tricalcium phosphate or sintered calcium phosphates, bioglass or sintered calcium sulphates. In contrast to HA, pure calcium phosphate or calcium sulphate is generally weaker in its composition and will presumably dissolve chemically in to its ions which may stimulate bone formation (Daculci 1998).

There are two major varieties of hydrated calcium phosphate (hydroxyapatite) preparations for use in bone applications: ceramics and cements. Hydroxyapatite ceramic implants can be manufactured in a variety of shapes, forms, structures and chemical compositions. They possess different mechanical and biological properties and the time of degradation may vary. Their similarity in composition to bone mineral,

their biodegradability and osteoconductivity were the rationales for development of CaPs (Hannink and Arts 2011). Hydroxyapatite ceramic implants have been extensively used within the field of oral implantology (Kwon et al. 1986; El Deeb et al. 1991). Unlike ceramic forms, hydroxyapatite cements are moldable, allowing for intraoperative contouring, however their use has been limited to low-stress regions in the craniofacial skeleton. Smiler and Holmes (1987) reported on a mean bone ingrowth of 21,1% after 5 months when using porous hydroxyapatite (HA) in sinus lifting procedures. In another study by Wheeler et al. (1996), HA was used with and without autogenous bone and the levels of newly formed bone ranged from 11 to 20% for the different groups.

Active glass is another type of allograft that has been tested and shows active bone formation and support for dental implants. In a study by Cordioli et al. (2000), mixture in a ratio of 4:1 of bioactive glass and autogenous bone was used as grafting material to the maxillary sinus. Biopsies harvested after 9 to 12 months revealed a mean of 30,6±5,7% bone tissue in grafted sites.

Tricalciumphosphate (TCP) has also been tested and has been found to be suitable for use as a maxillary sinus floor graft (Szabo et al. 2001).

β-tricalcium phosphate has been developed to achieve a more organized crystal structure in relation to pure calcium phosphate but will still dissolve into calcium and phosphate ions. Cerasorb® (Curasan AG, Kleinostheim, Germany) is the most used commercial β-tricalcium phosphate today for dental implant surgery and the material has been used for maxillary sinus floor augmentation. However drawbacks such as rapid dissolution and volume reduction have been reported (Lu et al. 2002).

In order to combine different properties, materials are sintered together. Bone Ceramic® (Straumann, Basel, Schweiz) is a novel, fully synthetic bone substitute, aimed for the market of implant surgery. It consists of 60% HA and 40% β-TCP. The HA component is supposed to protect the augmented area from resorption and the TCP component is supposed to dissolve relatively quickly in to its ions and stimulate bone formation (Lindgren et al. 2009).

In a randomized and controlled study, bilateral sinus floor augmentation was performed in 11 patients with severe atrophy in the posterior maxilla using biphasic calcium phosphate (Bone Ceramic®) at one side and deproteinized bovine bone (Bio-Oss®) at the contra lateral side acting as control. Micro implants were installed simultaneously and retrieved after 8 months with a surrounding bone core and analyzed by scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS). The conclusion was that the β-TCP component of BCP may be gradually substituted by calcium deficient hydroxyapatite over the healing period and might influence the progress of resorption and healing (Lindgren et al. 2010).

In another prospective study with 9 patients undergoing bilateral sinus floor augmentation with BoneCeramic® on one side and Bio-Oss® on the other side, similar results were found after 1 & 3 years of functional loading and implant success rate was not dependent on the biomaterial used for augmentation (Lindgren et al. 2012a,b)

Other examples of alloplastic materials are Easygraft CRYSTAL® (Degradable Solutions AG, Schiliern, Switzerland), Tricos® (Baxter healthcare corp., USA) and calcium sulphate ($CaSO_4$) (Surgiplaster, Ghimas, Bologna, Italy) with different tissue response to their resorption and dissolution (Hallman and Thor 2000, De Leonardis and Pecora 2000).

-Dentin as a bone substitute material

Dentin is a hard, elastic, avascular mineralized tissue comprising the major portion of the tooth, supporting enamel and enclosing the central pulp chamber. It is composed of approximately 70% mineral, 20% organic matrix and 10% water by weight, and 45%, 33%, and 22% by volume, respectively. The mineral phase in dentin mainly consists of carbonate-substituted hydroxylapatite. Furthermore the organic phase contains about 90% fibrous proteins (mainly type I collagen and a small percentage of type III collagen), while the rest of the organic phase is comprised of lipids and non-collagenous matrix proteins (Xu and Wang 2012). The organic and inorganic components of dentin are very similar to those of bone. In alveolar bone, the inorganic content is 65%, and the organic content is 25%. Type I collagen, which constitutes major part of the organic content of dentin, plays an important role in bone formation and mineralization. Dentin also contains bone morphogenetic protein (BMP), which promotes the differentiation of mesenchymal stem cells into chondrocytes and consequently enhance bone formation. Dentin matrix derived BMP is not exactly same as bone matrix derived BMP, but they are very similar. BMP content of dentin makes it to a viable bone substitute since it possesses both osteoinductive and osteoconductive properties compared to strictly osteoconductive bone substitutes. Furthermore, dentin contains non-collagenous proteins such as osteocalcin, osteonectin and dentin phosphoprotein, which are known to be involved in bone calcification (Urist and Strates 1971; Morotome et al. 1988). Since its introduction by Urist in 1965, BMP has been widely studied and used in clinical applications (Urist 1965). As a result other investigators showed the osteoinductivity of rabbit demineralized dentin matrix by BMP (Yeoman et al. 1967 and Bang et al. 1967).

The idea of using dentin as a bone substitute for clinical practice probably originates from the principle of dentoalveolar ankylosis and replacement resorption, which is seen in more severe types of dental trauma, after the PDL has been severely damaged e.g. after tooth avulsion and delayed replantation. During intrusion, lateral luxation or avulsion with subsequent replantation, contusion or drying of the PDL is a common occurrence. Wound healing is subsequently initiated when damaged tissue is removed by macrophage or osteoclastic activity. During these events, not only are necrotic PDL tissue remnants removed, but sometimes also bone and cementum (Andreasen 1966; Andersson et al. 1984). When large areas of the PDL are traumatized, competitive wound healing processes begin between bone marrow-derived stem cells destined to form bone and PDL-derived cells which are programmed to form PDL fibers and cementum resulting in fusion of the alveolar bone with dentin (ankyloses) which is followed by replacement resorption (Blomlöf & Lindskog 1994). IGF-1, TGF-β and BMPs in the dentin are released slowly and may serve as stimulators for osteoclasts. If the area of injury is limited healing with normal periodontal ligament can be seen (Andreasen and Kristerson 1981). However, when the area of injury is large ankyloses cannot be avoided (Andreasen and Kristerson 1981). When ankylosis has been established the dentin is replaced by bone (osseous replacement, replacement resorption) (Andreasen 1966, Andersson et al. 1984, Andersson et al. 1989). The rate of replacement resorption is related to the age of the patient with a higher rate in young individuals (Andersson et al. 1989)

Yeomans and Urist showed back in 1967 bone induction by decalcified dentin implanted into oral, osseous and muscle tissues in rabbits. Samples of allogenic tendon from the lower leg, quadriceps muscle, and either decalcified bone or decalcified dentin were implanted into three different

sites: (a) a pouch in the rectus abdominus muscle, (b) a drill-hole bone defect in the mandible, and (c) an empty tooth socket. Samples were recovered after 4, 8 and 12 weeks after operation. Tendon and muscle were rapidly resorbed over a 4- to 12 week period and did not induce osteogenesis. Samples of decalcified dentin in comparison were relatively slowly resorbed and always positive for bone induction.

Bang et al. (1972) used allogenic demineralized dentin implants in jaw defects of Java monkeys. Surgical defects of a certain diameter were made bilaterally in the region below root apices of the mandibular third molars in 16 Java monkeys. Allogenic demineralized dentin was placed in the defects on the right side while the contra lateral side served as controls. After a healing period of 1 week to 1 year histological studies were undertaken. Dentin implants, were shown to be tolerated very well and were gradually resorbed. They were both osteoinductive and osteoconductive and secured complete osseous healing. The control cavities exhibited incomplete osseous healing with persisting fibrous defects.

Su-Gwan et al. (2001) used a combination of particulated dentin and plaster as bone substitute material in calvarial bone defects in rats and compared it with Bio-Oss. They concluded that the combination of particulate dentin and plaster is an alternative bone substitute, although it is less effective than Bio-Oss. Carvalho et al. 2004 used homogenous demineralized dentin matrix as osteopromotive material in rabbit mandibles. They concluded that demineralized dentin matrix were biocompatible and were resorbed during the bone remodelling process. Bone repair was accelerated in the bone defects treated with demineralized dentin in comparison to the control group. Demineralized dentin blocks implanted in the palatal connective tissue in rats did not seem to induce any bone formation up to 4 weeks after implantation

(Miyaji et al. 2002). In a recent study in rats, it was concluded that demineralization of dentin blocks in 24% EDTA for 2 or more hours resulted in significantly higher rate of resorption and significantly lower rate of encapsulation (Mordenfeld et al. 2011).

Furthermore, there is some evidence in the literature that non-demineralized dentin may resorb fast with no signs of bone formation when implanted in animal muscle (Bang 1972; Machado et al. 2006). Hence, there is reason to believe that for dentin, besides the surgical technique, the demineralization procedure and the implant environment play important roles in osteoinduction and osteoconduction. However, there is limited information in the literature systematically analyzing the biological outcome of different clinically applicable preparations of dentin for augmentative use.

Analysis methods

-Bone histomorphometry

Bone histomorphometry is a quantitative histological examination of an undecalcified bone biopsy performed to obtain quantitative information on bone remodelling and structure (Kulak and Dempster 2010). It is considered a valuable and well-established clinical and research tool for studying the pathogenesis of metabolic bone diseases as well as in order to evaluate the healing process for different bone substitutes or implants (Parfitt 1983; Dempster 2001). Histomorphometry has traditionally been assessed in two dimensions by means of histology, where the structural and remodelling parameters are measured on sections, and the third dimension is extrapolated using standard stereology theory (Parfitt 1983). In the last two decades, there have been significant advances in

histomorphometric techniques with coupled stereology software which have largely substituted the manual techniques (Malluche et al. 1982). Remarkable advances in bone histomorphometry were made in the 1950's and 60's due to the discovery of plastic embedding allowing high quality histologic sections of mineralized bone (Frost 1958) and the use of labeling fluorochromes leading to a better understanding of the dynamic process of bone formation (Frost 1969).

Placement of microimplants is a well-established method to create a miniature model of the titanium-bone interface (Jensen and Sennerby 1998; Lundgren et al. 1999; Hallman et al. 2002). In brief, a titanium threaded microimplant is installed in the graft penetrating the residual bone. After a certain healing period the microimplant with surrounding bone core is retrieved using a trephine burr. The specimens are fixed by immersion in buffred formalin solution, dehydrated in alcohol and embedded in plastic resin. A specialized laboratory prepares undecalcified sections and histological and histomorphometrical analysis is carried out after the samples have been stained with certain dyes.

-Scanning electron microscopy

Scanning electron microscopy (SEM) is a well-known technique used for micro-anatomical imaging. The SEM is a microscope that uses electrons instead of light to form an image. A beam of electrons is generated from a scheelite cathode at the top of the microscope by an electron gun. The electron beam follows a vertical path through the microscope, which is held within a vacuum. The beam travels through electromagnetic fields and lenses, which focus the beam down toward the sample. Once the beam hits the sample, electrons and X-rays are ejected from the sample. Detectors collect these X-rays, backscattered electrons, and secondary electrons and convert them into a signal that is sent to a screen similar to a television screen. This produces the final image (Sriamornsak and

Thirawong 2003). Since their development in the early 1950's, scanning electron microscopes have developed new areas of study in the medical and physical science communities. The SEM has allowed researchers to examine a much bigger variety of specimens. The scanning electron microscope has many advantages over traditional microscopes. The SEM has a large depth of field, which allows more of a specimen to be in focus at one time. The SEM also has much higher resolution, so closely spaced specimens can be magnified at much higher levels. Because the SEM uses electromagnets rather than lenses, the researcher has much more control in the degree of magnification. SEM can exploit different types of samples such as dried specimens non-embedded or embedded in resin, frozen-wet tissue or damp-wet tissue. All of these advantages, as well as the actual strikingly clear images, make the scanning electron microscope one of the most useful instruments in research today (Slater et al. 2008).

AIMS

General aim:

The overall aim of the present thesis is to study dentin as a possible bone replacement and augmentation material prior to implant treatment.

Specific aims

Paper I

To evaluate and compare the host tissue response to autogenous and xenogenic non-demineralized dentin blocks implanted in non-osteogenic areas, the abdominal connective tissue and femoral muscle of rabbits.

Paper II

To evaluate the healing pattern of xenogenic non-demineralized dentin granules and dentin blocks grafted to maxillary bone of rabbits and secondarily to study integration of titanium micro-implants installed in grafted areas.

Paper III

To primarily investigate the morphological appearance and mineral content in decalcified dentin grafts and secondarily to study the healing pattern of xenogenic demineralized dentin blocks and granules grafted to cavities created in tibial bone of rabbits and subsequent integration of titanium micro-implants installed in the previously grafted areas.

Paper IV

To compare the host tissue response and remodelling of onlay grafts of demineralized xenogenic dentin in comparison to onlay autogenous bone grafts transplanted to the native tibial cortical bone wall.

Material and methods

Animals and anesthesia

New Zealand male white rabbits were used in all studies. The animals were kept in specially designed rooms in separate cages and fed pellets and water *ad libitum* throughout the duration of the study. The experiments were carried out at the Animal Research Centre, Health Sciences Centre, Kuwait University. The protocol for animal experiments by the Animal Research Centre of the Health Sciences Center, Kuwait was strictly adhered to.

Thirty minutes prior to the experimental surgery, the rabbits were sedated with Xylazine HCl(Rompun, Bayer, Leverkusen, Germany) 5mg/kg by intramuscular injection. Animals were anaesthetized by intravenous injection of 35mg/kg of Ketamine HCl (Tekan, Hikma, Amman, Jordan). A veterinarian was responsible for administering the sedation, anesthesia and for the intra- and postoperative care of the animals. To compensate for peri-operative and postoperative dehydration 10ml sterile saline solution was injected subcutaneously immediately following surgery and antibiotics (Pen-Hista-strep,Vetoquinol SA, Lure Cedex, France) 50mg/kg was administered by intramuscular injection. Antibiotic administration was continued during the first 3 days after surgery. After completion of healing, the animals were sacrificed by an overdose of Ketamine.

Implants

Study II and III

Screw-shaped micro implants (5 mm long, 2 mm in diameter), which were machined from medical grade Ti (grade IV) rods (Elos, Pinol, Gørløse, Denmark) were used.

Fig.1 Screw-shaped micro implants used in study II & III

Study IV

Titanium fixation screws, 6-mm long, 1.5-mm-wide (DePuy Synthes, Jonhsson & Johnsson) were used in this experiment.

Surgical Protocols

Study I

Fifteen 6-month old New Zealand male white rabbits were used in this study. The surgical areas were shaved and washed with iodine 7.5% solution and the animals were prepared for surgery. As a supplement to general anesthesia and for vasoconstriction purposes, local anesthesia 1ml Lidocainehydrochloride 1% + epinephrine 5µg/ml (Xylocain-adrenalin, Astra Zeneca, Södertälje, Sweden) was administered in each experimental area. Bilateral incisions, each 2,5cm long, on either side of the abdominal midline were performed. In each wound, dissection was carried out to create a pocket in the abdominal connective tissue. Bilateral incisions, each 2.5 cm long over the femoral muscle in the groin region on either side were performed. With the two pockets in the abdominal

region and the two pockets in the muscles prepared, attention was then focused on the oral region of the rabbit where an incision was made in the gingiva lateral to the right central incisor and a 5mm mucoperiosteal flap was raised. The incisor was luxated in a lateral direction, the thin lateral alveolar bone socket was fractured and the tooth was gently removed out through the lateral alveolar socket. The tooth was then removed by forceps to be used as autograft. The incision was closed by Vicryl 4-0 sutures.

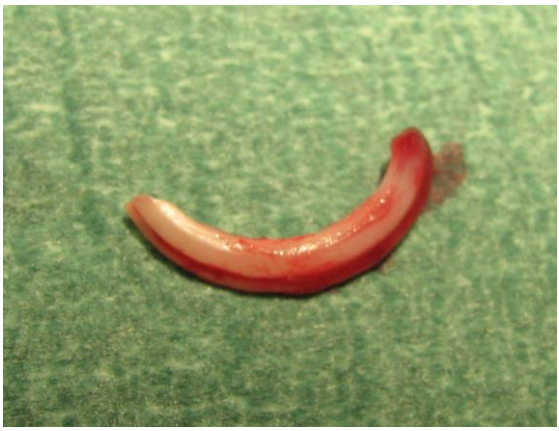

Fig.2 Rabbit central incisor, used as autograft.

The dentin grafts were taken from human premolars extracted for orthodontic reasons. The teeth were prepared in the following manner: The coronal part of the tooth was cut and removed with the help of rotary instruments. The pulp and periodontal ligament were removed with endodontic files and a scalpel blade respectively. The premolar xenografts were prepared similarly to the autografts by removing the coronal part of the tooth, its pulp and periodontal ligament and the root was also cut in two halves to facilitate pulp removal. The dentin autografts and xenografts were then grafted to the pockets created in the abdominal connective tissue and femoral muscles.

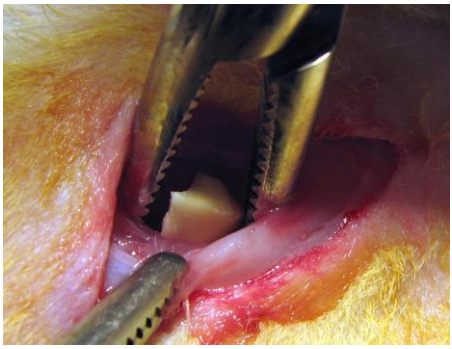

Fig.3 Dentin graft implanted in femoral muscle of rabbit.

The incisions were closed in layers with Vicryl 4/0 (Ethicon, Cornelia, USA). All rabbits were sacrificed after 12 weeks by an overdose of Ketamine and block biopsies were prepared.

Study II

Fifteen 6-month old New Zealand male, white rabbits were used in this study. As a supplement to general anesthesia and for vasoconstriction purposes, local anesthesia 1ml lidocainehydrochloride 1% + epinephrine 5μg/ml (Xylocain-adrenalin, Astra Zeneca, Luton, UK) was administered in each experimental area. The bilateral edentulous areas superior to and between incisors and posterior teeth of the maxilla were used as experimental sites. The bone surface was exposed via a 10 mm long incision between buccal and palatal mucosa. A muco-periostal flap was raised. A 5x5 mm wide and 3mm deep cavity was prepared penetrating through the maxillary cortical bone wall with the use of round burr (3mm in diameter) under irrigation with saline. Dentin grafts from human premolars, which were extracted for orthodontic reasons, were prepared in the following manner: The coronal part of the tooth was cut and removed with the help of rotary instruments so no enamel remained. The pulp and periodontal ligament were removed with endodontic files and a

scalpel blade respectively. With the help of a trephine burr (5mm diameter) cylinder shaped block was harvested from the premolar and the cylinder was sectioned into 3mm thick blocks. The rest of the premolar was cut into granules in sizes of 1-3mm. Granules and blocks were cleaned by being placed in 1% chlorhexidine and stored dry for one month. They were rinsed in saline for one hour before being used as grafts. The cavities on the right side were filled with dentin granules and the cavities on the left side with dentin blocks. No membrane or any other type of fixation was used. The incisions were closed with 4/0 Vicryl (Ethicon, Bridgewater, NJ,USA). After a healing period of 24 weeks, rabbits were anesthetized once again as described earlier. Surgical access was accomplished in a similar way and one micro implant was installed in each surgical site in such a way, that the apical half of the implant were placed in native bone, serving as control site, and the coronal part in dentin. All rabbits were sacrificed 24 weeks after the second surgery by an overdose of Ketamine and block biopsies were prepared.

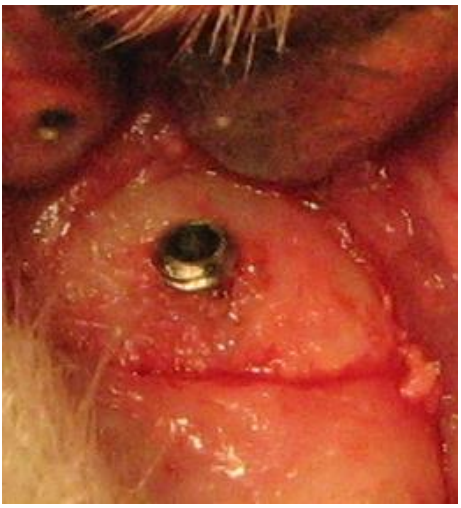

Fig.4 Micro-implant installed in grafted dentin

Study III

Twelve 6-month old New Zealand male, white rabbits were used in this study. The surgical areas were shaved and washed with iodine 7.5% solution and the animals were prepared for surgery. Local anesthesia 1ml lidocaine hydrochloride 2% + epinephrine 1:100 000 (Lignospan standard, Septodont, Saint Maur des Fosses Cedex, France) was administered in each experimental area. Bilateral incisions, 2-3 cm long over the lateral aspect of the tibia were performed and the tibial bone was exposed by surgical dissection. On each side, two cavities of 5mm diameter and 3mm depth were prepared penetrating through the tibial cortex with the use of round burr (3mm in diameter) under irrigation with saline. The dentin grafts were taken from human premolars, which had been extracted on orthodontic indications. The teeth were prepared in the following manner: The coronal part of the tooth was cut and removed with the help of rotary instruments. The pulp and periodontal ligament were removed with endodontic files and a scalpel blade respectively. Cylindrical dentin block grafts were prepared in standardized sizes by the use of a trephine bur, 5mm in diameter, and cut and trimmed to 2mm thick dentin blocks by a diamond dish. The thickness of 2mm was checked with a caliper. The rest of the premolar was cut into granules in sizes of 1-3mm. The dentin blocks and granules were placed in chlorhexidine to reduce bacterial growth. Twelve hours prior to grafting the dentin grafts were rinsed in saline and demineralized on its surface by being placed in 24% EDTA neutral, pH7, for 12 hours. On the right tibia, the proximal cavity was filled with demineralized dentin block and the distal cavity with demineralized dentin granules. On the left tibia non-demineralized dentin was used in the same manner. No membrane or any other type of fixation was used. The soft tissue was sutured in two layers, muscle and dermis.

After a healing period of 24 wks, rabbits were anesthetized once again as described earlier. Surgical access was accomplished in a similar way and one micro implant was installed in each surgical site in such a way that the coronal part of the micro implant was placed in dentin. All rabbits were sacrificed 24 weeks after the second surgery by an overdose of Ketamine and block biopsies were prepared.

Preparation and characterization of dentin grafts

Twelve dentin blocks were prepared as described (*vide supra*). Conventional dental x-rays (0.06 sec, f 22.5 cm) were taken on all graft samples prior to decalcification. All samples were conditioned in 24% EDTA neutral, pH 7, for 12 hours followed by a second x-ray analysis. Four samples were chosen for conventional SEM and energy dispersive X-ray analysis (EDX), both image mode and element analysis mode. Semi-quantitative data for the elements C, N, O, P, Ca and Au (100% in total together) were sampled for 25 min.

Study IV

Eight 6-month old New Zealand male white rabbits were used in the experiments. The surgical areas were shaved and washed with iodine 7.5% solution and the animals were prepared for surgery. Local anesthesia 1ml lidocaine hydrochloride 2% + epinephrine 1:100 000 (Lignospan standard, Septodont, Saint Maur des Fosses Cedex, France) was administered in each experimental area. Bilateral incisions, 2-3 cm long over the lateral side tibia of the rabbit were performed and the tibial bone was exposed by surgical dissection. With a 5mm diameter trephine the tibial medial cortex was penetrated through its full thickness and a bone block was harvested. The harvested bone block was measured and trimmed by a diamond dish to a thickness of 2mm as controlled with the help of a caliper. The dentin grafts were taken from human premolars,

which had been extracted on orthodontic indications. The teeth were prepared in the following manner: The coronal part of the tooth was cut and removed with the help of rotary instruments. The pulp and periodontal ligament were removed with endodontic files and a scalpel blade respectively. Cylindrical dentin block grafts were prepared in standardized sizes by the use of a trephine bur, 5mm in diameter, and cut and trimmed to 2mm thick dentin blocks by a diamond dish. The thickness of 2mm was checked with a caliper. The bone graft and the earlier prepared dentin blocks were anchored as onlay grafts on the tibial bone by a titanium screw in the centre of the graft so that the screw pulled the onlay graft tightly towards the tibia.

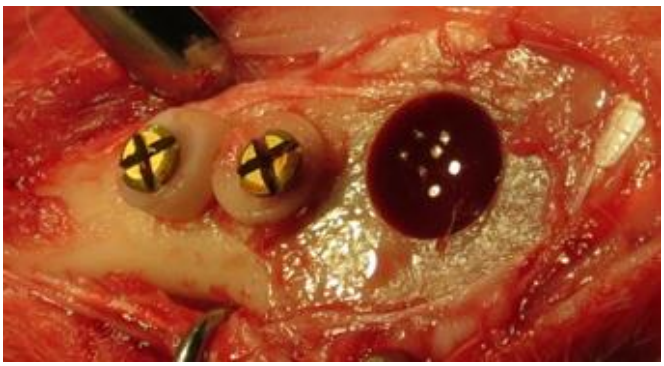

Fig.5 Bone block and dentin block fixated to the tibia with one titanium screw

Placement of the grafts were intentionally varied so that eight of bone blocks were placed close to the trephine cavity on the right tibia and the other eight more distant to the cavity on the left tibia in order to be able to evaluate any differences in healing pattern related to adjacency to the trephine cavity. The reversed order was applied for the dentin blocks. The soft tissue was sutured in two layers, muscle and dermis. All rabbits were sacrificed after 12 weeks.

Specimen preparation

Study I

Following surgical removal, the samples were immersed and fixed for 48 h in 10% neutral buffered formalin. The samples were then decalcified in neutral EDTA for 4-6 weeks, dehydrated in alcohol and embedded in paraffin under vacuum using standard histological methods. Serial sections were cut at 5µm thickness and were mounted on polylysine-coated slides and then stained with hematoxylin and eosin, and examined using light microscopy. The best sections including both dentin blocks and the surrounding soft tissue were selected and evaluated for thickness of fibrous capsule, number of inflammatory infiltrate and heterotopic bone formation.

Study II-IV

Directly after the sacrifice of the animals, the implants and surrounding tissue were removed *en bloc* and immediately fixed by an immersion of 10% neutral buffered formalin. The specimens were later dehydrated in a graded series of ethanol, infiltrated with plastic resin and polymerized prior to cutting along the long axis of the implant. A central ground section was prepared by cutting and grinding, and was subsequently stained with 1% toluidine blue.

Analysis and calculation

Study I

The thickness of the fibrous tissue surrounding the grafts was measured using a software program called Leica Application Suite v3.1 (Leica, Microsystems, Switzerland). Three areas were selected and the capsule thickness was measured in µm by drawing a straight line across the capsule. A magnification of 2.5 X was used for all the sections examined. The average of the three readings, were taken as capsule thickness. Using

the same image analysis system, round cells consistent with inflammatory cells were counted in the region of interest (ROI) close to the site where the thickness of the capsule was measured with a magnification of 20x. The area of this measurement was around 0.071mm^2. Then using the cell count tool, the cells were counted and the average of 3 readings, were taken as the cell count. In sections where heterotopic hard tissue formation was seen, the contact surface of heterotopic bone formation related to the total graft circumference was calculated using the same analysis system. The total circumference of the graft was measured as well as the sites of hard tissue formation. Heterotopic bone formation was expressed as a quotient of the sites of heterotopic hard tissue formation related to the total circumference of the graft and expressed as a percentage.

Study II

Two regions of interest were defined (ROI I and ROI II). ROI I (5 coronal threads) corresponded to the area where dentin blocks or granules were placed.

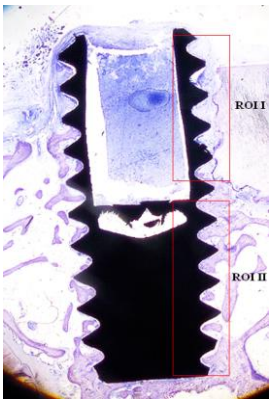

Fig.6 ROI I corresponds to the coronal threads embedded in dentin and ROI II corresponds to the apical threads embedded in host bone.

ROI II corresponded to the apical portion of the implants, which were installed in the maxillary host bone only (serving as control). The specimens were observed along their full length. The measurements of bone-to-implant contact (BIC) and the bone fill area (BA) within the threads were calculated on the mesial and distal aspect of each specimen. A mean value was then calculated for each specimen (ROI I and II respectively). The dentin and the bone-to-implant contact and the relative amount of bone and dentin within the threads, were determined using light microscopy (Nicon Eclipse E600) at 10 times magnification. The specimens were assessed using NIS Elements Microscope Imaging Software, Nikon.

Study III

One region of interest was defined (ROI). ROI (5 coronal threads) corresponded to the area where dentin blocks or granules were placed. The specimens were observed along their full length. The measurements of bone-to-implant contact (BIC) and the bone fill area (BA) within the threads were calculated on the mesial and distal aspect of each specimen. A mean value was then calculated for each specimen. The dentin and the bone-to-implant contact and the relative amount of bone and dentin within the threads, were determined using light microscopy (Nicon Eclipse E600) at 10 times magnification. The specimens were assessed using NIS Elements Microscope Imaging Software, Nikon.

Study IV

A central ground section was prepared by cutting and grinding, and was subsequently stained with toluidine blue. The specimens were observed along their full length. A defined region of interest (ROI) was constructed with equal dimensions as the respective grafts (5 x 2 mm). The presence

of the respective tissue types were calculated and presented as percentage values of the respective graft area (ROI). Furthermore resorption pattern of both graft types was assessed both on top and on the interface between the grafts and native bone. Descriptive histology as well as histomorphometric analysis of the remaining dentin, bone graft and soft tissue was determined using light microscopy (Nicon Eclipse E600) at 10 times magnification. The specimens were assessed using NIS Elements Microscope Imaging Software, Nikon.

Statistics

Study I

Host tissue reactions as expressed by the thickness of surrounding fibrous connective tissue capsule and counts of inflammatory cells adjacent to the grafted dentin were compared between the groups. The normal distribution assumption for variables; thickness and counts, was ascertained with Shapiro-Wilk test. The overall group differences were compared using non-parametric Kruskal-Wallis test, whereas Kolmogorov-Smirov Z test for comparing two independent variables. The two-tailed probability value $p < 0.05$ was considered statistically significant.

Study II

Results were reported as mean values and standard deviations. Bone-to-implant contact (BIC) and bone fill area (BA) in ROI I and ROI II were compared using the student's t-test. P-values of < 0.05 were considered statistically significant.

Study III

Results were reported as mean values and standard deviations. The Wilkoxon signed rank test was used for comparing bone-to-implant contact (BIC) and bone fill area (BA) between the groups. *P*-values of <

0.05 were considered statistically significant.

Study IV

The amount of resorption of dentin and bone on top versus on the interface, the amount of newly formed bone on top of the graft versus on the interface and the amount of newly formed soft tissue on top versus on the interface were assessed. A pairwise Wilcoxon rank sum test with Bonferroni correction for multiple testing was used to test for significant differences between the test sites. P-values of < 0.05 were considered statistically significant.

Results

Study I

The aim of this study was to evaluate and compare the host tissue response to autogenous and xenogenic non-demineralized dentin blocks implanted in non-osteogenic areas, the abdominal connective tissue and femoral muscle of rabbits.

Clinical observation

All animals recovered uneventfully and gained weight**.** The soft tissue healing in all 15 rabbits were uneventful and there were no signs of infection.

Histological observation

The thickness of the fibrous tissue surrounding the grafts was measured. There was no significant difference ($p=0.388$) in thickness between capsules of autografts and xenografts or between capsules in muscle or abdominal tissue.

Round cells consistent with inflammatory cells were counted in the region of interest (ROI) close to the site where the thickness of the capsule was measured. There were no significant differences between

autografts and xenografts. A significant difference (p=0.018) was seen with more inflammatory cells in abdominal grafts than muscle grafts among the autografts.

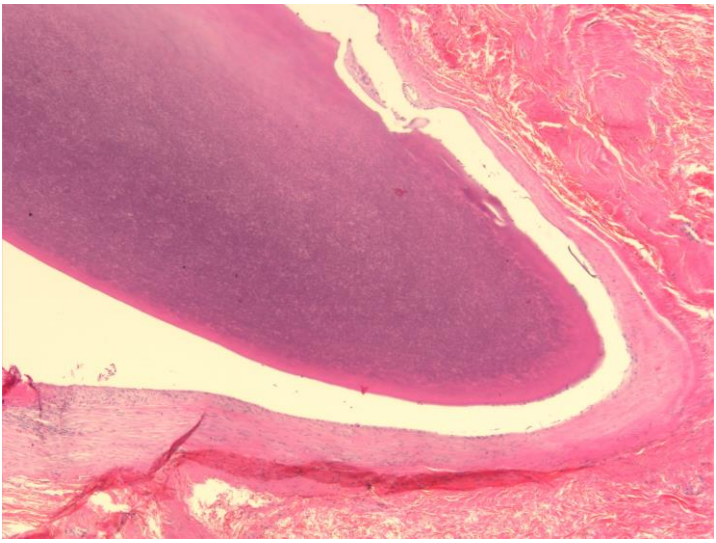

Fig.7 Connective tissue capsule surrounding the dentin with no evidence of any inflammatory infiltrate

In sections where heterotopic hard tissue formation was seen, the contact surface of heterotopic bone formation related to the total graft circumference was calculated.

In the abdominal grafts no heterotopic bone formation was seen in any of the sections except for one section in one autograft. Hard tissue formation was seen in 3.9% of the dentin graft circumference in this section. In the muscle grafts hard tissue formation was seen in 3 of the sections. The surface area was estimated to 2.2 and 5.7% respectively in two xenograft sections, and 3% of one section in the autograft group.

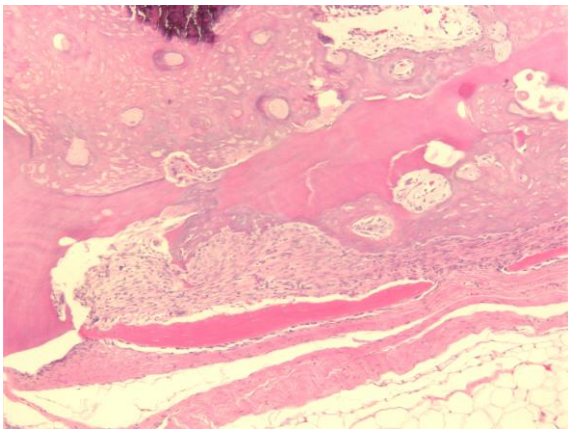

Fig.8 Dentin autograft in rabbit femoral muscle. Extensive resorption and evidence of hard tissue formation is seen.

Study II

The aim of this study was primarily to evaluate the healing pattern of xenogenic non-demineralized dentin granules and dentin blocks grafted to maxillary bone of rabbits and secondarily to study integration of titanium micro-implants installed in grafted areas.

Clinical observation

Three rabbits died during the healing period. The remaining 12 rabbits recovered uneventfully and gained weight. The soft tissue healing in all 12 rabbits was uneventful and there were no signs of infection. Three sites grafted with dentin granules and 3 sites grafted with dentin blocks were encapsulated by loose connective tissue which did not allow any implant installation due to lack of bone. Hence a total of 18 micro-implants were installed (Block group n=9, granulae group n= 9).

Histological observation

In general, the incorporation of the dentin blocks and granulae varied. In the block group, nine out of 12 available blocks were considered enough fused to the surrounding bone and suitable for implant placement. In the granulae group, less fusion to bone was seen. A common feature was that

granulae were encapsulated by means of fibrous tissue and only scarce contact between the xenogenic dentin granulae and blocks and the surrounding host bone was found. In general no or limited direct contact between xenogenic dentin and the microimplant surface could be noted. A few osteoclasts could be identified on the surface of the dentin, mostly located adjacent to present native bone tissue.

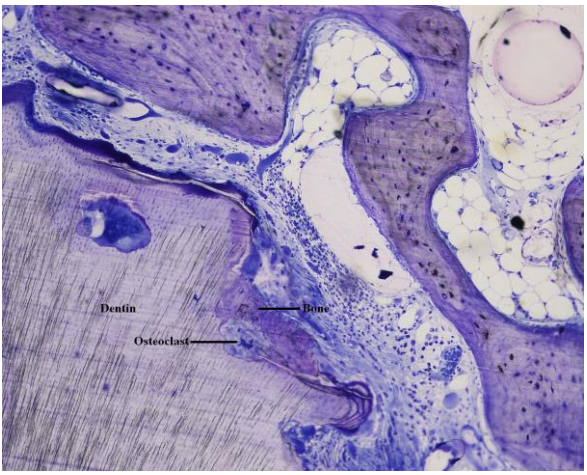

Fig.9 Osteoclast adjacent to implanted dentin and subsequent bone deposition.

The dentin particles were otherwise surrounded by fibrous tissue with scarce presence of cells. The dentin material per se, did not seem to induce bone apposition on the implant surface. Instead newly formed bone seemed to migrate into the microgap between the dentin and the titanium surface.

Histomorphometric analysis

ROI I comprised of the first 5 threads and the border between native bone (ROI II) and the dentin area was set at thread 5, where the interface could be assessed. The dentin specimens (ROI I) revealed a mean BIC of 17.8% and the native bone (ROI II) resulted in a BIC of 24.4% (p=0.188). The

percentages of new bone fill in the area (BA) within the threads (% bone fill) for the dentin specimens were 31.6% and 42.6% (P=0.360) for the native bone. Overall the BIC and percentage of new bone fill of the block specimens were higher than the same parameters for the particulate graft. Only fractional areas of direct contact between the dentin and the titanium surface could be noted.

Study III

The aim of this study was primarily to evaluate the healing pattern of xenogenic demineralized dentin granules and dentin blocks grafted to cavities created in tibial bone of rabbits, secondarily to study integration of titanium micro-implants installed in grafted areas and thirdly to investigate the morphological appearances and differences between decalcified and non-decalcified dentin by means of Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX).

Clinical observation

Four rabbits died during the healing period. The remaining 8 rabbits recovered uneventfully

and gained weight**.** The soft tissue healing in all 8 rabbits was uneventful and there were no signs of infection. Hence a total of 32 micro implants were placed (demineralized group n=16, non-demineralized n=16).

Histological observation

In general no or limited direct contact between xenogenic dentin and the microimplant surface could be noted. A few osteoclasts could be identified on the surface of the dentin, mostly located adjacent to present native bone tissue. The dentin particles were otherwise surrounded by fibrous tissue with scarce presence of cells. The dentin material per se, did not seem to induce bone apposition on the implant surface. Instead newly formed bone seemed to have migrated into the microgap between the dentin and the titanium surface.

Descriptive characterization of dentin block

In X-rays prior to decalcification, dentin grafts were clearly discernible. After a 12 h EDTA surface conditioning no differences in image contrast could be observed. After 4 weeks of decalcification, the dentin grafts could not be discerned in X-ray images.

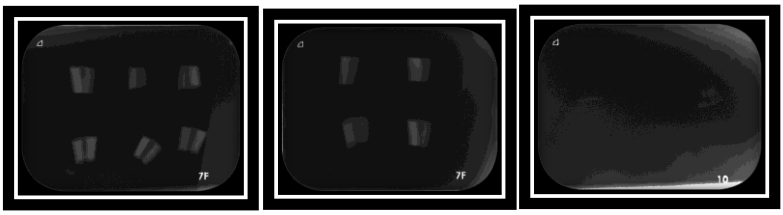

Fig.10 X-rays prior to, after 12h and after 4 weeks of decalcification

In longitudinal sections of dentin grafts densely packed tubules, approximately 3 µm wide, were observed. In transversal sections, tubules of approximately Ø 3 µm, devoid of odontoblast processes, were seen. In transversal close ups the dense peritubular walls were discerned, between which the demineralized collagenous intertubular dentin surface appeared.

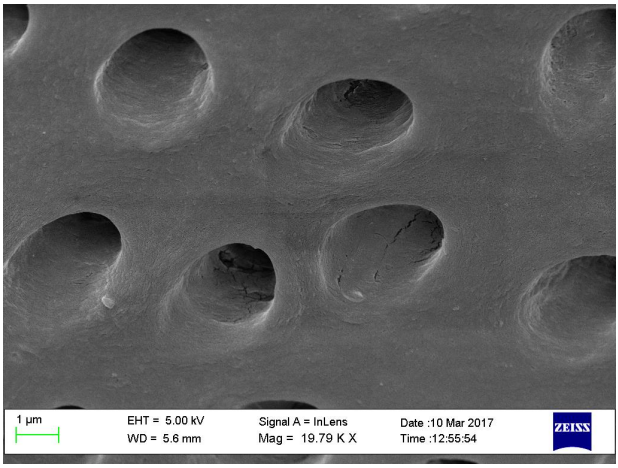

Fig.11 Close up of tubules with regular peritubular walls and irregular intertubular dentin, x $19.8.10^3$

On the demineralized dentin surface the organic marker element C dominated, as revealed by EDX image mode. The hydroxyapatite constituents Ca, P and O were close to devoid on the dentin surface. Remnant of surface gold coating was seen. A similar pattern was discerned from the semi-quantitative data analysis. The organic markers C and N dominated, 55 and 27%, respectively. Ca, P and O constituted less than 1% in the dentin surface layer.

	C	**N**	**O**	**P**	**Ca**	**Au**
Spectrum 1	57.95	23.68	14.70	-0.35	0.16	3.85
Spectrum 2	52.97	28.23	15.09	0.10	0.48	3.13
Spectrum 3	55.11	27.28	15.30	0.04	0.18	2.08
Spectrum 4	54.40	27.18	14.47	0.21	0.29	3.35
Mean	55.13	26.59	14.89	0.00	0.28	3.10
Std.deviation	2.08	2.00	0.38	0.24	0.14	0.75
Max	57.95	28.23	15.30	0.21	0.48	3.85
Min	52.97	23.68	14.47	-0.35	0.16	2.08

Table 1. EDX data. All elements analyzed and results shown in atomic %.

Histomorphometric analysis

After exclusion of specimen from the analysis due to the difficulty encountered to show a visible screw during specimen preparation, a total of 26 specimens were available for analysis. ROI comprised of the first 5 threads corresponding to the length placed in dentin. The non-demineralized group revealed a mean BIC of 36.2% and the demineralized group 40.4% (p=0.480). The percentages of new bone fill in the area (BA) within the threads (% bone fill) for the non-demineralized group were 67.4% and 72.4% (P=0.09) for the demineralized group. Overall the BIC and percentage of new bone fill of the demineralized group were higher than the same parameters for the non-demineralized specimens. Only fractional areas of direct contact

between the dentin and the titanium surface could be noted.

Study IV

The aim of this study was to compare the host tissue response and remodelling of onlay grafts of demineralized dentin in comparison to onlay bone grafts transplanted to the native tibial cortical bone wall.

Clinical observation

All animals tolerated the experiments very well and gained weight during the 12 weeks healing period. The soft tissue healing in all 8 rabbits was uneventful and there were no signs of infection.

Histological observation

After exclusion of specimen from the analysis due to the difficulties encountered in the histologic processing, a total of 14 and 12 specimens were available for analysis respectively.

In general both the dentin and bone block grafts were to a varying degree fused to the bone and replaced by bone and connective tissue. Both types of grafts were still present after 12 weeks. Resorption had occurred to approximately one third of the original sizes. Fusion of the graft to bone was more pronounced in the bone graft group compared to dentin group where some ingrowth of connective tissue was seen between the dentin graft and host bone surface. Zones of osseous replacement resorption of the dentin could be noted. New bone was formed on 4.55% of the surface of the resorption cavities (range 1.5-9.9). There was no significant difference with the connective tissue in contact with the dentin and bone grafts. Dentin had induced bone only in the resorption cavities in the interface and not on the surfaces facing the overlying soft tissues. Connective tissue in contact with the bone graft was seen in 63.3% (range 60.0-73.0), while the connective tissue in contact with dentin was 72.6% (range 54.4-89.6).

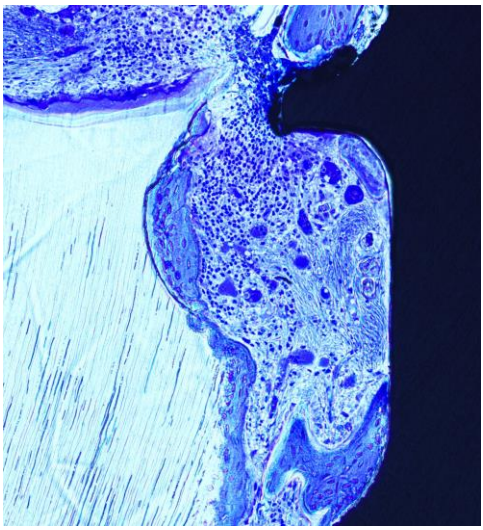

Fig.12 Replacement resorption taking place between dentin graft and titanium screw.

Inflammatory cells could not be seen to a large extent in any of the graft types. Towards the surrounding soft tissue, a thin layer of denser connective tissue ("scar tissue") could be noted. There was no difference in healing pattern if the bone or dentin graft had been placed most close to or most distant to the trephine cavity.

Histomorphometric analysis

ROI was assessed using a grid consisting of 10 equal (5 top and 5 interface) squares measuring 1000 micrometer each. Resorption pattern, degree of bone and soft tissue formation of each graft was calculated both on top and on the interface between the graft and native bone. There was no significant difference between top surface or the interface of dentin grafts in terms of resorption ($p= 0.209$). However, significantly more bone formation was seen in the interface compared to the top surface in the same group ($p=0.001$). Similar pattern was seen in the bone group regarding bone formation in the interface compared to the top surface

(p=0.002). Finally, both groups showed significantly higher degree of soft tissue formation towards the top compared to the interface (p=0.048 for dentin group and p=0.003 for the bone group).

Discussion

Study I

Comments on aim, material and methods

The aim of this study was to evaluate and compare the host tissue response to autogenous and xenogenic non-demineralized dentin blocks implanted in non-osteogenic areas, the abdominal connective tissue and femoral muscle of rabbits.

Fifteen New Zealand male white rabbits were used in the experiments. Dentin blocks of autogenous and xenogenic origin were implanted in pockets created in the abdominal connective tissue and femoral muscle. All animals survived throughout the healing period without any macroscopic signs of infection. They were sacrificed after 12 weeks.

A similar model has previously been used to study bone induction by implantation of human dentin and cementum into subcutaneous tissues of rats (Morris 1957). The use of rabbits in bone induction studies has been criticized since reactive ectopic bone formation is considered common in this animal (Morris 1957). However these objections, are not supported by others using the same animal model as in the present study (Gomes et al. 2002; Carvalho et al. 2004). This was confirmed in our experiment where we did not find any heterotopic bone formation in spite of using the rabbit as experimental model.

Previous studies have shown the property of BMPs to induce bone formation in a variety of models having many clinical applications in orthopedics and in oral and maxillofacial areas (Herford et al. 2007;

Herford and Boyne 2008; Sing et al. 2011). Dentin contains BMP and has been used in several experimental studies as bone substitute due to its osteoinductive and osteoconductive properties (Pinholt et al. 1992; Andersson et al. 2009; Andersson 2010). However, we do not know if bone formation seen around dentin is solely due to osteoinductivity or if the bone may also be due to osteoconductivity.

The reason for choosing non-demineralized dentin in our experimental model is that we wanted to continue the studies based on results reported from such grafts (Andersson 2010; Andersson et al. 2009), which has been based on the findings from replantation of teeth, where dento-alveolar ankylosis is a well-known phenomenon (Andreasen 1966, Andersson at al 1984, Andersson et al 1989). This phenomenon is a bone remodelling process but the possible role of active bone induction cannot be ruled out and the mechanism is not fully understood. To further study the mechanisms of dentin as an osteoinductive material, in our opinion, it had to be implanted in areas where there is no bone tissue present initially. Other factors of importance may be if the dentin is autograft or xenograft, or whether there is rich or poor vascularity in the region where the dentin is implanted.

Comments on results

The results of this experimental study showed that non-demineralized dentin grafts of xenogenic or autogenic origin implanted in the rabbit abdominal wall did not induce bone formation. It was further shown that a fibrous capsule displaying none or very little signs of inflammatory reactions surrounded all these grafts. This was probably due to graft mobility since dentin blocks were implanted in sites surrounded by mobile tissue.

Two specimens were lost during the healing period and could not be retrieved. The most likely explanation is that they had been lost through

the wound early in the healing phase and less likely due to resorption since resorption was a very uncommon finding in our study. Another five specimens were not possible to evaluate due to difficulties in the methodology when the histological sectioning was carried out. The specimens separated from the connective tissue and hence histological evaluation was not possible to carry out.

Apparently the bone inductive properties of dentin in the present experimental model were weak. The reasons for this may be several. Connective tissue of the rabbit abdominal wall has a sparse vascularity with few blood vessels as shown in the histological samples of our investigation. We believe that vascularity of the host bed plays a crucial role in recruiting pluripotent cells to the grafted area and subsequent bone formation.

A similar animal model had previously been used to study bone induction by implantation of human dentin and cementum into subcutaneous tissues of rats. In accordance to the findings in our study no bone formation was observed and hence it was suggested that direct cellular contact between osteocyte and mesenchymal cell is a prerequisite for bone induction and mesenchymal cells may not have been able to reach the site of the graft (Morris 1957). One could also discuss the role of implant environment for any significant expression of the osteogenic properties of dentin as recently demonstrated for different intrabony sites (Andersson 2010).When dentin has been experimentally implanted in direct contact with bone, fusion between bone and dentin has been reported with replacement resorption of the dentin by bone (Andersson et al. 2009). However, this phenomenon is mainly due to osteoconductive properties and may be a possible explanation why we could not find any heterotopic bone formation in our experiment where dentin was implanted far away from bone without assistance of osteoconductive properties.

The idea of using non-demineralized dentin blocks originates from previous findings claiming that bone morphogenetic properties of dentin are lost if the matrix is exposed to chemical solvents which denature or otherwise derange the three dimensional framework of BMP (Urist 1971). Also we hypothesized that non-demineralized dentin may have long term BMP releasing properties, possibly acting as a slow releasing carrier.

Dentin blocks in our study were harvested after a period of 12 weeks. The choice of healing time for this experiment was based on previous animal studies (Yeomans and Urist 1967; Bang and Urist 1967). However, it has been shown that resorption of non-demineralized dentin starts about 8-12 weeks later than totally demineralized dentin when implanted in abdominal muscle of rat (Bang 1971; Urist 1973). This finding could be another explanation to why we did not observe bone formation around our grafts.

Host tissue reactions, *i.e.* number of inflammatory cells around autogenous and xenogenic dentin blocks showed a statistically significant difference in numbers. The fact that the xenogenic grafts showed less inflammatory cells than autogenous grafts indicates that the inflammatory cells may not be due to an immunogenic response. A more plausible explanation may be an inflammatory reaction due to pulp remnants, which were more difficult to completely eliminate in a direct transplantation situation as compared to the xenografts in which the block grafts had been stored dry between the time of extraction/preparation and the time of implantation surgery.

We concluded that during the time frame of this study, non-demineralized dentin, whether autogenous or xenogenic did not have the potential to induce bone formation when implanted in the abdominal wall of rabbit

and did not induce inflammatory response suggesting that immunogenicity is not an important factor in dentin.

Study II

Comments on aim, material and methods

The aim of this study was to evaluate the healing pattern of xenogenic non-demineralized dentin granules and dentin blocks grafted to maxillary bone of rabbits. A further purpose was to study integration of titanium micro-implants installed in grafted areas. Limited data exists regarding the interaction between dentin as a bone substitute material and placement of dental implants in the same location. In vivo studies have demonstrated that successful implant integration can be obtained in the presence of intentionally retained root fragments (Schwartz et al. 2013). These findings mainly comprise of the establishment of newly formed root cementum and establishment of a periodontal ligament in the contact areas (Buser et al. 1990; Warrer et al. 1993; Hurzeler et al. 2010; Schwartz et al.2013). All these studies have in common that they involve a root with the presence of a viable root cementum, periodontal ligament and dentin with vascular support from the pulp.

In this experiment, fifteen New Zealand male white rabbits were used. The edentulous area superior and between the incisors and posterior teeth was chosen as experimental sites. One cavity on each side was prepared and filled either with non-demineralized dentin block or non-demineralized dentin granulae. No membrane or any type of fixation was used. After a healing period of 24 weeks one micro implant was installed in each grafted area. In order to optimize this study, it would have been desirable to have a control group. However, our intention was to install the micro-implants in such a way, that the apical half of the implants were placed in native bone, serving as control site, and the coronal part in dentin. Another aspect to think of would have been usage of a membrane

of to avoid in-growth of soft tissue in the grafted areas and particularly restricting movement of the grafted particles.

Dentin blocks in our study were not demineralized in anyway, because we wanted to use a similar experimental situation like in dentoalveolar ankylosis after trauma to be able to compare our result to studies using the same principle (Andersson et al. 1984; Andersson et al. 1989; Andersson et al. 2009; Andersson 2010; Al-Asfour et al. 2013; Al-Asfour et al. 2014).

Comments on results

The result of this experimental and descriptive study showed only limited or no bone contact between micro-implants and xenogenic dentin grafts. Furthermore, it was indicated that the granulae were encapsulated by means of a fibrous connective tissue in the majority of cases, whereas most dentin blocks were fused with the bone. One may speculate that granules might have been subjected to more mobility in the experimental cavity than a block and that this mobility could have promoted formation of fibrous tissue rather than bone.

During the specimen processing the ground sections were stained with toluidine blue. The staining procedure might present some problems, such as possible differences in the resulting color for different samples and the presence of artifacts, which sometimes are visible as stripes.

The result of the histomorphometric analysis revealed no significant difference between BIC and BA for the block group compared to the granulae group. This might be due to the limited number of micro-implants analyzed (18/30). Fibrous encapsulation encountered in 6 cases and death of another 3 rabbits rendered in additional six drop-outs. However, BIC and BA indicated higher values for the block group compared to the granulae group.

Since the retrieved biopsies contained micro-implants, the

osteoconductive properties of the titanium must also be considered. An interesting observation in this study was that there was no statistically significant difference between BIC and BA when comparing ROI I (dentin) and ROI II (native bone). This indicates that dentin shows comparable osteoconductive properties to bone.

Dentin grafts in our study were cleaned by being placed in chlorhexidine and stored dry for one month before implantation. It has been demonstrated that storage of dentin for more than 8-12 weeks may lead to decreased bone inducing capacity, especially if stored in room temperature (Bang & Johannessen 1972). In vitro studies have proven chlorhexidine to be toxic to fibroblasts and odontoblast-like cells (Lessa et al. 2010; Pucher and Daniel 1999). Since we cleaned our grafts with chlorhexidine, one might speculate that this fact has affected the integration of our grafts, however the very same processing protocol has been used in previous studies, without any adverse effects on healing of the dentin grafts (Andersson 2010; Al-Asfour et al. 2013).

The findings from our study raised the question for an alternative study trying to find answers to whether demineralization of the dentin grafts might affect the integration of the grafts to the surrounding bone and also the integration of the micro-implants to the grafted dentin (Paper III).

Study III

Comments on aim, material and methods

The aim of this experimental and descriptive study was to evaluate the morphological appearance and mineral content in decalcified dentin prior to grafting by means of energy dispersive x-ray (EDX) and scanning electron microscopy (SEM). A further purpose was to assess the integration of dental implants in conjunction with grafted dentin.

It has been shown that dentin possesses both osteoinductive assets due to its content of BMP among others as well as osteoconductive properties

(Bang 1967, Bang & Urist 1970). This may indicate that dentin might function as a bone substitute in the clinical setting. Previous studies have demonstrated that non–treated grafted dentin into non-osteogenic environments possess only minimal amount of bone formation capacity (Wedenberg 1994, Machado 2006, Al-Asfour 2014). When non-demineralized dentin is placed in direct contact with native cortical bone, the novel bone formation seems to be more of an osteoconductive resorption character (Al-Asfour et al. 2016). Several studies propose a preconditioning of the dentin block surface in order to facilitate release of BMP from the dentin (Bang & Urist 1967, Urist 1971, Bang 1972, Machado 2006, Mordenfeld et al. 2011).

EDTA at neutral pH was chosen since it selectively removes mineral from the dentin surface without any adverse effects on the integrity of the collagen matrix. Etchants operating at lower pH has been shown to have a negative impact on the integrity of the collagen matrix thereby affecting the bone formation capacity of dentin (Blomlöf 1997). It has also been claimed that bone morphogenetic properties are lost if the matrix is exposed to chemical solvents that denature or otherwise derange the three-dimensional framework of the fibrous proteins (Urist 1971). EDTA however seems to preserve the integrity of intercellular structures (Kiviranta et al. 1980).

Energy dispersive x-ray provides information about the chemical composition in a subject and changes in the chemical composition over time can also be assayed. Scanning electron microscopy (SEM) is a well-known technique used for micro-anatomical imaging. The scanning electron microscope has many advantages over traditional microscopes. The SEM has a large depth of field, which allows more of a specimen to be in focus at one time and the resolution is much higher compared to traditional light microscopy.

SEM and EDX used together is an easily affected method that requires technical support and continuous calibration, which must be considered.

Comments on results

After exclusion of specimen from the analysis due to the difficulty encountered to show a visible screw during specimen preparation, a total of 26 micro-implants were available for analysis. The non-demineralized group revealed a mean BIC of 36.2% and the demineralized group 40.4% (p=0.480). The percentages of new bone fill in the area (BA) within the threads (% bone fill) for the non-demineralized group were 67.4% and 72.4% (P=0.09) for the demineralized group. Overall, the BIC and percentage of new bone fill of the demineralized group were higher than the same parameters for the non-demineralized specimens.

Demineralization is a crucial step in dentin grafting. This process increases the bioavailability of matrix associated noncollagenous proteins such as osteocalcin, osteonectin, dentinphosphoprotein and BMP, which may enhance new bone formation (Gomes et al. 2006, Kim et al. 2013, Rezende et al. 20014). The purpose of the demineralization was not a complete removal of minerals from the dentin. The 12 h EDTA conditioning resulted in a surface decalcification, as seen from the EDX analysis. Notably, no difference in radiograph contrast was observed after the 12 h conditioning. It can be speculated about whether the surface decalcification promotes the initial graft-host response. The graft surface exposes its full organic composition to the wound area, possibly contributing to the rapid initiation of the replacement resorption process, where the initial inorganic tissue components are already devoid.

In radiographic images taken prior to decalcification, dentin grafts were clearly discernible. After a 12 h EDTA surface conditioning no differences in image contrast could be observed. After 4 weeks of decalcification, the dentin grafts could not be discerned in the images. In

longitudinal sections of dentin grafts densely packed tubules, approximately 3 µm wide, were observed by means of SEM. In transversal sections, tubules of approximately Ø 3 µm, devoid of odontoblast processes, were seen. In transversal close ups the dense peritubular walls were discerned, between which the demineralized collagenous intertubular dentin surface appeared. On the demineralized dentin surface the organic marker element C dominated, as revealed by EDX image mode. The hydroxyapatite constituents Ca, P and O were close to devoid on the dentin surface. Remnant of surface gold coating was seen. A similar pattern was discerned from the semi-quantitative data analysis. The organic markers C and N dominated, 55 and 27%, respectively. Ca, P and O constituted less than 1% in the dentin surface.

This study indicated that the bone inductive capacity of the dentin material seemed limited although demineralization by means of EDTA resulted in a higher BIC and BA value in conjunction with installed implants in the area. No implants were found to be integrated in direct contact with the dentin particles or blocks. The impact of dentin as a bone substitute material seems mostly to rely on a replacement resorption pattern.

Study IV

Comments on aim, material and methods

The aim of this study was to compare the host tissue response and remodelling of onlay grafts of demineralized dentin in comparison to onlay bone grafts transplanted to the native tibial cortical bone wall.

Autogenous bone is often used for grafting and bone augmentation prior to implant installation (Kahnberg 2010). Bone block grafts of autogenous origin seem to be more resistant to loss in bone volume than particulate grafts but several studies have shown that also onlay bone block grafts are prone to resorption and a large part of the bone graft can be lost

during the healing period (Gordh & Alberius 1999; Nyström et al. 2002). For this reason various alternatives of less resorption prone material such as deproteinized bovine bone, biphasic calcium phosphate and dentin, have been attempted for clinical use (Lindgren et al. 2012; Kim et al. 2010).

It has been shown previously that dentin xenografts implanted in rabbits had a potential to be incorporated in bone without inflammation and were then gradually resorbed and replaced by new bone (Andersson 2009; Andersson 2010). Based on these findings it was hypothesized that transplanted dentin onlay block grafts will undergo a comparable host integration as autogenous cortical bone blocks and hence may have a clinical potential as bone replacement material for augmentation of deficient alveolar bone.

Bone blocks and demineralized dentin blocks of certain thickness were transplanted to the rabbit tibia and rigidly fixated. Placement of the grafts were intentionally varied so that eight of bone blocks were placed close to the trephine cavity from where the bone block was harvested and the other eight more distant to the cavity in order to be able to evaluate any differences in healing pattern related to adjacency to the trephine cavity. The reversed order was applied for the dentin. After 12 weeks, all rabbits were sacrificed. A defined region of interest (ROI) was constructed with equal dimensions as the respective grafts (5 x 2 mm). The presence of the respective tissue types were calculated and presented as percentage values of the respective graft area (ROI). Furthermore resorption pattern of both graft types was assessed both on top and on the interface between the grafts and native bone. Descriptive histology as well as histomorphometric analysis of the remaining dentin, bone graft and soft tissue was determined using light microscopy (Nicon Eclipse E600) at 10

times magnification. The specimens were assessed using NIS Elements Microscope Imaging Software, Nikon.

Comments on results

Demineralized xenogenic dentin onlay grafts showed similar resorption patterns as autogenous bone onlay grafts and being resorbed in a similar rate during 12 weeks. Resorption had occurred to approximately one third of the original sizes. The replacement with connective tissue was more pronounced in the interface zone for the dentin grafts as compared to the bone grafts, which demonstrated a more marked fusion to the host surface. Zones of osseous replacement resorption of the dentin could be noted. New bone was formed on 4.55% of the surface of the resorption cavities (range 1.5-9.9). Connective tissue in contact with the bone graft was seen in 63.3% (range 60.0-73.0), while the connective tissue in contact with dentin was 72.6% (range 54.4-89.6). Inflammatory cells could not be seen to a large extent in any of the graft types. We could not detect any significant difference with the connective tissue in contact with the dentin and bone grafts.

Dentin had induced bone only in the resorption cavities in the interface and not on the surfaces facing the overlying soft tissues.

There was no significant difference between top surface or the interface of dentin grafts in terms of resorption ($p= 0.209$). However, significantly more bone formation was seen in the interface compared to the top surface in the same group ($p=0.001$). Similar pattern was seen in the bone group regarding bone formation in the interface compared to the top surface ($p=0.002$). Finally, both groups showed significantly higher degree of soft tissue formation towards the top compared to the interface ($p=0.048$ for dentin group and $p=0.003$ for the bone group).

Cortical bone blocks were characterized by an incomplete revascularisation, thus resulting in a composition of nonvital necrotic and

newly formed vital bone. In some areas fusion of bone to dentin was seen indicating a process of osseous replacement of the dentin by bone. This has been reported in previous studies on grafted dentin (Murata et al. 2011; Al-Asfour et al. 2013) and in experiments with delayed replantation of teeth (Andersson et al. 2009; Andersson 2010) and clinical studies (Andersson et al. 1980; Andreasen et al. 1995).

As seen from our previous studies, no inflammatory reactions were detected indicating that the use of xenogenic dentin was a factor of no or minor importance.

The choice of demineralization time of 12 hours prior to implantation in our study, was based on a previous study in rats where it was shown that dentin blocks demineralized for this time frame showed significantly higher rate of resorption and bone formation compared to demineralization time of 1, 2 or 6 hours (Mordenfeld et al. 2011)

One concern prior to the experiments was that, a graft located close to a harvest site could possibly be influenced by the vicinity to an open cavity, with cells migrating from the bone marrow. For this reason the placement of the grafts were intentionally varied so that some of the grafts were placed close to the trephine cavity and other more distant to the cavity. However, no difference was seen in healing pattern if the grafts were located closest to or more distant to the trephine cavity indicating that the nearby trephine cavity had little or no influence on the healing process. This may indicate that the replacement resorption is rather a local bone remodelling process than being under influence of specially recruited cells from the marrow.

Assessment of the top surface and interface of the grafts with native bone in terms of bone formation indicated significantly higher values in the interface for both groups. This confirms the fact that newly formed bone in dentin group is mainly due to replacement resorption and that dentin

per se does not induce bone formation (Al-Asfour et al. 2013; Al-Asfour et al. 2014). In both groups the newly formed bone originated mainly from the interface with native host tissue and not from the top surface of the graft.

Conclusions

Paper I

We conclude that during the timeframe of this study, non-demineralized dentin, whether autogenous or xeno- genic and regardless of being implanted in a vascular rich or vascular sparse environment, did not have the potential to induce bone formation and did not induce inflammatory response suggesting that immunogenicity is not an important factor in dentin when grafted. Furthermore, this study supports previous findings that replacement resorption of dentin in contact with bone is the prime mechanisms for bone formation and possible long-term BMP-releasing properties of non- demineralized dentin seems to be of less importance in the clinical setting.

Paper II

The result of this experimental study showed limited or no bone contact between micro- implants and xenogenic dentin grafts. Furthermore, it was indicated that the granulae were encapsulated by means of a fibrous connective tissue in the majority of cases, whereas most dentin blocks were fused with the bone.

Paper III

The bone inductive capacity of the dentin material seemed limited although demineralization by means of EDTA indicated a higher BIC and BA value in conjunction with installed implants in the area. The impact of dentin as a bone substitute material seems mostly to rely on a replacement resorption pattern. EDTA-conditioned dentin grafts exhibit all surface characteristics of human dentin. The dentin grafts were cell free, *i.e.* no discernible odontoblast processes. A 12 h EDTA treatment did not fully decalcify the grafts, as revealed by X-ray and the dentin

graft surfaces were almost devoid of calcium and phosphorus, as revealed by EDX.

Paper IV

Demineralized xenogenic dentin onlay grafts show similar resorption characteristics as autogenous bone onlay grafts, being resorbed in a similar rate during 12 weeks. Bone formation occurs mainly in terms of replacement resorption in the interface between dentin and native bone. Our findings indicate that dentin may be used as a bone grafting material, replacing bone or adding to the bone as grafting material.

Acknowledgements

I would like to express my sincere gratitude to the following people.

Christer Dahlin, professor and main supervisor, sharing deep experiences of research, guidance and encouragement throughout the thesis. For always being available, accepting me as a doctoral student and for all help in the planning of this dissertation.

Lars Andersson, professor and my co-supervisor, who introduced me to the field of OMFS and research in Västerås, where this journey started many years ago. Thanks for all support during these years and thank you and **Karin** for opening up your home to me so generously during my visits in Q8.

Adel Al-Asfour, **Bobby Joseph, Ala Al-Musawi,** my co-authors for all help during this project.

Lena Emanuelsson, Birgitta Nordlindh in particular and all other staff at the department of Biomaterials for technical assistance and support.

Severino Gabato, for taking good care of the animals throughout these trials.

Anna Dahlin, co-author in paper II, for helping me with the preparation and interpretation of the samples in this study.

Lovely James, for excellent technical assistance in paper I.

Ted Lundgren, associate professor and co-author in paper III, for valuable contribution to this paper, interesting discussions and comments on the project as a whole.

Anders Eriksson, Göran Gynther, Måns Jungner, former heads and **Daniel Danielsson** current head of the department of OMFS, Karolinska University hospital for providing me with leave of absence during completion of this work.

Finally my family,
My mum, for indescribable and endless patience, for always being there no matter what.

Bita & Neda, best sisters ever, for the support during the hard times…

Afshin, Neggi & Nav for being in my life and the later two for giving me the honor of being their uncle.

Kian and Arvin, my two most precious things in this world. It is a privilege to be your dad.

Last but not least, **Shabnam**, love of my life, for her endless support, for her patience, for being a role model to our kids and for simply being the wonderful person she is.

References

Aarden E, Burger E, Nijweide P. Function of osteocytes in bone. J Cell Biochem. 1994;55:287-299.

Al-Asfour A, Andersson L, Kamal M, Joseph B. New bone formation around xenogenic dentin grafts to rabbit tibia marrow. Dent Traumatol 2013; 29:455-460.

Al-Asfour A, Farzad P, Andersson L, Joseph B, Dahlin C. Host tissue reactions of non-demineralized autogenic and xenogenic dentin blocks implanted in a non-osteogenic environment. An experimental study in rabbits. Dent Traumatol 2014;30:198-203.

Albrektsson T, Johansson C. Osteoinduction, osteocunduction and osseointegration. Eur Spine J 2001;10:96-101.

Albrektsson T. Repair of bone grafts. A vital microscopic and histological investigation. Scand J plast Rec Surg 1980;14:1-12.

Andersson L, Blomlöf L, Lindskog S, Feiglin B, Hammarström L. Tooth ankylosis. Clinical, radiographic and histological assessments. Int J Oral Surg 1984;13:423-431.

Andersson L. Dentin xenografts to experimental bone defects in rabbit tibia are ankylosed and undergo osseous replacement. Dent Traumatol 2010; 26:306-310.

Andersson L, Bodin I, Sörensen S. Progression of root resorption following replantation of human teeth after extended extra-oral storage. Endodontics and Dent Traumatol 1989;5:38-47.

Andersson L, Ramzi A, Joseph B. Studies on dentin grafts to bone defects in rabbit tibia and mandible; development of an experimental model. Dent Traumatol 2009;1:78-83.

Andersson L, Bodin I, Sörensen S. Progression of root resorption following replantation of human teeth after extended extra-oral storage. Endod Dent Traumatol 1989;5:38-47.

Andersson L. Dentoalveolar ankylosis and associated root resorption in replanted teeth. Experimental and clinical studies in monkeys and man. Swedish Dental Journal Suppl 1988: 56:1-75.

Andersson L. Patient self-evaluation of intra oral bone grafting treatment to the maxillary frontal region. Dent Traumatol 2008;24:164-169.

Andreasen JO, Borum M, Jacosbsen H, Andreasen F. Replantation of 400 avulsed permanent incisors. Factors related to periodontal ligament healing. Endod Dent Traumatol 1995;11:76-89.

Andreasen JO, Hjorting-Hansen E. Replantation of teeth. Radiographic and clinical study of 110 human teeth replanted after accidental loss. Acta Odontol Scand 1966; 3:263-286.

Andreasen JO, Kristerson L. The effect of limited drying or removal of the periodontal ligament. Periodontal healing after replantation of mature permanent incisors in monkeys. Acta Odontol Scand. 1981;39:1-13.

Andreasen JO. The effect of extra-alveolar period and storage media upon periodontal and pulpal healing after replantation of mature permanent incisors in monkeys. Int J Oral Surg 1981;10:43-51.

Aparicio C, Perales P, Rangert B. Tilted implants as an alternative to maxillary sinus grafting: a clinical, radiological and prosthetic study. Clin Implant Dent Relat Res 2001;3:39-49.

Araújo M, Wennström J, Lindhe J. Modeling of the buccal and lingual bone walls of fresh extraction sites following implant installation. Clin Oral Implants Res 2006;17:606-614.

Azami-Aghdash S, Ebadifard Azar F, Pournaghi Azar F, Rezapour A, Moradi-Joo M, Moosavi A, Ghertasi Oskouei S. Prevalence, etiology, and types of dental trauma in children and adolescents: systematic review and meta-analysis. Med J Islam Repub Iran 2015;10:234-238.

Bahat O, Fontanessi R. Efficacy of implant placement after bone grafting for three-dimensional reconstruction of the posterior jaw. Int J Periodontics Restorative Dent 2001;21:220-223.

Bang G. Induction of heterotopic bone formation by demineralized dentin in guinea pigs: antigenicity of the dentin matrix. J Oral Pathol. 1972;4:172-185.

Bang G. Induction of heterotopic bone formation by demineralized dentin in guinea pigs: relation to time. Acta Path Microbiol Scand 1973; 236(Suppl): 60-70.

Bang G, Urist M. Bone induction in excavation chambers in matrix of decalcified dentin. Arch Surg 1967;94:781-789.

Barrett E, Kenny D. Avulsed permanent teeth: a review of the literature and treatment guidelines. Endod Dent Traumatol 1997;13:153-163.

Bays R. The pathophysiology and anatomy of edentulous bone loss. In: Fonseca RJ, Davis HW, eds. Reconstructive preprosthetic oral and maxillofacial surgery. Philadelphia: WB Saunders Company, 1986;1-17.

Becker W, Urist M, Tucker L, Becker B, Ochsenbein C. Human demineralized freeze-dried bone: inadequate induced bone formation in athymic mice. A preliminary report. J Periodontol 1995;66:822-828.

Beirne O. Comparison of complications after bone removal from lateral and medial plates of the anterior ilium for mandibular augmentation. Int J Oral & Maxillofac Surg 1986;15:269-272.

Benke D, Olah A, Möhler H. Protein-chemical analysis of Bio-Oss bone substitute and evidence on its carbonate content. Biomaterials. 2001;9:1005-1012.

Blair H, Athanasou N. Recent advances in osteoclast biology and pathological bone resorption. Histol Histopathol 2004;19:189-199.

Blomlöf L, Andersson L, Lindskog S, Hedström K-G, Hammarström L. Periodontal healing of replanted monkey teeth prevented from drying. Acta Odontol Scand 1983; 41:117-123.

Blomlöf L, Lindskog S. Quality of periodontal healing II. Dynamics of reparative cementum formation. Swed Dent J 1994;18:131-138.

Bloomquist D.S. (1980) Bone grafting in dentofacial defects. In: Surgical correction of dentofacial deformities: 1st edn., pp 1502-1523. Philadelphia: W.B Saunders Company.

Boëck-Neto R, Gabrielli M, Lia R, Marcantonio E, Shibli J.

Histomorphometrical analysis of bone formed after maxillary sinus floor augmentation by grafting with a combination of autogenous bone and demineralized freeze-dried bone allograft or hydroxyapatite. J Periodontol. 2002;73:266-270.

Bonewald L. The amazing osteocyte. J Bone Miner Res 2011;26:229-238.

Boyne P, James R. Grafting of the maxillary sinus floor with autogenous marrow and bone. J Oral Surg 1980;38.613-616.

Boyne R. The science of alveolar ridge augmentation. Compend Contin Educ Dent 1982;Suppl 2:49-51.

Brånemark P, Adell R, Breine U, Hansson B, Lindström J, Ohlsson A. Intra-osseous anchorage of dental prostheses. I. Experimental studies. Scand J Plast Reconstr Surg. 1969;3:81-100.

Buck B, Malinin T, Brown M. Bone transplantation and human immunodeficiency virus: an estimate of risk of acquired immunodeficiency syndrome (AIDS). Clin Orthop Realt Res 1989;240:129-136.

Buck D, Dumanian G. Bone biology and physiology: Part I. The fundamentals. Plast Reconstr Surg. Jun 2012;129:1314-1320.

Burchardt H. The biology of bone graft repair. Clin Orthop Relat Res 1983;174: 28-42.

Burger E, Klein-Nulend J. Mechanotransduction in bone –role of the lacunocanalicular network. FASEB 1999;13:101-112.

Burwell R, Friedlander G, Mankin H. Current perspectives and future directions: The 1983 invitational conference on osteochondral allografts. Clin Orthop 1985;197:141-157.

Buser D, Warrer K, Karring T. Formation of a periodontal ligament around titanium implants. Journal of Periodontology 1990;61: 597–601.

Buser D, Dula K, Hirt H, Schenk R. Lateral ridge augmentation using autografts and barrier membranes: a clinical study with 40 partially edentulous patients. J Oral Maxillofac Surg. 1996;54:420-432.

Cannalis E, Varghese W, McCarthy T, Centrella M. Role of platelet derived growth factor in bone cell function. Growth Regul 1992;2:151-155.

Capulli M, Paone R, Rucchi N. Osteoblast and osteocyte: Games without frontiers. Arch Biochem Biophys. May 14 2014.

Carlsson G. Responses of jawbone to pressure. Gerodontology. 2004;21:65-70.

Carvalho V, Tosello Dde O, Salgado M, Gomes M. Histomorphometric analysis of homogenous demineralized dentin matrix as osteopromotive material in rabbit mandibles. Int J Oral Maxillofac Impl 2004;5:679-686.

Chalmers J. Transplantation immunity in bone homografting. J Bone Joint Surg 1959;41:160-179.

Clokie C, Bell R. Recombinant human transforming growth factor beta-1 and its effects on osseointegration. J Craniofac Surg. 2003;14:268-77.

Cochran D, Rouse C, Lynch S, Graves D. Effects of platelet-derived growth factor isoforms on calcium release from neonatal mouse calvariae. Bone. 1993;14:53-58.

Cobb C, Eick J, Barker B, Mosby E, Hiatt W. Restoration of mandibular continuity defects using combinations of hydroxylapatite and autogenous bone: microscopic observations. J Oral Maxillofac Surg. 1990;48:268-275.

Cordioli G, Mazzocco C, Schepers E, Brugnolo E, Majzoub Z. Maxillary sinus floor augmentation using bioactive glass granulaes and autogenous bone with simultaneous implant placement. Clinical and histological findings. Clin Oral Impl Res 2001;12:270-278.

Daculci G. Biphasic calcium phosphate concept applied to artificial bone, implant coating and injectable bone substitute. Biomat 1998;19:1473-1478.

Dahlin C, Linde A, Gottlow J, Nyman S. Healing of bone defects by guided tissue regeneration. Plast Reconstr Surg. 1988;81:672-676

Dayi E, Aslan M, Simsek G, Yilmaz A. The effects of bone chips dehydrated with solvent on healing bone defects. J Int Med Res 2002;30:168-173.

Deal C. Future therapeutic targets in osteoporosis. Curr Opin Rheumatol 2009;21:380-385.

De Leonardis, Pecora G. Prospective study on the augmentation of the maxillary sinus with calcium sulphate: histological results. J Periodont 2000;71:940-947.

Dempster D, Shane E. Bone quantification and dynamics of bone turnover by histomorphometric analysis In: Becker KL (ed.). Principles and practice of endocrinology and metabolism, 3rd ed. Lippincott Williams and Wilkins; 2001. p. 541-548.

Ducy P, Schinke T, Karsenty G. The osteoblast: a sophisticated fibroblast under central surveillance. Science 2000:289;1501-1504.

Dudley H, Spiro D. The fine structure of bone cells. J Cell Biol. 1961;11:627-649.

El Deeb M, Tompach P, Morstad A, Kwon P. Long-term follow-up of the use of nonporous hydroxyapatite for augmentation of the alveolar ridge. J Oral Maxillofac Surg 1991;49:257-261.

Enneking W. Histological investigation of bone transplants in immunologically prepared animals. J Bone Joint Surg 1957;39-A:597-615.

Esposito M, Grusovin M, Felice P, Karatzopoulos G, Worthington H, Coulthard P. Interventions for replacing missing teeth: horizontal and vertical bone augmentation techniques for dental implant treatment. Cochrane Database Syst Rev. 2009 Oct 7;(4):CD003607. doi: 10.1002/14651858.CD003607.pub4.

Esposito M, Grusovin MG, Rees J, Karasoulos D, Felice P, Alissa R, Worthington HV, Coulthard P. Interventions for replacing missing teeth: augmentation procedures of the maxillary sinus. Cochrane Database Syst Rev. 2010 Mar 17;(3):CD008397. doi: 10.1002/14651858.CD008397.

Farzad P, Andersson L, Gunnarsson S, Johansson B. Rehabilitation of severely resorbed maxillae with zygomatic implants: an evaluation of

implant stability, tissue conditions, and patients' opinion before and after treatment. Int J Oral Maxillofac Implants. 2006;21:399-404

Felice P, Lizio G, Checchi L. Alveolar distraction osteogenesis in posterior atrophic mandible: a case report on a new technical approach. Implant Dent 2013;22:332-338.

Felice P, Marchetti C, Iezzi G, Piattelli A, Worthington H, Pellegrino G, Esposito M. Vertical ridge augmentation of the atrophic posterior mandible with interpositional bloc grafts: bone from the iliac crest vs. bovine anorganic bone. Clinical and histological results up to one year after loading from a randomized-controlled clinical trial. Clin Oral Implants Res 2009;12:1386-1393.

Frost H. Preparation of thin undecalcified bone sections by rapid method. Stain Technol. 1958;33:273-277.

Frost H. Tetracycline based analysis of bone dynamic. Calcif Tiss Res. 1969;3:211-237.

Froum S, Tarnow D, Wallace S, Jalbout Z, Cho S, Rohrer M, Prasad H. The use of mineralized allograft for sinus augmentation: an interim histological case report from a prospective clinical study. Compend Countin Educ Dent 2005;26:259-260, 262-264,266-268, quiz 270-271.

Froum SJ, Wallace SS, Elian N, Cho SC, Tarnow DP. Comparison of mineralized cancellous bone allograft (Puros) and anorganic bovine bone matrix (Bio-Oss) for sinus augmentation: histomorphometry at 26 to 32 weeks after grafting. Int J Periodontics Restorative Dent 2006;26:543-551.

Gapski R, Neiva R, Oh T, Wang H. Histologic analyze of human mineralized bone grafting material in sinus elevation procedures: a case series. Int J Periodont Res Dent 2006;26:59-69.

Goldberg V, Powell A, Shaffer J, Zika J, Bos G, Heiple K. Bone grafting: Role of histocompatibility in transplantation. J Orthop Res 1985;3:389-404.

Goldberg V, Stevenson S. Natural history of autografts and allografts. Clin Orthop 1987;225:7-16.

Gomes M, dos Anjos M, NogueiraTde O, Catanzaro Guimarães S.Autogenous demineralized dentin matrix for tissue engineering applications: radiographic and histomorphometric studies. Int J Oral Maxillofac Implants. 2002;17:488-497.

Gord M, Alberius P, Johnell O, Linde A. Osteopromotive membranes enhance onlay integration and maintenance in the adult rat skull. J Oral Maxillofac Surg 1998;27:67-73.

Gordh M, Alberius P. Some basic factors essential to autogeneic nonvascularized onlay bone grafting to the craniofacial skeleton. Scand J Plast Reconstr Surg Hand Surg. 1999;33:129-146.

Gross T, Jinnah R, Clarke H, Cox Q. The biology of bone grafting. Orthopedics 1991;14:563-568.

Hallman M, Hedin M, Sennerby L, Lundgren S. A prospective 1-year clinical and radiographic study of implants placed after maxillary sinus floor augmentation with bovine hydroxyapatite and autogenous bone. J Oral Maxillofac Surg 2002;60:277-284.

Hallman M, Sennerby L, Lundgren S. A clinical and histologic evaluation of implant integration in the posterior maxilla after sinus floor augmentation with autogenous bone, bovine hydroxyapatite, or a 20:80 mixture. Int J Oral Maxillofac Implants 2002;5:635-643.

Hallman M, Thor A. Bone substitutes and growth factors as an alternative/complement to autogenous bone for grafting in implant dentistry. Periodontology 2000 2008;47:172-192.

Hallman M. A prospective study of treatment of severely resorbed maxillae with narrow non submerged implants: results after one year of loading. J Oral Maxillofac Impl 2001;16:731-736.

Hammarstrom L, Blomlöf L, Lindskog S. Dynamics of dentoalveolar ankylosis and associated root resorption. Endod Dent Traumatol 1989;5:163-175.

Hannink G, Arts J. Bioresorbability, porosity and mechanical strength of bone substitutes: what is optimal for bone regeneration? Injury 2011;42:Suppl 2:S22-25.

Heiple K, Goldberg V, Powell A, Bos G, Zika J. Biology of cancellous bone grafts. Orthop Clin North Am 1987;18:179-185.

Herford A, Boyne P, Rawson R, Williams R. Bone morphogenetic protein-induced repair of the premaxillary cleft. J Oral Maxillofac Surg. 2007;11:2136-2141.

Herford A, Boyne P. Reconstruction of mandibular continuity defects with bone morphogenetic protein-2 (rhBMP-2). J Oral Maxillofac Surg 2008;4:616-624.

Heslop B, Zeiss I, Nisbet N. Studies on transference of bone: A comparison of autologous and homologous bone implants with reference to osteocyte survival, osteogenesis, and host reaction. Br J Exp Pathol 1960;41:269-287.

Higuchi K. The zygomaticus fixture: an alternative approach for implant anchorage in the posterior maxilla. Ann R Australas Coll Dent Surg 2000;15:28-33.

Hirsch J, Brånemark P. Fixture stability and nerve function after transposition and lateralization of the inferior alveolar nerve and fixture installation. Br J Oral Maxillofac Surg. 1995;33:276-281.

Hising P, Bolin A, Branting C. Reconstruction of severely resorbed alveolar ridge crests with dental implants using a bovine bone mineral for augmentation. Int J Oral Maxillofac Implants 2001;16:90-97.

Hock J, Cannalis E. Platelet-derived growth factor enhances bone cell replication but not differentiated function of osteoblasts. Endocrinology 1994;134:1423-1428.

Honig J, Merten H, Heinemann D. Risk of transmission of agents associated with Creutzfeldt-Jakob disease and bovine spongiform encephalopathy. Plast Reconstr Surg. 1999;103:1324-1325.

Hurzeler M, Zuhr O, Schupbach P, Rebele S, Emmanouilidis N, Fickl S. The socket-shield technique: a proof-of-principle report. Journ Clin Periodontol 2010;37:855–862.

Ike M, Urist M. Recycled dentin root matrix for a carrier of recombinant human bone morphogenetic protein. J Oral Implantol 1998;3:124-132.

Jaquiéry C, Rohner D, Kunz C, Bucher P, Peters F, Schenk RK, Hammer B. Reconstruction of maxillary and mandibular defects using prefabricated microvascular fibular grafts and osseointegrated dental implants -- a prospective study. Clin Oral Implants Res 2004;15:598-606.

Jensen O, Sennerby L Histologic analysis of clinically retrieved titanium microimplants placed in conjunction with maxillary sinus floor augmentation. Int J Oral Maxillofac Implants 1998;4:513-521.

Jensen S, Aaboe M, Pinholt E, Hjørting-Hansen E, Melsen F, Ruyter IE. Tissue reaction and material characteristics of four bone substitutes. Int J Oral Maxillofac Implants 1996;11:55-66.

Jensen T, Schou S, Stavropoulos A, Terheyden H, Holmstrup P.Maxillary sinus floor augmentation with Bio-Oss or Bio-Oss mixed with autogenous bone as graft: a systematic review. Clin Oral Implants Res 2012;3:263-273.

Jepson N, Thomsson J, Steele J. The influence of denture and denture design on patient acceptance of partial of partial dentures. Br dent J 1995;178:296-300.

Johansson B, Grepe A, Wannfors K, Hirsch J. A clinical study of changes in the volume of bone grafts in the atrophic maxilla. Dentomaxillofac Radiol 2001;30:157-161.

Kim Y, Kim S, Byeon J, Lee H, Um I, Lim S, Kim S. Development of a novel bone grafting material using autogenous teeth. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2010;4:496-503.

Kim Y, Nowzari H, Rich S. Risk of prion disease transmission through bovine-derived bone substitutes: a systematic review. Clin Implant Dent Relat Res 2013;15:645-653.

Kim Y, Rodriguez A, Nowzari H. The risk of prion infection through bovine grafting materials. Clin Implant Dent Relat Res 2016;18:1095-1102.

Knothe M, Adamson J, Tami A, Bauer T. The osteocyte. Int J Biochem Cell Biol 2003;36:1-8.

Krekmanov L. Placement of posterior mandibular and maxillary implants in patients with severe bone deficiency: a clinical report of procedure. J Oral Maxillofac Impl 2000;15:722-730.

Kubler N. Osteoinduktion und reparation. Mund Kiefer Gesichtschirurgie 1997;1:2-25

Kulak C, Dempster D. Bone histomorphometry: a concise review for endocrinologists and clinicians. Arq Bras Endocrinol Metab 2010;2:87-98.

Kular J, Tickner J, Chim S, Xu J. An overview of the regulation of bone remodelling at the cellular level. Clin Biochem 2012;45:863-873.

Kwon H, El Deeb M, Morstad T, Waite D. Alveolar ridge maintenance with hydroxyapatite ceramic cones in humans. J Oral Maxillofac Surg 1986;44:503-508.

Körloff B, Nylin B, Ritz K. Bone grafting of skull defects: A report of 55 cases. Plast Rec Surg 1973;52:378-383.

Lane J, Sandhu H. Current approaches to experimental bone grafting. Orthop Clin North Am 1987;18:213-225.

Lee D, Chen S, Darby I. Maxillary sinus floor elevation and grafting with deproteinized bovine bone mineral: a clinical and histomorphometric study. Clin Oral Implants Res. 2012;8:918-924.

Lerner U. Osteoclast formation and resorption. Matrix Biol 2000;19:107-120.

Lerner U. Bone remodelling in post –menopausal osteoporosis. J Dent Res 2006;85:584-595.

Lessa, F, Aranha A, Nogueira I, Giro E, Hebling J, Costa C. Toxicity of chlorhexidine on odontoblast-like cells. J Appl Oral Sci. 2010;18:50-58.

Lindgren C, Mordenfeld A, Johansson C, Hallman M. A 3-year clinical follow-up of implants placed in two different biomaterials used for sinus augmentation. Int J Oral Maxillofac Implants. 2012;27:1151-62.

Kahnberg K. Treatment of bone deficient ridges in implant rehabilitation. In Andersson L, Kahnberg K, Pogrel M, ed. Oral & Maxillofacial Surgery, Oxford, UK; Wiley-Blackwell: 2010:462-468.

Lindgren C, Hallman M, Sennerby L, Sammons R. Back-scattered electron imaging and elemental analysis of retrieved bone tissue following sinus augmentation with deproteinized bovine bone or biphasic calcium phosphate. Clin Oral Implants Res 2010;21:924-930.

Lindgren C, Mordenfeld A, Hallman M. A prospective 1-year clinical and radiographic study of implants placed after maxillary sinus floor augmentation with synthetic biphasic calcium phosphate or deproteinized bovine bone. Clin Implant Dent Relat Res 2012;14:41-50.

Lindgren C, Sennerby L, Mordenfeld A, Hallman M. Clinical histology of microimplants placed in two different biomaterials. Int J Oral Maxillofac Implants 2009;24:1093-1100.

Lindskog S, Blomlöf L. Mineralized tissue formation in periodontal wound healing. J Clin Periodontol 1992;19:741-748.

Lohman C, Andreacchio D, Koster G, Carnes D, Cochran D, Dean D, Boyan B, Schwartz Z. Tissue response and osteoinduction of human bonegrafts in vivo. Arch orthop Trauma Surg 2001;121:583-590.

Lu J, Descamps M, Dejou J, Koubi G, Hardouin P, Lemaitre J, Proust J. The biodegradation mechanism of calcium phosphate biomaterials in bone. J Biomed Mater Res 2002;63:408-412.

Lundgren S, Rasmusson L, Sjöström M, Sennerby L. Simultaneous or delayed placement of titanium implants in free autogenous iliac bone grafts. Histological analysis of the bone graft-titanium interface in 10 consecutive patients. Int J Oral Maxillofac Surg. 1999;1:31-37.

Machado M, Souza A, Araújo V. Histological evaluation of the osteoinduction capability of human dentine. Int Endod J. 2006;11:855-859.

Mackie E.Osteoblasts: novel roles in orchestration of skeletal architecture. Int J Biochem Cell Biol 2003;35:1301-1305.

Mackiewicz Z, Niklińska W, Kowalewska J, Chyczewski L. Bone as a source of organism vitality and regeneration. Folia Histochem Cytobiol. 2011;49:558-569.

Maiorana C, Santoro F, Rabagliati M, Salina S. Evaluation of the use of iliac cancellous bone and anorganic bovine bone in the reconstruction of the atrophic maxilla with titanium mesh: a clinical and histologic investigation. Int J Oral Maxillofac Implants. 2001;16:427-432.

Malevez C, Daelemans P, Adriaenssens P, Durdu F. Use of zygomatic implants to deal with resorbed posterior maxillae. Periodontol 2000. 2003;33:82-89.

Malluche H, Sherman D, Meyer W, Massry S. A new semiautomatic method for quantitative static and dynamic bone histology. Calcif Tiss Int. 1982;34:439-444.

Manolagas S. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocer Rev 2000;21:115-137.

Marx R, Carlson E, Eichstaedt R, Schimmele S, Strauss J, Georgeff K. Platelet-rich plasma: Growth factor enhancement for bone grafts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998;85:638-646.

Maslamani M, Almusawi A, Joseph B, Gabato S, Andersson L. An experimental model for studies on delayed tooth replantation and ankylosis in rabbits. Dent Traumatol 2016;32:443-449.

Mattson T, Köndell P-Å, Gynther G, Fredholm U, Bolin A. Implant treatment without bone grafting in severely resorbed edentulous maxillae. J Oral Maxillofac Surg 1999;57:281-287.

Miller S, de Saint-Georges L, Bowman B, Jee W. Bone lining cells; structure and function. Scamming Microsc 1989;3:953-960.

Miller S, Jee W. The bone lining cell: a distinct phenotype? Calcif Tissue Int. 1987;41:1-5.

Misch C. Maxillary autogenous bone grafting. Oral Maxillofac Surg Clin North Am. 2011;23:229-238.

Miyaji H, Sugaya T, Miyamoto T, Kato K, Kato H. Hard tissue formation on dentin surfaces applied with recombinant human bone morphogenetic protein-2 in the connective tissue of the palate. J Periodontal Res 2002;37:204-209.

Miyazono K. Positive and negative regulation of TGF-beta signaling. J Cell Sci. 2000;113:1101-1109. Review.

Mordenfeld A, Hallman M, Lindskog S. Tissue reactions to subperiosteal onlays of demineralized xenogenous dentin blocks in rats. Dent Traumatol 2011;27:446-451.

Morris M. The implantation of decalcified human dentin and cementum into subcutaneous tissues of the rat. J Periodont Res 1957;2:284-288

Mulliken J, Kaban L, Glowacki J. Induced osteogenesis: the biological principle and clinical applications. J Surg Res 1984;37:487-496.

Murata M, Akazawa T, Mitsugi M , In-Woong U, Kyung-Wook K, Young-Kyun K. Human dentin as novel biomaterial for bone regeneration. In: Rosario Pignatello R, ed. Biomaterials-Physics and Chemistry, InTech; 2011:127-140.

Muschler G, Lane J, Dawson E. The biology of spinal fusion, in Cotler JM, Cotler HP (eds): Spinal Fusion Science and Technique. Berlin: Springer-Verlag, 1990, pp 9-21

Muscolo D, Caletti E, Schajowicz F, Araujo E, Makino A. Tissue-typing in human massive allografts of frozen bone. J Bone Joint Surg 1987;69-A:583-595

Nisbet N. Antigenicity of bone. J Bone Joint Surg 1977;59-B:263-266

Nyström E, Ahlqvist J, Legrell P, Kahnberg K. Bone graft remodelling and implant success rate in the treatment of the severely resorbed maxilla: a 5-year longitudinal study. Int J Oral Maxillofac Surg 2002;31:158-164.

O'Connor R. Survival factors and apoptosis. Adv Biochem Eng

Biotechnol. 1998;62:137-166.

Ozaki W, Buchman S. Volume maintenance of onlay bone grafts in the craniofacial skeleton: micro-architecture versus embryologic origin. Plast Reconstr Surg. 1998;102:291-299.

Parfitt A. Stereologic basis of bone histomorphometry; theory of quantitative microscopy and reconstruction of the third dimension. In: Recker RR (ed.). Bone histomorphometry: techniques and interpretation. Boca Raton, FL: CRC Press, USA; 1983. p. 53-85.

Parfitt A. The physiologic and clinical significance of bone histomorphometric data. In: Recker RR (ed.). Bone histomorphometry: techniques and interpretation. Boca Raton, FL: CRC Press, USA; 1983. p. 143-244.

Phillips J, Rahn B. Fixation effects on membranous and endochondral onlay bone-graft resorption. Plast Reconstr Surg. 1988;82:872-877.

Phillips J, Rahn B. Fixation effects on membranous and endochondral onlay bone graft revascularization and bone deposition. Plast Reconstr Surg. 1990;85:891-897.

Piattelli M, Favero GA, Scarano A, Orsini G, Piattelli A. Bone reactions to anorganic bovine bone (Bio-Oss) used in sinus augmentation procedures: a histologic long-term report of 20 cases in humans. Int J Oral Maxillofac Implants 1999;6:835-840.

Pinholt E, Bang G, Haanaes H. Alveolar ridge augmentation by osteocunduction in rats. Scand J Dent Res 1990;98:434-441.

Pinholt E, Bang G, Haanaes H. Alveolar ridge augmentation by osteocunduction in rats by combined hydroxylapatitae and osteoconductive material. Scand J Dent Res 1991;99:64-74.

Pinholt E, Haanaes H, Roservik M, Donath K, Bang G. Alveolar ridge augmentation by osteoconductive materials in goats. Scand J Dent Res 1992;100:361-365.

Pinholt E, Solheim E, Talsnes O, Larsen T, Bang G, Kirkeby O. Revascularization of calvarial, mandibular, tibial, and iliac bone grafts in rats. Ann Plast Surg. 1994;33:193-197.

Pohl V, Thoma DS, Sporniak-Tutak K, Garcia-Garcia A, Taylor TD, Haas R, Hämmerle CH. Short dental implants (6 mm) versus long dental implants (11-15 mm) in combination with sinus floor elevation procedures: 3-year results from a multicentre, randomized, controlled clinical trial. J Clin Periodontol. 2017 Jan 12. doi: 10.1111/jcpe.12694. [Epub ahead of print].

Plotnicov N, Nikitiin A. Reconstruction of the mandible by bone alloplasty. 20th annual meeting, Scandinavian association oral maxillofacial surgeons, Tampere, 1985;abstract 18.

Prolo D: Biology of bone fusion. Clin Neurosurg 1990;36:135-146.

Pucher J, Daniel J. The effects of chlorhexidine digluconate on human fibroblasts in vitro. J Periodontol. 1992;63:526-532.

Raghoebar G, Louwerse C, Kalk W, Vissink A. Morbidity of chin bone harvesting. Clinical Oral Implants Research 2001;5:503-507.

Randow K, Glanz P, Zoger B. Technical failures and some related clinical complications in extensive fixed prosthodontics. An epidemiological study of long-term clinical quality. 1986;44:241-255.

Recker R. Embryology, anatomy and microstructure of bone. In: Coe FL and Favus M, ed. Disorders of bone and mineral metabolism. New York: Raven press, 1992:219-240.

Reddi A, Huggins C. Influence of geometry of transplanted tooth and bone on transformation of fibroblasts. Proc Soc Exp Biol Med 1973;143:634-647.

Reddi A, Wientroub S. Biologic principles of bone induction. Orthop Clin North Am 1987;18:207-212.

Reddy S. Regulatory mechanisms operative in osteoclasts. Crit Rev Eukaryot Gene Exp 2004;14:255-270.

Rodd H, Malhotra R, O'Brien C, Elcock C, Davidson L, North S. Change in supporting tissue following loss of a permanent maxillary incisor in children. Dent Traumatol 2007;23:328-332.

Robling A, Niziolek P, Baldridge L, Condon K, Allen M, Alam I, Mantila S, Gluhak-Heinrich J, Bellido T, Harris S, Turner C. Mechanical stimulation of bone in vivo reduces osteocyte expression of Sost/sclerostin. J Biol Chem 2008;283:5866-5875.

Rochefort G, Pallu S, Benhamou C. Osteocyte: the unrecognized side of bone tissue. Osteoporos Int 2010;21:115-137.

Rohner D, Jaquiéry C, Kunz C, Bucher P, Maas H, Hammer B. Maxillofacial reconstruction with prefabricated osseous free flaps: a 3-year experience with 24 patients. Plast Reconstr Surg. 2003;112:748-757.

Roos-Jansaker A, Lindahl C, Persson G, Renvert S. Long-term stability of surgical bone regenerative procedures of peri-implantitits lesions in a prospective case-control study over 3 years. J Clin Periodontol 2011;38:590-597.

Sailer H. Transplantation of lyophilized cartilage in maxilla-facial surgery. Experimental foundations and clinical success. Basel: S Karger 1983;1-138.

Sakkas A, Wilde F, Heufelder M, Winter K, Schramm A. Autogenous bone grafts in oral implantology-is it still a "gold standard"? A consecutive review of 279 patients with 456 clinical procedures. Int J Implant Dent. 2017;3:23-27.

Sampath T, Reddi A. Homology of bone-inductive proteins from human, monkey, bovine, and rat extracellular matrix. Proc Natl Acad Sci U S A. 1983;80:6591-6595.

Scarano A, Perotti V, Degidi M, Piatelli A, Lezzi G. Bone regeneration with algae-derived hydroxyapatite: a pilot histologic and histomorphometric study in rabbit tibia defects. Int J Oral and maxillofac Implants 2012;27:336-340.

Chappuis V, Engel O, Reyes M, Shahim K, Nolte L, Buser D. Ridge alterations post-extraction in the esthetic zone: a 3D analysis with CBCT. J Dent Res. 2013;92(12 Suppl):195S-201S.

Scheufler C, Sebald W, Hülsmeyer M. Crystal structure of human bone morphogenetic protein-2 at 2.7 A resolution. J Mol Biol 1999 19;287:103-115.

Schilling A, Linhart W, Filke S, Gebauer M, Schinke T, Rueger J, Amling M. Resorbability of bone substitute biomaterials by human osteoclasts. Biomaterials 2004;25:3963-3972.

Schlegel A, Donath K. BIO-OSS-a resorbable bone substitute? J Long Term Eff Med Implants 1998;8:201-209.

Schmid C. The regulation of osteoblast function by hormones and cytokines with special reference to insulin-like growth factors and their binding proteins. J Intern Med 1993;234:535-542.

Schmitz J, Hollinger J. The critical size defect as an experimental model for craniomandibulofacial nonunions. Clin Orthop Relat Res 1996;324:66-74.

Schopper C, Moser D, Sabbas A, Lagogiannis G, Spassova E, könig F, Donath K, Ewers R. The fluorohydroxyapatite (FHA) FRIOS® Algipore® is a suitable biomaterial for the reconstruction of severely atrophic human maxillae. Clin Oral Impl Res 2003;14:743-749.

Schropp L, Wenzel A, Kostopoulos L, Karring T. Bone healing and soft tissue contour changes following single-tooth extraction: a clinical and radiographic 12-month prospective study. Int J Periodontics Restorative Dent 2003;23:313-323.

Schwartz Z, Weesner T, van Dijk S, Cochran D, Mellonig J, Lohmann C, Carnes D, Goldstein M, Dean D, Boyan B. Ability of deproteinized cancellous bovine bone to induce new bone formation. J Periodontol. 2000;71:1258-1269.

Schwarz F, Mihatovic I, Golubovic V, Becker J. Dentointegration of a titanium implant: a case report. Oral and Maxillofacial Surgery 2013;3:235-241.

Sjöström M, Sennerby L, Nilson H, Lundgren S. Reconstruction of the atrophic edentulous maxilla with free iliac crest grafts and implants: a 3-year report of a prospective clinical study. Clin Implant Dent Relat Res 2007;9:46-59.

Senn M. On healing of aseptic bone cavities by implantation of antiseptic decalcified bone. Am J Med Sci 1889;98:219-243.

Singh K, Dumonski M, Stanley T, Ponnappan R, Phillips F. Repeat Use of Human Recombinant Bone Morphogenetic Protein-2 for Second Level Lumbar Arthrodesis. Spine 2011; 3:192-196.

Slater N, Dasmah A, Sennerby L, Hallman M, Piattelli A, Sammons R. Back-scattered electron imaging and elemental microanalysis of retrieved bone tissue following maxillary sinus floor augmentation with calcium sulphate. Clin Oral Implants Res. 2008;8:814-822.

Smiler D, Holmes R. Sinus lift procedure using porous hydroxylapatite: A preliminary clinical report. J Oral Impl 1987;13:239-252.

Smiler D, Johnson P, Lozada J, Misch C, Rosenlicht J, Tatum O, Wagner J. Sinus lift grafts and endosseous implants. Treatment of the atrophic posterior maxilla. Dent Clin North Am 1992;36:151-186.

Söder P, Otteskog , Andreasen J, Modéer T. Effect of drying on viability of periodontal membrane. Scand J Dent Res 1977;85:164-168.

Sogal A, Tofe A. Risk assessment of bovine spongiform encephalopathy transmission through bone graft material derived from bovine bone used for dental applications. J Periodontol. 1999;70:1053-1063.

Sriamornsak P, Thirawong N. Use of back-scattered electron imaging as a tool for examining matrix structure of calcium pectinate. Int J Pharm 2003;267:151-156.

Stevenson S, Emery S, Goldberg V. Factors affecting bone graft incorporation. Clin Orthop Relat Res 1996;324:66-74.

Su-Gwan K, Hak-Kyun K, Sung-Schul L. Combined implantation of particulate dentine, plaster of Paris, and a bone xenograft (Bio-Oss ®) for bone regeneration in rats. J Cranio-Maxillofac Surg 2001;29:282-288.

Szabo G, Suba Z, Hrabak K, Barabás J, Németh Z. Autogenous bone versus beta-tricalcium phosphate graft alone for bilateral sinus elevations (2- and 3-dimensional computed tomographic, histologic, and histomorphometric evaluations): Preliminary results. J Oral Maxillofac Impl 2001;16:681-692.

Tanaka-Kamioka K, Kamioka H, Ris H, Lim S-S. Osteocyte shape is dependent on actin filaments and osteocyte processes are unique actin-rich projections. J Bone Miner Res 1998;13:1555-1568.

Thorwarth M, Wehrhan F, Srour S, Schultze-Mosgau S, Felszeghy E, Bader R, Schlegel K. Evaluation of substitutes for bone: comparison of microradiographic and histological assessments. Br J Oral Maxillofac Surg. 2007;1:41-47.

Trope M. Avulsion of permanent teeth: theory to practice. Dent Traumatol 2011;27:281-94.

Urist M. Bone formation by autoinduction. Science 1965;150:893-899.

Urist M, Strates B. Bone morphogenetc protein. J Dent Res 1971;50:Suppl6:1392-1406.

Urist M. Bone histogenesis and morphogenesis in implants of demineralised enamel and dentin. J Oral Surg 1971;29:88-102.

Urist M. Bone transplants and implants. In Urist MR (ed). Fundamental and clinical physiology of bone. Philadephia, JB Lippicot Co. 1980;331-368.

Vaananen H, Laitala-Leinonen T. Osteoclast lineage and function. Archives of biochemistry and biophysics 2008;473:132-138.

Valentini P, Abensur D, Densari D, Graziani J, Hämmerle C. Histological evaluation of Bio-Oss in a 2-stage sinus floor elevation and implantation procedure. A human case report. Clin Oral Implants Res 1998;9:59-64.

Valentini P, Abensur D, Wenz B, Peetz M, Schenk R. Sinus grafting with porous bone mineral (Bio-Oss) for implant placement: a 5-year study on 15 patients. Int J Periodontics Restorative Dent 2000;203:245-253.

Voggenreiter G, Ascherl R, Blumel G, Schmit-Neuerburg K. Effects of preservation and sterilization on cortical bone grafts: a scanning electron microscopic study. Arch Orthop Trauma Surg 1994;113:294-296.

Wallace S, Froum S, Tarnow D. Histologic evaluation of a sinus elevation procedure: a clinical report. Int J Periodontics Restorative Dent 1996;1:46-51.

Warrer K, Karring T, Gotfredsen K. Periodontal ligament formation around different types of dental titanium implants. The self-tapping screw type implant system. Journal of Periodontology 1993;64:29–34.

Wheeler S, Holmes R, Calhoun C. Six-year clinical and histologic study of sinus lift grafts. J Oral Maxillofac Impl 1996;1:26-34.

Wood R, Moor D. Grafting of the maxillary sinus with intraorally harvested autogenous bone prior to implant placement. J Oral Maxillofac Impl 1988;3:209-214.

Xu C, Wang Y. Chemical composition and structure of peritubular and intertubular human dentine revisited. Arch Oral Biol. 2012;57:383-391.

Yeomans J, Urist M. Bone induction by decalcified dentine implanted into oral, osseous and muscle tissues. Arch Oral Biol. 1967;8:999-1008.

Yildirim M, Spiekermann H, Handt S, Edelhoff D. Maxillary sinus augmentation with the xenograft Bio-Oss and autogenous intraoral bone for qualitative improvement of the implant site: a histologic and histomorphometric clinical study in humans. Int J Oral Maxillofac Implants. 2001;16:23-33.

Zipfel G, Guiot B, Fessler R. Bone grafting. Neuro Surg 2003; 15:14:e8.