1. General

1.1 Bone anatomy and histology

Bone is a specialised form of dense connective tissue which forms the main component of the skeleton in the adult human. It is a dynamic biological tissue composed of metabolically active cells that are integrated into a rigid framework.The cellular components of bone consist of osteogenic precursor cells, osteoblasts, osteoclasts, osteocytes, and the hematopoietic elements of bone marrow [7, 8].

Osteoprogenitor cells, or stem cells of bone, consist of undifferentiated stromal cells from which the osteoblasts are derived. They present on all nonresorptive bone surfaces, and they make up the deep layer of the periosteum, which invests the outer surface of bone, and the endosteum, which lines the internal medullary surfaces. Osteoprogenitors are induced to differentiate under the influence of growth factors, in particular the bone morphogenetic protein (BMPs). Aside from BMPs, other growth factors including fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β) may promote the division of osteoprogenitors and potentially increase osteogenesis.
The periosteum is a tough, vascular layer of connective tissue that covers the bone but not its articulating surfaces. The thick outer layer, termed the "fibrous layer," consists of irregular, dense connective tissue. A thinner, poorly defined inner layer called the "osteogenic layer" is made up of osteogenic cells. The endosteum is a single layer of osteogenic cells lacking a fibrous component.

Osteoblasts are mononucleate, mature and metabolically active cells that are responsible for bone formation. They secrete the organic portion of the bone matrix called osteoid. This osteoid contains water, collagen, non-collagenous protein, and proteoglycans and is an unmineralized organic matrix that subsequently undergoes mineralization, giving the bone its strength and rigidity. When the osteoid becomes calcified, the water is replaced by mineral [9]. As their bone forming activity nears completion, some osteoblasts are converted into osteocytes whereas others remain on the periosteal or endosteal surfaces of bone as lining cells. This means osteoblasts avoid being buried by newly formed bone and remain on the same level as this new bone. It is believed that they are responsible for mineral transfer in and out of the bone. They are also believed to sense mechanical strain and to initiate bone remodelling in response to various chemical and mechanical stimuli[10]. Osteoblasts also play a role in the activation of bone resorption by osteoclasts.

Osteocytes are the most abundant cells found in bone. They are mature osteoblasts trapped within the bone matrix. Osteocytes are networked to blood vessels and each other via long cytoplasmic extensions that occupy tiny canals called

canaliculi, which are used for the exchange of nutrients and waste. The space that an osteocyte occupies is called a lacuna (Latin for a *pit*). There are approximately 15,000 lacunae per cubic mm of bone. Osteocytes are involved in the control of extracellular concentration of calcium and phosphorus, as well as in adaptive remodelling behaviour via cell-to-cell interactions in response to the local environment.

Osteoclasts are multinucleated, bone-resorbing cells controlled by hormonal and cellular mechanisms. These cells function in groups termed “cutting cones” that attach to bare bone surfaces and by releasing hydrolytic enzymes, dissolve the inorganic and organic matrices of bone and calcified cartilage. Osteoclasts demineralize adjacent bone with acids and attack collagen fibres with enzymes. This occurs at a rate of approximately 10 um a day [9]. This process results in the formation of shallow erosive pits on the bone surface called Howship lacunae[11].Osteoclasts are formed by the fusion of cells of the monocyte-macrophage cell line [12].Osteoclasts are regulated by several hormones, including parathyroid hormone (PTH) from the parathyroid gland, calcitonin from the thyroid gland, and growth factor interleukin 6 (IL-6). This last hormone, IL-6, is one of the factors in the disease osteoporosis, which is an imbalance between bone resorption and bone formation. Osteoclast activity is also mediated by the interaction of two molecules produced by osteoblasts, namely osteoprotegerin and RANK ligand. Note that these molecules also regulate differentiation of the osteoclasts [13].
There are three primary types of bone: woven bone, cortical bone, and cancellous bone [7, 11].

Woven bone is found during embryonic development, during fracture healing (callus formation), and in some pathological states such as hyperparathyroidism and Paget disease [8]. It is composed of randomly arranged collagen bundles and irregularly shaped vascular spaces lined with osteoblasts. Woven bone is normally remodelled and replaced with cortical or cancellous bone [14].

Cortical bone Cortical bone, also called compact or lamellar bone, is remodelled from woven bone by means of vascular channels that invade the embryonic bone from its periosteal and endosteal surfaces. It forms the internal and external tables of flat bones and the external surfaces of long bones. Compact bone consists almost entirely of extracellular substance, the matrix. Osteoblasts deposit the matrix in the form of thin sheets which are called lamellae. The primary structural unit of cortical bone is an osteon, also known as a haversian system. Osteons consist of cylindrical shaped lamellar bone that surrounds longitudinally orientated vascular channels called haversian canals. Horizontally oriented canals (Volkmann canals) connect adjacent osteons. The mechanical strength of cortical bone depends on the tight packing of the osteons.

Cancellous bone (trabecular bone) lies between cortical bone surfaces and consists of a network of honeycombed interstices containing haematopoietic elements and bony trabeculae. The matrix of trabecular bone is also deposited in the form of lamellae.
However, lamellae in trabecular bone do not form Haversian systems. Lamellae of trabecular bone are deposited on pre-existing trabeculae depending on the local demands on bone rigidity. The trabeculae are predominantly oriented perpendicular to external forces to provide structural support [15]. Cancellous bone is continually undergoing remodelling on the internal endosteal surfaces.

1.2 Bone biochemistry
The matrix is the major constituent of bone, surrounding the cells. It has inorganic and organic parts. By weight, bone is approximately 20% water [8].The weight of dry bone is made up of inorganic calcium phosphate (65–70% of the weight) and an organic matrix of fibrous protein and collagen (30–35% of the weight) [7,8]. Bone is composed of five non-cellular materials in the extra-cellular matrix. These materials are collagen, hydroxyapatite minerals, non-collagenous proteins, proteoglycans and water.

Organic elements of matrix is mainly composed of Type I collagen. Osteoid is the unmineralized organic matrix that is composed of 90% type I collagen and 10% ground substance, which consists of noncollagenous proteins, glycoproteins, proteoglycans, peptides, carbohydrates, and lipids [8]. Osteoid is secreted by osteoblasts and makes up about fifty percent of bone volume and twenty-five percent of bone weight. The basic function of collagen is to provide structural strength with its fibril configuration. Collagen gives bone flexibility and tensile strength. The mineralization of osteoid by inorganic mineral salts provides bone with its strength and rigidity [9].The function of both non-collagenous proteins and proteoglycans is unclear. It is believed that proteoglycans function to control mineralization rate and location in bone. This is due to their calcium binding properties. Examples of non-collagenous proteins are osteocalcin, which is secreted by the osteoblasts, osteopontin and osteonectin.
Water is mainly bound to collagen, due to the hydrophobic effects of the molecules. Also water can be found free in the bone matrix. It is the water that gives bone its compressive strength and viscoelastic properties [9].
Inorganic elements form one half of the bone volume and two thirds of the dry weight of bone [10]. The inorganic content of bone consists primarily of calcium phosphate and calcium carbonate, with small quantities of magnesium, fluoride, and sodium. The mineral composition is mainly hydroxyapatite crystals ($Ca_{10}(PO_4)_6(OH)_2$). These are rods or plates with hexagonal symmetry measuring approximately 50 x 50 x 400 A° and become deposited between collagen fibres of the osteoid. Calcification begins a few days after the deposition of organic bone substance (or osteoid) by the osteoblasts. Mineralisation involves osteoblasts

secreting vesicles containing alkaline phosphatase. This cleaves the phosphate groups and acts as the foci for calcium and phosphate deposition. The vesicles then rupture and act as a centre for crystals to grow on. About 75% of the hydroxyapatite is deposited on the collagen molecules in the first few days of the process, but complete calcification may take several months.

1.3 Regulators of bone metabolism

Bone metabolism is under constant regulation by a host of hormonal and local factors. Three of the calcitropic hormones that most affect bone metabolism are parathyroid hormone, vitamin D, and calcitonin. Parathyroid hormone increases the flow of calcium into the calcium pool and maintains the body's extracellular calcium levels at a relatively constant level. Osteoblasts are the only bone cells that have parathyroid hormone receptors. This hormone can induce cytoskeletal changes in osteoblasts. Vitamin D stimulates intestinal and renal calcium-binding proteins and facilitates active calcium transport. Calcitonin is secreted by the parafollicular cells of the thyroid gland in response to an acutely rising plasma calcium level. Calcitonin serves to inhibit calcium-dependent cellular metabolic activity.

Bone metabolism is also affected by a series of proteins or growth factors, released from platelets, macrophages, and fibroblasts. These proteins cause healing bone to vascularize, solidify, incorporate, and function mechanically. They can induce mesenchymal-derived cells, such as monocytes and fibroblasts, to migrate, proliferate, and differentiate into bone cells. The proteins that enhance bone healing include the BMPs, insulin-like growth factors, transforming growth factors, platelet derived growth factor and fibroblast growth factor among others [16]. The most well known of these proteins are the BMPs, a family of glycoproteins derived from bone matrix. Bone morphogenetic proteins induce mesenchymal cells to differentiate into bone cells. Although typically present in only minute quantities in the body, several BMPs have been synthesized using recombinant DNA technology and are currently undergoing clinical trials.Other proteins influence bone healing in different ways.Transforming growth factor–β regulates angiogenesis,bone formation, extracellular matrix synthesis, and controls cell-mediated activities. Osteonectin, fibronectin, osteonectin, and osteocalcin promote cell attachment, facilitate cell migration, and activate cells [9, 17, 18].

1.4 Bone regeneration

The principle of bone regeneration is independent of bone type (i.e. desmoid or chondral genesis) and how the defect came about. Three healing phases are observed for minor bone defects: early inflammatory phase, biological bone regeneration and remodelling.

1. Early inflammatory phase:

In the early inflammatory phase the first change is the presence of blood cells within the tissues which are adjacent to the injury site. Soon the blood vessels constrict, stopping any further bleeding [1].Thrombocytes in this phase play an important role, since they are the first wound closure cells to become active in the area of tissue destruction. They control the inflammatory and immune reactions by releasing growth factors and cytokine like proteins. During the first few hours and days the extravascular blood cells, are known as a "hematoma" form a blood clot. All of the cells within the blood clot degenerate and die [2]. Inflammatory cells (macrophages, monocytes, lymphocytes and polymorphonuclear cells) and fibroblasts infiltrate the bone under prostaglandin mediation. This results in the formation of granulation tissue, in growth of vascular tissue and migration of mesenchymal cells. Fibroblasts which were survived and replicated form a loose aggregate of cells, interspersed with small blood vessels which are called granulation tissue. This perfused, fibrous connective tissue replaces fibrin clot in the healing wound. Granulation tissue is composed of tissue matrix supporting a variety of cell types which can be associated with one of the following functions:

Extracellular matrix: which is is created and modified by fibloblasts. Initially, it consists of a network of Type III collagen, a weaker form of the structural protein that can be produced rapidly. This is later replaced by the stronger, long-stranded Type I collagen, as evidenced in scar tissue.

Immune system: The main immune cells active in the tissue are macrophages and neutrophiles although other leucocytes are also present. These work to phagocytize old or damaged tissue, and protect the healing tissue from pathogenic insult. This is necessary both to aid the healing process and to protect against invading pathogens, as the wound often does not have an effective skin barrier to act as a first line of defence.

Vascularization: It is necessary for a network of blood vessels to be established as soon as possible to provide the growing tissue with nutrients, to take away cellular wastes, and transport new leukocytes to the area. Fibroblasts, the main cells that deposit granulation tissue, depend on oxygen to proliferate and lay down the new extracellular matrix. The primary nutrient and oxygen supply of this early process is provided by the exposed cancellous bone and muscle. The use of antiinflammatory or cytotoxic medication during this 1st week may alter the inflammatory response and inhibit bone healing. In vascularisation, also called angiogenesis, endothelial cells quickly grow into the tissue from older, intact blood vessels.These branch out in a systematic way, forming anastomoses with other vessels. It is during this stage that the presence of nicotine in the system can inhibit this capillary in growth [3, 4].

2. **Biological bone regenerating or reparative phase**

In this phase the osteoprogenitor cells and osteoblasts become active in addition to the fibroblasts and angiogenetic cells and restore the bone to its original state. The periosteum is the primary source of precursor cells which develop into chondroblasts and osteoblasts that they are essential to the healing of bone. The bone marrow (when present), endosteum, small blood vessels, and fibroblasts are secondary sources of precursor cells.

As vascular in growth progresses, a collagen matrix is laid down while osteoid is secreted and subsequently mineralized, which leads to the formation of a soft callus around the repair site. Osteoid bone is newly-formed ground substance that is secreted by osteoblasts and has not yet calcified (glycoproteins and proteoglycanes). Osteocytes which are derived from osteoblasts are completely surrounded by bone ground substance. The periosteal cells proximal to the fracture gap develop into chondroblasts and form hyaline cartilage. The periosteal cells distal to the fracture gap develop into osteoblasts and form woven bone. The fibroblasts within the granulation tissue also develop into chondroblasts and form hyaline cartilage [1]. Woven bone is connective tissue, hardened and rigidified by inorganic and organic substances, with a random disposition of bone cells and collagen fibres. After formation of the callus, eventually the fracture gap is bridged by the hyaline cartilage and woven bone, restoring some of its original strength. Woven bone is weaker, with a small number of randomly oriented collagen fibres, but forms quickly. It is replaced by lamellar bone, which is highly organized in concentric sheets with a low proportion of osteocytes. Lamellar bone is stronger and filled with many collagen fibres parallel to other fibres in the same layer. The lamellar structures surround the vascular (Haversian) canals. Substitution of the woven bone with lamellar bone precedes the substitution of the hyaline cartilage with lamellar bone. The lamellar bone begins forming soon after the collagen matrix of either tissue becomes mineralized. At this point, "vascular channels" with many accompanying osteoblasts penetrate the mineralized matrix. These osteoblasts form new lamellar bone in the form of trabecular bone. Eventually, all of the woven bone and cartilage of the callus is replaced by trabecular bone, restoring much, if not all, of the bone's original strength.

3. Biological remodelling:

This process substitutes the trabecular bone with compact bone. The trabecular bone is first resorbed by osteoclasts which lyse bone with extracellular enzymes and absorb the products of this dissolution through pinocytosis. This osteoclastic-enzymatic bone tissue lysis creates a shallow resorption pit known as a 'Howship's lacuna'. The osteoblasts then deposit compact bone within the resorption pit. Eventually, the fracture callus is remodelled into a new shape which closely duplicates the bone's original shape and strength [5]. Bone healing is completed during the remodelling stage in which the healing bone is restored to its original shape, structure, and mechanical strength. Remodelling of the bone occurs slowly

over months to years and is facilitated by mechanical stress placed on the bone. This process continuously takes place in healthy bone and is maintained by the interaction of bone-lysing osteoclasts and bone-building osteoblasts. The bone mass is maintained in a continuous process of bone structure adaptation to biomechanical load according to the principles of Wolff's law of transformation: The bone trabecules always arrange themselves along the load lines to bear the applied forces [6].

2. Bone graft substitute materials

Introduction

2.1 Bone graft substitute materials

The filling of a bone defect is a significant issue in each day of clinical work. Restitutio ad integrum is achieved in minor defects through biological self healing. Self healing is limited by the size of the defect. In larger defects where the organism is unable to heal itself, the restoration of the original condition must be the objective of the therapy. Bone tissue is unable to regenerate defects larger than 1 mm in a single step. Weeks or even months are required to heal bone defects larger than 3 mm [20].

In many cases, the intended implant site is compromised because of poor bone quality (i.e., low bone density, in the case of highly cancellous bone, or low vascularity, in the case of primarily cortical bone) or insufficient quantity of bone (in terms of the width or height of the alveolar ridge). Lack of sufficient alveolar ridge height is often related to the proximity of the implant site to other anatomical structures (i.e., the maxillary sinus or the mandibular canal). In these situations separate preparatory procedures may be required to augment the available volume of bone before placement of the implant, using bone graft substitute materials [122].

Bone grafts are necessary to provide support, fill voids, and enhance the biologic repair of skeletal defects. They are used by orthopaedic surgeons, neurosurgeons, craniofacial surgeons and periodontists. Today, the trend is towards bioresorbable materials which are resorbed within a relatively short period simultaneously with autogenous bone formation. They are preferably termed bone regeneration or augmentation materials.

There are several physiological properties of bone grafts which can directly affect the success or failure of graft incorporation. These properties are osteogenesis, osteoinduction, and osteoconduction [18]. They are descriptive models or concepts involved with bone healing and regeneration. These terms may also be used to describe and classify the biological properties and clinical effects of graft materials.

Osteogenesis is the ability of the graft to produce new bone, and this process is dependent on the presence of living bone cells in the graft. Cells with osteogenic

potential include endosteal or cambial osteoblasts, perivascular cells, and undifferentiated stem cells from the bone and bone marrow. 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. Osteogenesis is a property found only in fresh autogenous bone and in bone marrow cells, although the authors of radiolabelling studies of graft cells have shown that very few of these transplanted cells survive [19].

Osteoconduction is the physical property of the graft to serve as a scaffold for viable bone healing [52]. Osteoconduction is a three-dimensional process that is observed when porous structures are implanted into or adjacent to bone. It allows for the in growth of neovasculature and the infiltration of osteogenic precursor cells into the graft site. Osteoconductive materials may also stimulate the recruitment and migration of potentially osteogenic cells to the site of matrix formation [21].Clinically, osteoconduction results in bone growth within a defect or on a surface which may otherwise repair with soft tissue. Osteoconductive properties are related to structural and material properties (porosity, pore size, shape, particle size, crystallinity) that influence cell attachment, migration, differentiation and vascularization. There have been a number of investigations which sought to identify the proper pore size for a tissue engineering scaffold, with results showing that pores ranging from 80 to 500 µm to be viable [22,23]. Osteoconductive properties are found in cancellous autografts and allografts, demineralized bone matrix, hydroxyapatite, collagen, polymers (HTR Polymer) bioglass and calcium phosphate [19].

Osteoinduction is the ability of graft material to induce stem cell differentiation into mature bone cells, or in more detail to induce differentiation of undifferentiated pluripotential cells toward an osteoblastic phenotype. The phenomenon of osteoinduction was first described in the classic works of Urist [49,50,51]. Clinically, implantation of an osteoinductive material stimulates bone formation; even in an ectopic site such as muscle.This process is typically associated with the presence of bone growth factors within the graft material or as a supplement to the bone graft. Bone morphogenic proteins [24,25,26,27] and demineralized bone matrix are the principal osteoinductive materials. To a much lesser degree, autograft and allograft bone also have some osteoinductive properties [19].
Several osteoinductive agents which are generally proteins have been identified. Among these compounds are transforming growth factor (TGF-β) [24], fibroblast growth factors (FGFs) [28,29], insulin-like growth factors (IGFs) [30], and platelet-derived growth factors (PDGFs) [31].

Bone Morphogenetic Proteins (BMP), are low-molecular-weight non-collagenous glycoproteins that belong to an expanding TGF-β super family of at least 15 growth and differentiation factors [96]. They are known for their ability to induce the formation of bone and cartilage [97,98]. BMP makes up only 0.1% by weight of all bone proteins and acts as an extracellular molecule that can be classified as a morphogen, as its action recapitulates embryonic bone formation. The human BMP is now produced by using recombinant techniques (rhBMP). Therefore, the available protein is free from the risk of infection or allergic reaction. Interestingly the amount of human rhBMP necessary to produce bone induction *in vivo* is more than ten times higher than that of highly purified native bone extracted BMP [99], suggesting that native BMP is a combination of different BMP's or represents a synergy between them [100]. Large and small animals have been used to study the influence of BMP on bone regeneration [101,102,103]. BMP has demonstrated the ability to heal many different varieties of critical sized defects including cranial vault defects, long bone defects and mandibular continuity defects [104,105,106] without the addition of a bone graft. Since morphogen BMP is rapidly absorbed into the surrounding tissues, many different carrier vehicles have been used to deliver BMP. The reaction of the soft tissues with notable edema, erythema and inflammation is the most remarkable problem with the use of BMP's [107]. Therefore The effects of BMP must be regulated. There is increasing evidence that serum protein fetuin may serve as one regulator of BMP's effects [107,108].

Transforming Growth Factor (TGF-β) is a osteopromotor protein agent which participates in all phases of bone healing [109]. TGF-β is the most extensively studied growth factor in the field of bone biology. It comprises an entire family of molecules that includes the BMPs. In an animal study, it was found that BMP, and not TGF-β, enhanced bone formation [114]. TGF- β may be more effective than BMP in those situations where enhanced bone healing is preferred to bone induction [95].
TGF-β is chemotatic for bone forming cells, stimulating angiogenesis and limiting osteoclastic activity at the revascularization phase. Once bone healing enters osteogenesis then TGF- β increases osteoblast mitoses, regulating osteoblast function and increasing bone matrix synthesis, inhibiting type II collagen but promoting type I collagen. Finally, during remodelling it assists in bone cell turn-over [107-113].

Platelet-Derived Growth Factor (PDGF) is dimeric glycoprotein growth factor that regulates cell growth and division by playing a role in embryonic development, cell proliferation, cell migration and angiogenesis. In particular, it plays a significant role in blood vessel formation (angiogenesis), the growth of blood vessels from already existing blood vessel tissue, and is known to stimulate the reproduction and chemotaxis of connective tissue cells, matrix deposition [115,116]. It was shown that PDGF had a stimulatory effect on fracture healing in rabbit [31].

Insulin-like growth factor (IGF) is a polypeptide that is secreted from many different cells. Their designation as "insulin-like" is due to the fact that they have high sequence similarity to insulin. Treatment of serum with antibodies to insulin failed to eliminate all insulin activity; the remaining activity was ultimately ascribed to the IGFs. Due to their growth promoting activity, they were formerly called somatomedins. Insulin-like growth factor 1 (IGF-1), is mainly secreted by the liver as a result of stimulation by growth hormone (GH) and is important for promotion of cell proliferation and the inhibition of cell death (apoptosis). Insulin-like growth factor 2 (IGF-2) is thought to be a primary growth factor required for early development and may be primarily fetal in action, it is also essential for development and function of organs such as the brain, liver and kidney. PDGF and IGF have shown an ability to work together during the reparative stages of bone healing in defects associated with dental implants and teeth [117,118,119].

Platelet rich plasma (PRP) is a modification of fibrin glue made from autologous blood and is used to deliver growth factors in high concentration to sites requiring osseous grafting. Calcium ions, resulting from the dissociation of bone augmentation material in the blood fluid, especially encourage the activation of thrombocytes. Growth factors released from degranulated platelets include PDGF, TGF-β, platelet-derived epidermal growth factor, platelet-derived angiogenesis factor, IGF-1 and platelet factor 4. These factors signal the local mesenchymal and epithelial cells to migrate, divide, and increase collagen and matrix synthesis [121]. PRP has been suggested for use to increase the rate of bone deposition and quality of bone regeneration. They are the potential source of concentrated platelets that could be used in bone regeneration [120]. PRP contains 500,000 to 1,000,000 platelets, which are mixed with a thrombin/calcium chloride (1,000units/10%) solution to form a gel [120]. This gel when used in combination with autogenous bone, reportedly causes PRP to increase the maturation rate of a bone graft and increase the bone density of the graft [120].

Basic fibroblast growth factor (bFGF) in normal tissue is present in basement membranes and in the sub endothelial extracellular matrix of blood vessels. It has been hypothesized that bFGF is activated by heparan sulphate-degrading enzyme and promotes angiogenesis. Additionally, bFGF is a critical component of human embryonic stem cell culture medium, which is necessary for the cells to remain in a undifferentiated state. Hyaluronic acid (Hy) is a viscoelastic polymer found throughout the body that cushions and protects soft tissues. The synergistic combination of bFGF and Hy appears to accelerate the fracture healing process.

2.2 Classification of bone grafting materials based on source

Autograft: also called autogenous or autologous, refers to a transplant of viable cortical or cancellous bone [53,54,55] from one location to another within the same patient. It is a gold standard grafting material for osseous regeneration, fulfilling all essential physicochemical and biological properties of ideal bone graft [32, 33]. It is osteogenic (living cells such as osteocytes or osteoblasts within a donor graft form new bone at the implantation site), osteoinductive (as bone matrix is broken down BMP are released and stimulate attraction, differentiation and proliferation of host mesenchymal stem cells into bone-forming osteoblasts), osteoconductive (the remaining non-vital bone matrix provides an scaffold which osseous tissue can regenerate bone by means of vascularization), biomechanically stable, disease free and contain no antigenic factors [34]. Autologous bone is typically harvested from intra-oral sources such as mandibular symphysis, maxillary tuberosity, ramus, exostoses and debries from an implant osteotomy or extra-oral sources such as iliac crest, cranial vault and ribs [56,57]. Cancellous bone contains a higher percentage of cells, and therefore has more osteogenic potential. Conversely, cortical bone is believed to have higher levels of BMP's, and is useful when structural support or three-dimensional augmentation is required. The disadvantages of autografts include the need for a separate incision for harvesting, increased operating time and blood loss, the risk of donor-site complications, post-operative pain and the frequent insufficient quantity of bone graft.

Allograft: refers to a transplant of non-vital osseous tissue from genetically no identical members of the same species. It has low or no osteogenicity, increased immunogenic activity and resorbs more rapidly than autogenously bone. In clinical practice, fresh allografts are rarely used because of immune response and the risk of transmission of disease. Bone allograft has the advantage of being available in far larger quantities than autograft and lack of additional morbidity to harvest the graft; however, the treatment process the bone goes through following harvest, which usually involves deep-freezing and may also involve demineralization, irradiation and/or freeze-drying, kills living bone or bone marrow cells. This significantly reduces the immunogenicity (risk of graft rejection) such that no anti rejection drugs are needed and, combined with appropriate donor screening practices, these processing and preservation practices can significantly reduce the risk of disease transmission. Despite this processing, cancellous allograft bone retains its osteoconductive properties. Furthermore, certain processing practices have been shown to also retain the acid-stable osteoinductive proteins in cortical bone grafts, so that many bone allografts can be considered both osteoconductive and only weakly osteoinductive. The disadvantages of allograft include delayed vascular penetration, slow bone formation, accelerated bone resorption, and delayed or incomplete graft incorporation [35,36,37]. Although transmission of infection and lack of histocompatibility are potential problems with allograft bone, improved tissue-banking standards have greatly reduced their incidence.

Demineralized freeze-dried bone is sourced from human cadavers screened for malignancy, HBV, HCV, HIV and associated lifestyle factors that place the recipient at risk for infectious disease. The risk of transmitting HIV with a properly screened demineralized freeze-dried bone allograft has been calculated to be 1 in 2.8 billion [58]. It is believed to have osteoinductive activity due to the presence of bone morphogenetic proteins [41] which was first demonstrated by Urist using animal models in the 1960's [40]. However, recent evidence suggests that this phenomenon is not universal among products from the various bone banks around the country [42]. It has been shown that the processing techniques and sterilization methods used may affect BMP activity, and there are those who suggest that exposing the bone to irradiation or ethylene oxide sterilization renders the BMP's inactive [59,60]. Donor age has also been shown to be a variable which may affect the amount of active BMP's within a given allograft.
Mineralized freeze-dried human bone allograft is available as cortical or cancellous granules. Due to the presence of a mineralized matrix, the graft is denser and the turnover time (resorption time) of this material is extended when compared to demineralized bone which may be of clinical benefit in larger defects in terms of gaining increased volume. Based on theory, the mineralized component must be resorbed in order to expose the BMP's and make them biologically active, the dissadventage of this type of graft is less bioavailabilty and activity of BMP's.

Xenograft: also called heterograft refers to a skeletal tissue that is harvested from an animal of one species and transferred to the recipient site of another species [61,62], such as the use of inorganic bovine bone or bovine collagen in human subjects [63,64]. Xenograft bone could be processed to be safe for transplantation in a human host [38]. It has the same inherent problems as allografts, and being from a different species, it may cause even more pronounced immunological problems.
Coralline hydroxyapatite is a naturally derived graft material prepared from sea coral. Corals made by marine invertebrates have skeletons with a structure similar to both cortical and cancellous bone, with interconnecting porosity [43]. Porites and Goniopora are the only two genera of corals meeting the required standards of pore diameter and interconnectivity [23]. Natural coral or biocoral are slowly resorbing, porous, coralline graft material in the form of aragonite (>98% $CaCO_3$) manufactured directly in calcium carbonate form which is not altered by processing. The other process is Replamineform process that converts calcium carbonate to hydroxyapatite. The material is essentially pure HA, with the balance consisting of TCP and is osteoconductive in its mechanism of action. They are simultaneously incorporated into the human bony skeleton and replaced by human bone. The enzyme carbonic anhydrase, liberated by osteoclasts is responsible for the breakdown of this material. The time for total replacement of this implant by bone in the human craniofacial skeleton is approximately 18 months [67].The natural porosity of the material facilitates cell attraction and in growth while the structural density prevents rapid resorption of the particle. They are brittle, difficult to

maintain micropores and highly potential for infection.
Anorganic bovine bone is a naturally derived hydroxyapatite originated from cows. Currently there are two processes used to prepare theses materials: one is low temperature (300°C) chemical extraction of organic components used by Bio-Oss., which maintains the exact trabecular architecture and porosity of the original bone and the other is high temperature (1100°C) sintering process used by Osteograft/N results in fusion of bone crystallites which possesses a large, non homogeneous crystal morphology, with decreased porosity and surface area. Bio-Oss provides the body with an osteoconductive matrix for bone cell migration and is integrated into the natural physiologic remodelling process due to preservation of the trabecular architecture and fine crystalline structure of the natural bone. The graft will undergo physiologic remodelling and become incorporated into bone over a period of time [44]. Their disadvantage is the concern with the possibility of future bovine spongiform encephalopathy due to potential slow virus transmission in bovine-derived products [65, 66].
BIO-GEN® It is a natural bone conductive material without collagen, deantigenised and derived from equine bone, which is completely absorbable. It elevates level of bone genesis thanks to the absence of the calcification process within the production process, that occurs through physical and chemical processes at a maximum temperature of 130°C in a humid environment. The final bone tissue, constituted of bone mineral matrix which is unmodified at the atomic structural level. The absorption time of spongy granular is 4-6 month and cortical granular type is 8-12 month. Granular mix is made up of a calibrated mixture of cortical and spongy bone tissue with cortical grains of 0.5 mm and spongy grains of 1 mm.

Alloplast: refers to implantation of synthetic materials which are available in different forms with variable density, porosity and crystallinity, all of which are dependent on the manufacturing process. Synthetic materials consist entirely of defined chemical substances and with consideration with regards to material risks of infection, immunological risk and physiological intolerance they are produced by suitable synthetic methods. An optimum synthetic bone augmentation material must fulfil certain requirements: (In the early healing phase, while an inflammatory reaction is acute, the material must remain stable. It must not disintegrate or lose stability due to lysis or dissolving), porosity (this requires macropores >100 µm [85,150-152], and micropores ≥ 5 µm [153] to ensure progressive angiogenesis), primary grain size (during the sintering process, particles are fused to each other by sinternecks,and thus ensure the mechanical stability of the structure as well as an interconnective microporosity. Primary grains size must be > 10 µm to avoid cellular degradation processes [154]), purity of phase (ensure uniform resorption behavior and complete degradation), and homogeneous solubility (provides a constant physiological concentration of calcium and phosphate ions which can be

assimilated by the bone-forming osteoblasts and processed in the anabolic metabolism).

Used alone as a single graft material, these materials can result clinically in improved bone density, and can lead to more complete bone fill of defects via osteoconduction. These materials are osteoconductive only, and as such, autogenous bone or an osteoinductive material should be combined with them when treating large defects or in defects with incomplete walls. Degradation of these materials occurs either by solution mediated or cell mediated resorption depending on the material used, graft volume, physical environment, number of adjacent bony walls, patient age, and local vascularity [39].

Hydroxyapatite (HA) ($Ca_{10}(PO_4)_6(OH)_2$) is a primary inorganic, natural component of bone, comprising about 65% of the calcified skeleton and 98% of dental enamel, with Ca:P ratio of 10:6. Hydroxyapatite in the mineral phase of bone is a natural biomimetic biomaterial. BMPs bind to collagens I and IV, heparin sulfate, heparin and hydroxyapatite [45, 46]. The geometry of the hydroxyapatite is critical for delivery of BMPs for bone induction. In subhuman primates hydroxyapatite appears to be osteoinductive [47]. It is likely that BMPs in circulation in the vascular system may bind to hydroxyapatite and secondarily induce bone formation. Thus, an osteoconductive biomaterial such as hydroxyapatite progressively becomes an osteoinductive substratum [48].Hydroxyapatite is a ceramic that depending upon its ability to resorb, can be divided into two groups [68,69,70,71]. Low-density HA is an amorphous and readily resorbable material which is used as a plasma sprayed, applied to implant surface. Hydroxyapatite coating of metal surfaces enhances ingrowth and direct bonding of bone to porous surface [81, 82]. Dense HA has higher crystallinity and lower restorability which results in excellent long term ridge maintenance and soft tissue support. The mechanism of action is via osteoconduction and the material exhibits bioactivity. HA can be also classified according to their internal pore size [72,73]. HA is brittle and its strength decreases exponentially with increase in porosity therefore implants can not be placed in a HA-treated ridge. Granular migration and incomplete resorption are other dissadvantages of HA [74, 75, 76].

Tricalcium Phosphate (TCP) ($Ca_3(PO4)_2$) like hydroxyapatite is a synthetic calcium phosphate ceramic with a different stoichiometric profile [77,78]. TCP is bioabsorbable and biocompatible material which is used in a variety of orthopaedic and dental applications since 1981. Osteoconduction is facilitated by the porous nature of the particles, with bone growth said to occur within and throughout the porous matrix. The mechanism of the effect of tricalcium phosphate is explained by an enrichment of the microenvironment by the biodegradable material and release of ions of calcium and phosphor which stimulates the activity and proliferation of cells [156]. The increased concentration of calcium and phosphate ions leads to the formation of carbonate apatite which is similar to bone apatite

which binds directly to the bone [155,156.] . It is important to remember that the two crystal modifications α-TCP and β-TCP show pronounced differences as to the mechanism and kinetics of resorption. α-TCP in contact with tissue fluid at 37 °C completely transforms into calcium deficient hydroxyapatite [79] and is therefore not resorbed completely [80].The particles of β-TCP are eventually resorbed and replaced by host bone in 9 to 12 months. To ensure a complete and timely resorption of TCP, it demands a phase purity of more than 95%. Two known examples of tricalcium phosphate preparations in the market are Bioresorb and Cerasorb

Bioglass or Bioactive glass is an amorphous synthetic material composed of calcium phosphate, sodium, and silicon. These materials have the ability to chemically bond with bone [83] and been used in dentistry as restorative materials such as glass ionomer cement. Hydroxycarbonate apatite is a bioactive layer which formed by biochemical transformation following implantation and is thought to be responsible for bone cell attraction and bonding. Bioactive glasses may have osteoinductive properties and have been tested in animal trials [84]. Turnover and resorption of bioactive glass is an area of controversy. Bioglass materials have been used extensively in periodontal regeneration with good results [85,86]. The primary indication of these materials is for the repair of small, localized intrabony defects.

Calcium Sulphate (CaSO4) also called Plaster of Paris or Gypsum, is a biologically inert, resorbable osteoconductive material. Their first internal use to fill bony defects was reported in 1892 by Dressmann [87]. The application of Plaster of Paris as bone void filler, and the use of antibiotic-laden plaster in the treatment of infected bony defects, has been supported by various studies [88,89,90,91]. Calcium sulphate resorbs quickly (4 to 8 weeks). Medical grade calcium sulphate is crystallised in highly controlled environments producing regularly shaped crystals of similar size and shape. It possesses a slower, more predictable solubility and reabsorption [96].

Polymer is a large molecule composed of many smaller repeating units (the monomers) bonded together. They are non-biologic materials and have the advantage of the ability to control all aspects of the matrix, avoidance of immunologic reaction, and excellent biocompatibility. Polylactic acid (PLA) and Polyglycolic acid (PGA) polymers have been used extensively as suture materials [92], and biodegradable fracture fixation implants [93,94]. They can be integrated with growth factors, drugs, and other compounds to create multiphase delivery systems. The turnover rate and resorbability of this material is not clearly understood. The future of bone regeneration could lie with this class of synthetic materials [95].

2.3 Property requirements of bone augmentation materials:
The ideal bone substitute material should have properties similar to that of human bone. Bone augmentation materials used to support the biological bone regeneration process must fulfil various functions in the different phases of regeneration (please refer back to 'Bone remodelling' on pg 6).

In the early inflammatory phase the bone substitute materials must have the following properties:

- *Biocompatibility of bone augmentation*: displays a very high integration within the natural bone without any soft tissue encapsulation or pathological reactions. Possible effects of biomaterials on the living environment due to a lack of blood compatibility are thrombogenicity, and the induction of haemolysis. In addition, the biomaterial must not be carcenogenic, immunogenic, antileukotactic or mutagenic. In turn, the environment should not cause degradation or corrosion of the biomaterial that would result in loss of physical and mechanical properties. No synthetic material will be completely harmonious or inert with the living environment, however, materials do have different levels of inertness.
There are many factors which influence biocompatibility.
For a material to be deemed biocompatible, any adverse reactions which may ensue at the blood/material or tissue/material interface must be minimal. This requires a biomaterial to interact as a natural material would in the presence of blood and tissue. Materials should not:
- Cause thrombus formations
- Destroy or sensitize the cellular elements of blood
- Alter plasma proteins (including enzymes), so as to trigger undesirable reactions
- Cause adverse immune responses
- Cause cancer
- Cause teratological effects
- Produce toxic and allergic responses
- Deplete electrolytes
- Be affected by sterilization

At present date, there are no known materials which totally satisfy these criteria so when a foreign material is placed into a biological environment, inevitable reactions occur which are detrimental to both host and material.

Materials all possess inherent morphological, chemical, and electrical surface qualities which elicit reactionary responses from the surrounding biological environment. In fact, biocompatibility can be described as multifactorial in that simultaneous stimuli from any of these material properties can affect the host response.

- *Osteoconductivity:* allows bone to directly grow into the interconnecting pores prior to the resorption process. (Please refer back to 'osteoconduction' on pg 8).

- *Haemostatic effects*: Refers to the ability of the augmentation to prevent or absorb blood from the surrounding tissue. This often means the material used is open-pored and designed to take up body fluids, so that undesired body fluids are withdrawn from the area surrounding the defect. This leads, for example, to possible seromas or accumulations of fluid being limited to the area of the projection of the material and to fibrin deposition in this area with optimized wound healing; haemostatic effects are also obtained.

- *Encouragement of angiogenesis*: Angiogenesis is a physiological process involving the growth of new blood vessels from pre-existing vessels. Angiogenesis is a normal process in growth and development, as well as in wound healing. Examples of such bone augmentation materials are Fibrin implants containing "vascular endothelial growth factor (VEGF)". VEGF from fibrin implants may present a therapeutically safe and practical modality to induce local angiogenesis. The encouragement of angiogenesis can also be achieved by implanting gelfoam sponges saturated with different angiogenic growth factors.

-*Porosity*: consist of percentage porosity, pore diameter, interconnecting pore diameter, orientation of pores. Surgically installed bone augmentation as replacements for bone sections, load bearing joints, roots of teeth and the like must be structurally sound, capable of knitting into existing bone structure and chemically compatible with body tissue and fluids. Bone knitting and tissue attachment to the material is encouraged by employing structures containing sufficient, correctly sized porosity to provide a penetrable host for the infusion and growth of new tissue and bone material. From a biomedical point of view, a bone augmentation material must feature porous connectivity to ensure progressive angiogenesis (penetration of the bone augmentation material by blood vessels). This requires macropores >100 µm (Klawitter et al. 1971, Eggli et al. 1987, Cornell 1999, Nasr et al. 1999) and micropores ≥ 5 µm (Foitzik and Merten 1999). In granular materials the macropores are primarily formed by the intergranular spaces. Rounded granules provide a good basis for macropore formation. In molded pieces, predetermined macropores (i. e. with a defined diameter) are the ideal pre-requisite for rapid angiogenesis in the bone.
Currently, calcium phosphate cements (CPCs)--water-based pastes of powdered calcium and a phosphate compound that form hydroxyapatite, a material found in natural bone are used for reconstructing or repairing skeletal defects, but only in bones that are not load-bearing (such as those in the face and skull). Macropores built into the CPCs make it easier for new bone cells to infuse and, eventually, solidify the implant. Until this happens, however, the macropores leave the implant brittle and susceptible to failure.

- *Physical stability*: strength needs to be high enough to resist fragmentation before the cells synthesizes their own extracellular matrix. Modulus of elasticity or stiffness needs to be high enough to resist compressive forces that would collapse the pores. In the early healing phase, whilst an inflammatory reaction is acute, the material must remain stable. It must not disintegrate or lose stability due to lysing or dissolving. Premature disintegration into micro particles provokes the activity of phagocytozing macrophages and polymorphic polynuclear cells and thus intensifies the nonspecific immune defense reaction which is detrimental to regeneration.

There are various methods of strengthening and stabilizing a bone augmentation. An altogether different technique for enhancing bone density at the region of the implant, involves effectively transferring loading stress from an implant to the surrounding bone through the use of an implant having a tapered body shape. Application of a vertical force on the tapered implant produces a sheer force component in addition to the normal force component acting on the surrounding bone.
So in conclusion the augmentation material must be hard enough to withstand forces from surrounding structures and at the same time be porous enough to allow the growth of bone in to it, this is achieved by many different methods. For example by the use of material consisting of different layers, all having different rolls in the process of bone growth. The material used must have slight elastic properties that prevent it from cracking when under stress.

- *No stimulation of immune reaction*: Another factor of great importance when selecting the bone substitution materials is that the material cannot stimulate the immune system to react to its presence. If the immune system reacts to the foreign bodies presence as something that should not be there it will reject it preventing any interaction between the material and the bone. To achieve the wanted effects and prevent the immune system from reacting different methods are used, such as autograft or autogenous bone graft. (Please refer to 'Classification of bone grafting materials based on source' on pg 10).

- *No pathological concentration of chemical substances*

In the <u>reparative phase</u> of bone healing, the substitute materials must have the following properties:

- *Simultaneous resorption for the purpose of bone regeneration*: excessively rapid degradation rate does not allow the proper regenerative processes to occur. If too slow however, degradation rate interferes with remodelling.

- *No pathological pH values*

-*Physiological supplementation with growth factor* (Please refer back to´osteoinduction´on pg 8)

- *Osteoinductivity* (Please refer back to 'osteoinduction' on pg 8)

In the biological remodelling phase the bone substitute materials must have the following properties:

- *Complete resorption of the material:* An ideal bone augmentation material adapts its resorption rate to the surrounding conditions. The time required for final resorption is also the crucial quality criterion of the bone augmentation material. It should fulfil its space holder function only until the bone can regenerate itself, so that it can react to changing biomechanical loads freely and undisturbed by remodelling.

- *No disturbing influence on biological remodelling*

2.4 Indications and usage of bone substitute materials in dentistry:

. Augmentation or reconstruction of the alveolar ridge
. Elevation of the maxillary sinus floor (sinus lift)
. Filling of extraction sockets to enhance preservation of the alveolar ridge: Extraction of teeth may result in 40% to 60% alveolar bone loss in a period of two to three years [132].
. Filling of periodontal defects
. Filling of peri-implant defects
. Filling of defects after root resection, apicectomy and cystectomy

2.5 Experimental studies using augmentation materials:

2.5.1 Study of statistical data of augmentation materials- Experiment 1

Aim

To determine the most common indicators for using a particular type of augmentation material and to obtain some statistical data concerning 3 different bone substitute materials as well as providing radiographic images of some patients treated by augmentation materials which is useful for clinical practice.

Materials and Methods

This study is based on the statistical data of different augmentation materials as used when indicated in patients of the Maxillofacial Surgery Department, Faculty

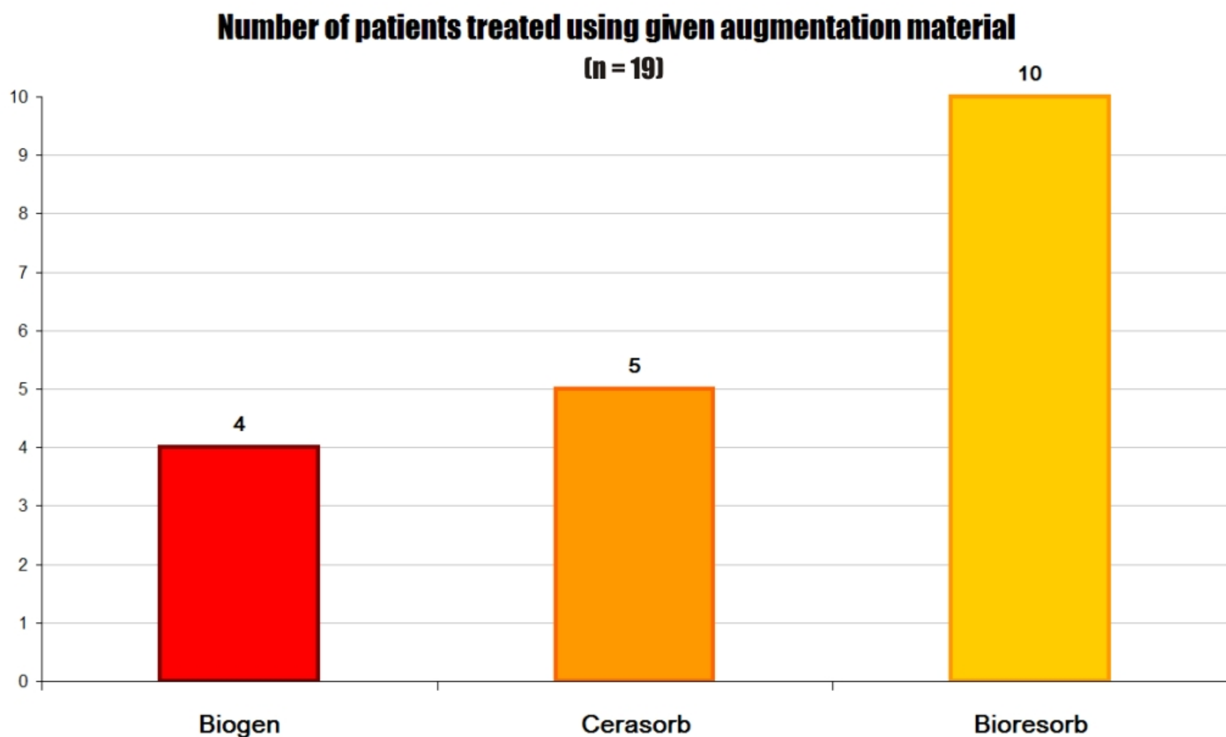
Hospital in Pilsen, during the period of 2003-2007.

During this period we treated 19 patients with 3 different augmentation materials; Biogen, Bioresorb and Cerasorb.

It is worth mentioning that 1 patient could have been treated by different augmentation materials or by one augmentation material in different regions of the jaw. In such an instance we record the result as a case rather than an individual patient.

We compiled the data obtained, into a form that one can easily interpret, and the results can be seen below as graphs and pie charts. We focused on the number of patients being treated with any given augmentation material, the indications for use of augmentation materials, as well as whether the sex of the patient was a determining factor.

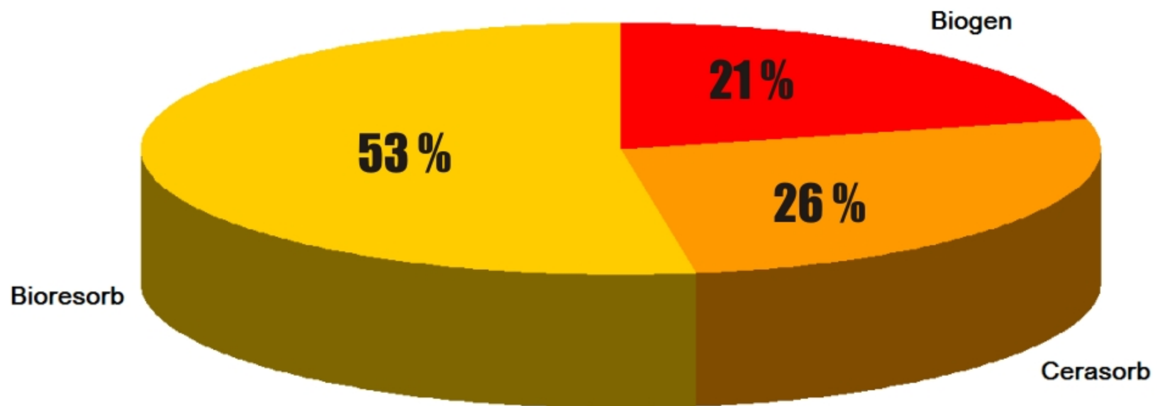
Results



Graph 1

Ratio of patients treated using chosen augmentation material

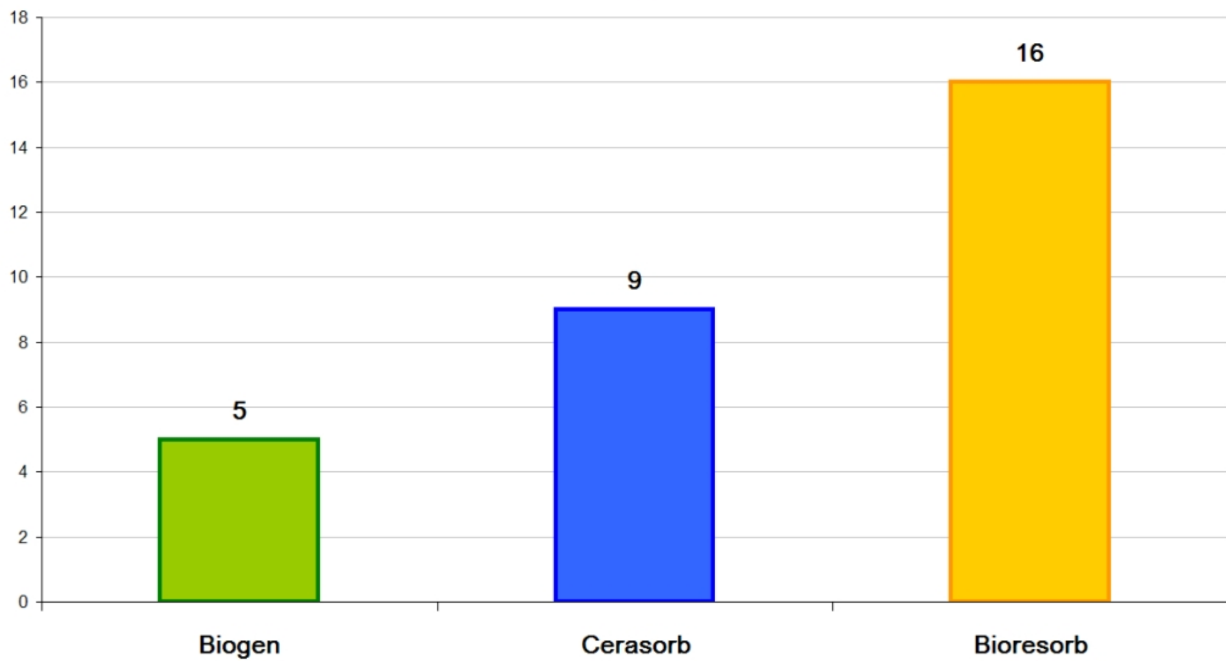
(n = 19)



Graph 2

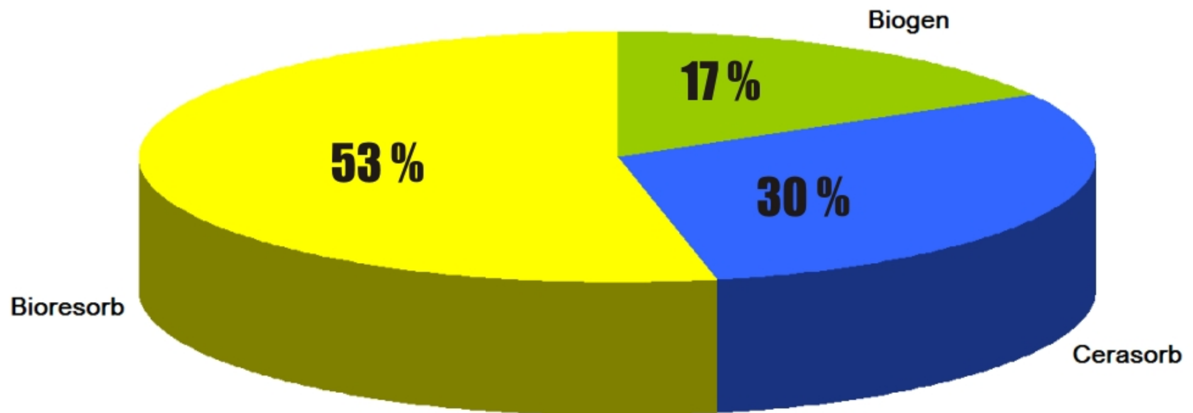
Number of cases treated with chosen augmentation material

(n = 30)



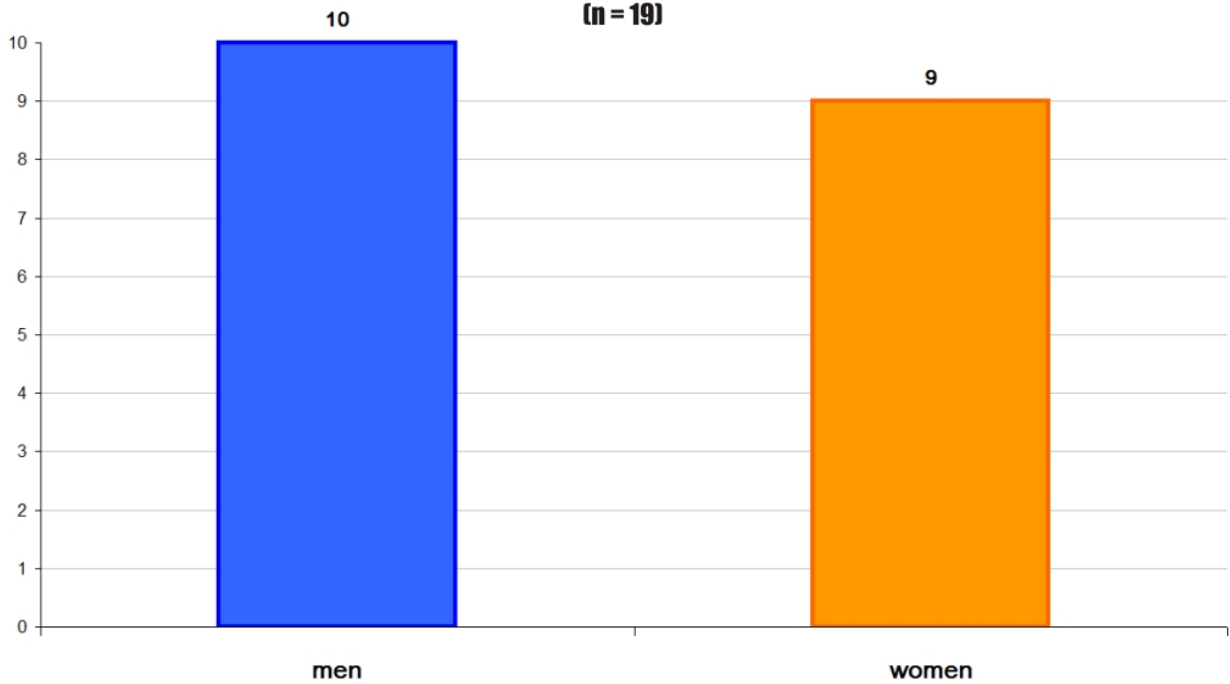
Graph 3

Ratio of cases treated using different kinds of augmentation materials
(n = 30)

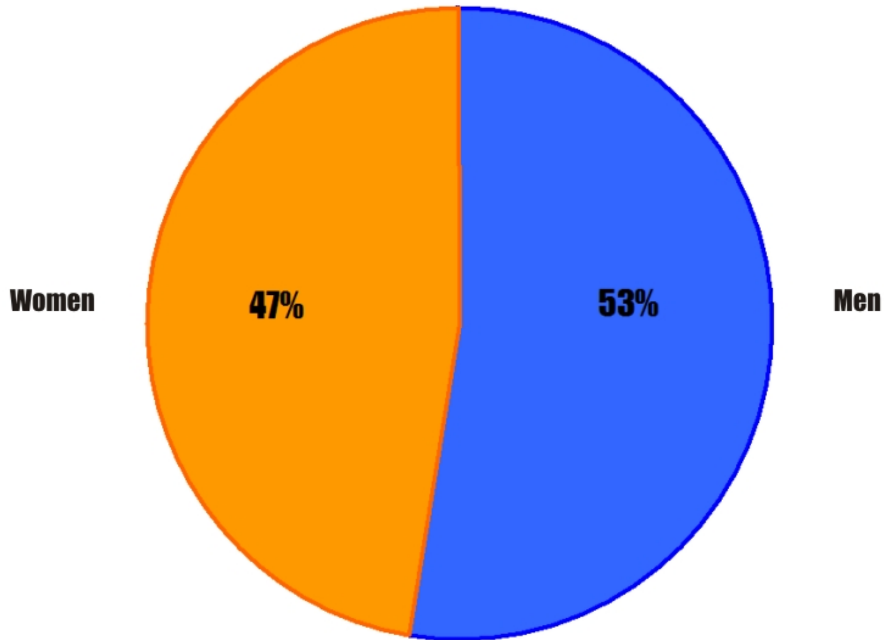


Graph 4

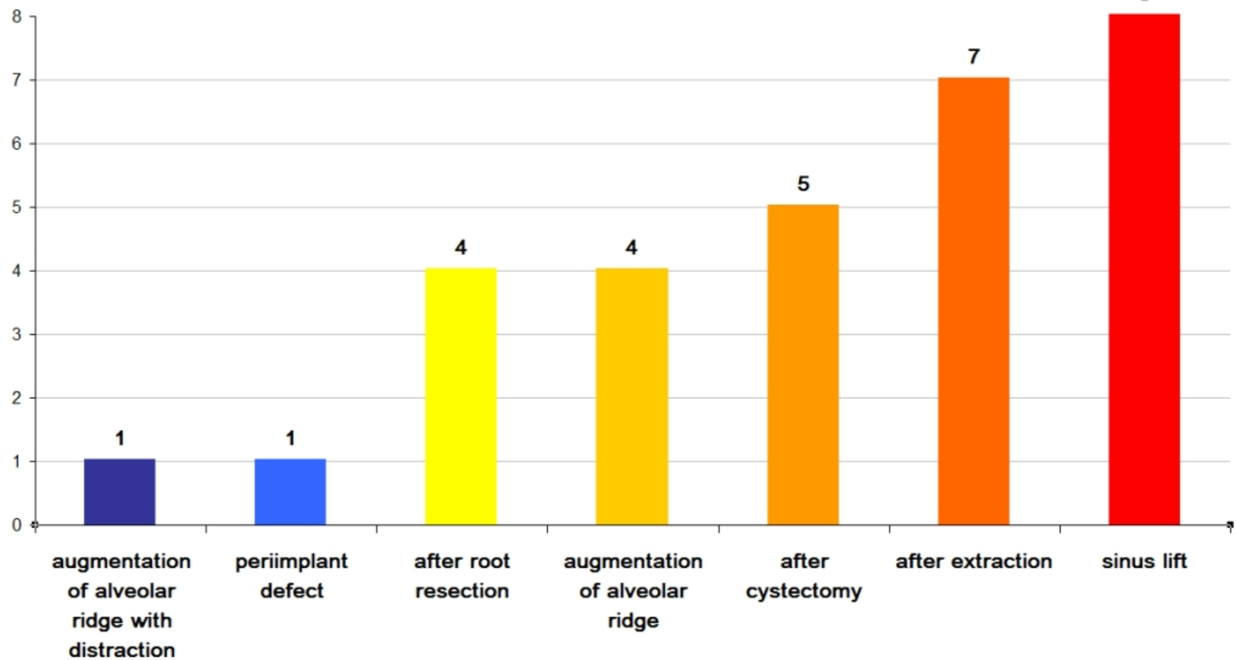
Number of women and men treated using augmentation materials
(n = 19)



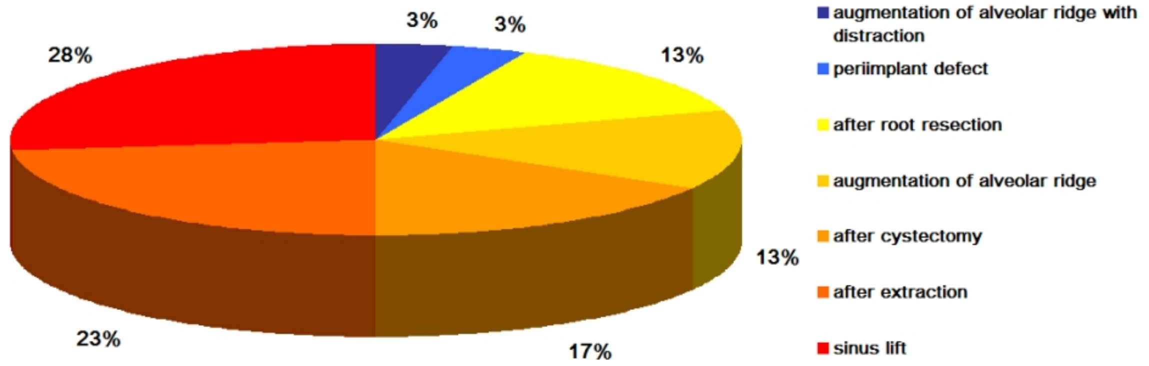
Graph 5



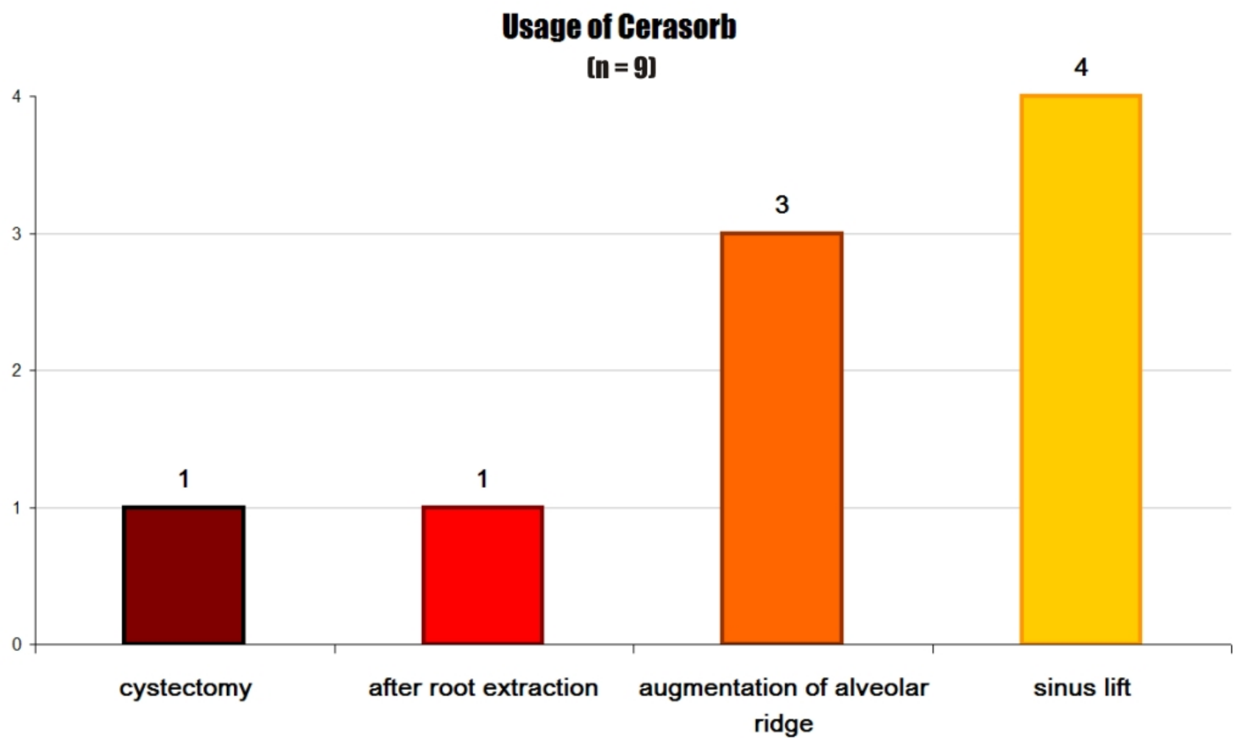
Graph 6



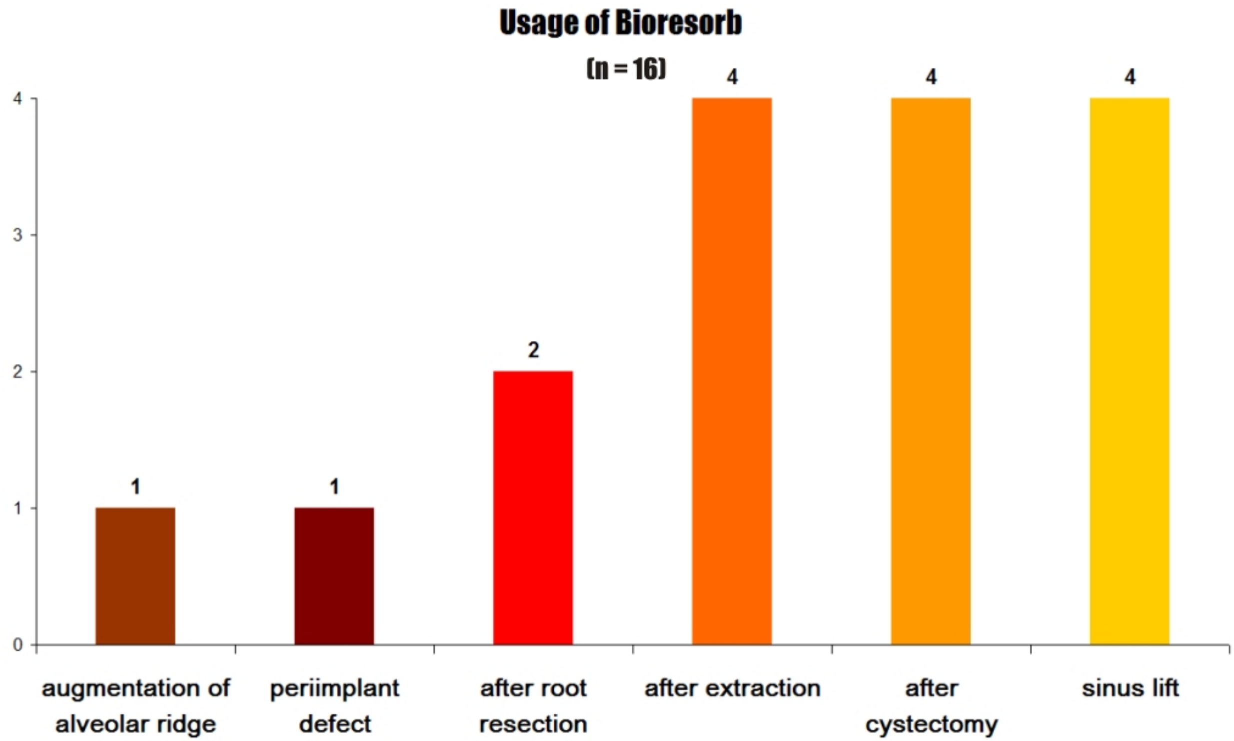
Graph 7



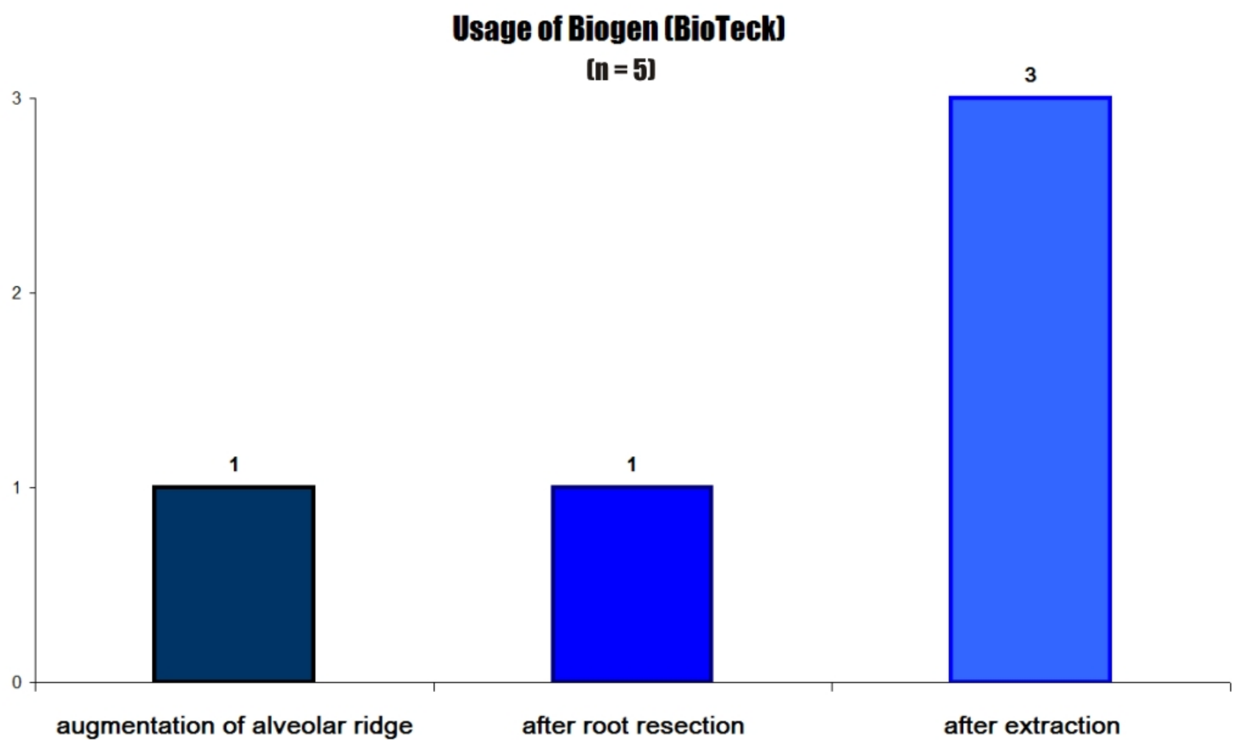
Graph 8



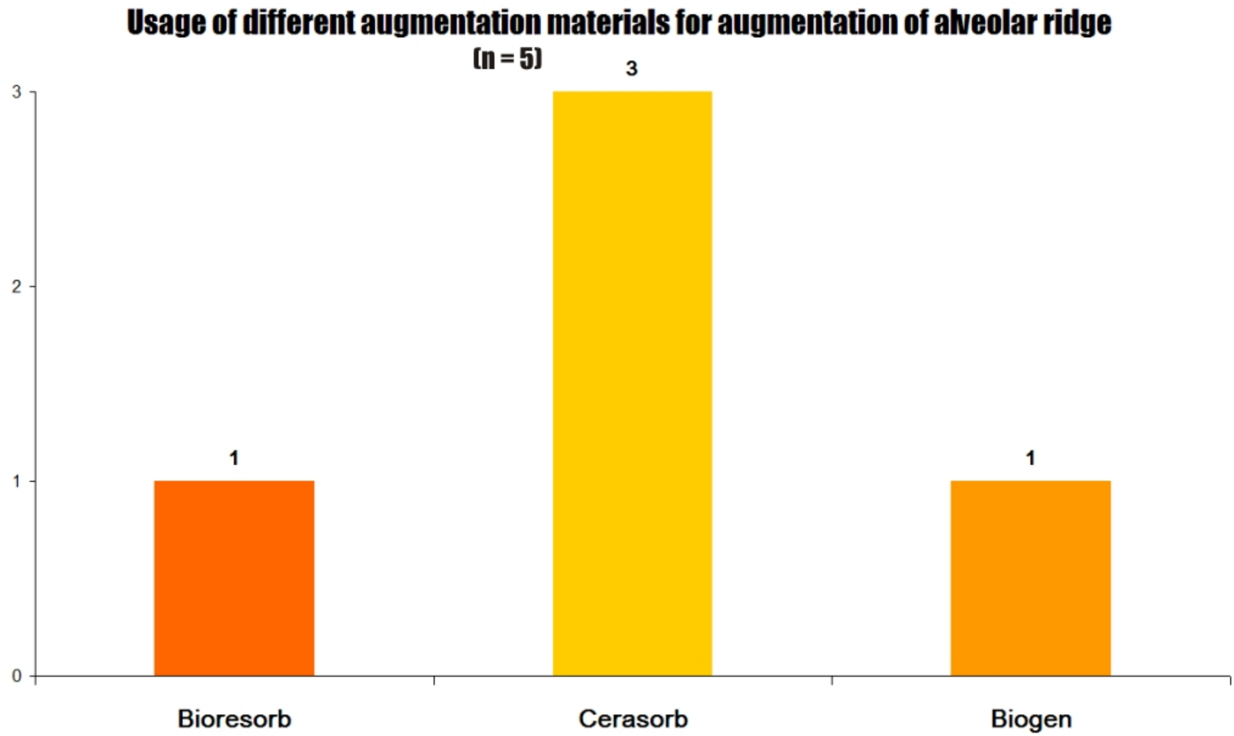
Graph 9



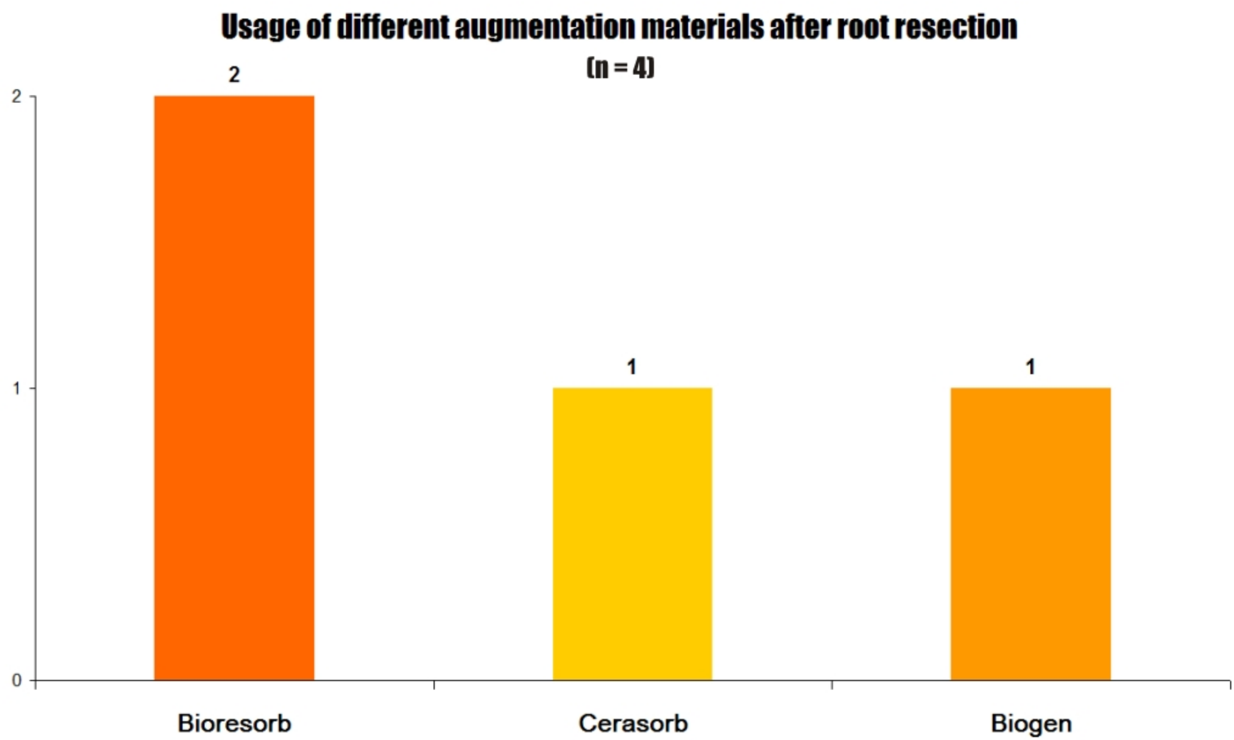
Graph 10



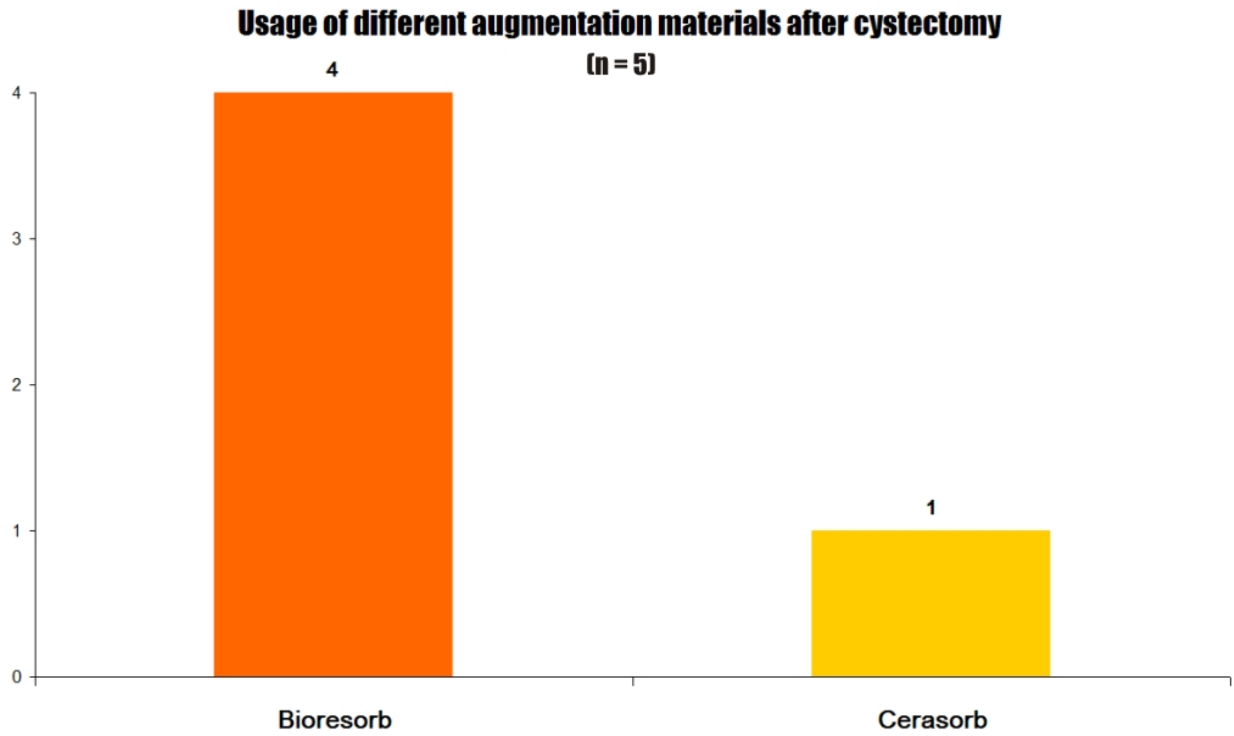
Graph 11



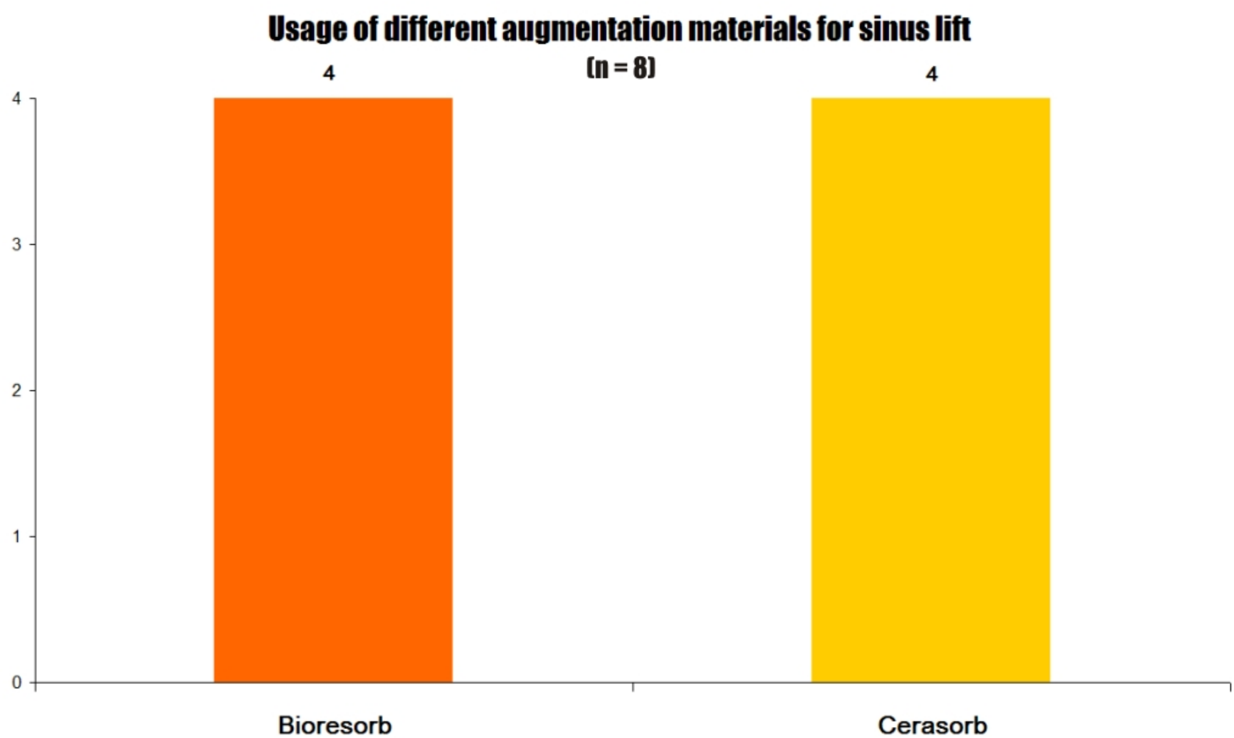
Graph 12



Graph 13



Graph 14



Graph 15

2.5.2 Radiographic images of 4 patients treated with different augmentation materials:

Patient 1

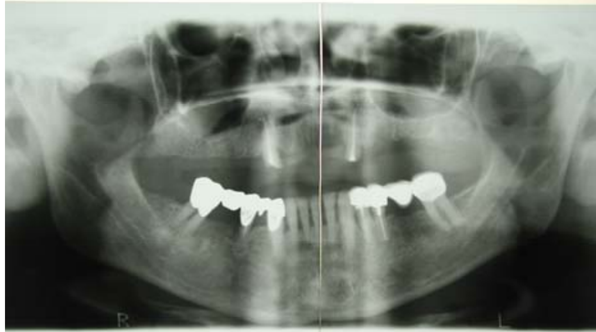
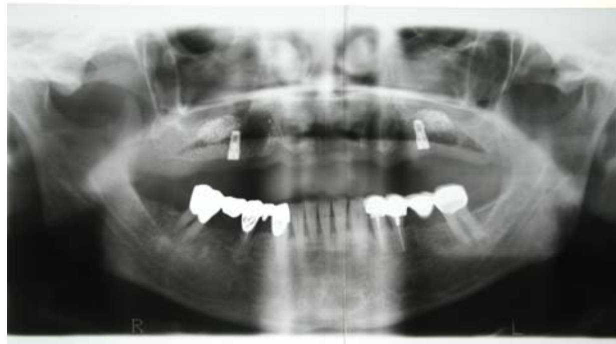
	

Fig 1. Panoramic radiograph taken before bilateral sinus lift operation.	**Fig 2. Panoramic radiograph taken after sinus lift using Cerasorb, and insertion of two implants at (26, 15).**

Patient 2 [201]

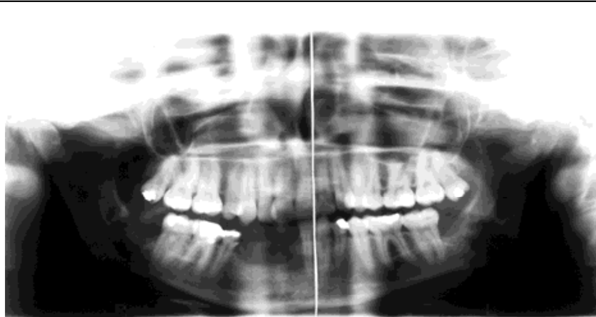
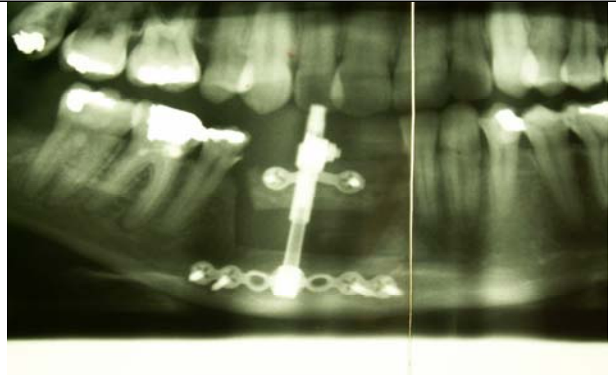
	

Fig 3. Atrophy of alveolar ridge (44- 31) after marginal resection of mandible due to osteosarcoma.	**Fig 4. Augmentation of alveolar ridge, using Distractor and Biogen**

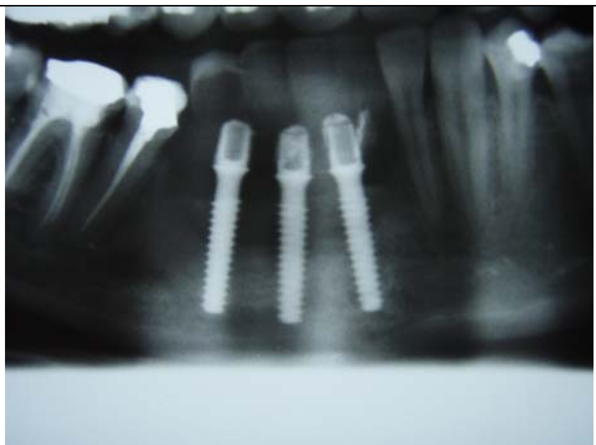
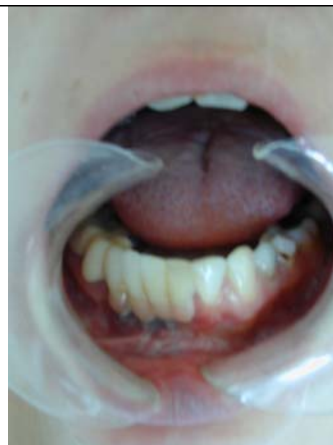
	

Fig 5. Inserted 3 nanoimplants. 2 years after augmentation.	**Fig 6. Clinical photo documentation. 2 years after augmentation.**

Patient 3

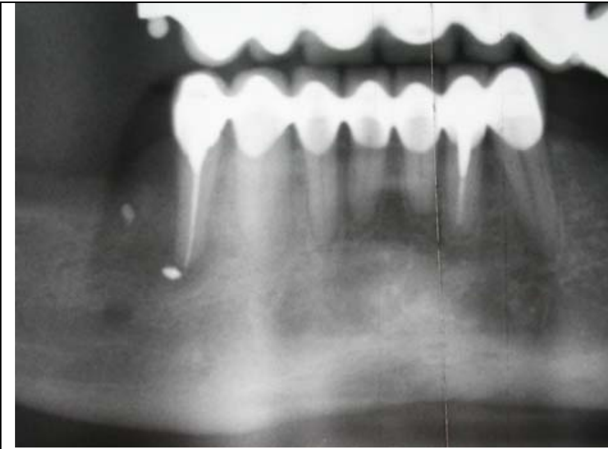


Fig 7. Panoramic radiograph taken before root resection of 44.

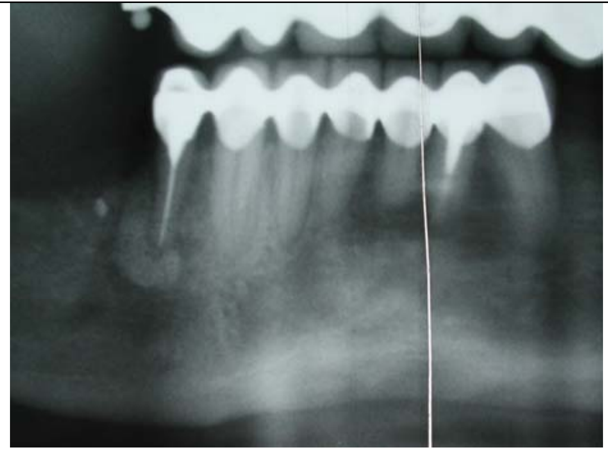


Fig 8. Panoramic radiograph after root resection of 44 and with defect filled with Cerasorb.

Patient 4

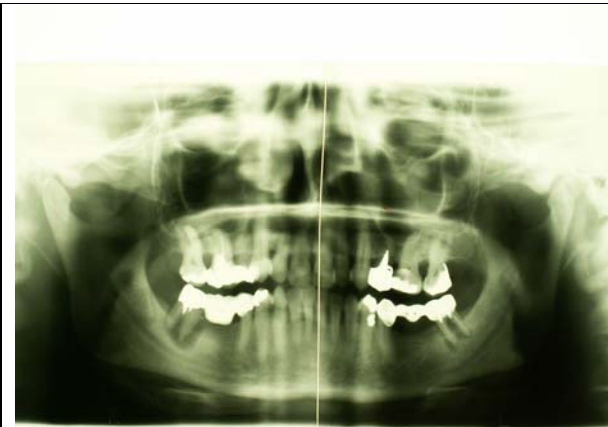


Fig 9. Teeth 37, 47 indicated for extraction due to periapical inflammation.

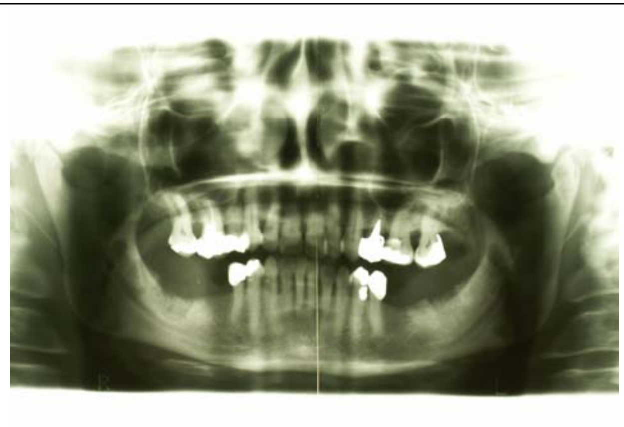


Fig 10. Extraction sockets filled by biogen.

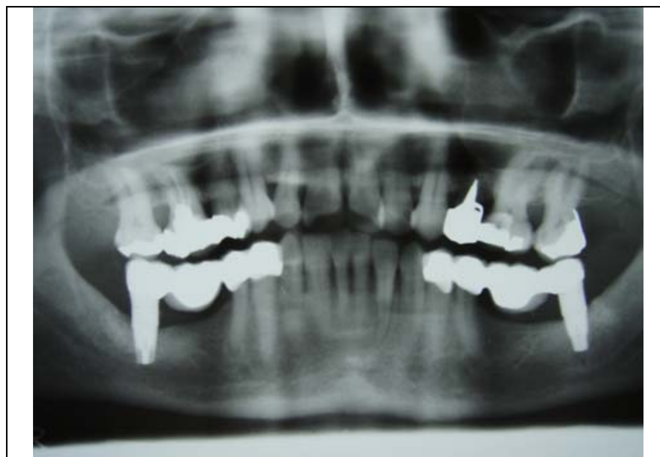


Fig 11. Two years after augmentation.

Discussion and Conclusion

The choice of augmentation materials was dependant on several factors, of principal importance was the experience of the operator and the economic situation of the patient.

It is important to note that the range of our results is limited. This is due to the very recent introduction of these materials in the hospital, as well as the high expense of the augmentation materials, which were not covered by the patients insurance. The range of results would be significantly higher in a private practice.

Based on our results, the use of augmentation materials were slightly more common in treatments carried out on males than in females.

Bioresorb was generally the most commonly used augmentation material.

The principal indicators for use of augmentation materials were concluded to be primarily sinus lifts followed closely by their use in post-extraction cases. It is also indicated in other minor surgical procedures, such as following root resections and cystectomy.

It was established that in cases of sinus lift, Bioresorb and Cerasorb were most commonly used. Bioresorb was also the principal material used after root resection and cystectomy, whereas Cerasorb was preferred in alveolar ridge augmentation. Indications for use of Biogen were in post extraction cases.

2.5.3 Experimental study in pigs with the purpose of evaluating the possible osteogenic activity of bone augmentation materials [198]- Experiment 2

All our experiments that were carried out on living specimens were approved by the Animal Ethics Committee of the Faculty of Medicine in Pilsen. All funding towards the experiments were provided by the author of this study, and the supplier of the materials used in the experiments.

Introduction

Experiments for development of new procedures for bone regeneration are a topic of modern research programs. Some scientists have demonstrated that demineralized bone matrix could increase the ectopic formation of cartilage and bone [133]. The active component of the matrix was identified as a growth factor protein that induces ectopic bone formation and it known as a bone morphogenetic protein (BMP) [134]. Recombinant human bone morphogenetic proteins (rhBMPs) can be used for formation of cartilage and bone when implanted in muscle with insoluble bone matrix by inducing muscle cells to differentiate into chondrocytes and osteoblasts [135].

Fluorescent labelling: The tetracyclines are a group of antibiotics which chelate with the calcium ions in bones and teeth [136].The uptake in vivo of antibiotic drugs of the tetracycline series at sites of bone mineral deposition provides a way of demonstrating regions of active bone formation and mineralisation [137]. The drug rapidly localizes at these sites so that in sections it appears as a bright fluorescent line. Milch et al. (1957, 1958) showed that tetracycline-induced fluorescence in

soft tissues disappeared within a matter of hours, but persisted in long and flat bones for long periods of time [136, 137]. They noted that deposition of tetracycline was limited to areas of new bone growth. Almost instantaneously after intravenous administration, and within 30 min of intraperitoneal injection, yellow-gold fluorescence was induced by ultraviolet light in soft tissues, with the exception of the brain. This disappeared from soft tissues within 6 h, but persisted in bone. Subsequent experimental evidence revealed that tetracycline is deposited where bone or cartilage matrix is mineralizing and its pattern is the same as that of radiolabelled calcium deposition [138, 139].

Aim

To evaluate in vivo, the possible osteoinduction potential and osteogenic activity of two different bone augmentation materials, Cerasorb (used since 1981 [140]) and BIO-GEN and if ectopic bone formation could be induced when implanted subcutaneously to the extremities of the pigs. We also aimed to label the regions of active bone formation and mineralization by means of fluorescent labelling by tetracycline, and then analysing the results with a laser confocal microscope.

Materials and methods

Animals: Two piglets were used in this study. Cerasorb and Biogen augmentation materials were applied at the ulnar region between muscular and cutaneus tissue. The animals were fed with commercial pig granules and had access to water *ad libitum*.

General anesthesia: The experiment was performed under general anesthesia. 24 hours before surgery the piglets were given only water. 30 minutes before surgery Azaperon (buthyrephenon) 6-10 ml was applied as a premedication. The general anaesthesia was initiated by application of Atropin (0.5mg) and Diprivan (propopholum 2%), 5-8ml intravenously according to the weight of animals. Antibiotic prophilaxy of Unasyn 1.5 g i.v. applied after the initiation of general anaesthesia.

Orotracheal intubation was performed. Buprenorphin (1-2ml) was applied as an analgesic solution. There was no need of application of myorelaxant. Anaesthesia was prolonged by pumping 5-15ml/h Diprivan (propopholum 2%) according to the depth of general anaesthesia.

During the operation 10ml/kg/h Plasma Lyte (Hartmann - Ringer's solution) was infused.

Ventilation: volume controlled 10 – 15 ml/kg, with the frequency of 30 – 40/min, pressure PEEP 4.

During the anaesthesia the following parameters were monitored by the anaesthesiologist:

SpO2 (oxygen saturation accuracy range) 95 – 99%

ET CO_2 (end tidal carbon dioxide) 4.5 – 5.0 kPa
Heart beat frequency 80 – 110/min
Anaesthesia was performed by an experienced anaesthesiologist from the I. internal medicine department of the Faculty Hospital in Pilsen.

Surgical procedure: Surgery was performed using aseptic techniques. An incision was made through the skin, subcutaneous tissue and extremity muscles were exposed.
Cerasorb microporous granules (Ø 500-1000 µm) and Biogen granules mix (Ø 0.5-1 mm) were applied to the subcutaneous tissue. The fascia and subcutaneous tissue was sutured by resorbable suture material and finally the skin was closed by Monofil suture materials (Fig.12.).

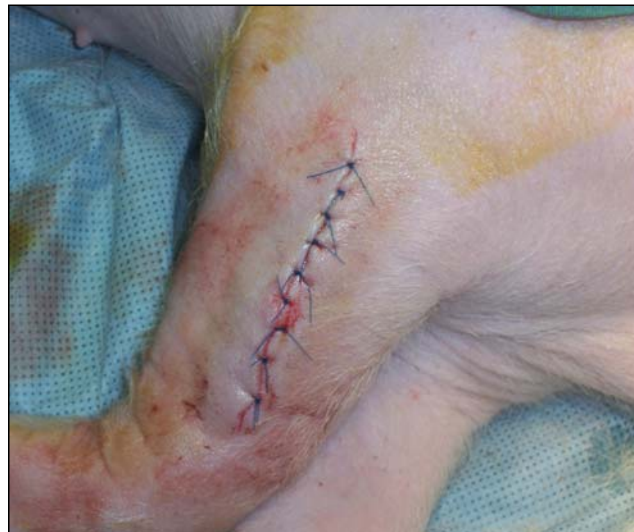


Fig 12. Representative photograph showing sutured incision line after subcutaneous application of augmentation material.

We performed application of antibiotic Tetracycline (20mg/kg, 100mg/1ml) 6.3ml i.m. at the weight of 30kg, 16 days after operation time.

Sacrificing of animal: After 52 days, the pigs were terminated by application of 6ml Stresnil (azaperonum), and 7ml T 61 (embutramidum, mebezonium iodatum, tetracainii hydrochloridum).
The sites of application of the augmentation materials were observed. At the site of application of Biogen there was no evidence of formation of any hard tissue and all the material was completely absorbed (Fig.13). At the site of application of Cerasorb, hard tissue materials were found in some places (Fig.14-15). The observed hard tissue was separated and immersed in 10% paraformaldehyde for 2 weeks.

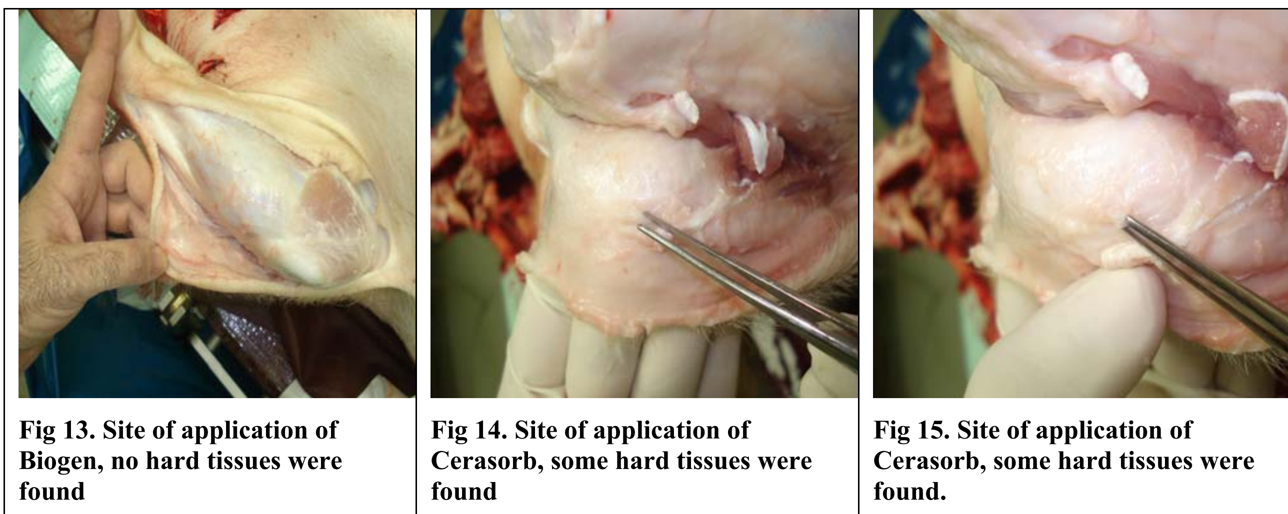


Fig 13. Site of application of Biogen, no hard tissues were found	Fig 14. Site of application of Cerasorb, some hard tissues were found	Fig 15. Site of application of Cerasorb, some hard tissues were found.

Histological processing: The specimens were sectioned and stained with hematoxylin-eosin and Verhoeff's hematoxylin with green trichrome. The histological sections were evaluated with the objective of verifying if it is possible to observe the newly formed bone in this tissue. An optical microscope (Olympus CX41) was used.

Results and discussion

At the site of the application of Cerasorb, hard tissue material was observed using the optical microscope. Three foci of accumulated multinucleate Langerhans giant cells were found in the dermis 0.5-2.2mm below the epidermis. These epitheloid macrophages surrounded the small Cerasorb residual granules and indicated a granulomatous condition. The diameter of the residual particles ranged between 7-15µm. Some of these corpuscles were phagocyted by Langerhans cells (fig.16-18). In another part of dermis, we found another three foci formed by incompletely mineralized osteoid material. The surface of these trabecular foci were covered by connective tissue cells rather than active osteoblasts (fig.19-20). During the sectioning of the specimens some part of the tissues were lost due to differences in the consistency of tissues around the dermis. In the remaining preserved tissue, chondroid tissue mass with chondrocytes or chondroblast cells were found (fig.21-22).

Summary of histological findings:

1. Tissue reaction similar to chronic granulomatosis from foreign material with partially encapsulated connective tissue.
2. Layers of chondroosteoid tissue.

For analysing the histological findings, the specimens were sectioned and stained with Toluidine blue stain and a mix of alizarin red (stains calcified tissue) and toluidine blue stains (Fig. 23-26). We also tried to stain the two above mentioned augmentation materials with Toluidine blue. Cerasorb was not stained at all and BIOGEN was stained sporadically.

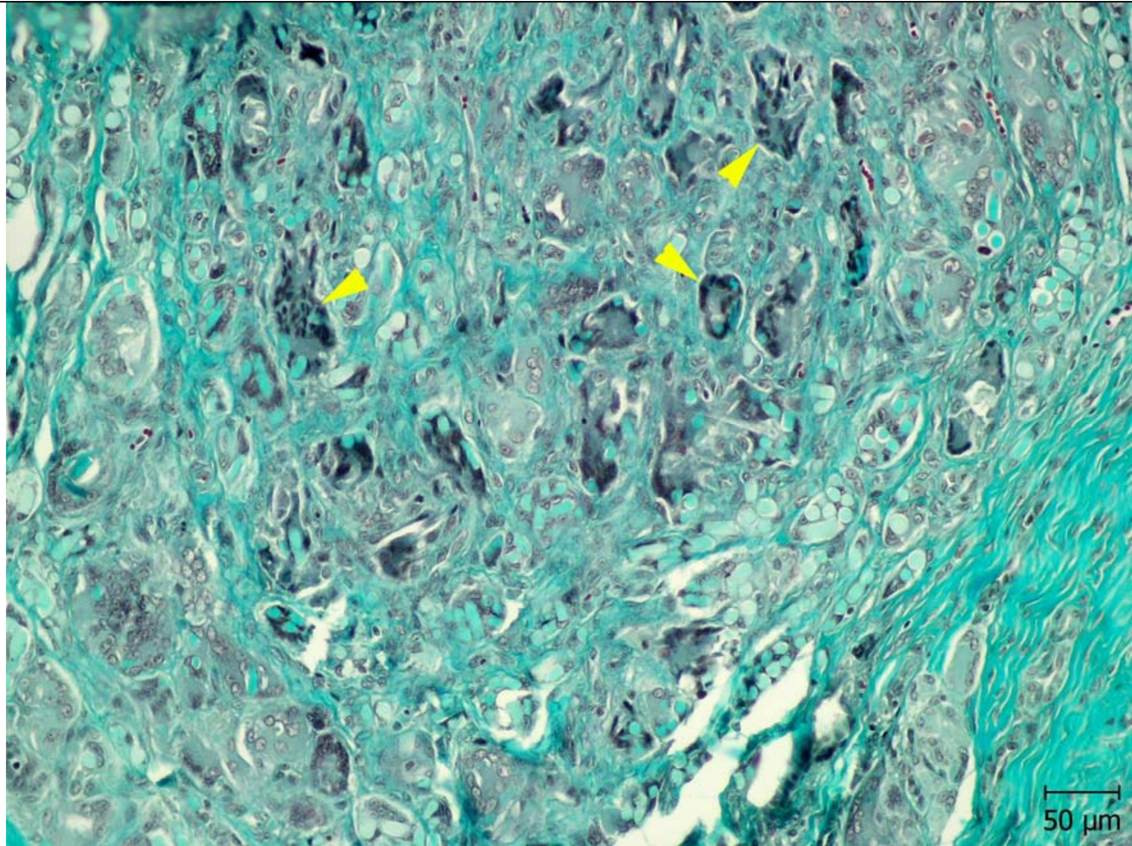


Fig 16. The presence of multinucleated Langerhans giant cells formed by fusion of epitheloid macrophages (indicated by arrowheads) surrounding the cerasorb particles indicates a granulomatous condition. Verhoeff's hematoxylin and green trichrome stain.

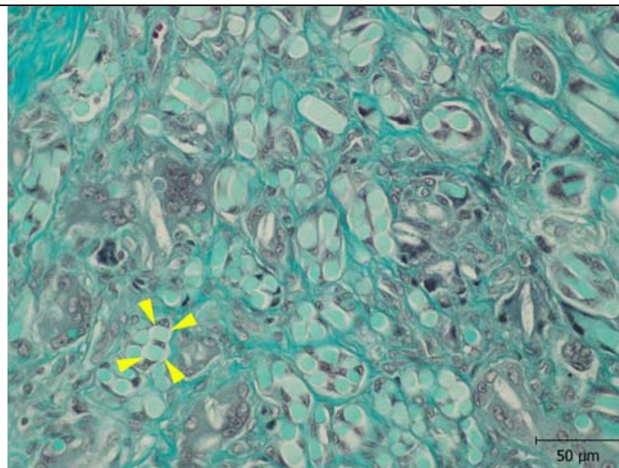


Fig 17. Cerasorb residual particles (two of them indicated by arrowheads) dispersed within the collagenous connective tissue. Verhoeff's hematoxylin and green trichrome stain.

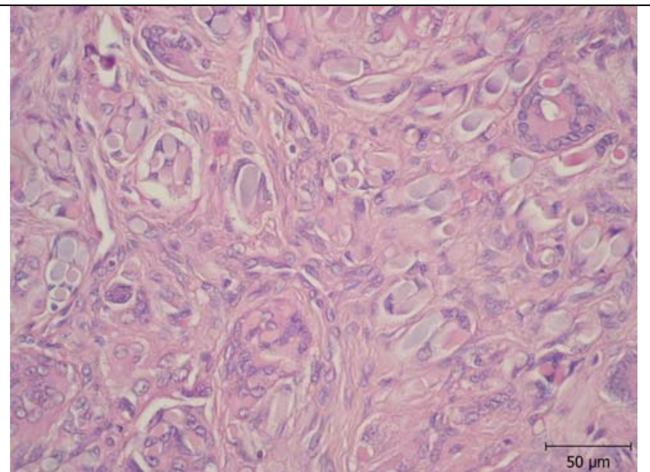


Fig 18. The same as in Fig 17, H.E stain.

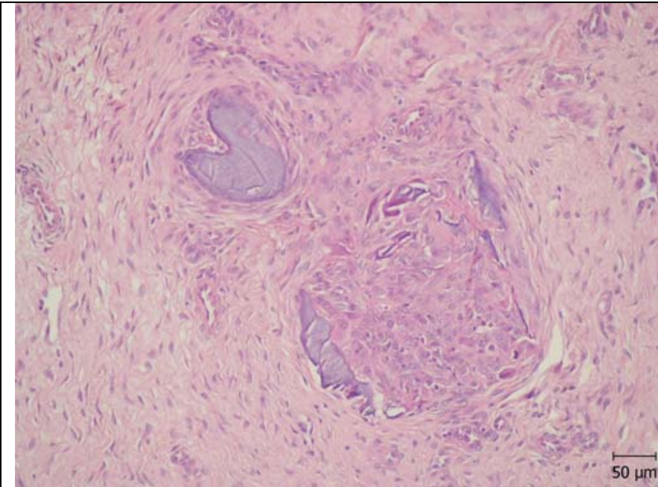


Fig 19. Incompletely mineralized osteoid tissue in the dermis. H.E stain.

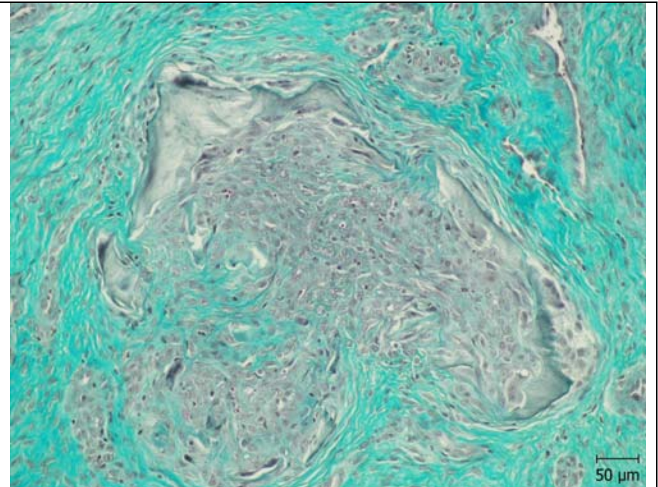


Fig 20. Incompletely mineralized osteoid stained with Verhoeff's hematoxylin and green trichrome.

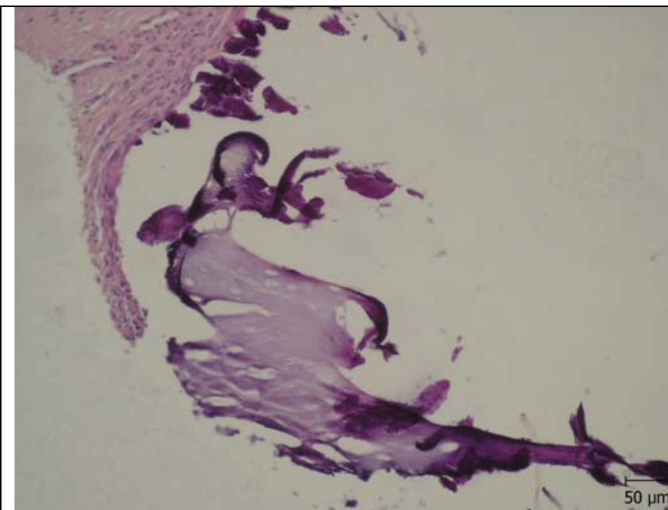


Fig 21. Remnants of chondroid tissue (most of the material was lost during the histological processing), H.E stain.

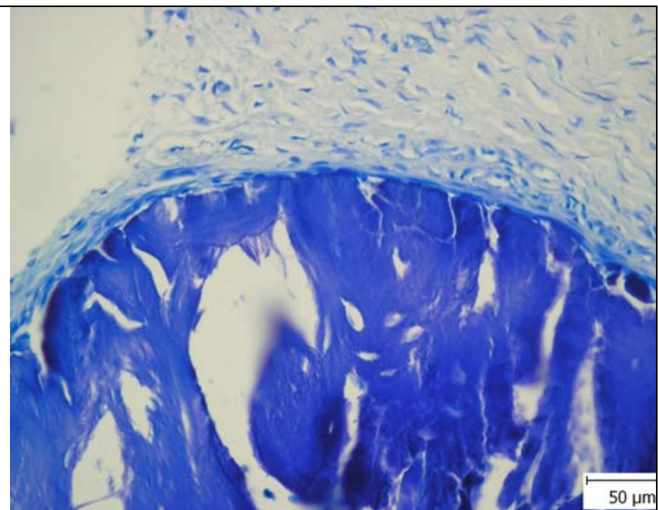


Fig 22. Remnants of chondroid tissue stained with Toluidine blue.

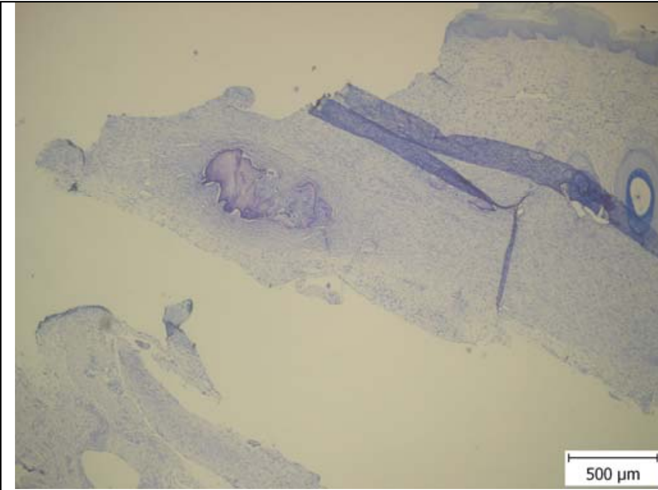


Fig 23. Partially calcified tissue in the dermis was located approximately 1.2-1.44 mm below the epidermis. Alizarin red and toluidine blue stain.

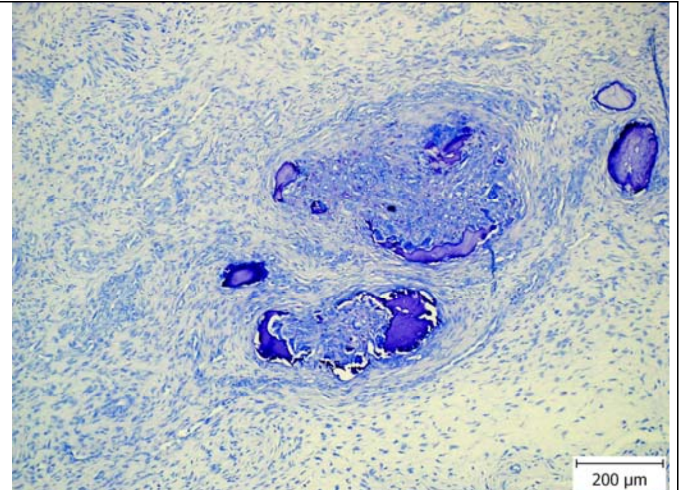


Fig 24. Most of the trabecular tissue was not stained with Alizarin red, and therefore can be considered as not calcified. Alizarin red and toluidine blue stain.

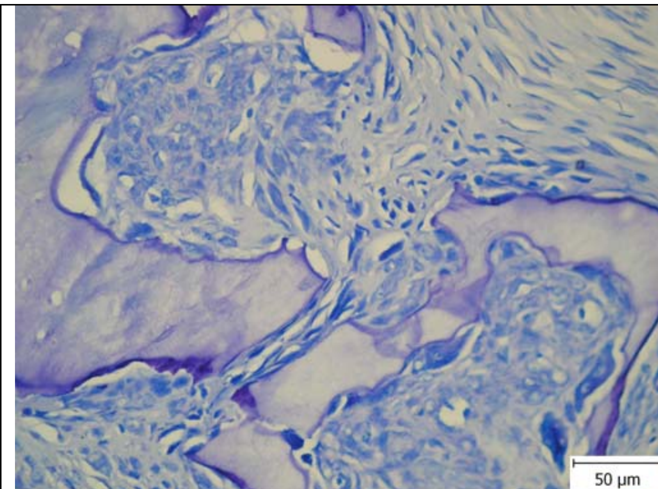


Fig 25. The trabecules were stained with toluidine blue, but not with alizarin red, i.e they were not calcified. Alizarin red and toluidine blue stain.

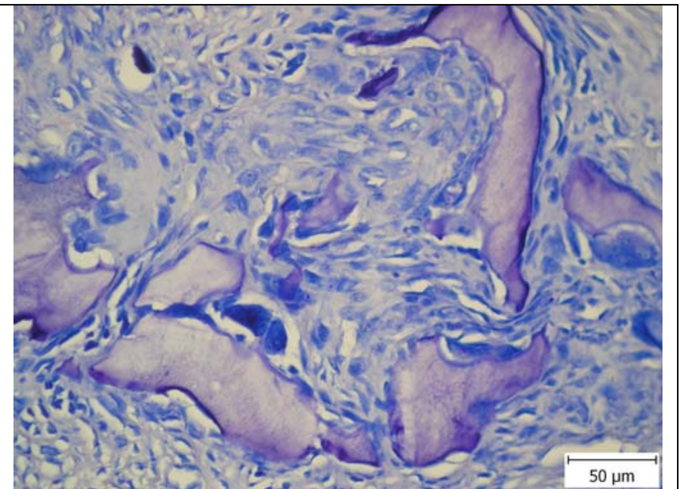


Fig 26. The surface of the trabecules was partially covered by connective tissue cells (see also fig. 25). Alizarin red and toluidine blue stain.

Photomicrographs from confocal laser microscope of tetracycline labelled tissue (fig. 27-28).

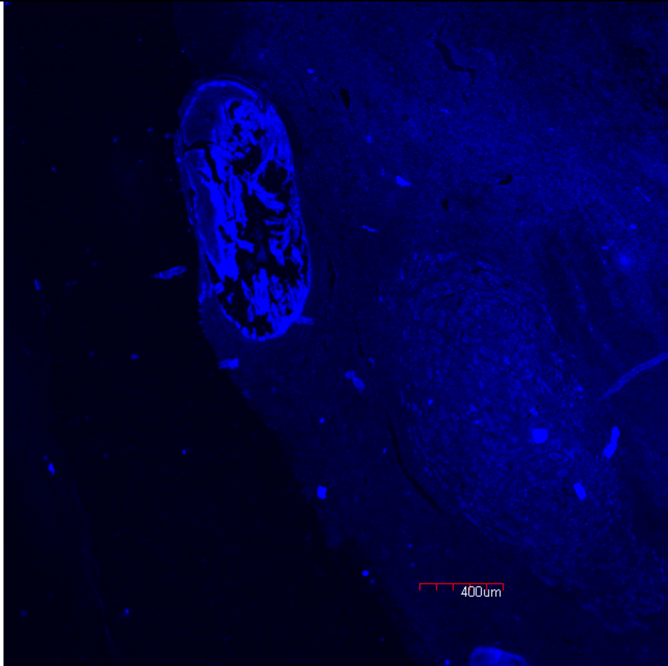


Fig 27 a. Area of mineralization with formation of hard tissue mass observed as a tetracycline labelled bright blue structure.

Fig 27 b. Contrast lighting of the same hard tissue mass.

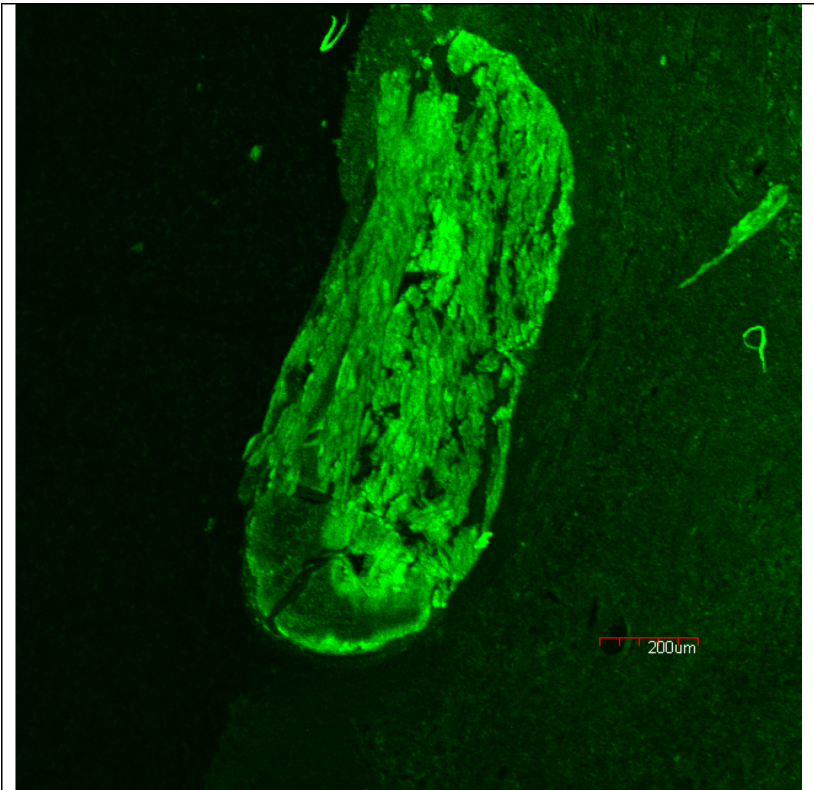


Figure 28. Again we can see the successful labelling of newly formed chondroosteoid structure with tetracycline, this time under a green filter.

Conclusion

Based on our results, it is possible to use this sequential methodology in soft tissues, with the objective to verify the osteoinduction potential of different bone augmentation materials. With respect to this original method, we can analyse different augmentation materials to establish the most successful one with the best osteinductive properties.

We also established that our results from the labelling of the tissue by tetracycline exhibited the same results as that shown in previous publication by Mitch et al [136, 137].

These results highlight the potential future use of augmentation materials in the creation of hard tissues, in areas where soft tissues are normally present. This would be invaluable, for example, in the clinical setting of cosmetic surgery or maxillofacial surgery where bone reconstruction must take place following oncological or traumatic destruction of bone.

2.5.4 Further experimental study in pigs to evaluate the ability of Cerasorb as a bone augmentation material for healing of bone defects -Experiment 3

Aim

To evaluate efficiency of Cerasorb bone augmentation material in bone healing when applied into the artificial hole made in the extremities of the pigs.

Materials and Methods

Simultaneously as we carried out experiement 5, we used the agumentation material Cerasorb in 2 of the pigs (Pigs 3 and 4) in order to evaluate its efficiency in bone healing. The method of this experiment can be seen below with further details in the protocol of experiment 5.

The experiment was performed under general anaesthesia, using aseptic techniques. Two artificial holes were made at the tibia of two pigs. In each tibia one hole was filled by microporous granules (Ø 500-1000 µm) of Cerasorb, and the second hole was kept empty as a control hole for further comparison. Two holes are covered by Biocollagen membrane to prevent outside influence. See figures 29-32 and 35-37

After 52 days the pigs were terminated and radiograms were obtained from the sites of the artificial holes (See figures 33-34 and 38-40).

The specimens were observed using an optical microscope after histological processing.

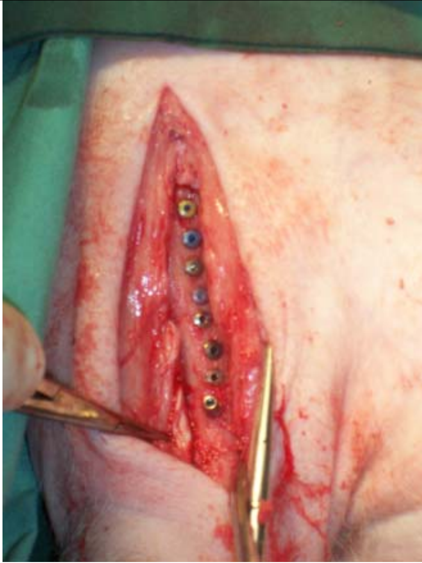


Fig 29. Proximal hole filled by Cerasorb.

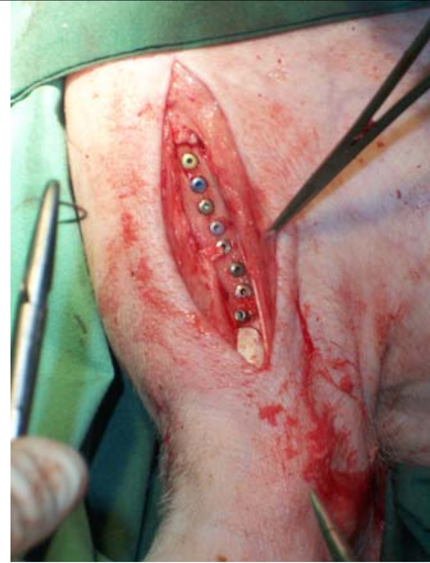


Fig 30. Two holes are covered by Biocollagen membrane to prevent outside influence.

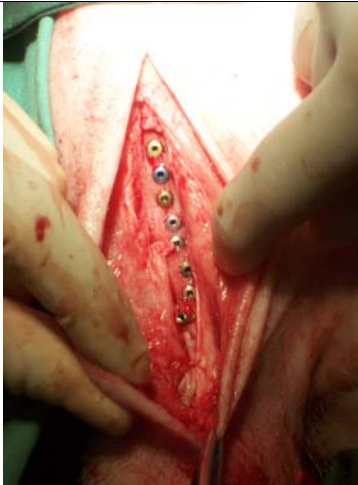


Fig 31. Periosteum sutured by resorbable suture material.

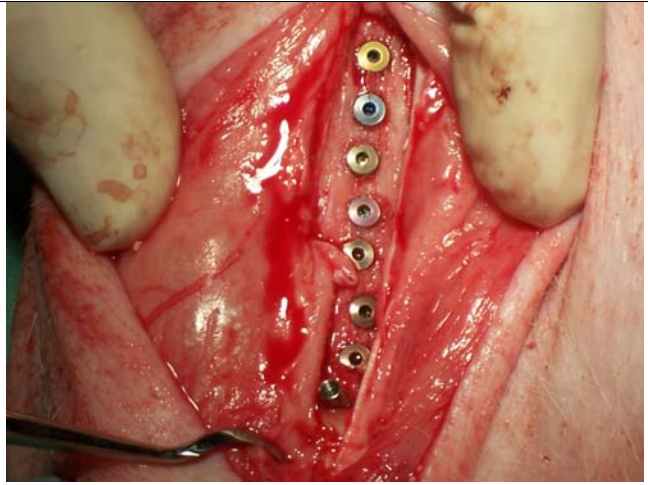


Fig 32. Sutured periosteum over Biocollagen membranes and holes.

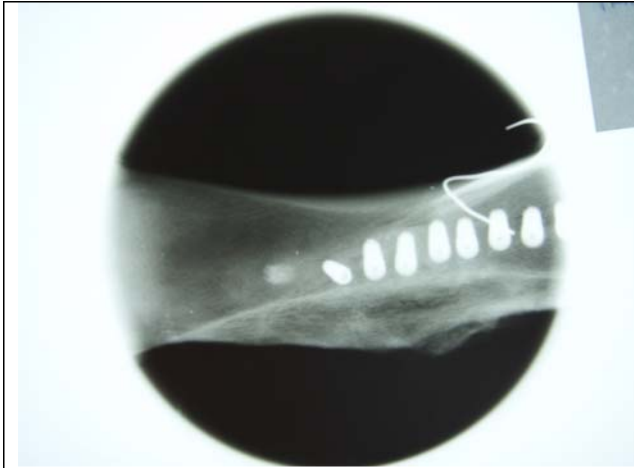


Fig 33. Radiogram of pig tibia, 52 days after operation.

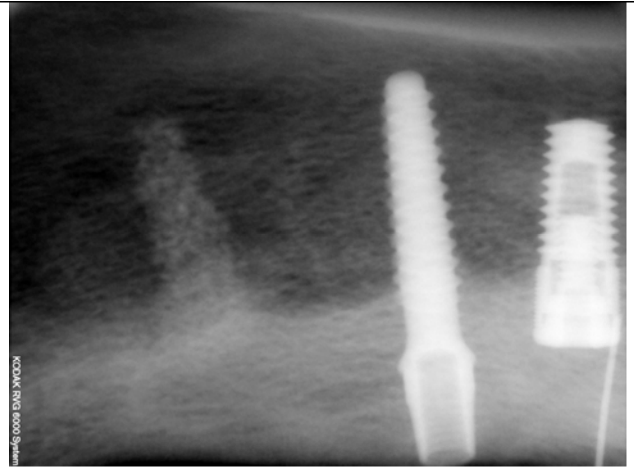


Fig 34. Radiogram shows artifical hole filled by Cerasorb, nanoimplant and implant F.

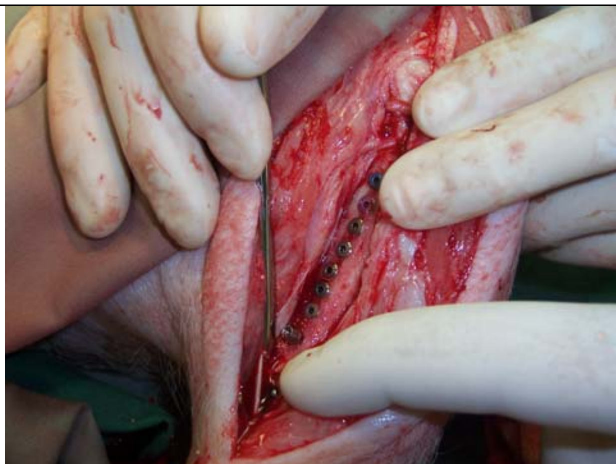


Fig 35. Application of Cerasorb into the artifical hole made in the distal part of the tibia of pig 4.

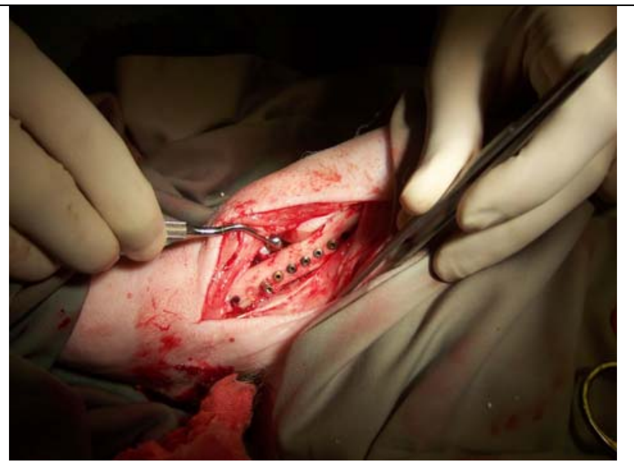


Fig 36. Proximal hole filled by Cerasorb, distal hole kept empty as a control hole.

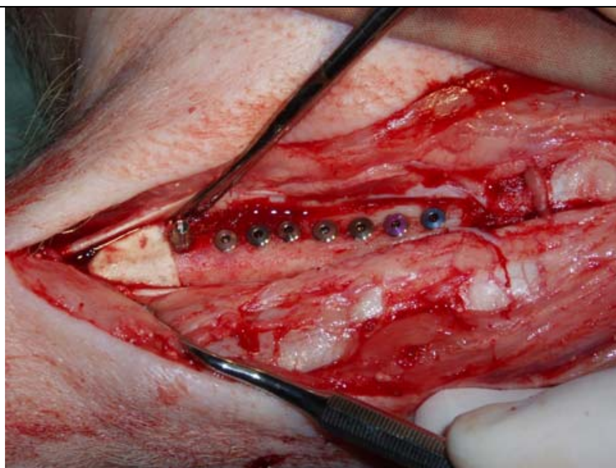


Fig 37. Two holes are covered by Biocollagen membrane.

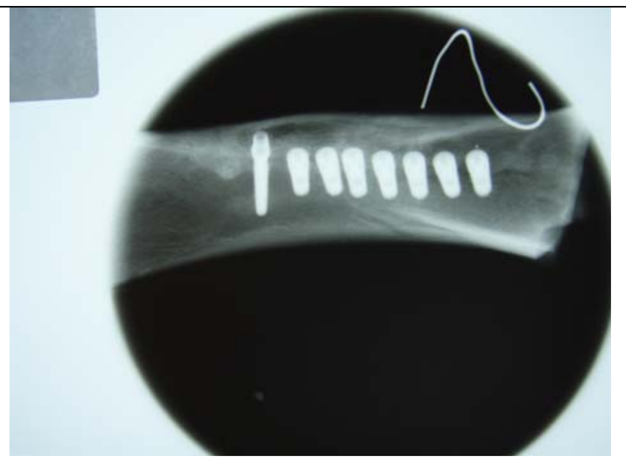


Fig 38. Radiogram of pig tibia, 52 days after operation.

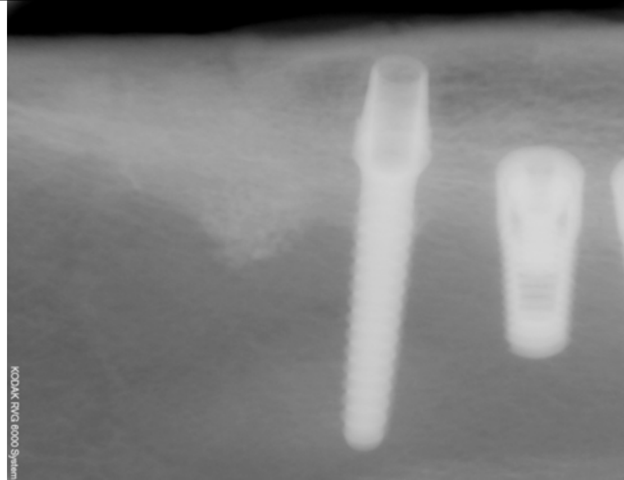


Fig 39. Radiogram shows artifical hole filled by Cerasorb, nanoimplant and implant F.

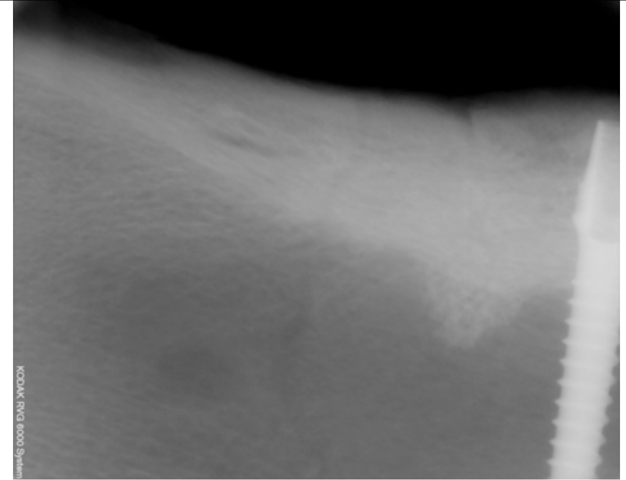


Fig 40. Radiogram shows hole filled by Cerasorb, control hole (radioluscent) and part of nanoimplant.

Histological results

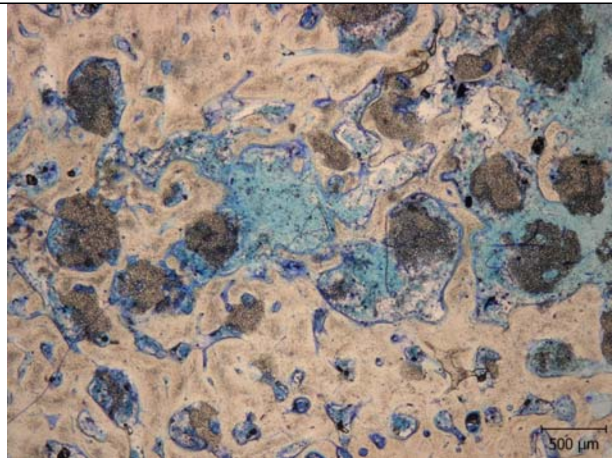


Fig 41. Histological image of dark brown roundish Cerasorb granules surrounded by blue stained connective tissue and osteoblasts demonstrating new bone formation.There is also a surrounding presence of light brown lamellar bone. (Please note that the smaller the foci of Cerasorb the greater the bone formation).

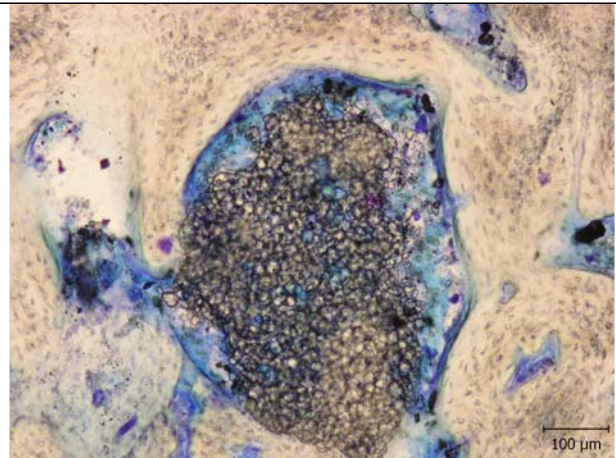


Fig 42. Higher magnification view of microporous Cerasorb granules surrounded by connective tissue with osteoblasts causing peripheral resorption of Cerasorb, with new bone formation. There is also some central penetration of Cerasorb granules by connective tissue.

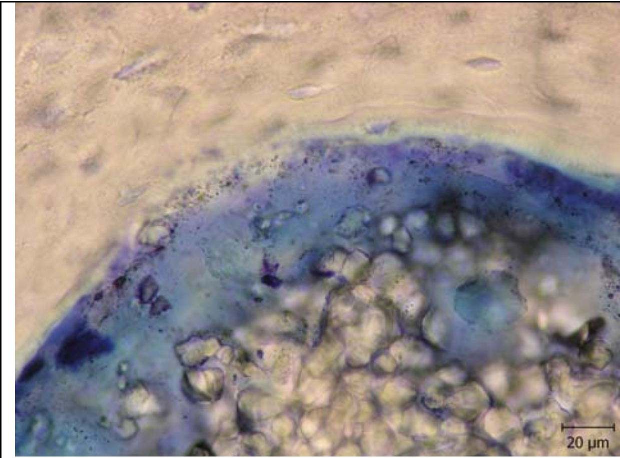


Fig 43. Formation of new bone causing gradual resorption of Cerasorb seen in this highly magnified image.

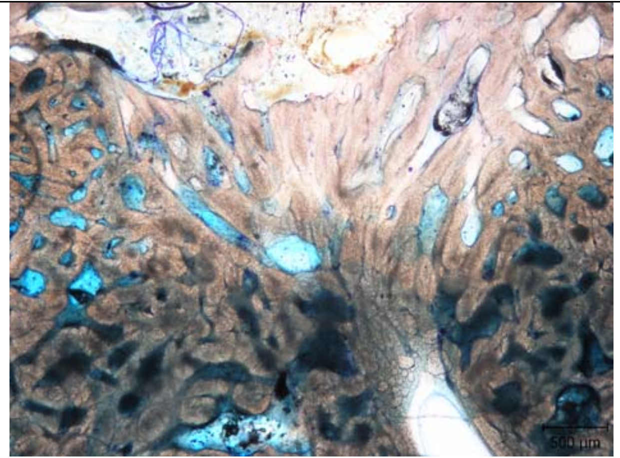


Fig 44. Histological image of the control hole with normal bone response to trauma. (Some osteoblasts can be seen).

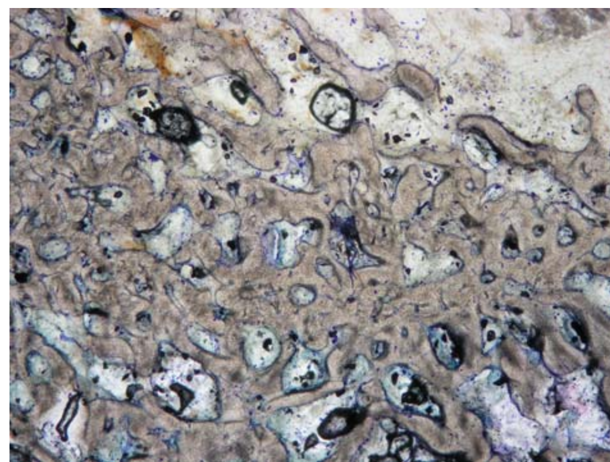


Fig 45. Similar image to fig. 44.

Discussion

Based on the results that we acquired from experiment 3 and after a thorough and careful analysis of the radiographic and histological images, we can conclude that Cerasorb gradually resorbs and is taken over by new bone formation and as such could be successfully used clinically.

The brown roundish microporous granules of Cerasorb, as seen in fig. 41-43 show different stages of penetration mostly peripherally by vascularized connective tissue leading to new bone formation[149]. Therefore we can say, with support from other publications by authors such as Plenk H Jr that Cerasorb has very good bone augmentating properties and can be readily used for healing of bone defects.

3. Dental implantology

Introduction

3.1 Dental implants:

A dental or oral implant is defined as a device which is either surgically placed **into** the jaw bone to replace one or several lost roots of teeth. It can also be defined as a device which is placed **onto** the bone, replacing several, if not all roots of lost teeth. Missing teeth can lead to speech difficulties, an unattractive smile, embarrassment from loose dentures, and pain or difficulty with eating. Atrophy of the jaw bone is another problem associated with the loss of teeth. Extraction of teeth may result in 40 to 60% alveolar bone loss in a period of two to three years [169,170].Oral implants have increased the treatment possibilities for patients and improved the functional results of their treatment. Patients who had to compromise with their aesthetic appearance, chewing and nutritional intake due to complete or partial loss of teeth can now be restored back to various degrees of normal aesthetics and function to improve the quality of their life. Research efforts from many different disciplines such as material science, physics, medicine, biochemistry and others, form a foundation for continual improvements in the field of Oral Implantology.

3.2 History of dental implants:

The history of the implantology dates back to the Egyptian times, over 1,350 years before the famous Per Brånemark started working with titanium. Trimmed and tooth-shaped pieces of seashells were applied into the lower jaw to replace three missing incisors. The calcium carbonate content within the shells was the reason why they could potentially fuse with the bone. Archeological findings showed that the ancient Egyptian and South American civilizations had already experimented with re-implanting lost teeth with hand-shaped ivory or wood substitutes. Although recently, metal is thought to have been introduced as an implantable material. A 'Wrought iron' dental implant for the right upper second premolar from a Gallo-Roman necropolis at Chantambre (Essonne, France), during the first or second century AD was reported [157]. The implant and the socket was said to fit perfectly together and the Osseo integration appears viable. In 1809, Maggiolo fabricated a gold implant which was placed into fresh extraction sockets to which he attached a tooth after a certain healing period. In 1886, platinum posts were implanted into the jawbone. Many other implantation attempts were made along the same lines,

experimenting with different metal alloys and porcelain formulations, however, and the long-term success rates were still with poor results.

In the early 1930s there was greater emphasis on the tissue tolerance along with the reaction of bone towards metal implants. The introduction of stainless steel and the development of a cobalt-chromium-molybdenum alloy (Vitallium) gave new light to surgical implants. Strock succeeded in anchoring a Vitallium screw within the bone and immediately mounting a porcelain crown to the implant, achieving a fifteen year survival of the implant for the first time in history.[158]. Histological sections from the trial animals showed a remarkable tissue tolerance to the vitallium implants. In 1937, Müller placed the first subperiostal implant, made of an iridium-platinum alloy [159]. In 1955, Marziani used a one-step procedure by placing a perforated deformable tantalum plate implant [160]. In 1952, the Swedish orthopedic surgeon, Per-Ingvar Brånemark, developed a threaded implant design made of pure titanium that increased the popularity of implants to new levels. He adopted titanium chambers in the femur of a rabbit and found that he was unable to remove them following several months. He observed that bone had grown into such close proximity to the titanium implant that it had effectively adhered to the metal. Discovering almost by chance the high compatibility and strong anchorage of titanium in the bone marrow of a rabbits fibula, he and his co-workers were the first to introduce the term *Osseo integration,* which can be defined as the tenacious affinity between living bone and the titanium oxides [161] as a direct and stable anchorage of an implant by the formation of bony tissue without the growth of fibrous tissue at the bone-implant interface [168]. He carried out many further studies and clinical examinations into this phenomenon, as well as the design of the implants, including biological, mechanical, physiological, and functional properties, using both animal and human subjects, which all confirmed this unique property of titanium [162]. In 1965, he was placed the first titanium dental implant into a human volunteer, which was characterized by its machined titanium surface, a thread pitch and an external hex point.

Leonard I.Linkow was one of the first who aimed to increase the contact area between the implant surface and the peri-implant bone. He also adapted implants to the respective anatomical structures with minimal surgical burden on the patient [163]. The Osseo integration concept evolved in conjunction with the design of a cylindrical titanium screw with a specific surface treatment to enhance its bioacceptance. To present, over 7 million Brånemark System implants have been placed and hundreds of other companies produce dental implants.

Since 1970, a number of new implant materials and designs have been developed, including the use of polymers [164], porcelain, high-density aluminum oxide (alumina) [165], bioactive glass (Bioglass) and carbon materials. Although nowadays, the most frequently used implant material is titanium which has become the gold standard in implant dentistry.

Andre Schroëder also focused his attention on the properties of titanium and he introduced the titanium plasma spray (TPS) coating concept with the one-piece transmucosal screw [166].These studies were the introduction to the early ITI system presenting the engineering concepts, design and clinical results with the hollow cylindrical implants.

In the mid 1980s, Stig Hansson developed a new implant concept with a micro textured surface [167].This was achieved by a pure titanium grit-blasting technique which roughened the implant to within 1–5 microns. The micro-thread was located on the coronal collar, which improved, the load distribution and retention of vital crestal bone.

3.3 **The criteria required for osteointegration of oral implants into bone:**

To be considered successful, an Osseo integrated oral implant has to meet certain criteria in terms of function, tissue physiology, and user satisfaction [171]. The following include certain requirements: **1)** immobility in any direction; **2)** the average radiographic marginal bone loss should be less than 1.5 mm during the first year of function and less than 0.2 mm annually following thereafter; **3)** the radiograph should not demonstrate any evidence of periimplant radiolucency; and **4)**The individual implant performance must be characterized by absence of signs and symptoms such as pain, infection, paresthesia, or violation of the mandibular canal [172,173,174]. Esthetic requirements, patient's and dentist's satisfaction with the implant prosthesis should also be considered as criteria of success [175]. For osteointegration of dental implants the correct surface modifications, shape and materials are vital.

3.4 The problems with materials used:

All materials used clinically in dental medicine are evaluated according to two groups of simultaneous parameters:

. The physical and mechanical properties

. The biological properties

The success of an endosteal implant depends on, whether the material used, complies with the requirements above.

In the case of endosseous oral implants, high mechanical stability is essential [176]. The aim of material research is to develop a material with sufficient mechanical properties but with brittleness similar to that of bone. The relevant physical properties are tensile strength and Youngs modulus.

Biocompatibility can be defined as the compatibility of any (foreign) material with a living organism. More specifically, biocompatible materials are those for which the interaction between the material and vital tissue is so minimal that the material is not detrimentally affected by the tissue or vice-versa. The materials used in clinics should not be toxic, carcinogenic, allergenic or radioactive.

Factors which can influence biocompatibility include chemical, mechanical, electrical and the surface-specific properties[123] .The biocompatibility of materials being considered for endosteal implants are evaluated primarily by the reaction of bone to the material, although the reaction with the mucosa at the implant neck is also significant.

Classification of materials with respect to their compatibility in bone according to Strunz: Metals, such as stainless steel, Co-Cr-Mo alloys, noble metal alloys, polymethylmethacrylate (PMMA), and other polymers are tolerated by bone to a certain extent, but cannot be said to integrate with it. Histological appearance ″interface″ shows that the bone keeps its distance and in this case the term ″distant osteogenesis″ is used to describe the situation in which there is a thick and fibrous layer of connective tissue between the implant and the bone. [124]

Titanium, Tantalum and other metals (e.g., niobium) as well as aluminum oxide ceramics are described as being bioinert. When an optimal fit is achieved between the implant bed and the implant, new bone formation and bone remodeling occurs up to the implant surface. This is termed ″contact osteogenesis″

A full ″bond osteogenesis, ″ in which a chemical reaction plays an important role, is characteristic of the so-called bioactive materials; these are described in literature under the generic term ″glass ceramics.″

3.5 Titanium and response of bone:

Titanium exists in nature as a pure element with an atomic number of 22 in the periodic table and an atomic weight of 47.9. It is the ninth most abundant element and the fourth most abundant structural metallic element in the earth’s crust, following aluminum, iron and magnesium. The metal exists as rutile (TiO_2) or ilmenite ($FeTiO_3$) and requires specific extraction methods to be recovered in its elemental state. The metal melts at 1,665ºC [183]. The normal level of titanium in human tissue is 50 ppm. Values of 100 to 300 ppm are frequently observed in soft tissues surrounding titanium implants. At these levels, tissue discoloration with titanium pigments can be seen. This rate of dissolution is one of the lowest of all passivated implant metals and seems to be well tolerated by the body [192].

Commercially pure titanium (CP) comes in different grades, from CP grade I to CP grade IV, which vary mostly in the oxygen content.

Titanium alloys of interest to dentistry exist in three forms: alpha, beta and alpha-beta. These types originate when pure titanium is heated, mixed with elements such as aluminum and vanadium in certain concentrations, and then cooled mainly for the purposes of improving strength, high temperature performance, creep resistance, response to ageing heat treatments and formability [184]. Aluminium stabilizes the alpha phase of titanium and serves to increase the strength and decrease the weight of the alloy. Vanadium stabilize the beta phase and decrease the alloys susceptibility to corrosion [183,185].The most commonly used alloys for dental implants are of the alpha-beta variation contain 6% aluminum and 4% vanadium (Ti 6Al 4V). These alloys possess many favorable physical and mechanical properties that make them excellent implant materials [185]. Compared with Co-Cr-Mo alloys, titanium alloy is almost twice as strong and has half the elastic modulus.

Corrosion leads to the release of compounds into biological environments, which may then cause adverse effects, such as toxic or allergic reactions. Corrosion resistance is, therefore, a prerequisite for biocompatibility [186,187]. Current data have shown that titanium can be corrosive as many other base metals under mechanical stress, oxygen deficit, or at a low pH level [187]. Fluoride ions can infiltrate and dissolve the stabilizing oxide layer [188]. It has been shown that caries-preventive fluoride gels increase the corrosion and surface roughness of titanium due to their pronounced adhesion [189]. In the oral environment, when the pH is almost neutral (pH=7) the passive layer dissolves at such a slow rate that the resultant loss of mass is of no consequence for the implant [190]. It has been found that acids in the absence of fluoride ions can cause electrochemical corrosion [191].

Pure titanium is one of the bioinert implant materials. Its biocompatibility has been known for a long time and has been repeatedly confirmed in literature. This biocompatibility is due to a surface oxide layer, which prevents direct contact of the metal with the surrounding tissue. Titanium in contact with oxygen is immediately covered by a titanium oxide layer, starting as a titanium monoxide and ending up as rutile surface, titanium dioxide [177,178].

Titanium does not simply behave passively in tissue and bone. Bone grows into the rough surface and bonds to the metal, a reaction which is normally only attributed to so-called bioactive materials. This ankylotic anchoring, forms the best possible basis for a functional dental implant, as it can withstand all possible load types, E.g.) tensile, compressive and shear forces. This ankylotic anchorage, also termed osteointegration, is accepted today as the most promising method of stabilizing endosteal implants and endoprostheses. The cancellous bone that is laid down first is replaced within a few weeks by mature lamellar bone. This regenerated tissue is both qualitatively and quantitatively indistinguishable from bone that would have been formed if no implant had been placed.

High-resolution electron microscopy to study the bone-titanium interface shows a layer or matrix material about 20 nm thick, and then, at a distance of about 100 nm, massively mineralized collagen fibers. In between these, the fibrils and mineral deposits were not organized [179,180].The main components of the bone ground substance were proteoglycans, giant molecules about the same size as the contact region. Biochemists have identified these substances as the "glue" between cells and between and other surfaces, including those of foreign bodies.

A border zone of necrosis at the defect site is inevitable, even when implant bed preparations are carried out as atraumatically as possible [181].The author begins: "Following severe trauma from a physical, chemical, or other nature, the osseous tissue heals through the formation of an irreversible layer of fibrous tissue. Healing is completely impeded if revascularization of the necrotic area does not occur."

Experimental findings, which have been repeatedly reproduced over many years, have led to the conclusion that new bone is laid down directly upon the implant surface, provided that the rules for atraumatic implant placement are followed, E.g.) rotation of the cutting instrument at less than 800 rpm, cooling with sterile physiologic saline solution. Further research suggests drill geometry plays a major role in heat production and explains the correlation between increased temperatures with increased use [182].

3.6 Types of oral implants:

Many clinicians designed implants to fit certain needs and properties. There are several types of implants available which are chosen for a particular patient depending upon a number of factors. The size and condition of the patient’s natural jawbone is probably the main factor: Some jawbones can be wide and deep and others narrow and shallow with many variations in between.

In general, Oral Implants can be categorized into three main groups:

1. Endosseous Implants: are implants that are surgically inserted into the jawbone. They are moulded or shaped to fit in a cavity in the jaw rather than sit on top of the jaw. These implants are the most frequently used implants today and based on their shape, function, surgical placement and surface treatment could be further categorized into several sub-categories:

1a. Root Form Implants: also called cylindrical or screw type implants, resemble in shape the natural root of a tooth with a surface area designed to promote good attachment to the bone. They are the most widely used design and are the most popular type of dental implants. They are formed like a self-tapping screw which gives it a good surface area for maximum fusion to the living bone and used to be called endosseous or endosteal implants, meaning they are placed in the bone. The

bone grows in and around the implant creating a strong structural support. In fact, this bond can be even stronger than the original teeth. However, this type of implant can only be used if there is sufficient width and depth to the jawbone. Sometimes it is necessary to graft additional bone to the jawbone before dental implants can be considered.

1b. Plate Form Implants: also known as blade form implants, they are flat rectangles which take the form of a long narrow strip of titanium which is inserted between the jawbone and the gum and will fuse with the jawbone giving a foundation for a number of new prosthetic crowns if required. They are usually used when there is insufficient live bone structure because the jawbone is too narrow or not deep enough or certain vital anatomical structures prevent conventional implants from being placed.

1c. Ramus Frame Implants: belong in the category of endosseous implants. They are designed for the toothless lower jaw only and are surgically inserted into the jaw bone in three different areas: the left and right back area of the jaw (the approximate area of the wisdom teeth), and the chin area in the front of the mouth. The part of the implant that is visible in the mouth after the implant is placed looks similar to that of the subperiosteal implants. This type of implant is usually indicated for a severely resorbed, toothless lower jaw bone, which does not offer enough bone height to accommodate root form implant as anchoring device and when the jaws are even resorbed to the point where subperiosteal implant will not suffice anymore. Ramus frame implants once integrated can stabilize and protect weak jaws and help to prevent them from fracturing.

2. Subperiosteal Implants: are designed to sit on top of the bone, but under the gums, fusion takes place between the jawbone and the subperiosteal implant. They are an option used in cases of advanced bone loss where the condition of the jawbone is such that an insert is not possible and a bone graft is not feasible.

3. Transosseous implants: were designed to be used in people who had very little bone in their lower jaws and who had no lower teeth. These implants are not in use that much any more, because they necessitate an extraoral surgical approach to their placement and requires extensive surgery, general anesthesia and hospitalization. They are used in the mandible only by inserting two metal rods from below the chin, through the chin bone, until they are exposed inside the mouth. The rods that can be seen inside the mouth are used to attach a denture.

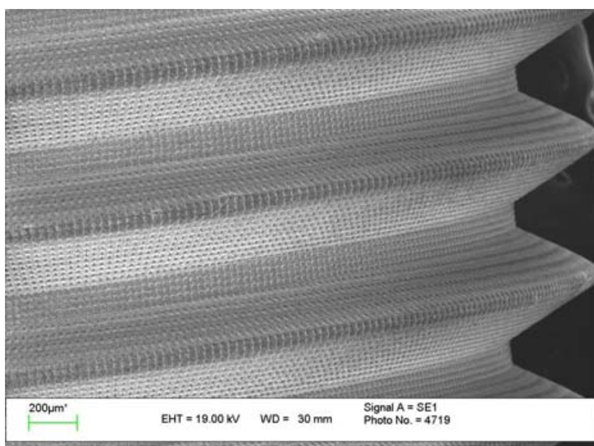
Most clinicians nowadays however, prefer to use bone grafts and one of the other endosseous implant methods described earlier.

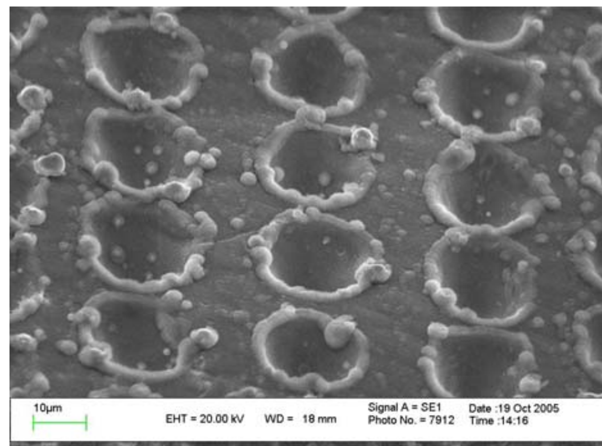
3.7 Implant surfaces:

Future dental implant requirements should include proper surface preparation and surface quality maintenance of the implants themselves. Types of surface categories include:

Laser treated

There are different laser sources used in implantology. In our experiment we used Nd:YAG diode pumped laser operating in Q-switching for treatment of the implant surface. This laser radiates electromagnetic energy which interacts with the titanium taking it to a plasma state from its solid state.
The wavelength of the laser and the extremely concentrated energy pulses allow for micro-fabrication of the implant surface without any dangerous effects, preventing thermal changes of material properties which can induce micro-fractures or alteration of the metal structure. This controlled micro-ablation was obtained using a low power setting.
An important goal of laser treatment of implant surface is to produce a surface with thousands of hemispheric pores for bone apposition.





Acid-etching

Acid etching of titanium is of particular interest because it creates a microtextured surface (fine rough surface with micro pits of 1-3µm and larger pits of approximately 6-10µm) that appears to enhance early endosseous integration and the stability of the implant. [126] This may be related to a change in surface roughness and/or chemical composition. [127]
Studies by Wenneberg et al. [128,129,130,131] demonstrated the optimal surface roughness of different particles of 75µm, made surface more resistant to torque and greater bone-to-metal contact than small (25µm) or coarse (250µm) particles. The optimal surface had an average height deviation of about 1.5µm resulting in a surface enlargement of 50%.

Plasma-coated spray

Coating is to produce a rough implant surface that significantly improves the anchorage of the implant in bone. This process can be used for both metal and ceramics.
Plasma coating works by blowing an inert gas through an intense electric arc . Down the arc The coating material is introduced in th form of a extremely hot gas. The inert gas is broken down into ions and electrons in the arc. This state is known as plasma. The titanium hydride (coating material), decomposes in the gas stream forming droplets of molten metal that are projected on to the implant surface to build up a coating. The layer is typically 20-30µm thick with a roughness of approximately 15µm.
Plasma coating is economically compatible, and its biological compatibility is at least as good as that of normal titanium. Gases in titanium harden the metal which is an enhancement that is advantageous for the surface of an implant. The bond strength between the porous plasma layer and the substrate is limited, but excessive treatment is required to cause this bond to fail (exposure to an ultrasound source).

Tianium implants with coatings have an average bone-implant contact in cancellous bone of nearly 40%, which was significantly higher than smoothly polished or finely structured titanium implants which had values of slightly over 20%.[125]

Sand-blasted

In this case the sand-blasting roughens the surface of the implant achieving both microretentive topography and increased surface area[195,196].
Sandblasting treatment consists of mechanical abrasion of surfaces using oxide particles shot against the implant. The treatment produces a surface with a roughness depending on size, shape and kinetic energy of the particles. However this increased roughness may reduce the endurance properties of metals [197].
Healing around the titanium sandblasted implants are similar to that observed around the plasma-spray surfaces. No statistical differences are observed between the 2 groups. In conclusion, a high bone-contact percentage was obeserved in the 2 groups of implants.

Overall Conclusion:

Texture was the most remarkable isolated feature, regarded as an osteointegration promoter. In a review of the effects of implant surface topography on cell behavior, one can verify that there is bone apposition onto the implant surface independant of whether it is polished or rough, made of titanium or ceramic. Roughness is not necessary for bone apposition. Osteoblasts have higher probability to adhere to a rough titanium surface while fibroblasts and epithelial cells adhere mainly to very smooth surfaces[193,194]. However, it has been shown that roughness may play an important role in the percentage of bone apposition as well as in the velocity of apposition. Roughness or acid conditioning of the surfaces can therefore significantly improve shear strength. Besides optimizing the procedure, these surface characteristics may allow for an earlier loading of the implant and extend the indications for implants in low-density alveolar bone and in regenerated bone.

3.8 Experimental studies with dental implants:

3.8.1 Experiment to establish the most efficient evaluation method of a bone-implant interface in a dead rabbit (experiment 4), and if successful to use this method to evaluate the actual osteointegration in a living rabbit (experiment 5).

Introduction and Aim

When looking to evaluate osteointegration, the preparation time can exceed 7 months when following the traditional method of preparation of the histological specimens. This procedure involves primarily fixing the specimen in a natural buffered 10% solution. This is followed by dehydration and staining by a basic fuchsin 5% and alcohol mix of concentration 60,80,90 100% for 24 hours each.
This is followed with a washing and rinsing procedure in 5 to 6 different acetone baths, separated by a minimum of 4 weeks in between each.
Finally the specimen is embedded in acrylic using a methyl methacrylic acid ester as the embedding medium (acrylic bath changed after 24hrs, 72 hrs, 20 days and 4 weeks.)[199]
The main difficulty in the traditional method is the ability to obtain a very thin section of bone-implant, without any displacement and micro-movements of osteointegrated implants from the bone, to be able to observe true bone-implant interface.
Our aim in this experiment was therefore to find an alternative method for preparation of the bone-implant interface for evaluation, and in order to improve cost efficiency, reduce the preparation time whilst obtaining reliable and useful results.

Experiment 4

Materials and Methods

In order to find out which staining agent would be optimum for use in our alternative experiment, we carried out a preparation of a human tibia and mandible using the traditional method and using erythrosine and basic fuchsin dyes.

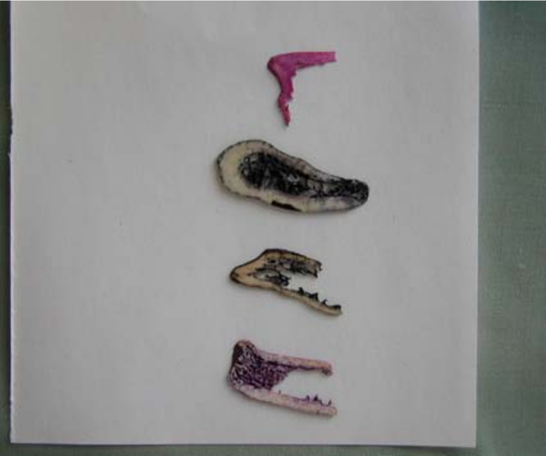


Fig 46. Sliced specimens of mandible.

Fig 47. Specimens of mandible stained.

Fig 48. Prepared slide of mandible with erythrosine (top) and basic fuchsin (bottom slide).

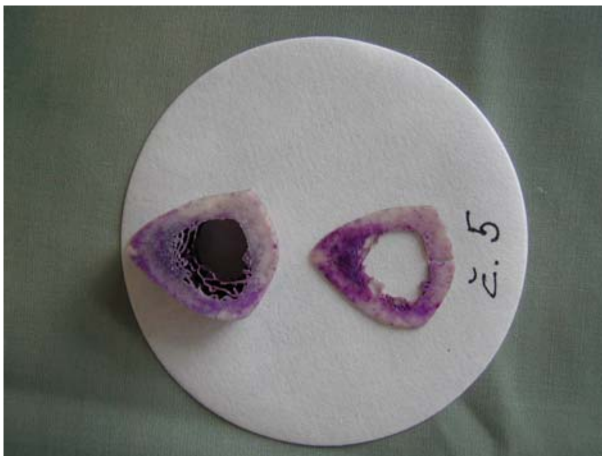


Fig 49. Tibia stained with Basic Fuchsin.

Fig 50. Prepared slide of tibia stained with Basic Fuchsin.

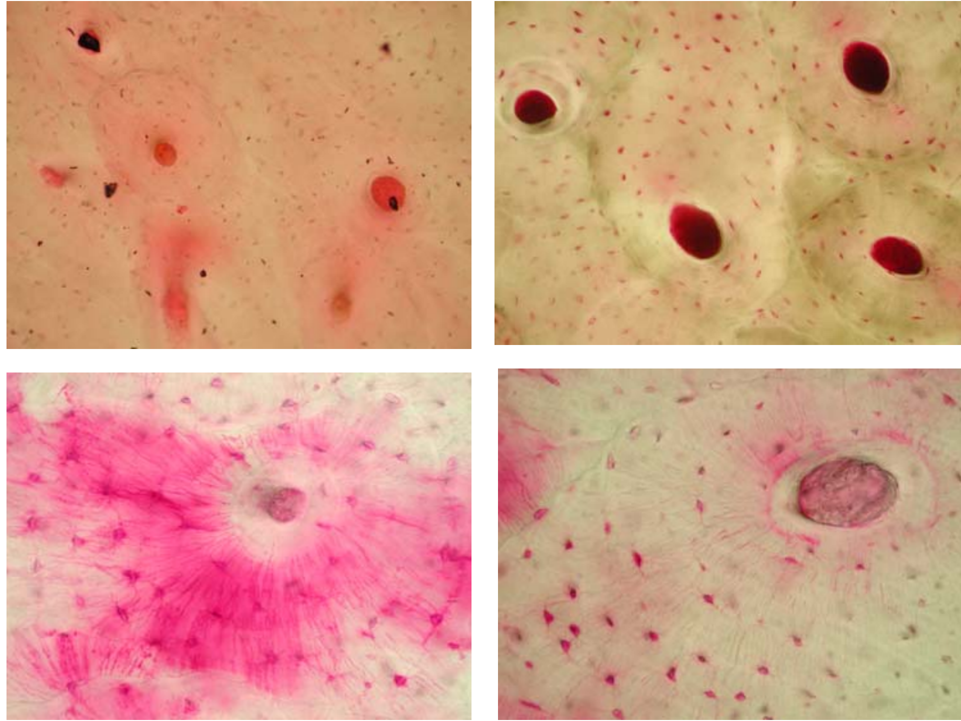


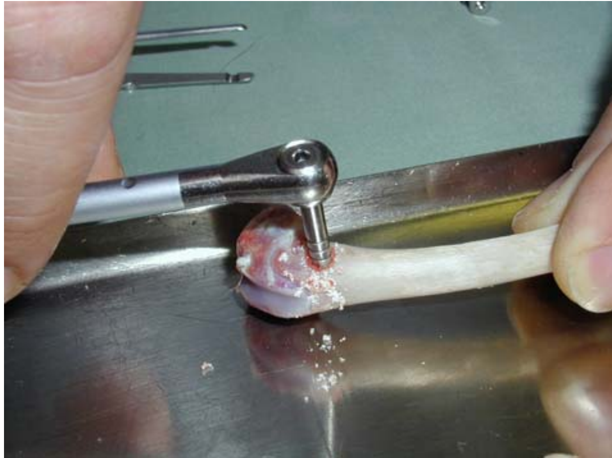
Figure 51. Showing staining of Haversian systems of bone by basic fuchsin and erythrosine.

We established that both staining materials could be used and then continued with our own alternative method of preparation using the femur of a non-living rabbit.

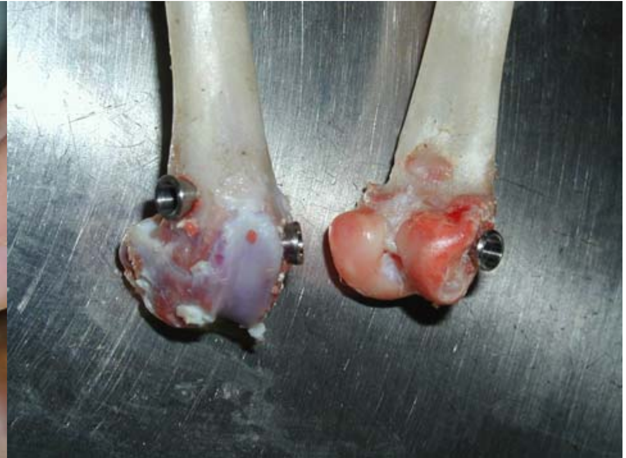
We prepared the implantation bed in the proximal part of the femur (A), and inserted the implant in combination with an insertion device (B), using a ratchet (C). Note the inserted implant in the bone (D). We also placed a Titanium screw in this part of the femur.

A

B



C



D

In the next step, grinding of the bone and implant was done until the bone-implant interface was seen (Fig 52 a, b). This was then stained with basic fuchsin (Fig 53 a, b), ready for histological evaluation under the laser confocal microscope.

Fig 52 a

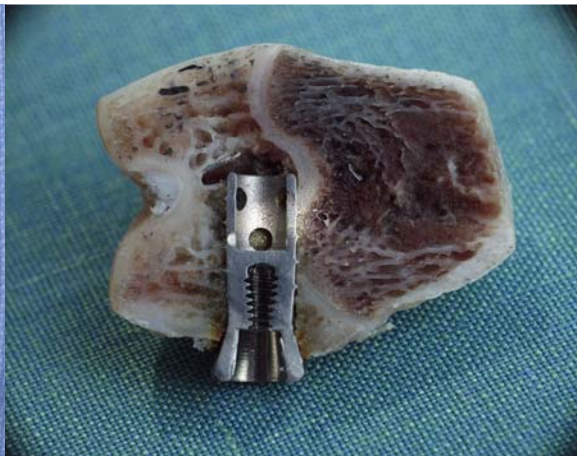


Fig 52 b

Fig 53 a

Fig 53 b

Results

The results, as can be seen in fig 54, 55 and 56 under a confocal laser microscope are very positive. Although, as expected, no osteintegration had taken place due to implantation into nonliving tissue, the bone-implant interface can clearly be seen. We can also observe black spaces between the surface of the implant and the bone (the same case between the thread of the screw and the bone).

When carrying out this implantation in a living animal, we will expect to see these dark spaces replaced by areas of osteoblast activity with subsequent bone formation and osteointegration.

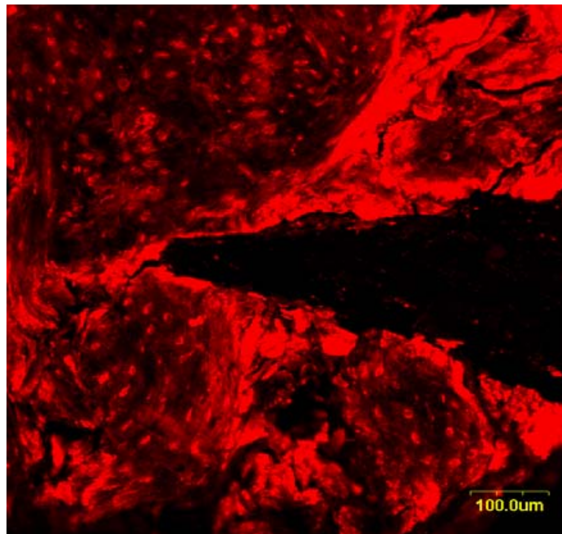


Fig 54. Interface seen between the thread of the titanium screw (in black) and the bone (red).

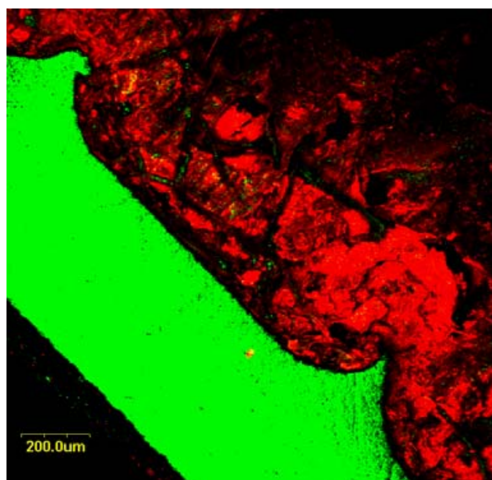


Fig 55. Bone-implant interface seen with green implant structure and red surrounding bone. Please make note of black space area between surface of implant and bone.

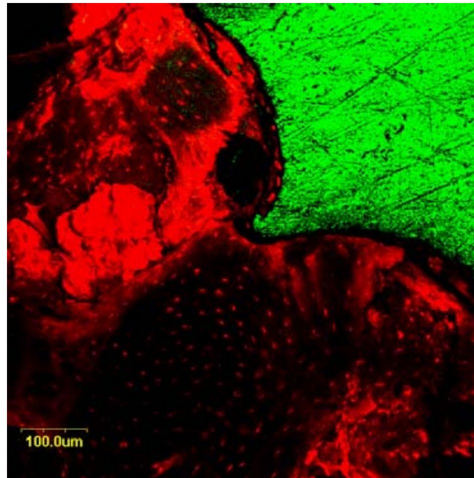


Fig 56. Further magnification of Fig 55, putting emphasis on observed black spaces between the implant structure in green and surrounding bone in red.

Conclusion

We ascertained that the traditional method of producing histological specimens of bone and implant is very time consuming and complicated.
The experimental method that was developed utilizes the confocal laser microscope, allowing accurate results and more rapid specimen preparation time whilst being easier to perform. The advantage of confocal laser scanning microscope (CLSM) is that the specimen should not be sectioned as thin as for a light microscope and its ability to produce in-focus images of thick specimens, a process known as *optical sectioning*.

Having established the success of the experimental method, we decided to repeat our experiment but with the use of a living rabbit.

Experiment 5

Materials and Methods

The rabbit was placed under general anesthesia and aseptic conditions. A titanium implant (Timplant) was surgically inserted into the femur. The incision wound was sutured.

Fig 57. Inserted implant can be seen.

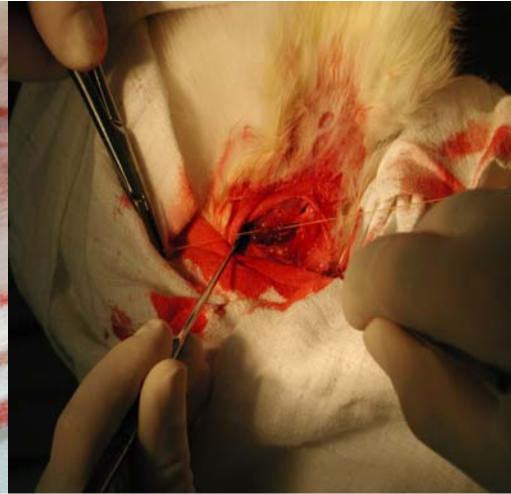


Fig 58. Suturing of wound.

After 45 days, the rabbit was sacrificed and a series of radiographical images were taken and using the alternative method mentioned previously, we prepared the histological specimen ready for the confocal laser microscope.

Results

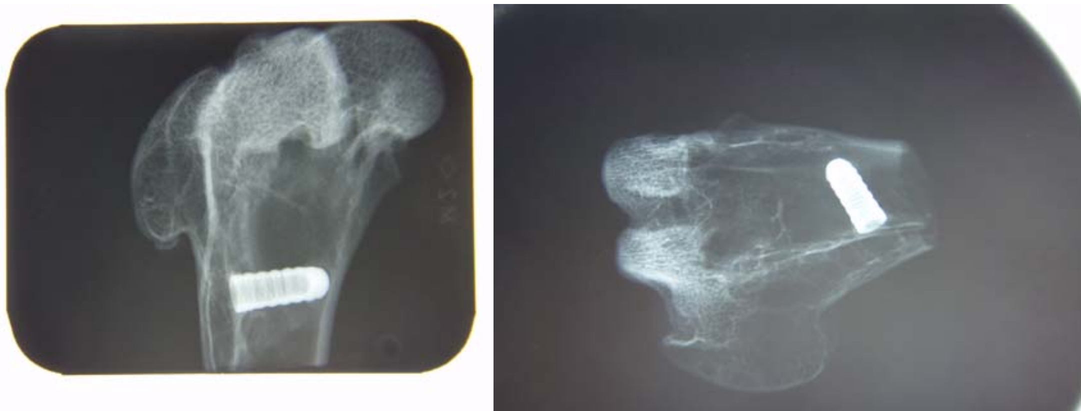


Fig 59. Radiographic images of implant in femur of rabbit after sacrifice.

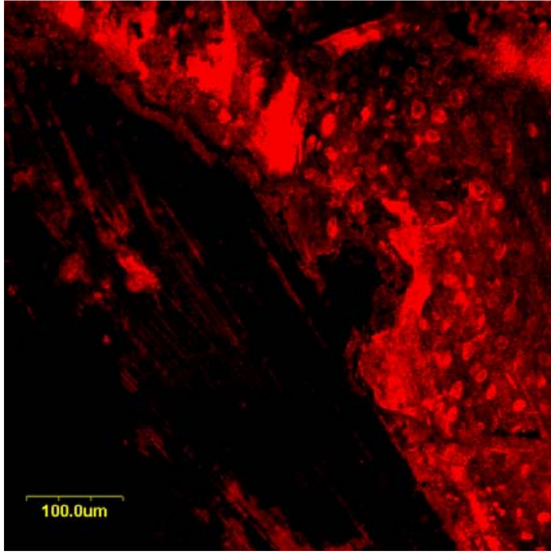


Fig 60

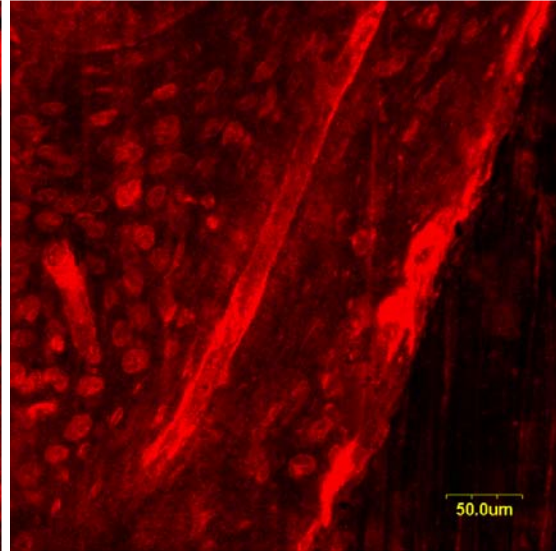


Fig 61

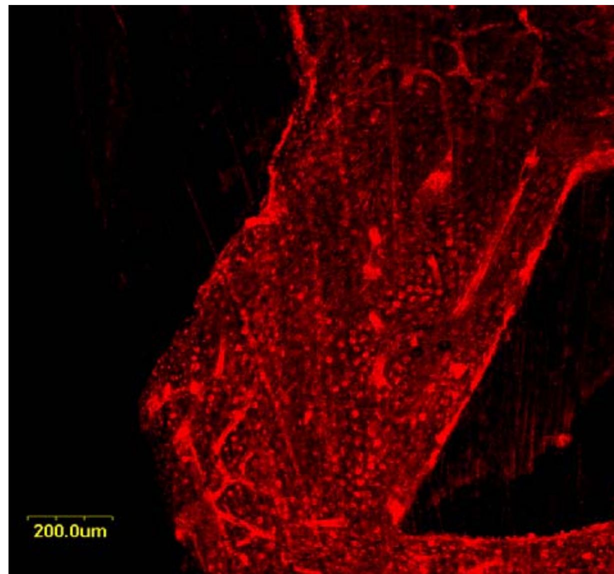


Fig 62

We also performed the analysis of our results by the traditional histological method, in order to confirm the confocal microscope results picture:

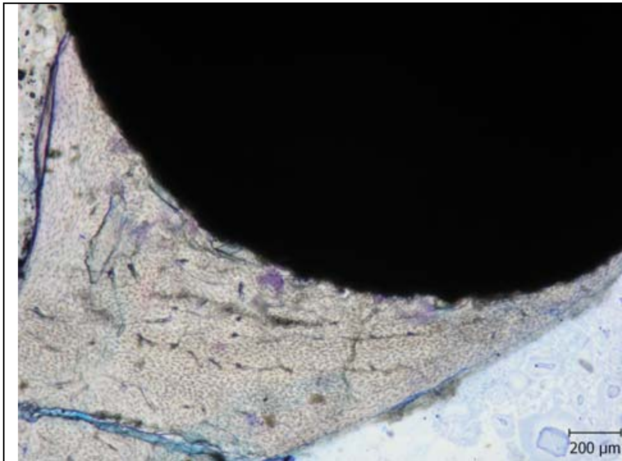


Fig 63. Histological view of implant in the rabbit femur (Transverse section).

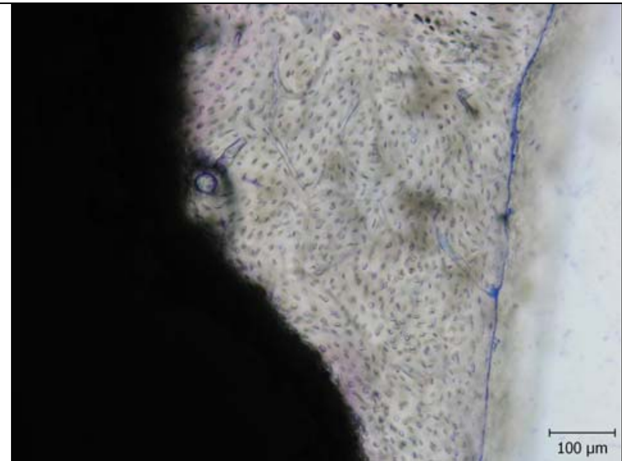


Fig 64. Histological view of the implant in the rabbit femur.

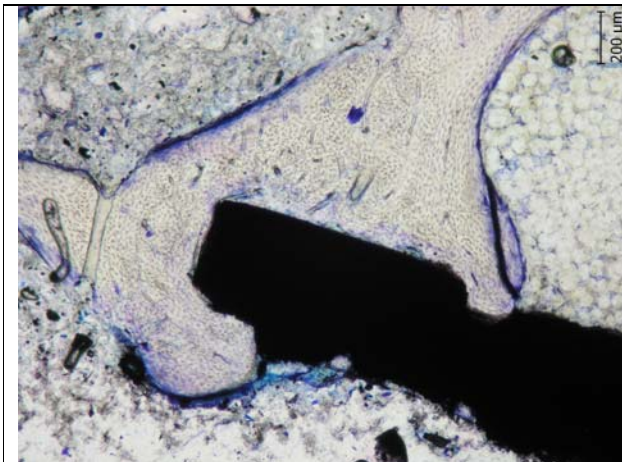


Fig 65. Histological view of the implant in the rabbit femur (Longitudinal section).

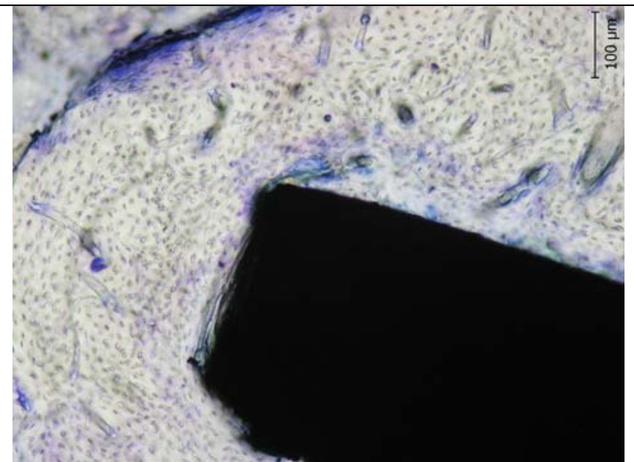


Fig 66. Histological view of the implant in the rabbit femur (Longitudinal section).

Conclusion

Once again the results as seen on Fig 60, 61 and 62, were highly successful and provided an excellent view of the bone-implant interface. The images made by laser confocal microscope showed osteointegration between the bone in red, and the dark black implant structure.
Evaluating figures 60, 61 and 62 we can establish that there are no spaces, showing good osteointegration.
Based on our results, in both the nonliving and living tissues, we can deduce that the alternative method is a very successful way in preparing and evaluating histological specimens in order to show the osteointegration between the bone and implant surface. Our results were similar to those provided by the traditional

method, however, our method was significantly less time consuming, more cost efficient and easier to carry out.

3.8.2 Experimental study in pigs with the purpose to evaluate and examine osteointegration of dental implants with differently treated surfaces [200]- Experiment 6

Aim

Examination and evaluation of osteointegration of dental implants with different laser treated surfaces, and the comparison of those results with sandblasted and machine treated implant surfaces.
Our aim is also to apply tetracycline (TTC) in order to demonstrate regions of active bone formation, mineralisation and to demonstrate the quantity of newly formed bone at the implant interface with the help of confocal microscope due to its fluorescent property. During the period of application, the TTC accumulates and labels the newly formed bone. Our expectation is to demonstrate two levels of bone formation labelled as fluorescent lines.

Materials and Methods

Different types of surface treated implants inserted into the long bones (ulna and tibia) and mandible in piglets.

Following the surgical insertion of implant in the piglets, the piglets will be allowed to mature and then be sacrificed. At this stage in all cases, radiographic images will be taken for analysis. Following this procedure, the bones will be prepared for histological evaluation by CLSM, and to also establish Bone Implant Contact (BIC). The protocol for the histological preparation can be seen in title 3.9 (bone processing protocol), and 3.10 (staining protocol), figures 107-133b.

For our experiment we used five piglets – one piglet died during the endotracheal intubation.
Experiment started in May 22nd, 2007.

Protocol of general anaesthesia

Protocol for each pig was the same.
24 hours before the experiment the piglets were only given water.
Premedication was applied 30 minutes before operation – Azaperon (buthyrephenon) 6-10 ml.
The general anaesthesia was initiated by application of Atropin 0.5mg and Diprivan (propopholum 2%) 5-8ml intravenously according to the weight of

animals.
The intubation was provided with orotrachael tube 6.0 or 7.0.
Analgesic solution used in this experiment was buprenorphin 1- 2ml.
Application of myorelaxant was not necessary.
Anaesthesia was prolonged by pumping 5-15ml/h Diprivan (propopholum 2%) according to the depth of general anaesthesia.
During the operation 10ml/kg/h Plasma Lyte (Hartmann - Ringer's solution) was infused.

Ventilation:
Volume controlled 10-15 ml/kg, with the frequency of 30-40/min, pressure PEEP 4.

During the anaesthesia the following parameters were monitored by the anaesthesiologist:
SpO_2 (oxygen saturation accuracy range) 95 – 99%
ET CO_2 (end tidal carbon dioxide) 4.5 – 5.0 kPa
Heart beat frequency 80 – 110/min

Anaesthesia was performed by an experienced anaesthesiologist from the I. internal medicine department of the faculty hospital in Pilsen.

The duration of the general anaesthesia was approximately 120 minutes.

Implantation scheme of oral implants

A = laser 5µm pores, pitch 15µm

B = laser 10µm pores, pitch 20µm

C = laser 20µm pores, pitch 30µm

D = laser 5µm pores, pitch 5µm

E = laser 10µm pores, pitch 10µm

F = laser 20µm pores, pitch 20µm

S = sandblasted

M = machined

Fig 67. Colour and code scheme of implants used.

COVERING SCREW COLOR	SURFACE
Grey: code 110	M
Brown: code 230	S
Yellow: code 130	A
Dark green: code 620	D
Dark blue: code 430	B
Dark purple: code 410	E
Light purple: code 530	C
Copper yellow: code 510	F

Piglet No.1, weight 14.7 kg and an experiment period of 64 days
Operation date: 22.5.2007
7.6.2007 application of antibiotic Tetracycline (20mg/kg, 100mg/1ml) 5.0ml i.m at the weight of 24.5 kg.
2nd dose 6.2ml in 21.6. 2007 at the weight of 32kg.

Pig No.1
22.05.07
14.7 kg

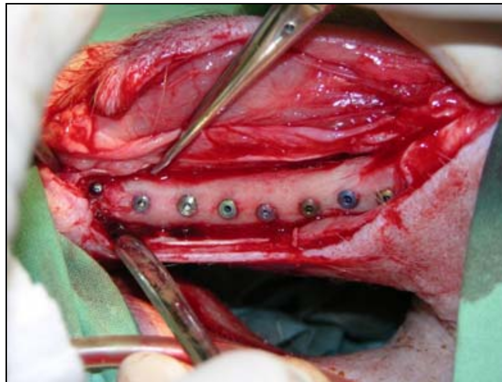


Fig 68. Implants inserted into the tibia.

Right Mandible
Distal placement of implant progressing mesially: A,B,S,D,E
Left Mandible
Distal placement of implant progressing mesially: C,S,M,D,E

Right Tibia
Proximal placement of implant progressing distally: A,B,S,C,D,M,E,F
Left Tibia
Proximal placement of implant progressing distally: A,B,S,C,D,M,E,F

Right Ulna
Proximal placement of implant progressing distally: A,B,S,D,E
Left Ulna
Proximal placement of implant progressing distally: C,S,M,D,E

Sacrifice of animal life

26.7.2007 at the weight of 49.5kg, application of 6ml Stresnil (azaperonum) and 7 ml T 61 (embutramidum, mebezonium iodatum, tetracainii hydrochloridum).

After the termination, the animal bones were removed from the soft tissue.

Radiographic documentation

Radiographic examination of individual prepared bone using intraoral x-ray and radiogram 13x18cm showing all inserted implants.
Detail radiographic examination of each implant using intraoral rvg (see figures 79-106).

Fixation

10% formaldehyde.

Pig No.2, weight 15.4 kg and an experiment period of 64 days
Operation: 22.5. 2007

At the 5th post-operative day there was swelling in the region of the right ulna, with elevation of the body temperature to 38.5 °C.
Application of antibiotic solution Unasyn 1.5 g every 8 hours i.m. Disappearance of inflammatory complication after 4 days of application. Formation of fistula in the region of incision in right ulna.

Tetracycline – same protocol as for pig No.I

Termination: 26.7.2007, weight 50kg, same protocol as pig No.I

(Excluded due to the inflamation: right ulna p2).

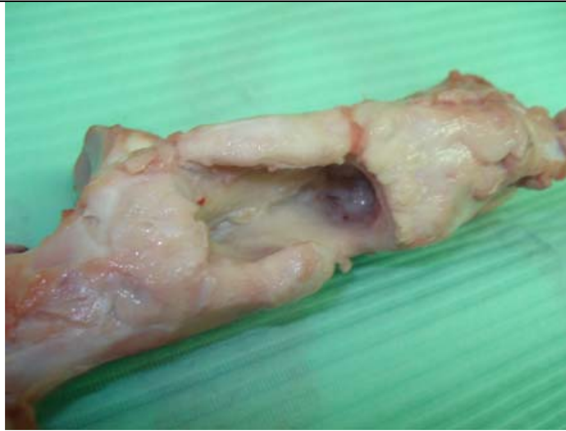


Fig 69. Inflammation of the ulna.

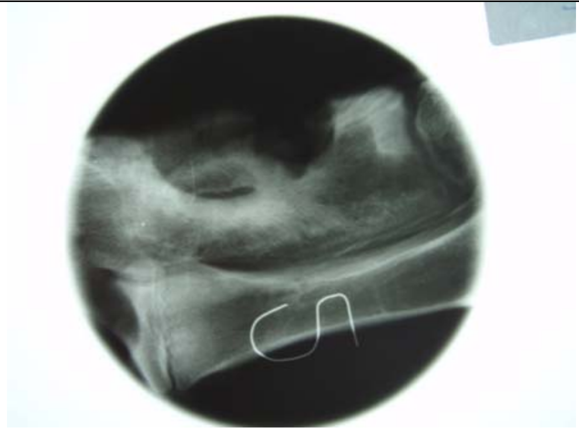


Fig 70. Radiogram of inflamed ulna.

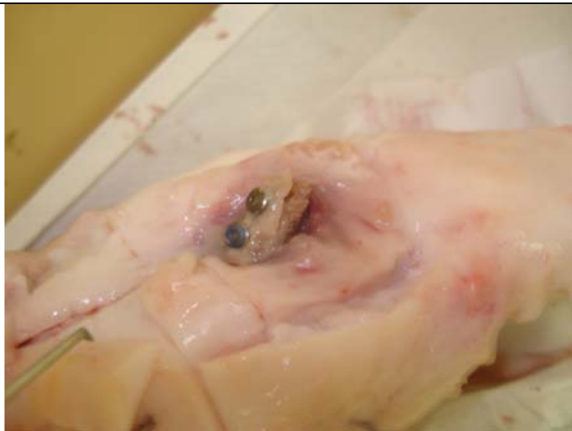


Fig 71. Rejected implants due to inflammation.

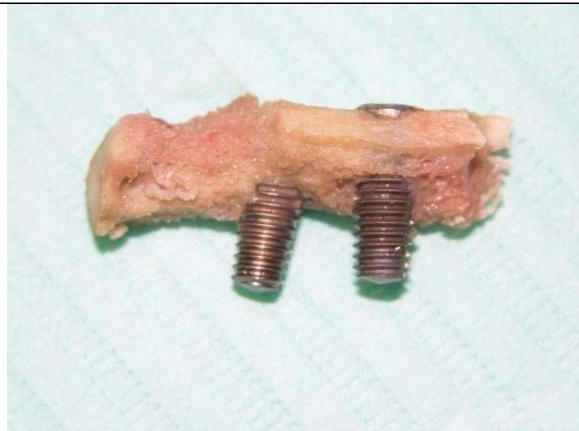


Fig 72. Rejected implants due to inflammation.

Pig No.2
22.05.07
15.4 kg

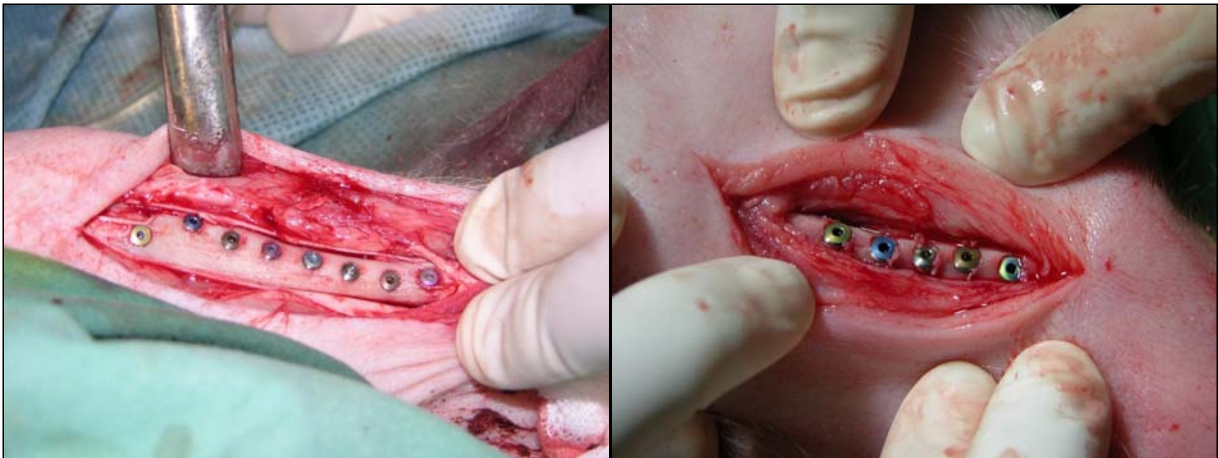


Fig 73. Implants inserted into tibia. **Fig 74. Implants inserted into the mandible.**

Right Mandible
Distal placement of implant progressing mesially**:** A,B,S,C,M
Left Mandible
Distal placement of implant progressing mesially: A,B,M,S,D

Right Tibia
Proximal placement of implant progressing distally: A,B,S,C,D,M,E,F
Left Tibia
Proximal placement of implant progressing distally: A,B,S,C,D,M,E,F

Right Ulna
Proximal placement of implant progressing distally: A,B,S,C,M
Left Ulna
Proximal placement of implant progressing distally: A,B,M,S,D

22.5.2007 intubation of the 3rd piglet was complicated by perforation of trachea into the mediastinum which caused death due to pneumomediastinum.

Pig No III, weight 24 kg and an experiment period of 52 days
Operation: 5.6.2007 under antibiotic prophylaxis of Unasyn 1.5g i.v. applicated after the initiation of general anaesthesia.

In the distal part of left tibia we inserted a nanoimplant.

- In the distal part of the left tibia we made two artificial holes. The most distal hole kept empty as a control hole and covered by Biocollagen membrane and the other hole filled by Cerasorb granules 500-1000μm and covered by Biocollagen membrane.

In the subcutaneus region of the right ulna we applied augmentaion material Cerasorb.

21.6.2007 application of antibiotic Tetracycline (20 mg/kg, 100 mg/1ml) 6,3ml i.m. at the weight of 33 kg.
The 2nd dose 7.0ml in 26.7. 2007 at the weight of 52.5kg.

Sacrifice:
27.7.2007, weight 52.5kg

Pig No 3
05.06.07
24 kg

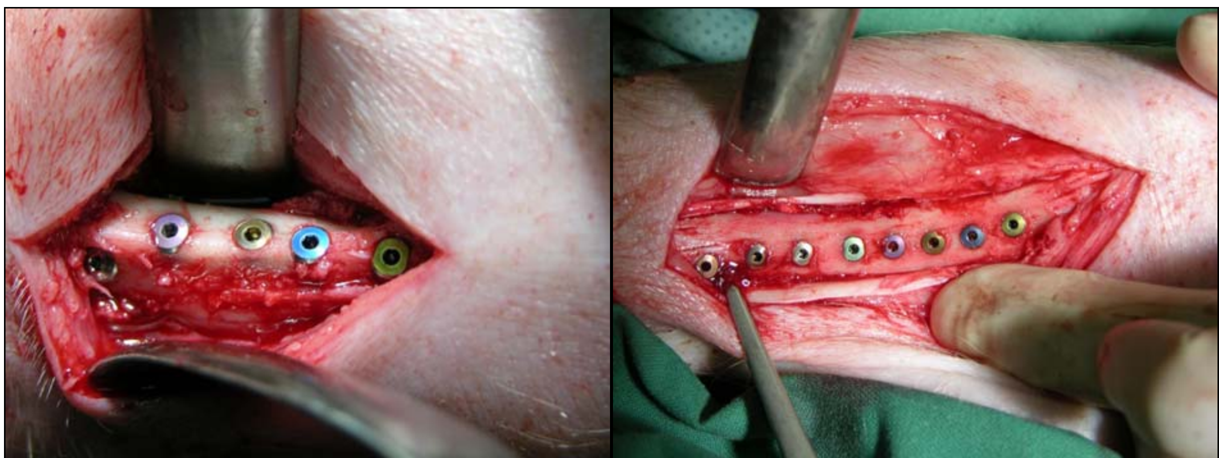


Fig 75. Implant inserted into the mandible. **Fig 76. Implants inserted into the tibia.**

Right Mandible
Distal placement of implant progressing mesially: A,B,S,C,M
Left Mandible
Distal placement of implant progressing mesially: A,C,S,D,M

Right Tibia
Proximal placement of implant progressing distally: A,B,S,C,D,M,E,F
Left Tibia
Proximal placement of implant progressing distally: A,B,S,C,M,E,F,N

Right Ulna
Proximal placement of implant progressing distally: A,C,S,D,M (M= subcutanous application of Cerasorb)
Left Ulna
Proximal placement of implant progressing distally: B,C,S,F,M

Pig No IV, weight 18kg and an experiment period of 52 days
Operation: 5.6.2007 under antibiotic prophylaxis of Unasyn 1.5g i.v. applicated after the initiation of general anaesthesia.

In the right mandible we applied augmentation material Cerasorb around implant A.

- In the distal part of the left mandible we made two artificial holes. The most distal hole was filled with Dentalgel collagen resorb and covered by Biocollagen membrane and the other hole filled by Biogen and covered by Bicollagen membrane.

- In the distal part of right tibia we inserted a nanoimplant

- In the distal part of the right tibia we made two artificial holes. The most distal hole was kept empty as a control and covered by Biocollagen membrane with the other hole filled by Biogen and covered by Biocollagen membrane.

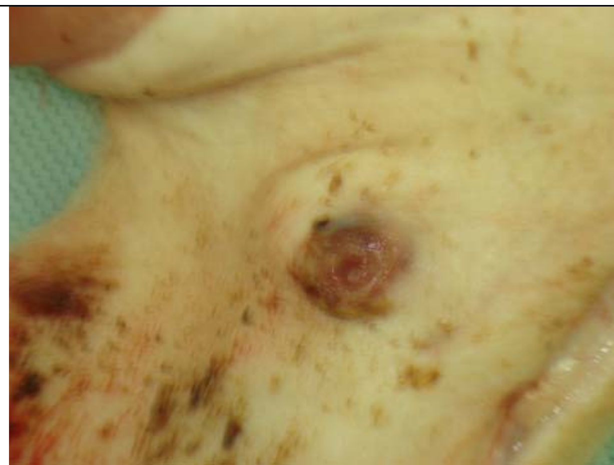
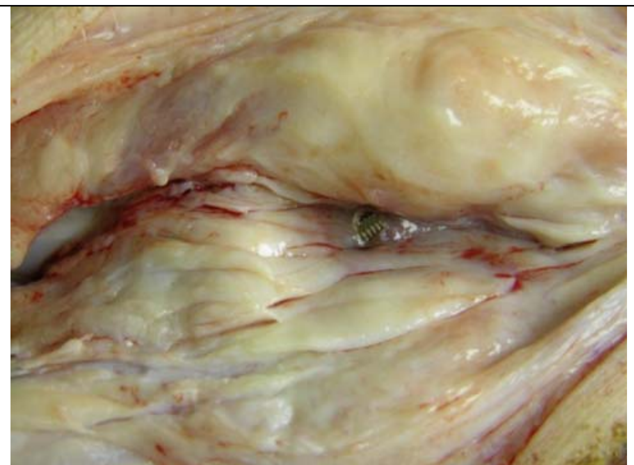
- In the distal part of left tibia we inserted a Nano implant

- In the distal part of the left tibia we made two artificial holes. The most distal hole kept empty as a control hole and covered by Biocollagen membrane and the other hole filled by Cerasorb granules 500-1000µm and covered by Biocollagen membrane.

- In the subcutaneus region of the right ulna we applied augmentaion material Biogen.

21.6.2007: application of antibiotic Tetracycline (20 mg/kg, 100 mg/1ml) 6,3ml i.m. at the weight of 30kg.
The 2nd dose 7.0 ml in 26.7. 2007 at the weight of 50.0kg.
During the application of TTC the soft tissues of the left leg was injured. The wound was treated by placing a suture provided under local anaesthesia.

Termination:
27.7.2007, weight 52.5kg
Right tibia was excluded due to the inflammation.

 **Fig 77. Cutaneous fistula of tibia due to inflammation.**	 **Fig 78. Rejected implant due to inflammation.**

Pig No 4
05.06.07
18 kg

Right Mandible
Distal placement of implant progressing mesially: A (around Cerasorb 500-1000),B,S,C,M
Left Mandible
Distal placement of implant progressing mesially: A,B,S,C,M,Hole Dentalgel collagen res + Bioc, Hole Biogen + Biocollagen mem

Right Tibia
Proximal placement of implant progressing distally: A,B,S,D,M,E,F,N, Hole Biogen + Biocollagen membrane, Hole Control + Biocollagen membrane
Left Tibia
Proximal placement of implant progressing distally: B,C,S,F,M,E,F,N, Hole Cerasorb+Biocoll.mem, Hole Control+Biocoll.mem

Right Ulna
Proximal placement of implant progressing distally: A,C,S (subcutanous application of Biogen),D,M
Left Ulna
Proximal placement of implant progressing distally: B,C,S,F,M

Excluded: right tibia p4

Radiographic Results and Discussion

These images were taken after the sacrifice of the animals.

The images taken, reveal that opposition of bone occurred above the implant head, where the largest part of the implant remained inside the bone marrow cavity due to the osteoclastic activity of osteoclasts. This lead to resorption of bone at the distal part of implants.

Osteoblastic activity lead to opposition of bone at the proximal part of implants. However since the primary stability of the implants at the time of operation were good, this can be explained by utilization of young animals for these experiments.

Due to the rapid growth of the animals, a thick layer of bone formed above the level of the implants.
The bone which the implants were inserted into at the time of the operation resorbed during the growth of the animals. Due to this problem we decided to evaluate osteointegration by focusing on the proximal part of the implants which were engaged with bone.
For performing a similar experiment, the author recommends the use of older animals or miniature pigs that have a slower rate of growth.

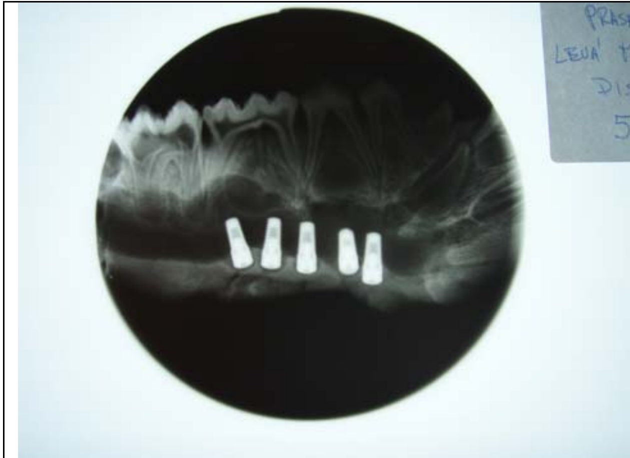


Fig 79. Pig 1, left mandible.

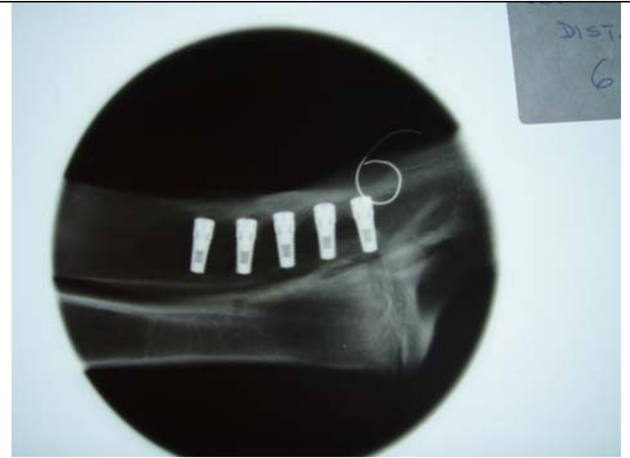


Fig 80. Pig 1, left ulna.

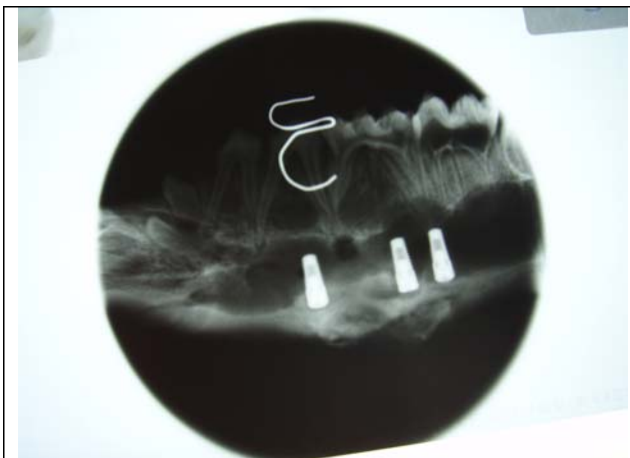


Fig 81. Pig 1, right mandible.

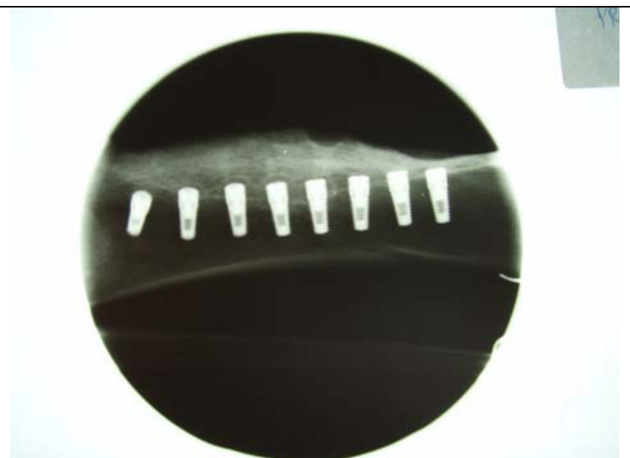


Fig 82. Pig 1, right tibia.

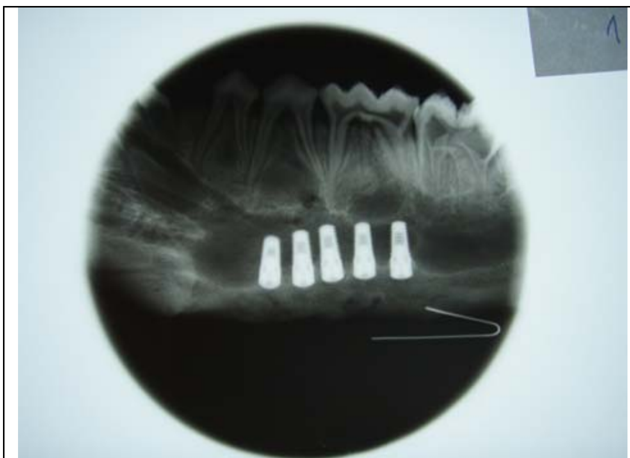


Fig 83. Pig 2, left mandible.

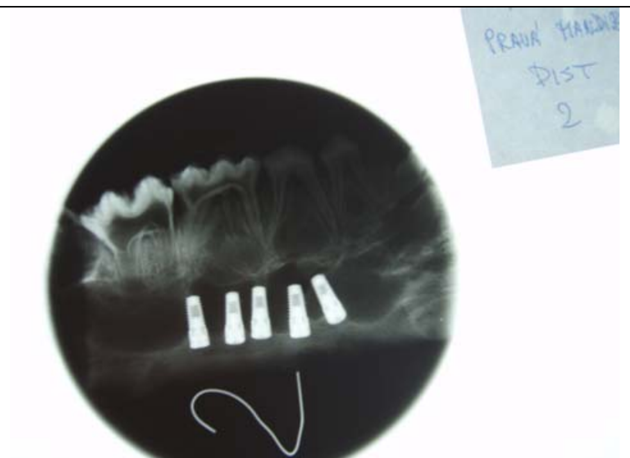


Fig 84. Pig 2, right mandible.

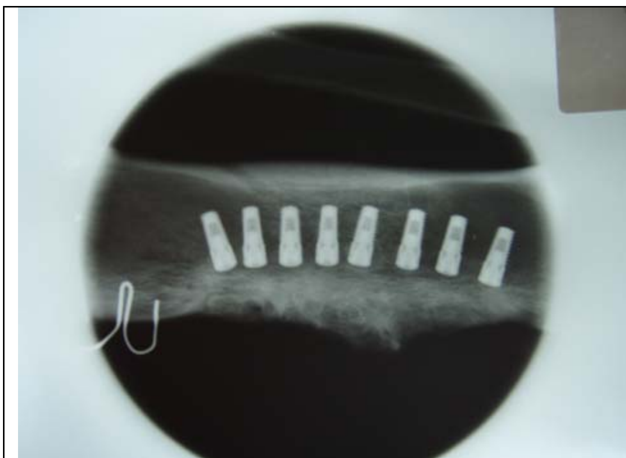


Fig 85. Pig 2, right tibia.

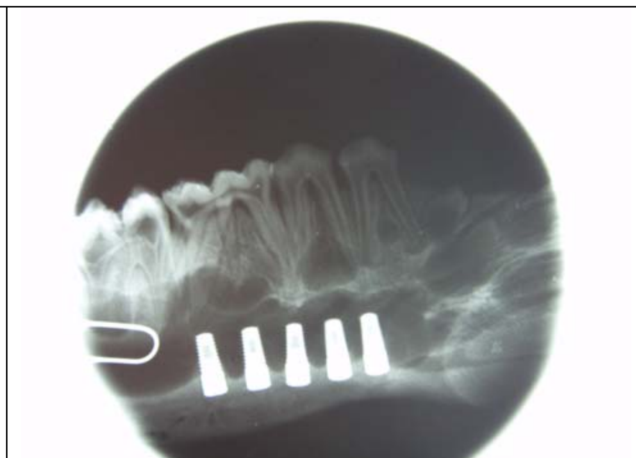


Fig 86. Pig 3, left mandible.

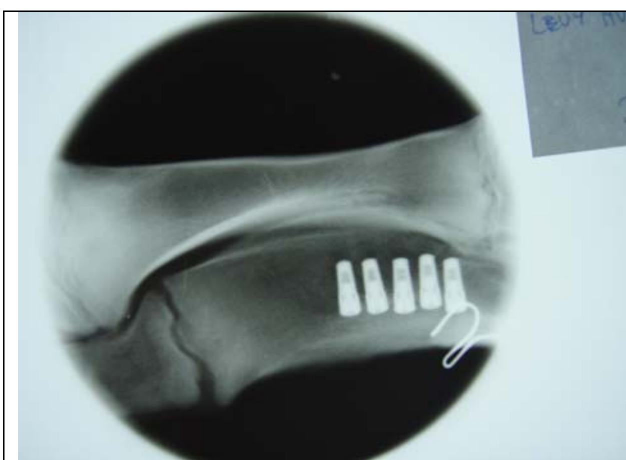


Fig 87. Pig 3, left ulna.

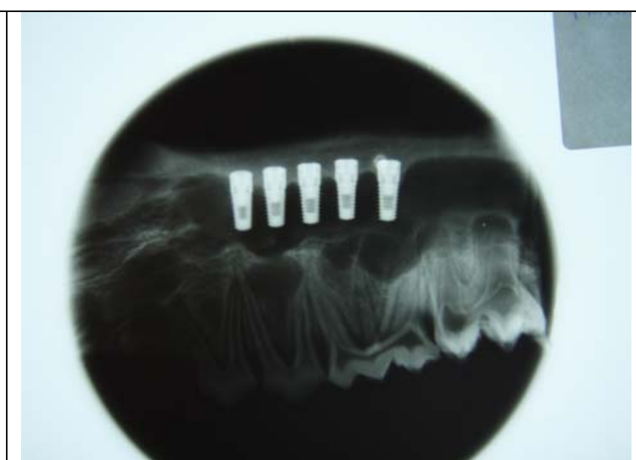


Fig 88. Pig 3, right mandible.

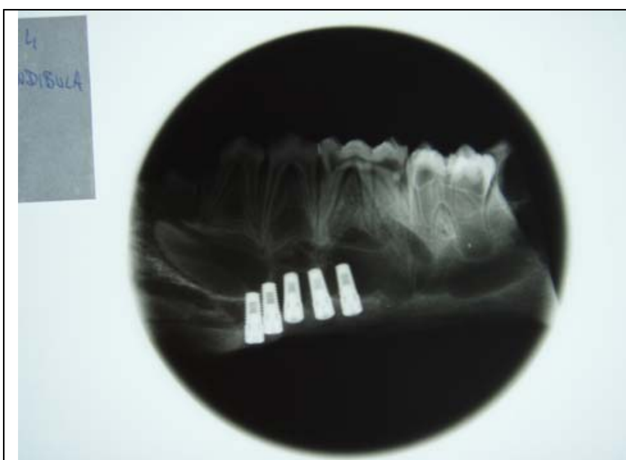


Fig 89. Pig 4, left mandible.

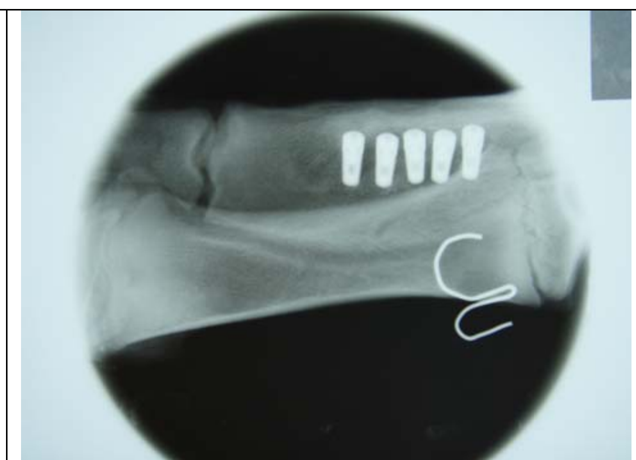


Fig 90. Pig 4, right ulna.

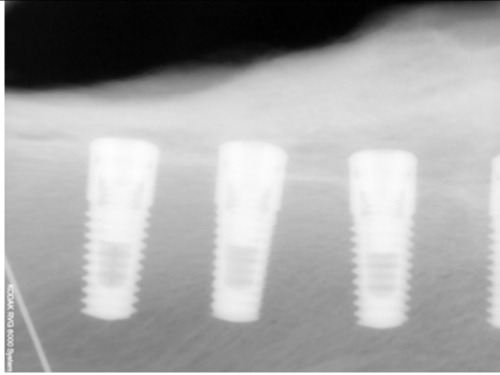


Fig 91. Pig 1, right tibia.

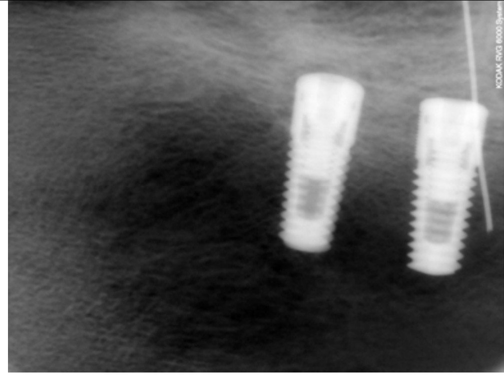


Fig 92. Pig 1, left tibia.

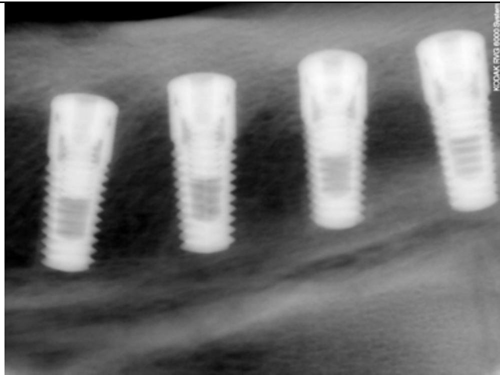


Fig 93. Pig 1, left ulna.

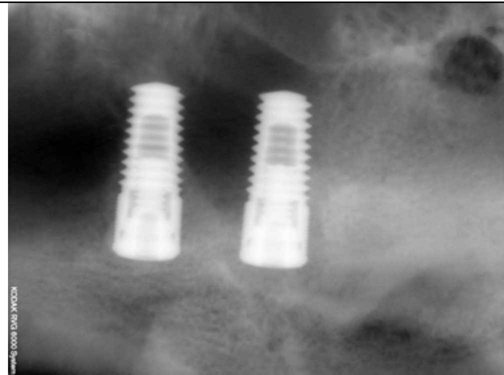


Fig 94. Pig 1, right mandible.

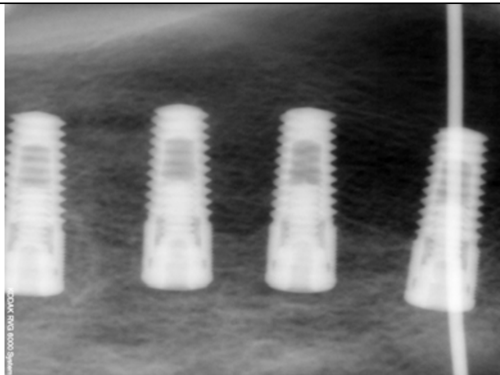


Fig 95. Pig 2, right tibia.

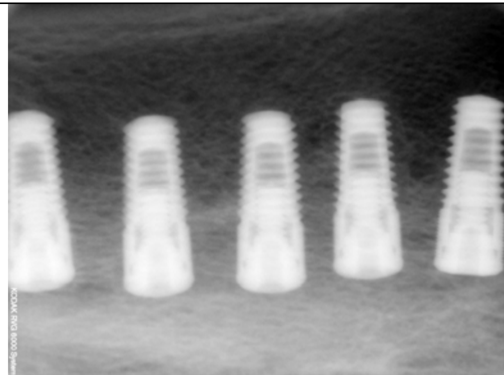
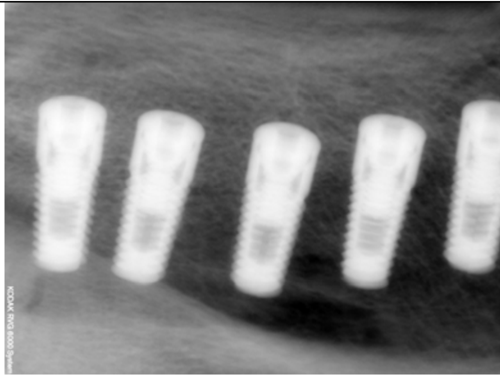
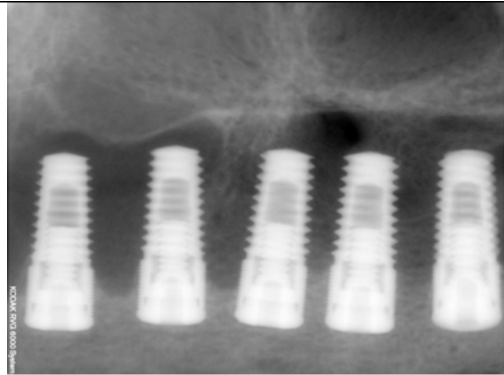
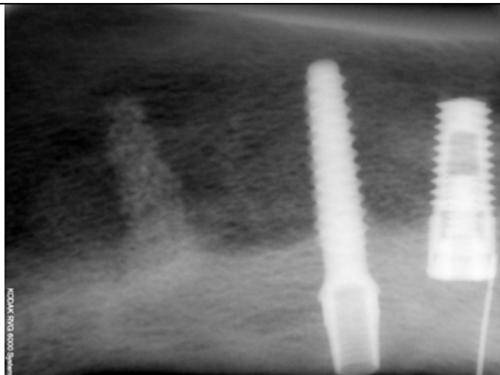
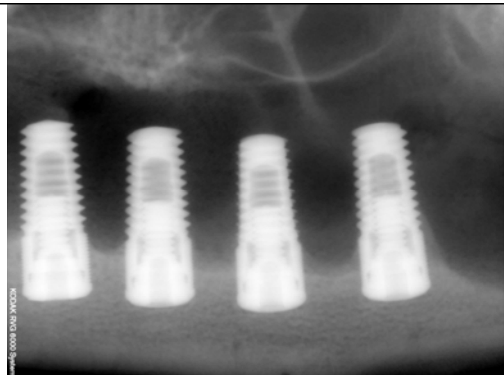
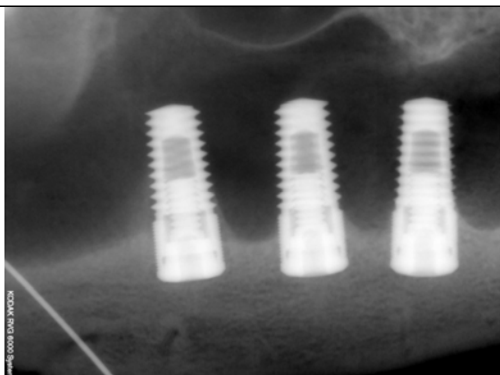
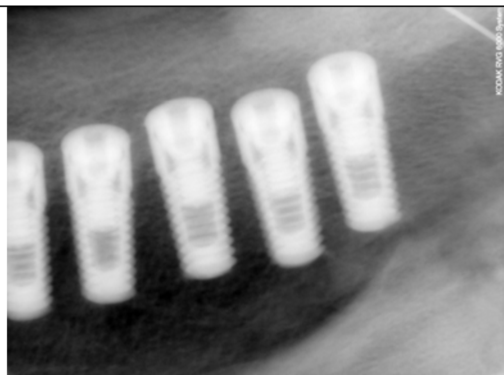


Fig 96. Pig 2, left tibia.

 **Fig 97. Pig 2, left ulna.**	 **Fig 98. Pig 2, left mandible.**

 **Fig 99. Pig 3, left tibia.**	 **Fig 100. Pig 3, right mandible.**
 **Fig 101. Pig 3, left mandible.**	 **Fig 102. Pig 3, left ulna.**

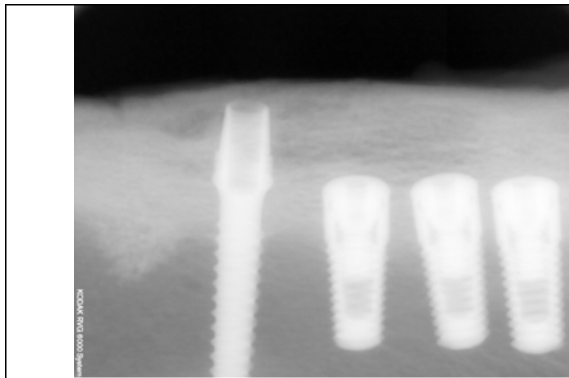
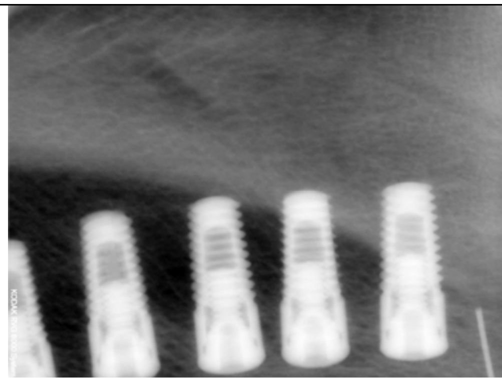
	

Fig 103. Pig 4, left tibia.	**Fig 104. Pig 4, left ulna.**

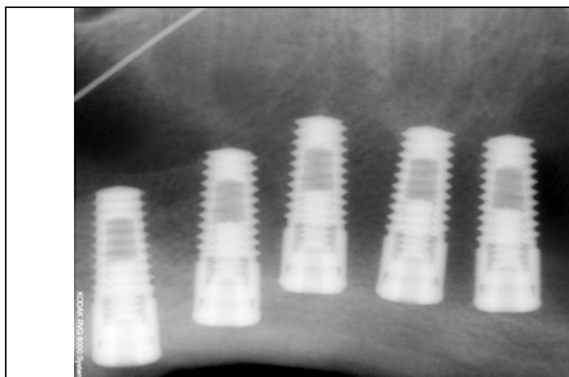
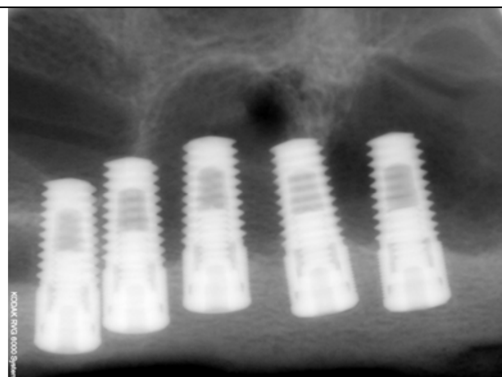
	

Fig 105. Pig 4, right mandible.	**Fig 106. Pig 4, left mandible.**

3.9 Protocol – Bone processing for eventual histological analysis

1. Embedding in Epon 812

Fig 107. The Chemicals Used.	**Fig 108. Potting embedding media + Marking the samples.**

Fig 109. Embedding media in marked thin-walled flat-based containers.

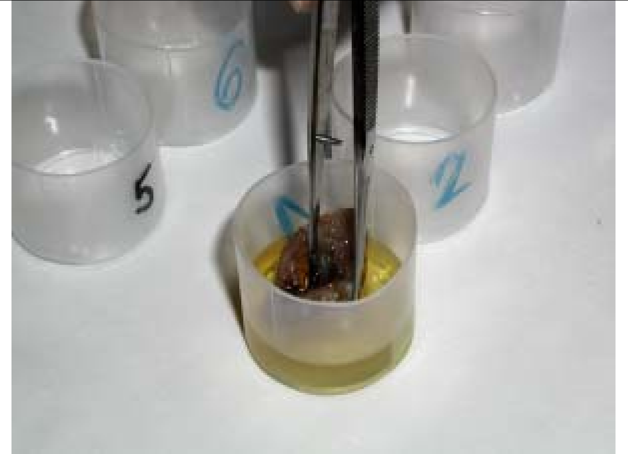


Fig 110. Embedding of the specimen.

Fig 111. Curing process first at 37°C, then at 60°C for 3 to 5 days.

Fig 112. Cured blocks ready for cutting.

2. Cutting and grinding

Fig 113. Samples ready for cutting.

Fig 114. Sectioning a bone-implant specimen to the thickness of 1-2 mm using a metal saw.

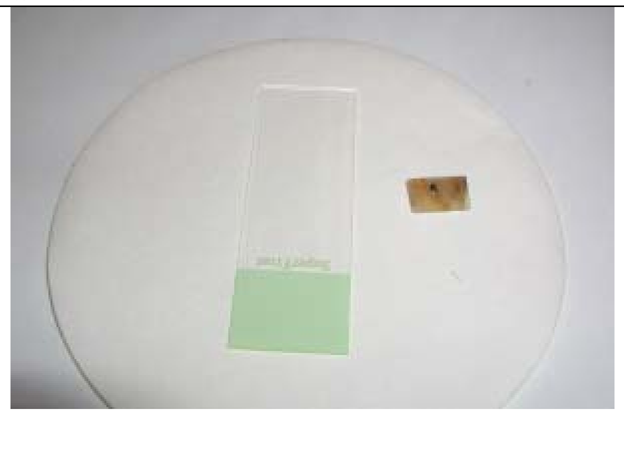
Fig 115. Section of bone-implant, ready for grinding.	**Fig 116. Grinding of bone-implant section with sandpaper from lower to higher grain size (Grain: 100, 200, 240, 800, 1200, and 2000).**
Fig 117. Manual grinding of bone-implant section.	**Fig 118. During grinding with sandpaper with the 1200 and 2000 grain, it is better to moisten sample with water.**
	
Fig 119. During grinding with sandpaper with 1200 and 2000 grain, pressure is applied very gently to avoid formation of grooves on the sample surface. After finishing grinding, the section is rinsed with water and left to dry.	**Fig 120. Bone-implant section is ready for placing on the surface of the slide.**

Fig 121. On the underlay surface of the slide, and on the polished surface of the prepared section Solacryl is placed (it is possible to use superglue.) This causes the polished surface of the section to stick to the underlay surface of the slide.

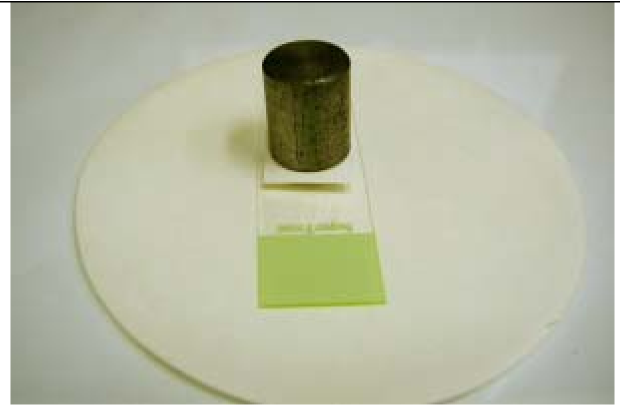


Fig 122. The glued on section has a weight load applied for about 12 hours. After hardening of Solacryl, it is possible to grind the section from the other side. (in the case of using superglue it is possible to grind immediately).

Fig 123. The glued on bone-implant section is ready for grinding.

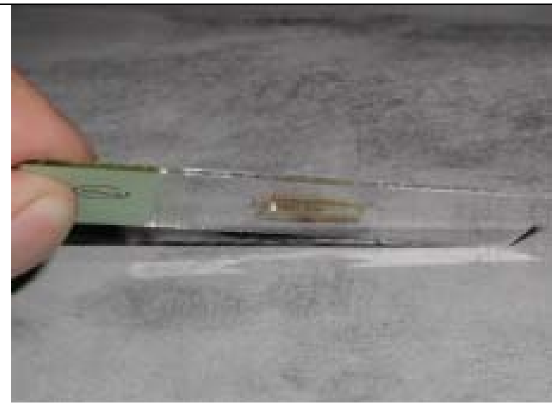


Fig 124. Grinding of glued section from nonpolished side. Grinding is done by sandpaper from lower to higher grain size (Grain: 100, 200, 240, 800, 1200, 2000). The same as the other side of section.

Fig 125. Grinding of the glued bone-implant section to the slide by sandpaper.

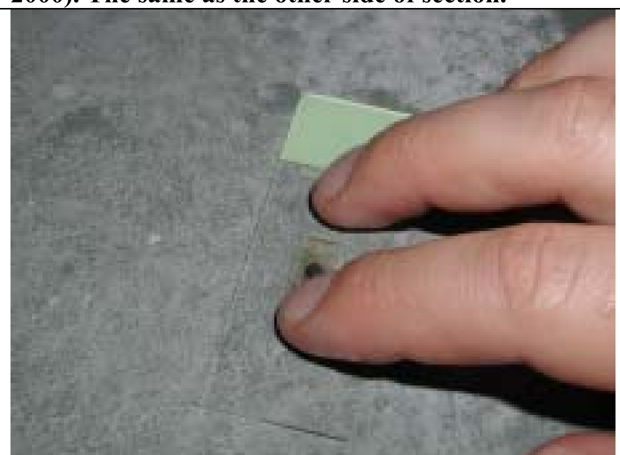


Fig 126. During grinding with sandpaper with 1200 and 2000 grain, pressure is applied very gently to avoid formation of grooves on the sample surface. After finishing grinding, the section is rinsed with water and left to dry.

3. Staining and Mounting

Fig 127. Staining – see the protocol for staining with Alizarin red and toluidine blue.	**Fig 128. Staining.**
Fig 129. Stained and dried preparation is covered by Solacryl.	**Fig 130. Application of cover glass (mount).**
Fig 131. Apply cover glass on the slide.	**Fig 132. After mounting, a small weight load is placed on top of the cover glass for one day while the Solacryl dries and hardens, in order to keep the section flat. (This prevents distortion of the preparation).**

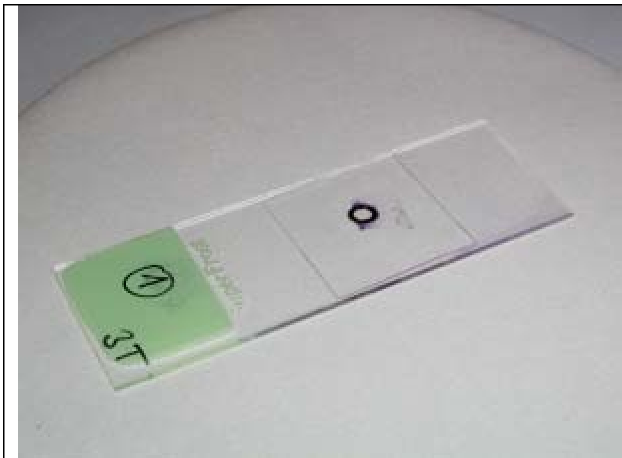


Fig 133a. Preparation is ready for light microscope.	**Fig 133b. Preparation is ready for light microscope.**

3.10 Protocol – Staining Methacrylate- embedded bone with Alizarin red and toluidine blue

(Procedure is according to Dr. Kirsti Winter, Veterinary Medical University in Vienna).

Solutions:

Alizarin red: 0.1% solution in 0.5% solution of NaOH

Toluidine blue:	borax	1g
	toluidine blue	1g
	distilled water	100ml

The borax makes the stain alkaline so it will penetrate the epoxy. It dissolves in the water resulting in a final pH of solution of 11. This is then mixed and added to toluidine blue. Thereafter we filter the solution.

Method:

Staining takes place at room temperature.

1. Alizarin red (30min – 60min).
2. Rinse in distilled water
3. Toluidine blue (10min – 20min).
4. Rinse in distilled water
5. Blot dry and leave in drying oven for about 15min.

Results:

Mineralised bone – red-pink/pink-purple
Osteoid – bright blue
Nuclei – Dark blue

3.11 Microphotographic results obtained by light microscope

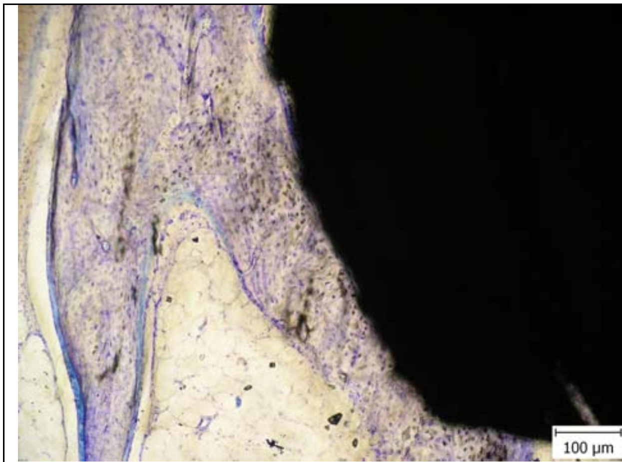


Fig 134. Histological view of implant E, left ulna, pig 1 (Transverse section).

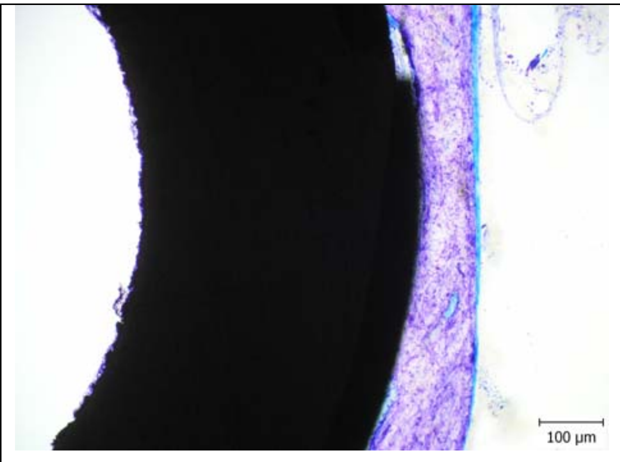


Fig 135. Implant E, left ulna, pig 1 (Transverse section).

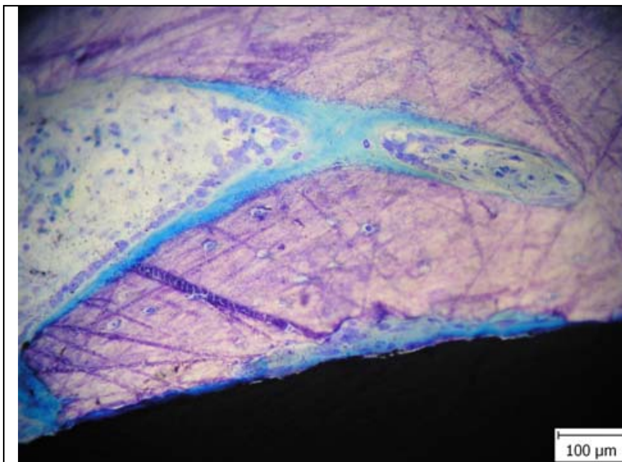


Fig 136. Implant S, left ulna, pig 1 (Transverse section).

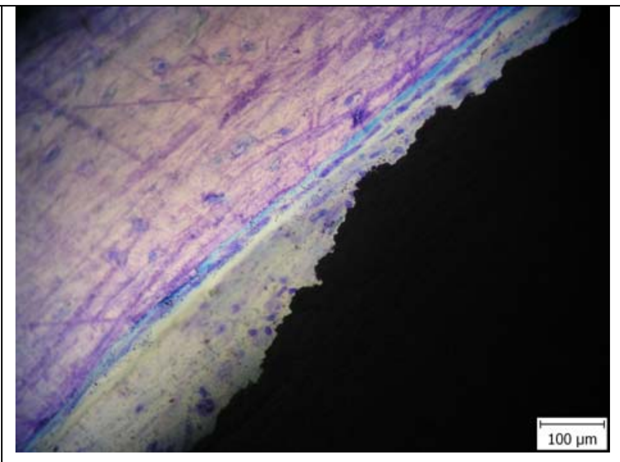


Fig 137. Implant S, left ulna, pig 1 (Transverse section).

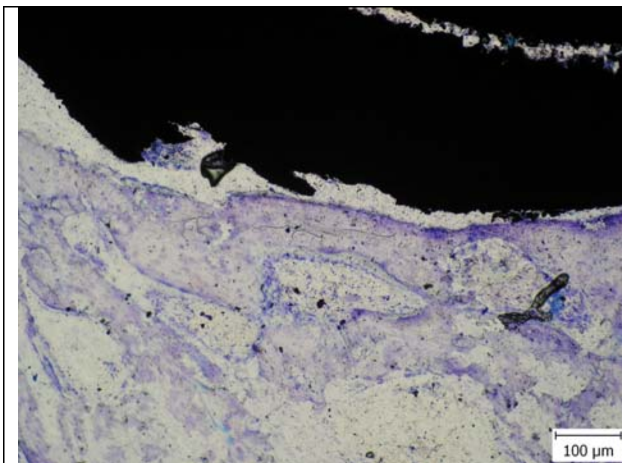


Fig 138. Implant A, left tibia, pig 1 (Transverse section).

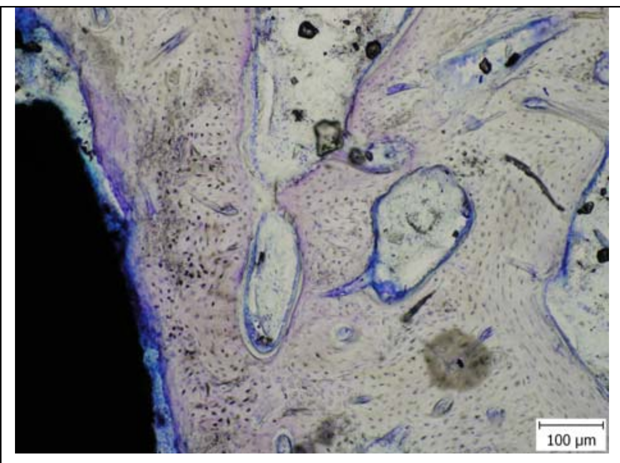


Fig 139. Implant B, left tibia, pig 1 (Longitudinal section).

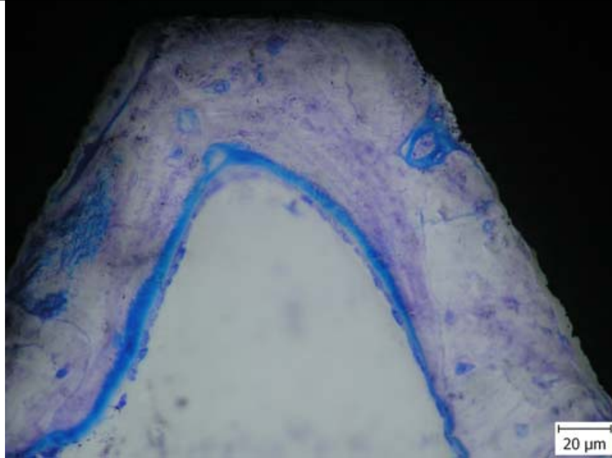


Fig 140. Implant C, left tibia, pig 1 (osteointegration between two threads - longitudinal section).

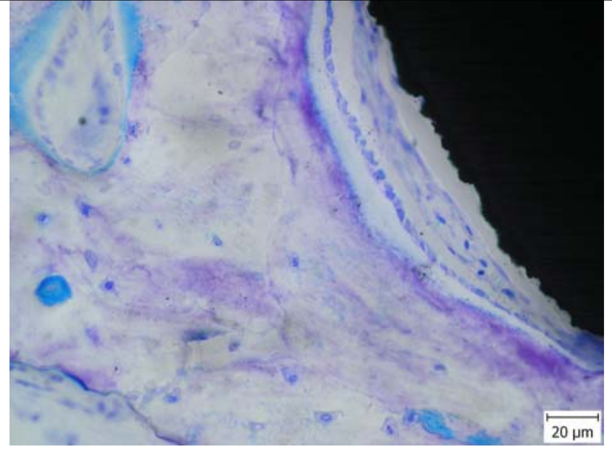


Fig 141. Implant C, left tibia, pig 1 (longitudinal section).

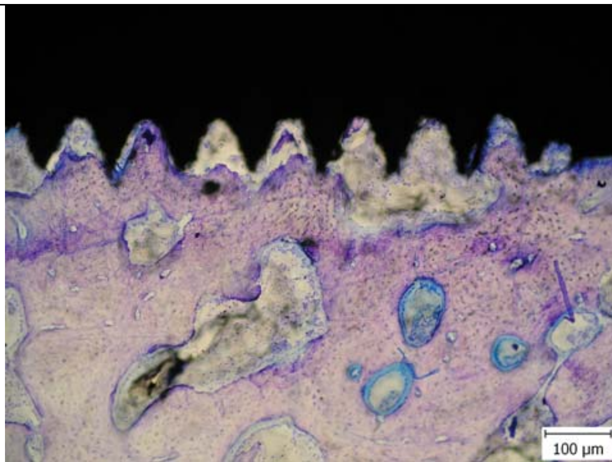


Fig 142. Implant C, left ulna, pig 3 (longitudinal section).

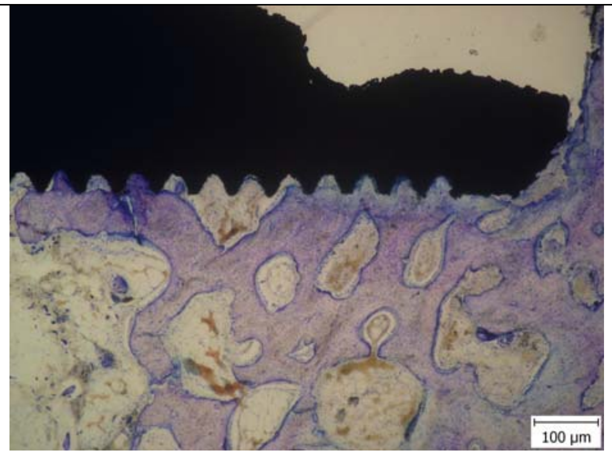


Fig 143. Implant S, left ulna, pig 3 (longitudinal section).

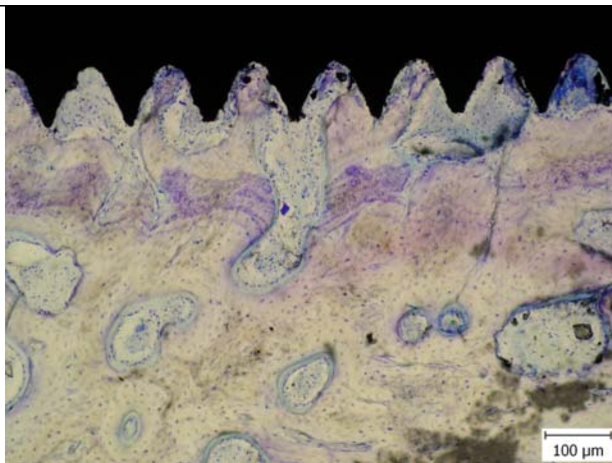


Fig 144. Implant A, right ulna, pig 3 (longitudinal section).

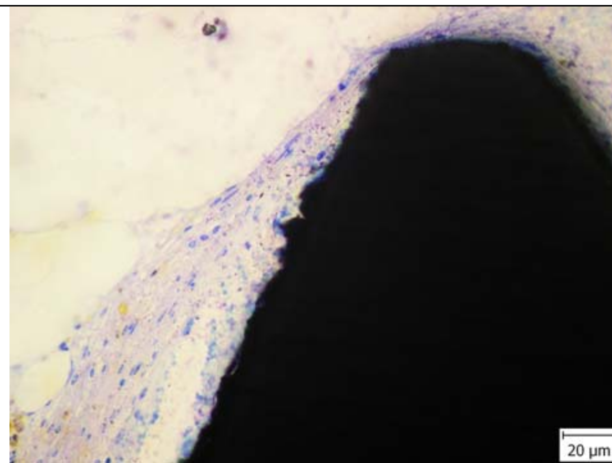


Fig 145. Implant A, right ulna, pig 3 (longitudinal section).

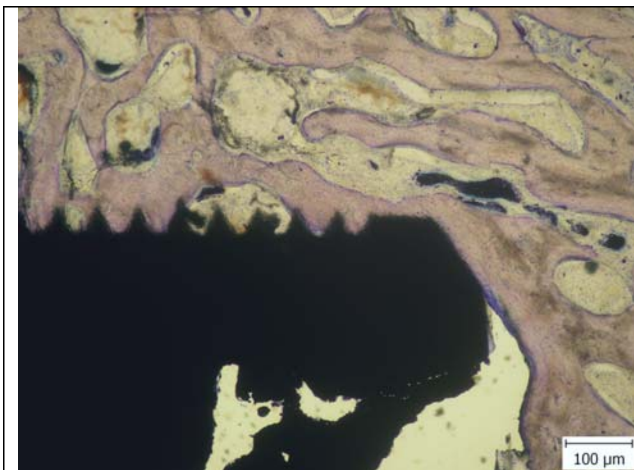


Fig 146. Implant D, right ulna, pig 3 (longitudinal section).

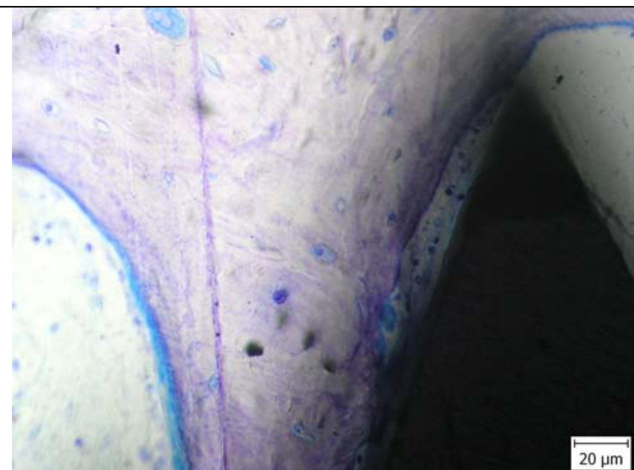


Fig 147. Implant D, right ulna, pig 3 (longitudinal section).

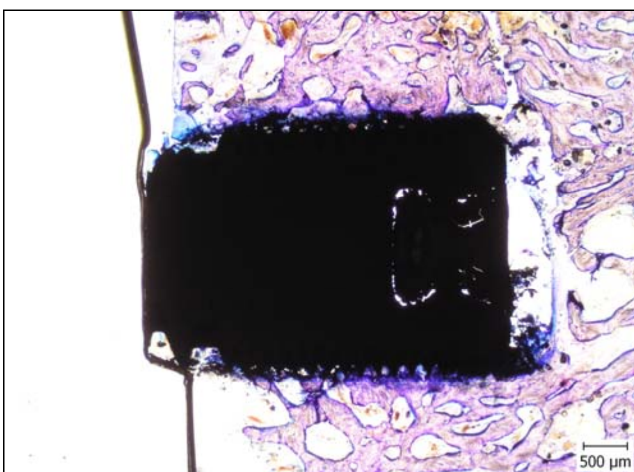


Fig 148. Implant M, right ulna, pig 3 (longitudinal section).

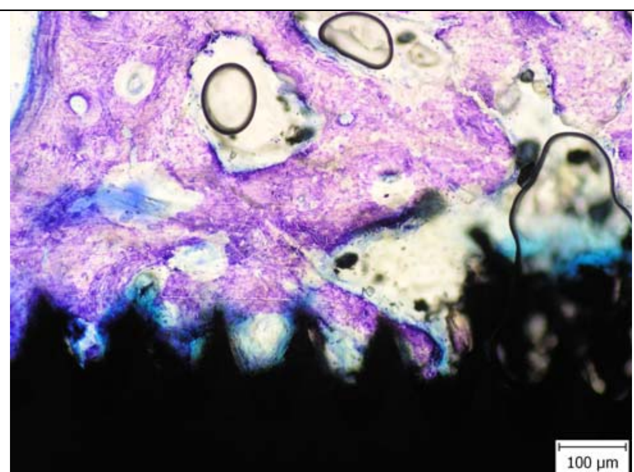


Fig 149. Implant M, left mandible, pig 3 (longitudinal section).

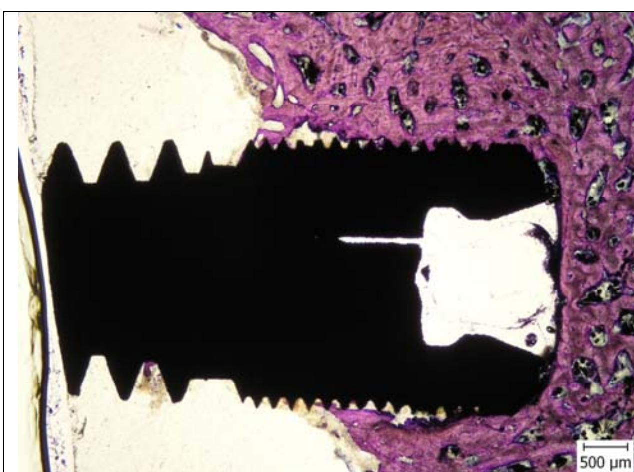


Fig 150. Implant D, left mandible, pig 3 (longitudinal section).

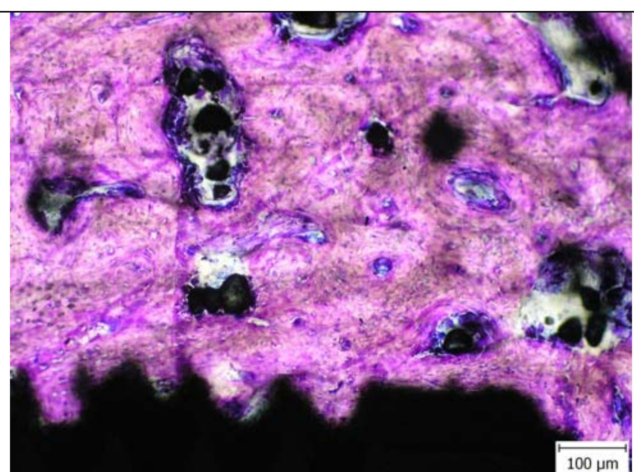


Fig 151. Implant D, left mandible, pig 3 (longitudinal section).

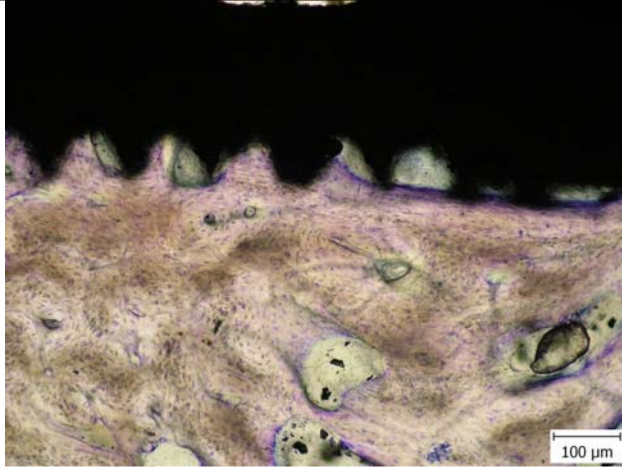


Fig 152. Implant C, left mandible, pig 3 (longitudinal section).

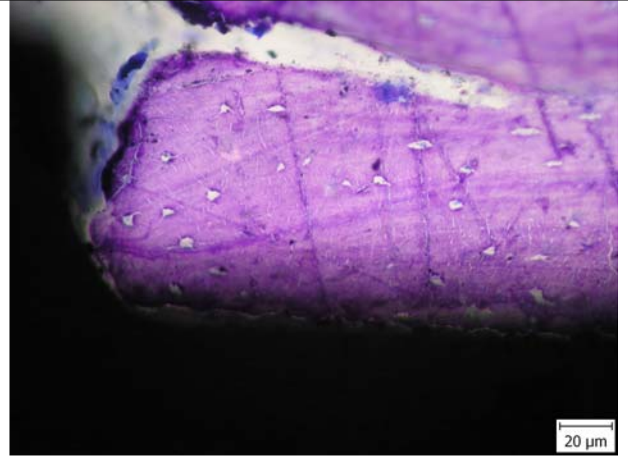


Fig 153. Implant C, left mandible, pig 3 (longitudinal section).

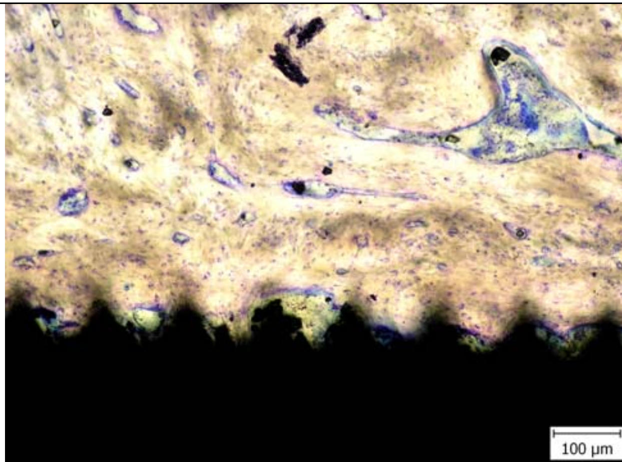


Fig 154. Implant A, left mandible, pig 3 (longitudinal section).

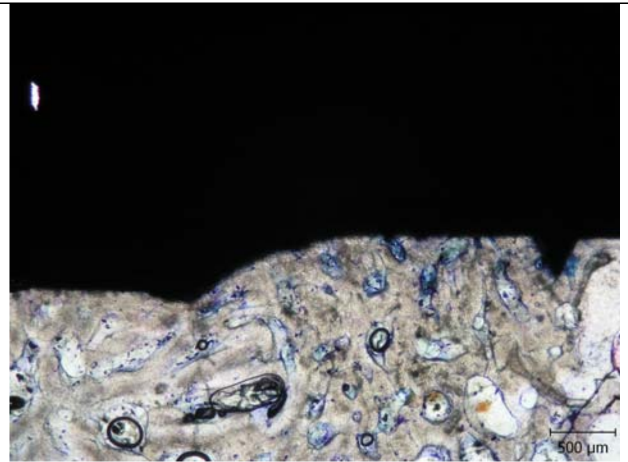


Fig 155. Nanoimplant, left tibia, pig 4 (longitudinal section).

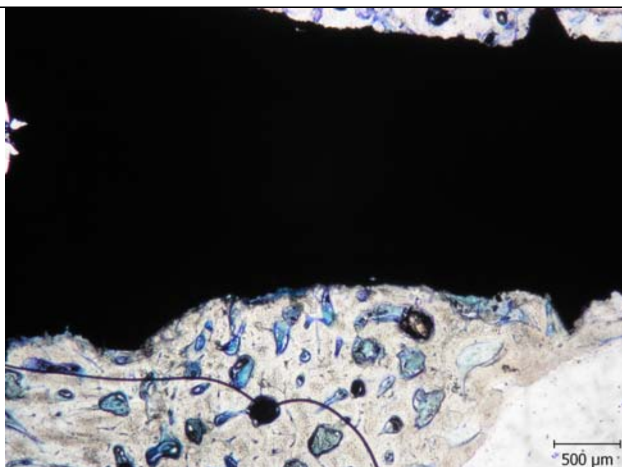


Fig 156. Nanoimplant, left tibia, pig 4 (longitudinal section).

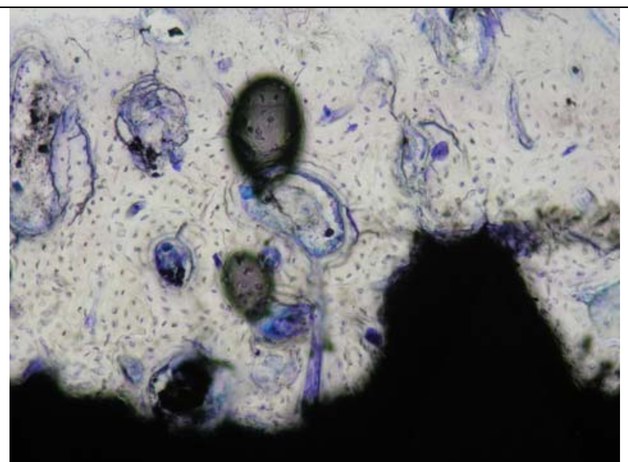


Fig 157. Same image as in figure 156 but with a higher magnification.

3.12 Microphotographic results obtained by confocal laser scanning microscopy (CLSM or LSCM) as well as an introduction to this concept

Introduction

This is a technique for obtaining high-resolution optical images. The key feature of confocal microscopy is its ability to produce in-focus images of thick specimens, a process known as *optical sectioning*. Images are acquired point-by-point and reconstructed with a computer, allowing three-dimensional reconstructions of topologically-complex objects. The principle of confocal microscopy was originally patented by Marvin Minsky in 1957, but it took another thirty years and the development of lasers for CLSM to become a standard technique toward the end of the 1980's.

<u>Principle of confocal microscopy.</u>

In a conventional widefield microscope, the entire specimen is bathed in light from a mercury or xenon source, and the image can be viewed directly by eye or projected onto an image capture device or photographic film. In contrast, the method of image formation in a confocal microscope is fundamentally different. Illumination is achieved by scanning one or more focused beams of light, usually from a laser or arc-discharge source, across the specimen. This point of illumination is brought to focus in the specimen by the objective lens, and laterally scanned using some form of scanning device under computer control. The sequences of points of light from the specimen are detected by a photomultiplier tube (PMT) through a pinhole (or in some cases, a slit), and the output from the PMT is built into an image and displayed by the computer. Although unstained specimens can be viewed using light reflected back from the specimen, they usually are labeled with one or more fluorescent probes. The following is a more detailed description of how a confocal microscope works:

A laser is used to provide the excitation light (in order to get very high intensities). The laser light reflects off a dichroic mirror. From there, the laser hits two mirrors which are mounted on motors; these mirrors scan the laser across the sample. Dye in the sample fluoresces, and the emitted light gets descanned by the same mirrors that are used to scan the excitation light from the laser. The emitted light passes through the dichroic and is focused onto the pinhole. The light that passes through the pinhole is measured by a detector, ie. a photomultiplier tube.

So, there never is a complete image of the sample at any given instant, only one point of the sample is observed. The detector is attached to a computer which builds up the image, one pixel at a time. In practice, this can be done perhaps 3 times a second, for a 512x512 pixel image. The limitation is in the scanning mirrors.

Confocal microscopy also provides a substantial improvement in lateral resolution and the capacity for direct, noninvasive, serial optical sectioning of intact, thick, living specimens with a minimum of sample preparation. Because CLSM depends on fluorescence, a sample usually needs to be treated with fluorescent dyes to make objects visible. However, the actual dye concentration can be low to minimize the disturbance of biological systems: some instruments can track single fluorescent molecules. Also, transgenic techniques can create organisms that produce their own fluorescent chimeric molecules (such as a fusion of GFP, green fluorescent protein with the protein of interest).

<u>What is the advantage of using a confocal microscope?</u>

By having a confocal pinhole, the microscope is really efficient at rejecting out of focus fluorescent light. The practical effect of this is that the image comes from a thin section of the sample (because of the small depth of field). By scanning many thin sections through your sample, you can build up a very clean three-dimensional image of the sample.

Also, a similar effect happens with points of light in the focal plane, but not at the focal point -- emitted light from these areas is blocked by the pinhole screen. So a confocal microscope has slightly higher resolution horizontally, as well as vertically. In practice, the best horizontal resolution of a confocal microscope is about 0.2 microns, and the best vertical resolution is about 0.5 microns.

Results

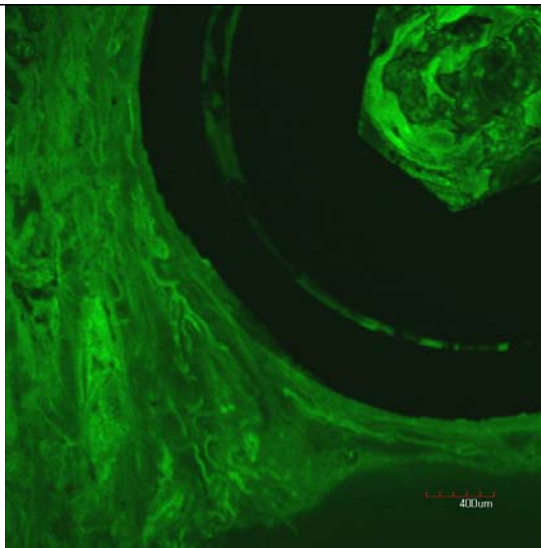


Figure 158. Implant M, left ulna, pig 1.

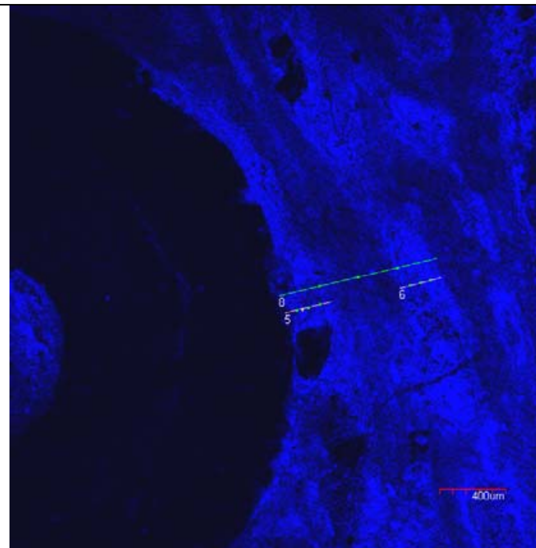


Fig 159. Implant M, left ulna, pig 1 (5=292µm), (6=257µm), (8=975µm).

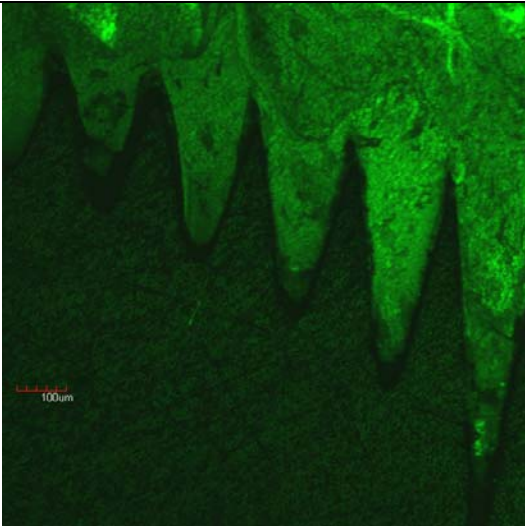


Fig 160. Implant D, left tibia, pig 1.

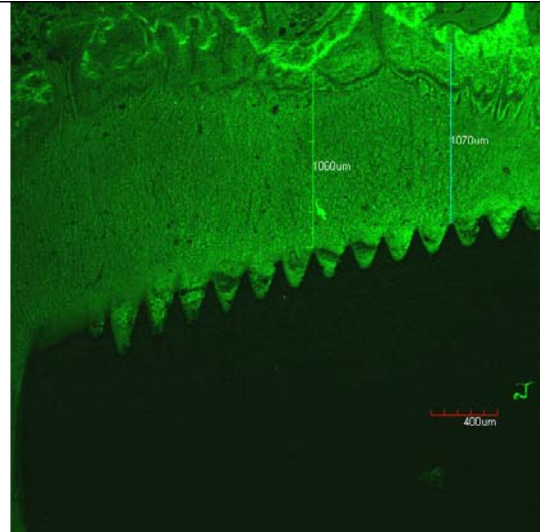


Fig 161. Implant E, left tibia, pig 1.

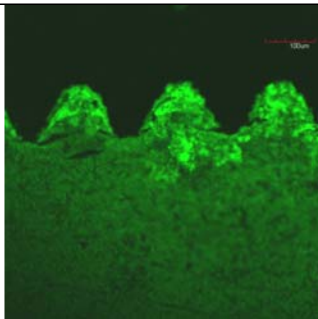


Fig 162. Implant S, left tibia, pig 1.

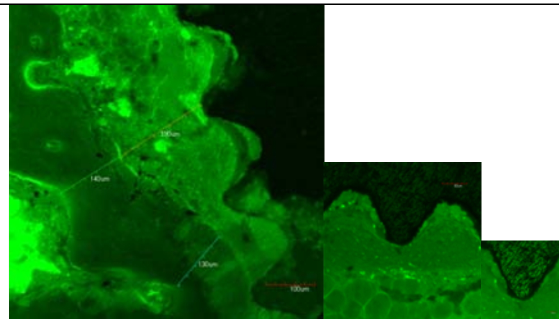


Figure 163. Implant S, left ulna, pig 3.

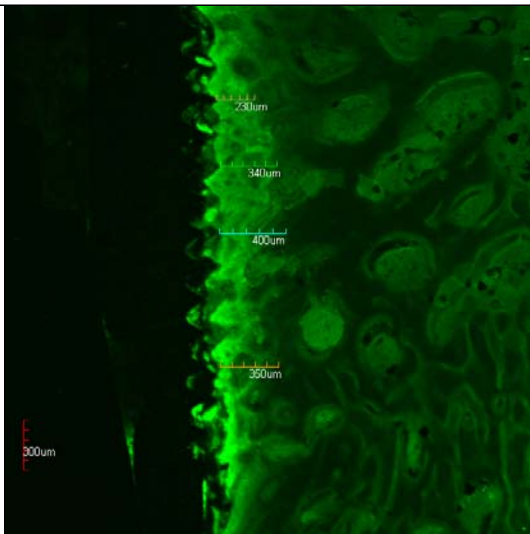


Fig 164. Implant C, left ulna, pig 3.

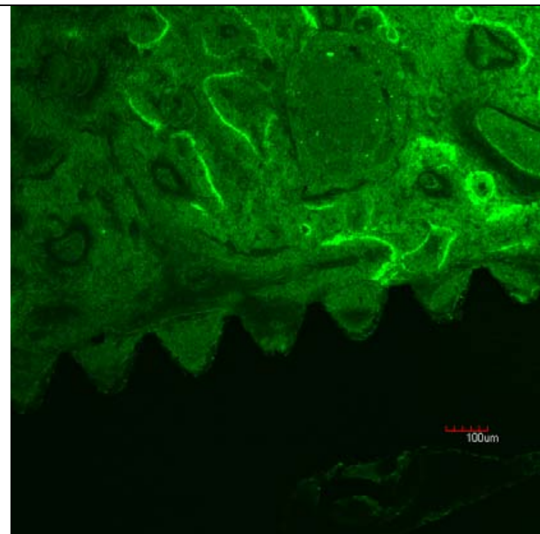


Fig 165. Implant M, left tibia, pig 1.

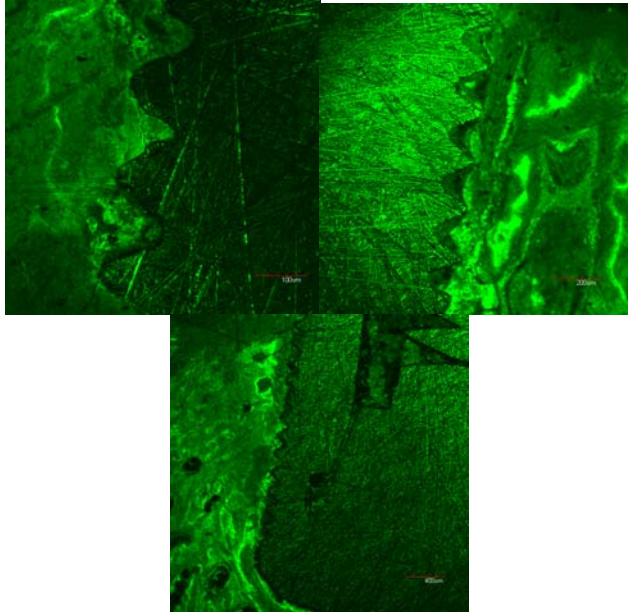


Fig 166. Implant D, left mandible, pig 3.

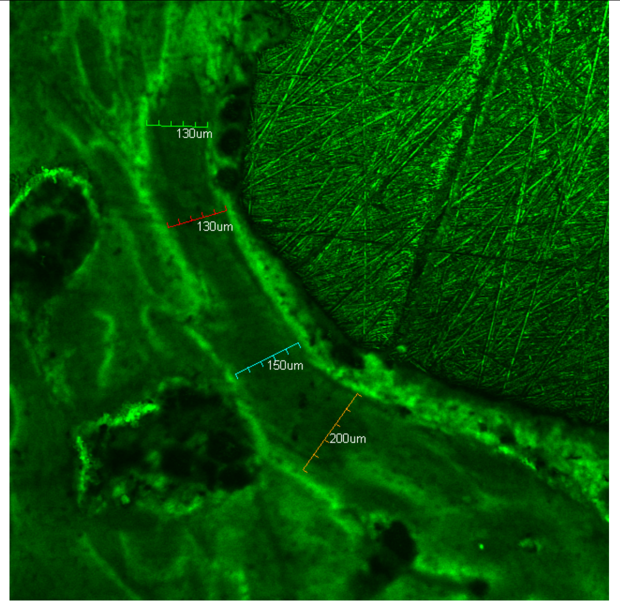


Fig 167. Implant D, left mandible, pig 3.

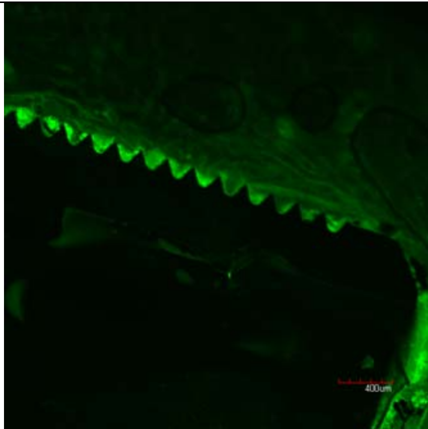


Fig 168. Implant A, left mandible, pig 3.

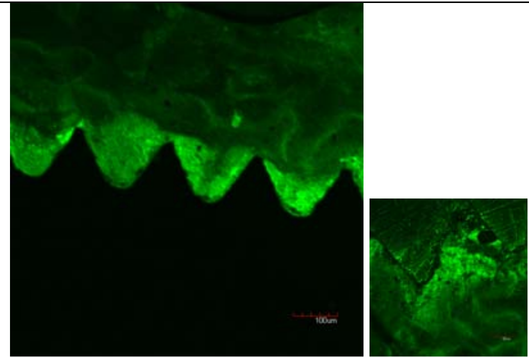


Fig 169. Implant A, left mandible, pig 3.

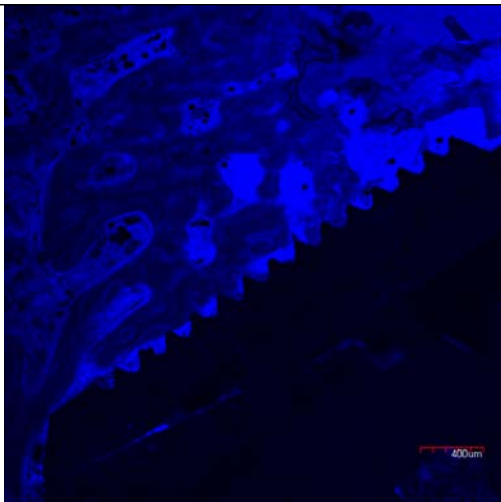


Fig 170. Implant S, left mandible, pig 3.

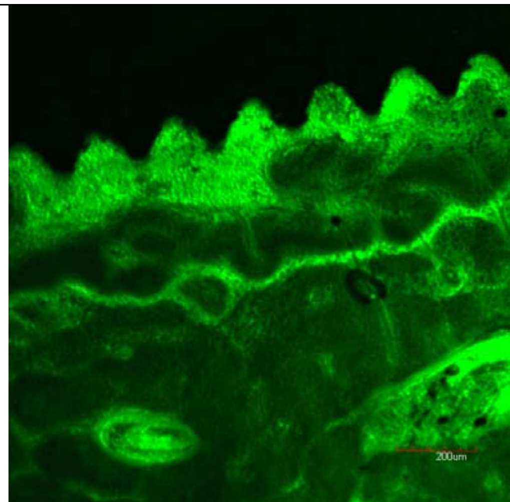


Fig 171. Implant S, left mandible, pig 3.

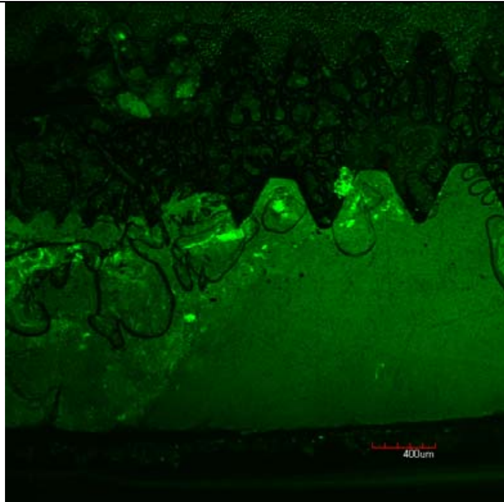


Fig 172. Implant M, left mandible, pig 3.

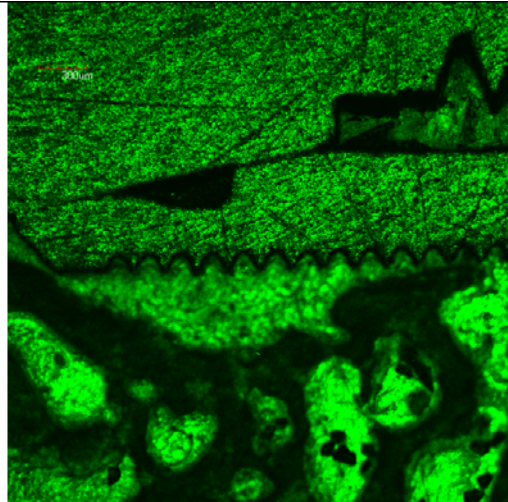


Fig 173. Implant S, right ulna, pig 1.

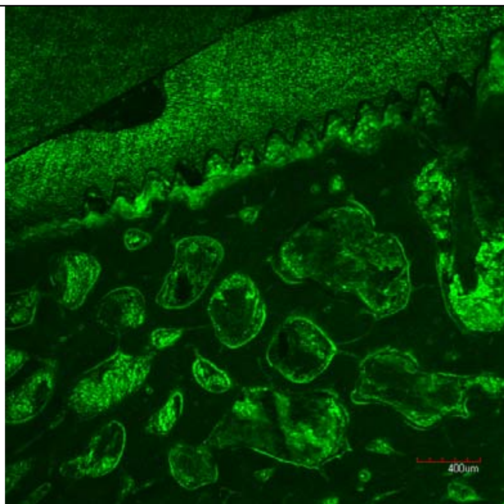


Fig 174. Implant A, right ulna, pig 1.

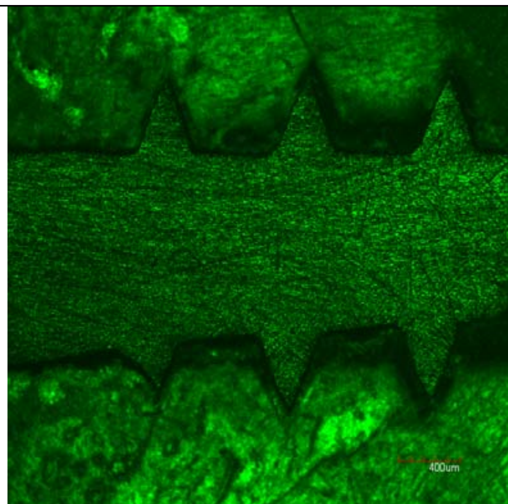


Fig 175. Nano implant, left tibia, pig 4.

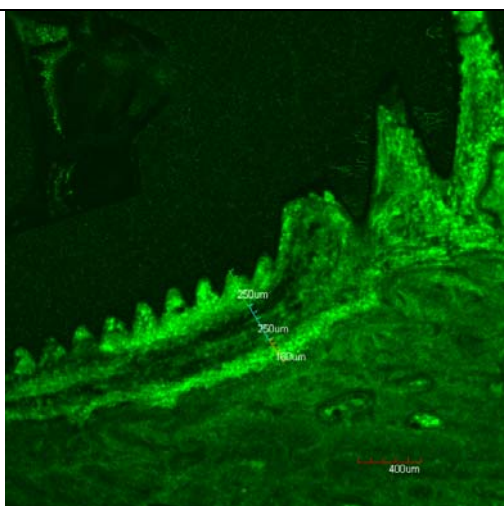


Fig 176. Implant E, right mandible, pig 1.

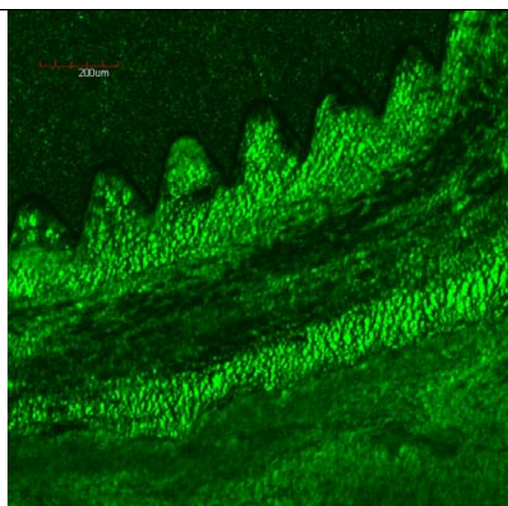


Fig 177. Two lines of tetracycline labelled bone.

3.13 Bone-Implant Contact (BIC):

Osteointegration is defined in histological implant studies as the direct contact between living bone and implant, on the level of a light microscope. Although this definition does not state the proportion of bone in contact with the implant surface, one of the most used variables in histomorphometric analysis of implants is the fraction of surface areas of mineralized bone in contact with the implant surface (BIC).

Implant ground sections in situ are relatively thick sections (20-40μm) and therefore only a few sections are obtainable from a bone-implant block. When serial sections of bone-implant specimens are cut exhaustively, a 'shadow effect' will occur at the peripheral section [147].

Histomorphometry is an established method to determine the extent of osteointegration and the rate of healing of dental implants. We can use this to measure the percentage of bone implant contact (BIC). Experimental trials on animal models revealed that implants with roughened surfaces had a better early anchorage in bone tissue and a higher percentage of BIC than implants with smooth surfaces [141,142]. Repeating the same experiments in human studies produced the same results.[143-146].

The successful clinical use of micro-rough titanium implant surfaces have laid the foundations for developing further surface topographies to promote enhanced peri-implant bone apposition, during the early stages of bone regeneration.

The aim of the present studies was to determine the effects of different surface treatments, through a histological evaluation of the implant-bone interface. In order to evaluate serial optical sections of the under-calcified ground sections and to reconstruct the three-dimensional images of the peri-implant bone tissue, labeled by tetracycline antibiotic (by setting the excitation wavelength at 405nm and emission wavelength at 519nm [148]), a confocal laser scanning microscope was used.

Histology and CLSM results, in most regions revealed, that there was a lack of direct connecting bridges between the peri-implant bony trabeculae and the implant surface. Only in a few regions, was the presence of the implant roots in close proximity to the bone visible. However, in other regions, bone appeared to end in a rather perpendicular orientation to the implant surface, and had started to grow in the interthread region, without coming into direct contact with the implant roots.

In the majority of these cases, peri-implant bone was not always continuous and rarely followed the entire perimeter of the threads.

3.14 Protocol for BIC measurement, in order to obtain results for the evaluation of osteointegration

This was done by the use of the programme Elipse produced by ViDiTo Košice org. module line system.

The BIC was evaluated in a rectangular network with each retangle being 75 x 75 px to 150 x 150 px.

The size of the network allows a great number of evaluation points from only one preparation. Stereologically there are 200 evaluation points for the 1 histological preparation. In our experiment this point refers to the meeting point between each rectangular network with the margin of the implant.

For our experiement we evaluated the meeting of network-margin of implant by the following formula:

Contact of network-Bone implant contact / network-margin of implant x 100

Or alternatively: **Bone/Implant x 100**

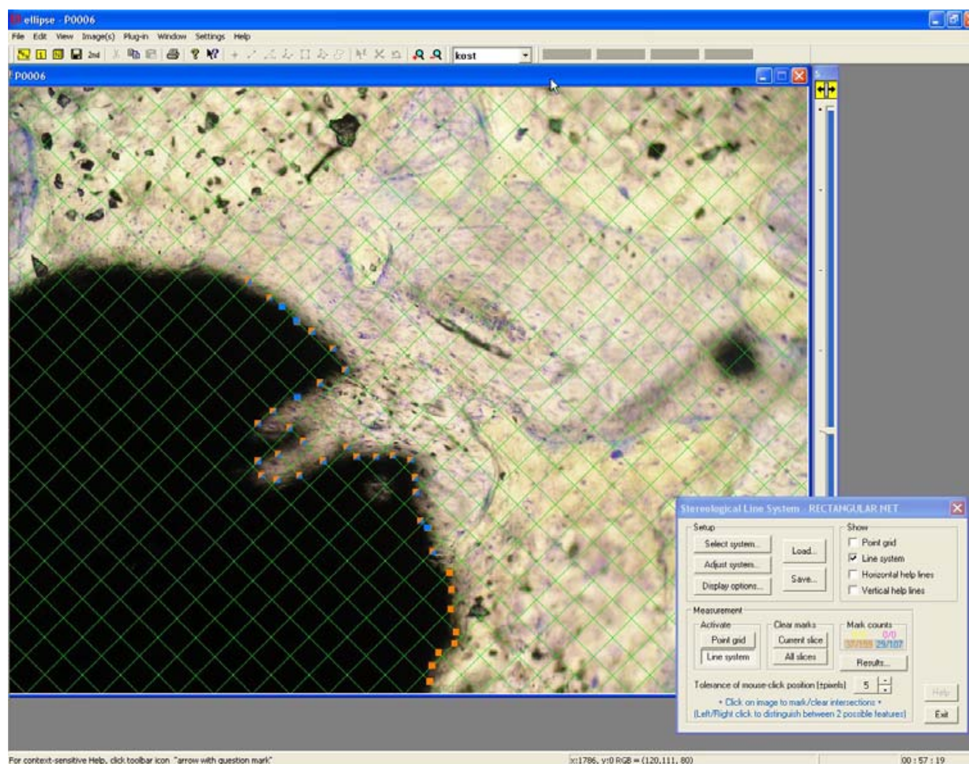


Fig 178. A Green rectangular network can be seen. The meeting point from which the formula can be used can be seen as small orange squares (margin of implant contact) and small blue squares (Bone contact with implant)

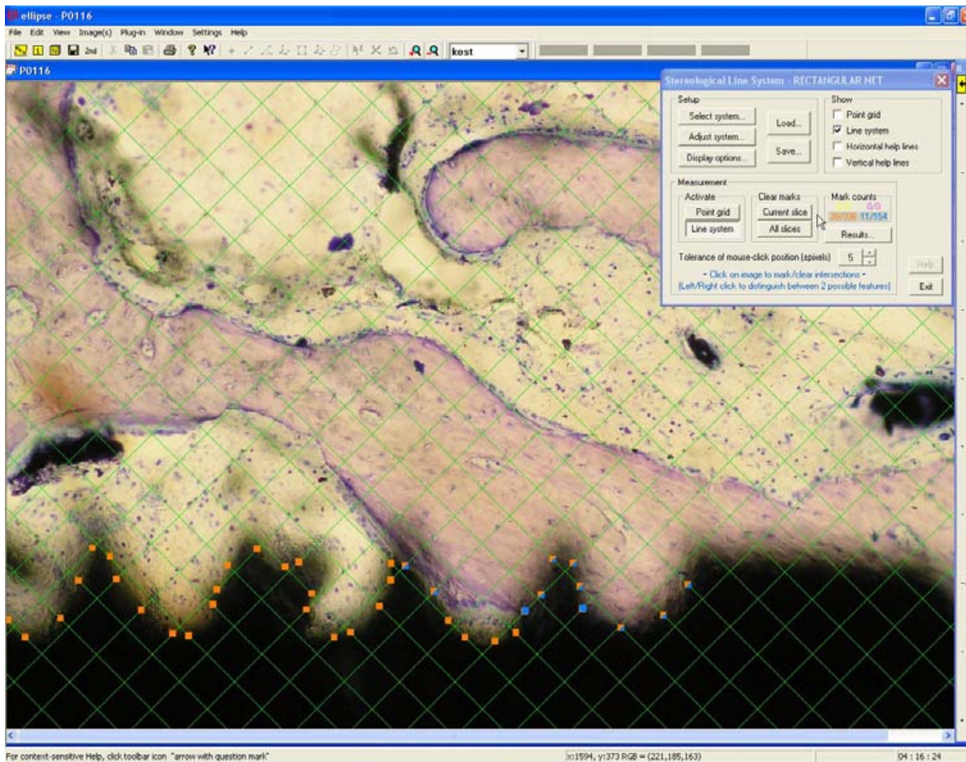


Fig 179. A higher magnification view of the evaluation points

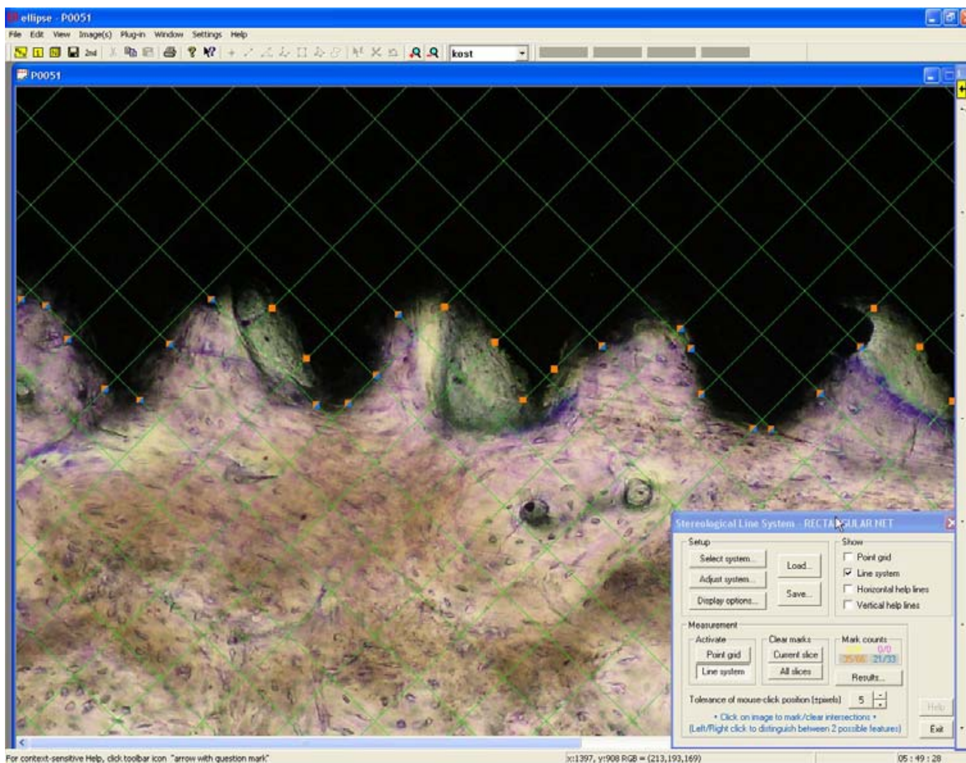
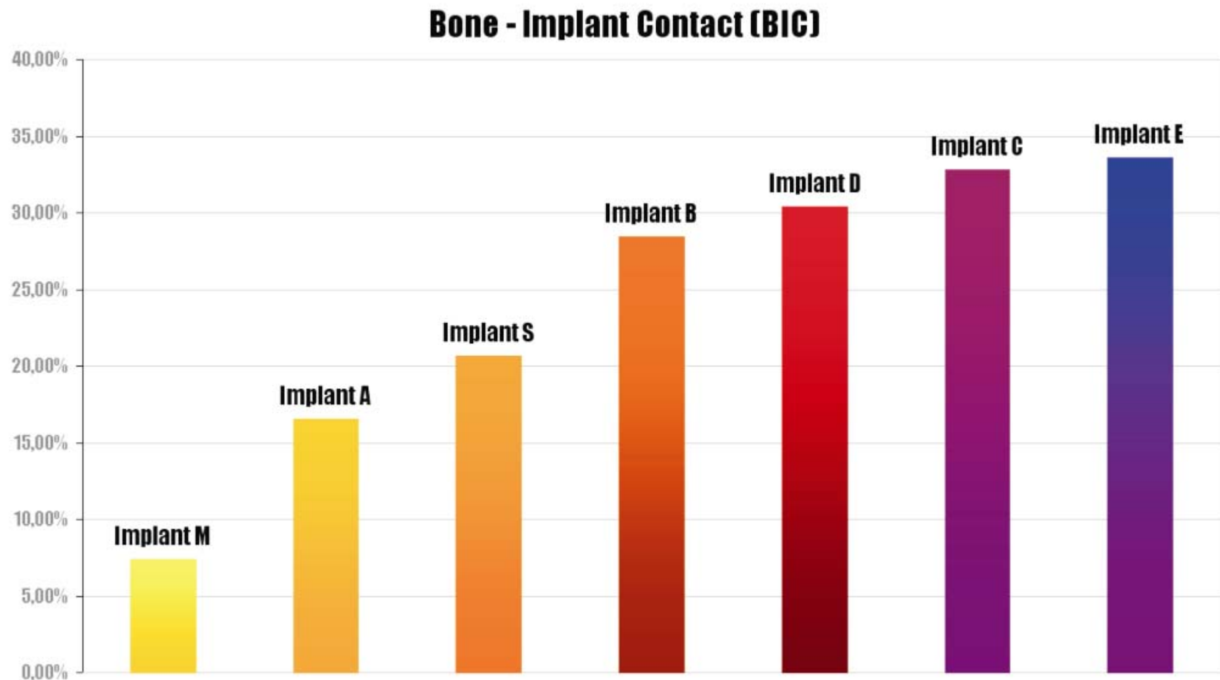


Fig 180. Same as figure 179

3.15 BIC results and discussion

where **A** = laser 5 µm pores, pitch 15 µm; **B** = laser 10 µm pores, pitch 20 µm; **C** = laser 20 µm pores, pitch 30 µm; **D** = laser 5 µm pores, pitch 5 µm; **E** = laser 10 µm pores, pitch 10 µm; **F** = laser 20 µm pores, pitch 20 µm; **S** = sandblasted; **M** = machined



Graph 16

Average BIC (%) for each type of implant:
M = 7.36
A = 16.54
S = 20.64
B = 28.44
D = 30.36
C = 32.80
E = 33.58

Conclusion

Based on these results we can conclude that the surface of the implant has a very important role in osteointegration, and that mechanically added roughness to these surfaces significantly increases the contact area between the implant surface and the peri-implant bone. We can see that the different types of laser treated and sandblasted implant surfaces had the highest percentage of Bone-Implant Contact whilst the machined surface had the lowest, as expected. However in comparison between laser treated and sandblasted surfaces, the laser treated surfaces had a higher BIC % with the exception of implant A which had a lower % than the sandblasted implant. The results show that laser treatment produced the highest BIC%, and is therefore the method of choice when treating the surface of the implant. This may be due to the laser source used for manufacturing the implants, which was provided by Synthegra Technology. It was able to produce surfaces with biometric characteristics without altering the characteristics of the implant material itself, in this case, titanium. The use of the laser allowed the creation of a highly controlled surface which helped to improve the integration of the implant with the surrounding tissue, as it allowed the creation of a vast number of highly accurate niches for each osteoblast. The laser source resulted in the ability to micro-roughen the titanium surface in a controlled and uniform manner without chemically altering it, and also assisted in stimulating adhesion, proliferation and differentiation of the osteogenic cells in contact with the implant surface

Its important to note that there are many physiological and pathological influences which can affect the results, so it is vital to repeat the method several times in order to increase the accuracy of the results.

Implant technology is a rapidly progressing science, with very frequent production of new designs, materials, shapes and surface treatments. Therefore based on the success of our method for this experiment, we could in the future utilize this method in order to analyse these new modifications. The current method that was used allowed the evaluation of osteointegration to be more efficient, accurate and less time consuming. Further scientific research that is currently ongoing will further help to improve the benefits of laser technology. An important area of research involves the creation of a 'biomimetic surface', a surface that closely resembles that of real tissue, which would help assist the stimulation and proliferation of the bone tissue due to the fact that it stimulates the regularity and dimensions of the bone tissue itself, without altering the properties of the titanium.

4. Summary and discussion of the experiments

Summary and discussion of experiment 1- study of statistical data of augmentation materials

Aim

To understand what the most common indications of using augmentation materials are and to obtain some statistical data concerning 3 different bone substitute materials.

Materials and Methods

This study is based on the statistical data of different augmentation materials as used when indicated in patients of the Maxillofacial Surgery Department, Faculty Hospital in Pilsen, during the period of 2003-2007.

During this period we treated 19 patients with 3 different augmentation materials; Biogen, Bioresorb and Cerasorb. Regarding this fact that one patient could have been treated by different augmentation materials or by one augmentation material in different regions of the jaws, we record the results of indicated material as a case which were a total of 30.

Results

1. Of all 19 patients, 10(53%) were treated by Bioresorb, 5(26%) by Cerasorb and 4(21%) patients by Biogen. See graphs 2 and 1.
2. Of all 30 cases, 16(53%) Bioresorb, 9(30%) Cerasorb and 5(17%) Biogen were used. See graphs 3 and 4.
3. Of all 19 patients, 10(53%) were male, and 9(47%) were women. See graphs 5 and 6.
4. Of all 30 cases of indication for treatment using augmentation materials 8(28%) were for sinus lift, 7(23%) for augmentation of bone after extraction, 5(17%) after cystectomy, 4(13%) for augmentation of alveolar ridge, 4(13%) after root resection, 1(3%) for augmentation of periimplant defect and 1(3%) for augmentation of alveolar ridge with distraction. See graphs 7 and 8.
5. Of all 9 cases treated by Cerasorb, 4 cases were indicated for sinus lift, 3 for augmentation of alveolar ridge, 1 for augmentation of bone after extraction and 1 after extraction. See graph 9.
6. Of all 16 cases teated by Bioresorb, 4 cases were indicated for sinus lift, 4 for augmentation after cystectomy, 4 for augmentation of bone after extraction, 2 after root resection, 1 for augmentation of per implant defect and 1 for augmentation of alveolar ridge. See graph 10.
7. Of all 5 cases treated by Biogen, 2 cases were indicated for augmentation of bone after extraction, 1 after root resection and 1 for augmentation of alveolar ridge. See graph 11.
8. In total 5 augmentation materials were used for augmentation of alveolar ridge, among them in 3 cases Cerasorb were used and 1 case for Bioresorb and Biogen each. See graph 12.

9. In total 4 augmentation materials were used after root resection, among them in 2 cases were used Bioresorb and 1 case for Cearsorb and Biogen each. See graph 13.
10. In total 5 augmentation materials were used after cystectomy, among them in 4 cases were used Bioresorb and for 1 case Cerasorb. See graph 14.
11. In total 8 augmentation materials were used for sinus lift operation, among them in 4 cases were used Bioresorb and 4 cases were used Cerasorb. See graph 15.

Conclusion

The use of augmentation materials were slightly more common in treatments carried out on males than in females.

Bioresorb was generally the most commonly used augmentation material.

The principal indicators for use of augmentation materials were concluded to be primarily sinus lifts followed closely by their use in post-extraction cases. It is also indicated in other minor surgical procedures, such as following root resections and cystectomy.

It was established that in cases of sinus lift, Bioresorb and Cerasorb were most commonly used. Bioresorb was also the principal material used after root resection and cystectomy, whereas Cerasorb was preferred in alveolar ridge augmentation.

Indications for use of Biogen were in post extraction cases.

Summary and discussion of experiment 2- Experimental study in pigs with the purpose of evaluating the possible osteogenic activity of bone augmentation materials

Aim

To evaluate in vivo the possible osteoinduction potential and osteogenic activity of two different bone augmentation materials, and if ectopic bone formation could be induced when implanted subcutaneously to the extremities of the pigs.

Materials and Methods

The experiment was performed under general anaesthesia, using aseptic techniques. Two bone augmentation materials: microporous granules (Ø 500-1000 μm) of pure phase ß-tricalcium phosphate ceramic Cerasorb® and granular mix (Ø 0.5-1 mm) derived from equine bone BIOGEN® were applied at the ulnar region between muscular and cutaneus tissue. We performed two intramuscular applications of the antibiotic Tetracycline at 16 days after operation. Tetracycline is deposited where bone or cartilage matrix is mineralizing and can therefore demonstrate regions of active bone formation and mineralization. After 52 days the pigs were terminated. At the site of application of Cerasorb some hard tissue material was found (none at BIOGEN®).See figures 13-15.

The specimens were observed by optical and confocal laser microscope after histological processing.

Results

The observation revealed incompletely mineralized osteoid material and layers of chondroid tissue mass. See figures 17-26

Bright fluorescent zones showed the deposition of tetracycline-induced fluorescence limited to area of active hard tissues formation. See figures 27-28

Conclusion

Based on our results, it is possible to use this sequential methodology in soft tissues, with the objective to verify the osteoinduction potential of different bone augmentation materials. With respect to this original method, we can analyse different augmentation materials to establish the most successful one with the best osteinductive properties.

We also established that our results from the labelling of the tissue by tetracycline exhibited the same results as that shown in previous publication by Mitch et al [136, 137].

These results highlight the potential future use, of augmentation materials in creation of hard tissues, in areas where soft tissues are normally present. This would be invaluable, for example, in the clinical setting of cosmetic surgery or maxillofacial surgery where bone reconstruction must take place following oncological or traumatic destruction of bone.

Summary and discussion of experiment 3- experimental study in pigs to evaluate the ability of Cerasorb as a bone augmentation material for healing of bone defects

Aim

To evaluate the efficiency of Cerasorb bone augmentation material in bone healing when it is applied into the artificial hole made in the extremities of the pigs.

Materials and Methods

The experiment was performed under general anaesthesia, using aseptic techniques. Two artificial holes were made at the tibia of two pigs. In each tibia one hole was filled by microporous granules (Ø 500-1000 µm) of Cerasorb and the second hole was kept empty as a control hole for further comparison. Two holes are covered by Biocollagen membrane to prevent outside influence. See figures 29-32 and 35-37.

After 52 days the pigs were terminated and radiograms were obtained from the sites of artificial holes. See figures 33-34 and 38-40.

The specimens were observed by optical microscope after histological processing.

Results and Discussion

Based on careful analysis of the radiographic and histological images, we can conclude that Cerasorb gradually resorbs and is taken over by new bone formation. The brown roundish microporous granules of Cerasorb show different stages of peripheral penetration by vascularized connective tissue, leading to new bone formation. Histolological findings revealed that Cerasorb granules were surrounded by connective tissue and osteoblasts and new bone formation is

replaced by gradual resorption of cerasorb mainly from the peripheral site. Therefore we can conclude that Cerasorb has very good bone augmentating properties and can be readily used for healing of bone defects.

Summary and discussion of an experiment to establish the most efficient evaluation method of a bone-implant interface in a dead rabbit (experiment 4), and if successful to use this method to evaluate the actual osteointegration in a living rabbit (experiment 5).

Aim

For evaluation of osteointegration between bone and dental implants, the preparation time can exceed 7 months when following the traditional method of preparation of the histological specimens. The main difficulty in the traditional method is to get a very thin section of bone-implant, without any displacement and micro-movements of osteointegrated implants from the bone, to be able to observe true bone-implant interface. Our aim in these experiments was to find an alternative method for preparation of the bone-implant interface for evaluation, by using a confocal laser microscope in order to improve cost efficiency and reduce the preparation time whilst obtaining reliable and useful results. The advantage of CLSM is that speciment should not be sectioned as thin as for light microscope and its ability to produce in-focus images of thick specimens, a process known as *optical sectioning*. Images are acquired point-by-point and reconstructed with a computer, allowing three-dimensional reconstructions of topologically-complex objects.

Materials and Methods

Experiment 4: In the first step we tried to determine the best staining method for staining of different sections of various bone by different staining methods and materials.See figures 46-51.

We inserted the titanium implant and screw in to the femur of the dead rabbit. In the next step, grinding of the bone and implant was done until the bone-implant interface was seen. This was then stained with basic fuchsin ready for histological evaluation under the confocal laser scanning microscope (CLSM). See figures 52 and 53.

Experiment 5: Under general anesthesia titanium implant was surgically inserted into the femur.See figures 57-58

After 45 days the rabbit was sacrificed and a series of radiographical images were taken. See fig.59

Results

Experiment 4: The results, as can be seen in fig 54-56 under a confocal laser microscope are very positive. Although, as expected, no osteintegration had taken place due to implantation into nonliving tissue, the bone-implant interface can clearly be seen.

In conclusion we ascertained that the traditional method of producing histological

specimens of bone and implant is very time consuming and complicated.
The experimental method that was developed utilizes the confocal laser microscope, allowing accurate results and more rapid specimen preparation time whilst being easier to perform.
Having established the success of the experimental method, we decided to repeat our experiment but with the use of a living rabbit.
Experiment 5: After histological preparation the specimens were observed under CLSM. The results as seen on Fig 60, 61 and 62, were highly successful and provided an excellent view of the bone-implant interface for evaluation of osteointegration.
We confirmed the results obtained from CLSM pictures by performing the traditional method and analysed our results under light microscope. See figures 63-66.

Conclusion

Experiment 4**:** We ascertained that the traditional method of producing histological specimens of bone and implant is very time consuming and complicated.
The experimental method that was developed utilizes the confocal laser microscope, allowing accurate results and more rapid specimen preparation time whilst being easier to perform.
Experiment 5: The results as seen on Fig 60, 61 and 62, were highly successful and provided an excellent view of the bone-implant interface. The images made by laser confocal microscope showed osteointegration between the bone in red, and the dark black implant structure.
Evaluating figures 60, 61 and 62 we can establish that there are no spaces, showing good osteointegration.
Based on our results, in both the nonliving and living tissues, we can deduce that the alternative method is a very successful way in preparing and evaluating histological specimens in order to show the osteointegration between the bone and implant surface. Our results were similar to those provided by the traditional method, however, our method was significantly less time consuming, more cost efficient and easier to carry out.

Summary and discussion of experiment 6- Experimental study in pigs with the purpose to evaluate and examine osteointegration of dental implants with differently treated surfaces.

Aim

Examination and evaluation of osteointegration of dental implants with different laser treated surfaces, and the comparison of those results with sandblasted and machine treated implant surfaces.

Materials and Methods

Different types of surface treated Implants inserted into the long bones (ulna and tibia) and mandible of 4 piglets under general anesthesia. Two doses of antibiotic

Tetracycline (TTC) were applied in different periods of time to demonstrate regions of active bone formation, mineralisation and to demonstrate the quantity of newly formed bone at the implant interface with the help of a confocal microscope due to its fluorescent property.
The pigs were sacrificed after the periods of 52 and 64 days. A Series of radiographical images were taken and revealed that opposition of bone occurred above the implant head, where the largest part of the implant remained inside the bone marrow cavity due to the osteoclastic activity of osteoclasts. (See figures 79-106). Note: For performing a similar experiment, the author recommends the use of older animals or miniature pigs that have a slower rate of growth.

Results

After bone processing and staining for eventual histological analysis (fig.107-133), a series of microphotographic images were taken under both light and CLSM showing the bone-implant interface. See figures 134-177
Images from CLSM reveal that during the period of application, the TTC accumulated and labelled the newly formed bone demonstrated two levels of bone formation labelled as fluorescent lines. (See figures 159,167,176 and 177)
We evaluate the osteointegration of different types of surface treated implants by measuring of bone-implant contact (BIC) % according to the Elipse programme explained in fig. 178-180.

Conclusion

We found out that the different types of laser treated and sandblasted implant surfaces had the highest percentage of Bone-Implant Contact, whilst the machined surface had the lowest, as expected. However in comparison between laser treated and sandblasted surfaces, the laser treated surfaces almost had a higher BIC % than the sandblasted implant. The results show that laser treatment produced the highest BIC%, and is therefore the method of choice when treating the surface of the implant. This may be due to the laser source used for manufacturing the implants, which was provided by Synthegra Technology. It was able to produce surfaces with biometric characteristics without altering the characteristics of the implant material itself, in this case, titanium. The use of the laser allowed the creation of a highly controlled surface which helped to improve the integration of the implant with the surrounding tissue, as it allowed the creation of a vast number of highly accurate niches for each osteoblast. The laser source resulted in the ability to micro-roughen the titanium surface in a controlled and uniform manner without chemically altering it, and also assisted in stimulating adhesion, proliferation and differentiation of the osteogenic cells in contact with the implant surface

Based on these results as seen in graph 16, we can conclude that the surface of the implant has a very important role in osteointegration, and that mechanically added roughness to these surfaces significantly increases the contact area between the implant surface and the peri-implant bone.

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6. Literature

1. Brighton, Carl T. and Robert M. Hunt, "Early histologic and ultrastructural changes in microvessels of periosteal callus", Journal of Orthopaedic Trauma, (1997), 11 (4), p.244-253

2. Brighton, Carl T. and Robert M. Hunt, "Early histologic and ultrastructural changes in medullary fracture callus", Journal of Bone and Joint Surgery, (1991), 73-A (6), p.832-847

3. Daftari TK, Whitesides TE Jr, Heller JG, et al: Nicotine on the revascularization of bone graft. An experimental study in rabbits.Spine 19, (1994) p.904–911

4. Riebel ED, Boden SD, Whitesides TE, et al: The effect of nicotine on incorporation of cancellous bone graft in an animal model. Spine 20, (1995) p.2198–2202

5. Ham, Arthur W. and William R. Harris, "Repair and transplantation of bone",The biochemistry and physiology of bone, New York: Academic Press, (1972), p.337-399

6. Wolff, J. Das Gesetz der Transformation der Knochen. Verlag Hirschwald, Berlin. (1892)

7. Copenhaver WM, Kelly DE, Wood RL. The connective tissues: cartilage and bone, Wood RL (eds): Bailey's Textbook of Histology. Baltimore: Williams & Wilkins, (1978), ed 17, p.170–205

8. Recker RR: Embryology, anatomy, and microstructure of bone, in Coe FL, Favus MJ (eds): Disorders of Bone and Mineral Metabolism. New York: Raven, (1992), p.219–240

9. Martin BR, Burr DB, Sharkey NA: Skeletal Tissue Mechanics.Ed by Smith R. Springer-Verlag, (1998).

10. Kiebzak GM: Age related bone changes.Experimental Gerontology, (1991). 26, p.171-187

11. Dee R: Bone healing, in Dee R, Mango E, Hurst E, (eds): Principles of Orthopaedic Practice. New York: McGraw-Hill, (1988), p. 68–73

12. Netter, Frank H., Musculoskeletal system: anatomy, physiology, and metabolic disorders. Summit, New Jersey: Ciba-Geigy Corporation, (1987), p.169

13. Schoppet M, Preissner K, Hofbauer L. "RANK ligand and osteoprotegerin: paracrine regulators of bone metabolism and vascular function".Arterioscler Thromb Vasc Biol, (2002). 22 (4), p.549-553

14. Iain H. Kalfas, M.D., F.A.C.S. Principles of bone healing, Neurosurg. Focus 10(4). © 2001 American Association of Neurological Surgeons. (2001)

15. Hirsch C, White AA III: An experimental study of the immediate load bearing capacity of some commonly used iliac bone grafts. Acta Orthop (1971).Scand 42, p.482–490

16. Wozney JM, Rosen V, Celeste AJ, et al: Novel regulators of bone formation: molecular clones and activities. Science 242, (1988), p.1528–1534

17. Habal MB: Different forms of bone grafts, in Habal MB, Reddi AH (eds): Bone Grafts and Bone Substitutes. Philadelphia: WB Saunders, (1992), p.6–8

18. Prolo DJ: Biology of bone fusion. Clin Neurosurg, (1990), 36: p.135–146

19. Muschler GF, Lane JM, Dawson EG: The biology of spinal fusion, in Cotler JM, Cotler HP (eds): Spinal Fusion Science and Technique. Berlin: Springer-Verlag, (1990), p.9–21

20. Buser, D., Ch. Dahlin and R.K. Schenk , Guided bone regeneration in Implant Dentristry,Quintessence Publishing Co, Inc., Chicago, Berlin, London, Tokyo, Moscow, Prague, Sofia, and Warsaw,(1994), p.69

21. Davies JE, Hosseini MM. Histodynamics of endosseous wound healing.Davies JE, editor.Bone engineering.Toronto: em squared incorporated, (2000), p.1-14

22. Baksh D, Davies JE. Design strategies for 3-dimensional in vitro bone growth in tissue engineering scaffolds.Davies JE, editor.Bone Engineering.Toronto: em squared Incorporated, (2000), p.488-95

23. White E, Shors EC.Biomaterial aspects of Interpore-200 porous hydroxyapatite. Dent Clin North Am (1986), 30, p.49-67

24. Joyce ME, Jingushi S, Bolander ME.Transforming growth factor- in the regulation of fracture repair. Orthop Clin North Am (1990), 21, p.199-209

25. Bostrom MP, Lane JM, Berberian WS, Missri AA, Tomin E, Weiland A, et al. Immunolocalization and expression of bone morphogenetic protein 2 and 4 in fracture healing. J Orthop Res (1995), 13, p.357-67

26. Onishi T, Ishidou Y, Nagamine T, Yone K, Imamaru T, Kato M, et al. Distinct and overlapping patterns of localization of bone morpho-genetic protein (BMP) family members and a BMP type II receptor during fracture healing in rats. Bone (1998), 22, p.605-12

27. Sakou T. Bone morphogenetic proteins: From basic studies to clinical approaches. Bone (1998), 22, p.591-603

28. Bourque WT, Gross M, Hall BK.Expression of four growth factors during fracture repair.Int J Dev Biol (1993), 37, p.573-79

29. Nakamura T, Hara Y, Tagawa M, Tamura M, Yuge T, Fukuda H, et al. Recombinant human basic fibroblast growth factor accelerates fracture healing by enhancing callus remodeling in experimental dog tibial fracture. J Bone Miner Res (1998), 13, p.942-9

30. Trippel SB. Potential role of insulinlike growth factors in fracture healing. Clin Orthop (1998), 355S, p. 301-13

31. Nash TJ, Howlett CR, Martin C, Steele J, Johnson KA, Kicklin DJ.Effect of platelet-derived growth factor on tibial osteotomies in rabbits. Bone (1994), 15, p.203-8

32. Rueger JM.Bone replacement materials–state of the art and the way ahead.Orthop.ade (1998), 27(1), p.72–9

33. Greenwald AS, Boden SD, Goldberg VM, Khan Y, Laurencin CT, Rosier RN. Bone-graft substitutes: facts, fictions, and applications. J Bone Joint Surg (2001), 83A: p.98–103

34. Den Boer FC, Wippermann BW, Blokhuis TJ, Patka P, Bakker FC, Haarman HJTM. Healing of segmental bone defects with granular porous hydroxyapatite augmented with recombinant human osteogenic protein-1 or autologous bone marrow.J Orthopaed Res (2003), 21, p.521–8.

35. Brooks DB, Heiple KG, Herndon CH, et al: Immunological factors in homogenous bone transplantation. IV. The effect of various methods of preparation and irradiation on antigenicity. J Bone Joint Surg (1963) Am 45, p.1617–1626

36. Friedlaender GE, Strong DM, Sell KW: Studies on the antigen city of bone. II. Donor-specific anti-HLA antibodies in human recipients of freeze-dried allografts. J Bone Joint Surg (1984) Am 66, p.107–112

37. Herron LD, Newman MH: The failure of ethylene oxide gas sterilized freeze-dried bone graft for thoracic and lumbar spinal fusion. (1989) Spine 14, p.496–500

38. Poumarat G, Squire P, (1993) Biomaterials 14, p.337-340

39. Barry Kyle Bartee: Implant Site Development & Extraction Site Grafting Technique & Selection of Bone Grafting Materials (2005)

40. Urist MR. Bone: Formation by autoinduction. Science (1965), 150, p.893-8

41. Tuli SM, Singh AD. The osteoinductive property of decalcified bone matrix: An experimental study. J Bone Joint Surg Br (1978), 60, p.116-23

42. Adkisson HD, Strauss-Schoenberger J, Gillis M, Wilkins R, Jackson M, Hruska KA.A rapid quantitative bioassay of osteoinduction. J Orthop Res (2000), 18, p.503-11

43. Chiroff RT, White EW, Weber KN, Roy DM. Tissue ingrowth of replamineform implants. J Biomed Mater Res (1975), 6, p.29-45

44. HISLOP W. S.; FINLAY P. M.; MOOS K. F.: A preliminary study into the uses of anorganic bone in oral and maxillofacial surgery, Br J Oral Maxillofac Surg, (1993) vol. 31, p.149-153

45. Reddi AH. Role of morphogenetic proteins in skeletal tissue engineering and regeneration.Nat Biotechnology (1998), 16, p.247-52

46. Paralkar VM, Nandedkar AK, Pointer RH, Kleinman HK, Reddi AH.Interaction of osteogenin, a heparin binding bone morphogenetic protein, with type IV collagen. J Biol Chem (1990), 265, p.17281-4

47. Ripamonti U, Van Den Heever B, Sampath TK, Tucker MM, Rueger DC, Reddi AH.Complete regeneration of bone in the baboon by recombinant human osteogenic protein-1(hOP-1, bone morphogenetic protein-7). Growth Factors (1996), 13, p.273-89

48. Eds. N. Ashammakhi & P. Ferretti, Topics in Tissue Engineering, University of Oulu, (2003)

49. Urist MR, McLean F. Osteogenic potency and new bone formation by induction in transplants to the anterior chamber of the eye. J Bone Joint Surg (1952), 34A, p.443

50. Urist MR, Mikulski AJ, Nakagawa M, Yen K.A bone matrix calcification-initiator noncollagenous protein. Am J Physiol (1977), 232, p.115-127

51. Urist MR, Mikulski A, Lietze A. Solubilized and insolubilized bone morphogenetic protein.Proc Nat Acad Sci USA (1979), 76, p.1828-1832

52. Buchardt H. The biology of bone graft repair. Clinical Orthop (1983), 174, p.28-42

53. Bonutti PM, Cremens MJ, Miller BG. Formation of structural grafts from cancellous bone fragments. Am J Orthop (1998), 27, p.499-502

54. Keller EE, Tolman D, Eckert S. Endosseous implant and autogenous bone graft reconstruction of mandibular discontinuity: A 12-year longitudinal study of 31 patients. Int J Oral Maxillofacial Implants (1998), 13: p.767-780

55. Vinzenz KG, Holle J, Wuringer E, Kulenkampff KJ, Plenk H Jr. Revascularized composite grafts with inserted implants for reconstructing the maxilla: Improved flap design and flap prefabrication. Brit J Oral Maxillofac Surg (1998), 36, p.346-352

56. Kainulainen VT, Sàndor GKB, Caminiti MF, Clokie CML, Oikarinen KS.Extraoral bone harvesting sites for oral and maxillofacial surgery. Suomen Hammaslääkärilehti (Finnish Dental Journal) (2002) b, 10-11, p.570-576

57. Kainulainen VT, Sàndor GKB, Oikarinen KS, Clokie CML. The Intraoral bone harvesting sites for osseous reconstruction in oral and maxillofacial surgery. Oral Health (2003) a, 93(5), p.10-24

58. Russo R, Scarborough N. Inactivation of viruses in demineralized bone matrix. FDA Workshop on Tissue for Transplantation and Reproductive Tissue, Bethesda, MD, USA, (1995), p.20-21

59. Zhang M, Powers RM Jr, Wolfinbarger L Jr.A quantitative assessment of osteoinductivity of human demineralized bone matrix. J Periodontal (1997) a, 68, p.1076-1084

60. Zhang M, Powers RM Jr, Wolfinbarger L Jr. Effect(s) of the demineralization process on the osteoinductivity of demineralized bone matrix. J Periodontal (1997)b, 68,p.1085-1092

61. Van Den Bogaerde J, White DJ. Xenogeneic transplantation.Brit Med Bull (1997), 53, p.904-20

62. Hammer C, Linke R, Wagner F, Diefenbeck M. Organs from animals for man. Int Arch Allergy Immunol (1998), 116, p.5-21

63. Block JE, Poser J. Does xenogeneic demineralized bone matrix have clinical utility as a bone graft substitute? Med Hypotheses (1995),45, p.27-32

64. Jensen SS, Aaboe M, Pinholt EM, Hjorting-Hansen E, Melsen F, Ruyter IE.Tissue reaction and material characteristics of four bone substitutes.Int J Oral Maxillofac Impl (1996), 11, p.55-66.

65. Bons N, Lehmann S, Mestre-Frances N, Dormont D, Brown P. Brain and buffy coat transmission of bovine spongiform encephalopathy to the primate Microcebus murinus. Transfusion (2002), 42, p.513-516

66. Hunter N. Laboratory studies of bovine spongiform encephalopathy. Lancet (2002), 360, p. 488-489

67. Roux FX, Brasnu D, Loty B, George B, Guillemin G. Madreporic coral: A new bone graft substitute for cranial surgery. J Neurosurg (1988) a, 69, p.510-513

68. Jarcho M. Biomaterial aspects calcium phosphates: Properties and applications. Dent Clin North Am (1986), 30, p.25-43

69. Alexander H, Parsons JR, Ricci J, Bajpai PK. Calcium-based ceramics and composites in bone reconstruction. CRC Crit Rev Biocompat (1987), 4, p.43-77

70. Ricci JL, Spivak JM, Alexander H, Blumenthal NC, Parsons JR. Hydroxyapatite ceramics and the nature of bone-ceramic interface. Bull Hosp Joint Dis Orthop Inst (1989), 49, p.178-191

71. Brown PW, Constantz B. Hydroxyapatite and Related Materials. CRC (1998), 25, p.1036-1040

72. Guillemin G, Meunier A, Dallant P, Christel P, Puliquen JC, Sedel L. Comparison of coral resorption and bone apposition with 2 natural corals of different porosities. J Biomed Mater Res (1989), 23, p.765-779

73. Guillemin G, Patat JL, Meunier A. Natural corals as bone graft substitutes. Bulletin de L'Institut Océanographique (1995), 14, p.67-77

74. Byrd HS, Hobar PC, Shewmake K. Augmentation of the craniofacial skeleton with porous hydroxyapatite granules. Plast Reconstr Surg (1993), 91, p.15-22

75. Mercier P, Failures in ridge reconstruction with hydroxyapatite.Analysis of cases and methods for surgical revision. Oral Surg Oral Med Oral Path Oral Radiol Endod (1996), 81, p. 376-384

76. Prousaefs P, Lozada J, Valencia G, Rohrer MD. Histologic evaluation of a hydroxyapatite onlay bone graft retrieved after 9 years: A clinical report. J Prosthet Dent (2002), 87, p.481-484

77. Mors W, Kaminski E. Osteogenic replacement of tricalcium phosphate ceramic implants in the dog palate. Arch Oral Biol (1975), 20, p.365-367

78. Hollinger JO, Schmitz JP, Mizgala JW, Hassler C.An evaluation of two configurations of tricalcium phosphate for treating craniotomies. J Biomed Mater Res (1989), 23, p.17-29

79. K. Takahashi, Y. Fujishiro, S. Yin and T. Sato Preparation and compressive strength of α-tricalcium phosphate based cement dispersed with ceramic particles Ceramics International, Volume 30, Issue 2, (2004), p.199-203

80. Merten, H-A., J. Wiltfang, J.-F. Hönig, F. Funke und H.-G.Luhr .Intraindividueller Vergleich von - und-TCP-Keramik im Tierexperiment. Mund Kiefer GesichtsChir (2000), 4 (Suppl. 2), p.509 - 515

81. Soballe K, Hansen ES, Brockstedt-Rasmussen H, Bunger C Hydroxyapatite coating converts fibrous tissue to bone around loaded implants J Bone Joint Surg Br (1993),75, p.270-8

82. Tisdel CL, Goldberg VM, Parr JA, Bensusan JS, Staikoff LS, Stevenson S.The influence of a hydroxyapatite and tricalcium phosphatae coating on bone growth into titanium fiber-metal implants. J Bone Joint Surg Am (1994), 76, p.159-71

83. Ziffe D, Moroni A, Pezzuto V. Histological and Physico-Chemical Analyses on Transformations of Some Bioactive Glasses Implanted in Long Bones of Rabbit and Sheep. Vincenzini P, editor.Ceramics in Substitute and Reconstructive Surgery. Amsterdam: Elsevier Science Publishers (1991), p.115-132

84. Turunen T, Petola J, Helenius H, Yli-Urpo A, Happonen RP. Bioactive glass and calcium carbonate granules as filler material around titanium and bioactive glass implants in the medullar space of the rabbit tibia. Clin Oral Implants Res (1997), 8, p.96-102

85. Nasr HF, Aichelmann-Reddy ME, Yukna RA. Bone and bone substitutes. J Periodontal (1999), 19, p.74-86

86. Yukna RA, Evans GH, Aichelmann-Reddy MB, Mayer ET. Clinical comparison of bioactive glass bone replacement graft material and expanded polytetraflouroethylene barrier membrane in treating human mandibular molar class II furcations. J Periodontal (2001),72, p.125-133

87. Dressmann H. Ueber Knochenplombierung bei Hohlenformigen Defekten des Knochens. Beitr Klin Chir (1892), 9, p.804-10

88. Mousset B, Benoit MA, Bouillet R, Gillard J. Plaster of Paris: A carrier for antibiotics in treatment of bone infections. Acta Orthop Belg (1993), 59, p.239-48

89. Mousset B, Benoit MA, Delloye C, Bouillet R, Gillard J. Biodegradable implants for potential use in bone infection: An in-vitro study of antibiotic - loaded calcium sulphate. Int Orthop (1995), 19, p.157-61

90. Peltier LF .The use of plaster of Paris to fill large defects in bone. Am J Surg (1959), 97, p.311-15

91. Sidqui M, Collin P, Vitte C, Forest N. Osteoblast adherence and resorption activity of isolated osteoclasts on calcium sulphate hemihydrate. Biomaterials (1995), 16, p.1327-32

92. Aderriotis D, Sàndor GKB.Outcomes of vicryl rapide fast-absorbing suture in 80 oral and 42 scalp wounds. J Can Dent Assoc (1999), 65, p.345-347

93. Suuronen R, Haer PE, Lindqvist C, Sailer HF.Update on resorbable plates in maxillofacial surgery. Facial Plast Surg (1999), 15, p.61-72

94. Suuronen R, Kallela I, Lindqvist C. Bioabsorble plates and screws: Current state of the art in facial fracture repair. J Craniofac Trauma (2000), 6, p.19-27

95. Clokie CML, Sàndor GKB. Bone: Present and Future. Babush C, editor. Dental implants: The art and science. Philadelphia: W.B. Saunders Company (2001), p.59-84

96. Parikh SN. Bone graft substitutes: past, present, future. J Postgrad Med. (2002), 48, p.142-8

97. Wozney JM.Bone morphogenetic proteins.Prog in Growth Factor Res (1989), 1, p.267-280

98. Wozney JM, Rosen V, Byrne M, Celeste AJ, Moutsatsos I, Wang EA. Growth factors influencing bone development. J Cell Science Suppl (1990), 13, p.149-156

99. Tuominen T. Native bovine bone morphogenetic protein in the healing of segmental long bone defects.Thesis. Acta Universitatis Ouluensis (2001), D 641

100. Wang EA, Rosen V, D'Alessandro JS, Bauduy M, Cordes P, Harada T, Israel DI, Hewick RM, Kerns KM, LaPan P. Recombinant human bone morphogenetic protein induces bone formation. Proc Nat Acad Sci USA (1990), 87, p.2220-2224

101. Nilsson OS, Urist MR, Dawson EG, Schmalzried TP, Finerman GA. Bone repair induced by bone morphogenetic protein in ulnar defects in dogs. J Bone Joint Surg Br (1986), 68, p.635-642

102. Yamazaki Y, Oida S, Akimoto Y, Shioda S. Response of the mouse femoral muscle to an implant of a composite of bone morphogenetic protein and plaster of Paris. Clin Orthop(1988), 234, p.240-249

103. Johnson EE, Urist MR, Schmalzried TP, Chotivichit A, Huang HK, Finerman GA. Autogeneic cancellous bone grafts in extensive segmental ulnar defects in dogs. Effects of xenogeneic bovine bone morphogenetic protein without and with interposition of soft tissues and interruption of blood supply. Clin Orthop (1989), 243, p.254-265

104. Lindholm TC, Lindholm TS, Alitalo I, Urist MR. Bovine bone morphogenetic protein (bBMP)induced repair of skull trephine defects in sheep. Clin Orthop (1988), 227, p.265-268

105. Lindholm TC. Calvarial reconstruction with implants of hydroxyapatite, autogenous bonemarrow, allogenic demineralized bone matrix and bovine bone morphogenetic protein. Thesis.Annales Universsitatis Turkuensis (1995), D.193

106. Covey DC, Albright JA. Clinical induction of bone repair with demineralized bone matrix or a bone morphogenetic protein. Orthopaedic Review (1989), 18, p.857-863

107. Rittenberg B, Moghadam HG, Sandor GKB, Clokie CML. Mandibular reconstruction with BMP-7. A prospective clinical study. J Oral Maxillofac Surg (2003), 61(Suppl 1), p.92

108. Barkin S, Clokie CML, Baker G, Sandor GKB, Tenenbaum H. Fetuin inhibition of heterotrophic ossification in vitro. J Oral Maxillofac Surg (2003), 61(Suppl 1), p.57

109. Celeste AJ, Iannazzi JA, Taylor RC, Hewick RM, Rosen V, Wang EA, Wozney JM. Identification of transforming growth factor beta family members present in bone-inductive protein purified from bovine bone. Proc Nat Acad Sci USA (1990), 87, p.9843-9847

110. Mohan S, Baylink DJ. Bone growth factors.Clin Orthop (1991),263, p.30-43

111. Roberts AB, Sporn MB. Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta).Growth Factors (1993), 8, p.1-9

112. Miyazono K, Ten-Dijke P, Ichiyo H, Heldin CH. Receptors for transforming growth factor-beta. Adv Immunol (1994), 55, p.181-220

113. Cunningham NS, Jenkins NA, Gilbert DJ, Copeland NG, Reddi AH, Lee SJ. Growth/differentiation factor-10: a new member of the transforming growth factor-beta superfamily related to bone morphogenetic protein-3. Growth Factors (1995),12, p.99-109

114. Heckman JD, Ehler W, Brooks BP, Aufdemorte TB, Lohmann CH, Morgan T, et al. Bone morphogenetic protein but not transforming growth factor-β enhances bone formation in canine diaphyseal nonunions implanted with a biodegradable composite polymer. J Bone Joint Surg Am (1999), 81, p.1717-39

115. Antonaides HN, Williams IT. Human platelet-derived growth factor: Structure and functions.Federation Proc (1983), 42, p.2630-2634

116. Ross R, Raines EW, Bowen-Pope DF. The biology of platelet-derived growth factor. Cell (1986), 46, p.155-169

117. Giannobile WV, Hernandez RA, Finkelman RD, Ryan S, Kiritsy CP, D'Andrea M, Lynch SE.Comparative effects of platelet-derived growth factor-BB and insulin-like growth factor-I, individually and in combination, on periodontal regeneration in Macaca fascicularis. J Periodontal Res (1996), 31(5), p.301-312

118. Giannobile WV, Whitson SW, Lynch SE. Non-coordinate control of bone formation displayed by growth factor combinations with IGF-I. J Dent Res (1997),76, p.1569-1578

119. Howell TH, Fiorellini JP, Paquette DW, Offenbacher S, Giannobile WV, Lynch SE. A phase I/II clinical trial to evaluate a combination of recombinant human platelet-derived growth factor-BB and recombinant human insulin-like growth factor-I in patients with periodontal disease. J Periodontal (1997)b, 68, p.1186-1193

120. Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, Georgeff KR. Platelet-rich plasma: Growth factor enhancement for bone grafts. Oral Surg Oral Med Oral Path (1998), 85, p.638-646

121. Sanchez AR, Sheridan PJ, Kupp LI: Is platelet-rich plasma the perfect enhancement factor? A current review. Int J Oral Maxillofac Implants (2003), 18, p.93–103

122. Ziv Simon,Philip A.Watson, Biomimetic Dental Implants — New Ways to Enhance Osteointegration. J Can Dent Assoc (2002), 68(5), p.286-8

123. Kasemo: Metallauswahl und oberflachenbeschaffenheit. Gewebeintegrierter Zahnersatz. Quintessenz, Berlin (1985), p.110

124. Strunz: Enossale implantatmaterialien in der Mund- und kieferchirurgie. Hanser, Munchen (1985), p.111

125. Buser, D., RK Schenk, S. Steinenann, J.P. Fiorellini, C.Fox, H.Stich: Influence of surface characteristics of bone integration of titanium implants.A histomorphometric study in miniature pigs. Journal of Biomed, (1991), p.889-902

126. Davies J.E. Mechanisms of endosseous integration.Int. J.Prosthodont. (1998), vol.11, p. 391-401

127. Lazzara R., Testori T., Trisi P., Porter S. A human histologic analysis of Osseotite and machined surface using implants with 2 opposing surfaces .Int. J. Periodontics Restorative Dent. (1999), vol.19, p.117-129

128. Wennerberg A., Hallgren C., Johansson C., Danelli S. A histomorphometric evaluation of screw-shaped implants each prepared with two surfaces roughness's.Clin. Oral Implants Res. (1998), vol.9, p.11-19

129. Wennerberg A., Albrektsson T. Andersson B.,Krol J.J. A histomorphometric and removal torque study of screw-shaped titanium implants with three different surface topographies. Clin. Oral Implants Res. (1985), vol. 6, p.24-30

130. Wennerberg A., Albrektsson T. Andersson B. Bone tissue response to commercially pure titanium implants blasted with fine and coarse particles of aluminium oxide.Int. J.Oral Maxillofac.Implants. (1996), vol. 11, p.38-45

131. Cheang P., Khor K.A. Addressing processing problems associated with plasma spraying of hydrxyapatite coatings .Biomaterials (1996), vol. 17, p.537-544

132. Tallgren A. The continuing reduction of the residual alveolar ridges in complete denture wearers: a mixed longitudinal study covering 25 years. J Prosthet Dent (1972), 27, p.120-32

133. Urist, M. R. Bone: Formation by autoinduction. Science, (1965), 150, p.893-99

134. Urist, M. R. & Strates, B.S. Bone morphogenetic protein. J. Dent. Res,(1971), 50, p.1392-406.

135. Wang, E. A.; Rosen, V.; D´Alessandro, J. S.; Bauduy, M.; Cordes, P.; Harada, T.; Israel, D.; Hewick, R. M.; Kerns, K.; Lapan, P.; Luxenberg, D. P.; McQuaid, D.; Moutsatsos, I.; Nove, J. & Wozney, J.M.Recombinat human bone morphogenetic protein induces bone formation. Proc. Natl. Acad. Sci. USA. (1990), 87, p.2220-4

136. Samuel B. Linhart, James J. Kennelly : Fluorescent Bone Labeling of Coyotes with Demethylchlortetracycline The Journal of Wildlife Management, (1967),Apr, Vol.31, No.2, p. 317-321

137. Milch RA, Rall DP, Tobie JE Bone localization of the tetracyclines. J. Natl Cancer Inst. (1957), 19, p.87–93

138. Frost et al.: Tetracycline Bone Labeling, J Clin Pharmacol. (1961), 1, p.206-216

139. T. C. Lee, S. Mohsin, D. Taylor, R. Parkesh, T. Gunnlaugsson, F. J. O'Brien, M. Giehl and W. Gowin: Detecting microdamage in bone, J. Anat.(2003), 203, p.161–172

140. Barry Kyle Bartee, DDS, MD Implant Site Development and Extraction Site Grafting Technique and Selection of Bone Grafting Materials ESR (2005)

141. Cordioli G, Majzoub Z, Piattelli A, Scarano A. Removal torque and histomorphometric investigation of 4 different titanium surfaces: An experimental study in the rabbit tiba. Int J Oral Maxillofac Implants (2000), 15, p.668-674

142. Piattelli M, Scarano A, Paolantonio M, Lezzi G, Petrone G, Piattelli A. Bone response to machined and resorbable blast material titanium implants: An experimental study in rabbits. J Oral Implantol (2002), 28, p.2-8

143. Khang W, Feldman S, Hawley CE, Gunsolley J. A multi-center study comparing dual acid-etched and machined-surfaced impalnt in various bone qualities .J Periodontal (2001), 72, p. 1384-1390

144. Davarpanah M, Martinez H, Etienne D, et al. A prospective multicenter evaluation of 1,583 3i implants: 1- to 5-year data. int J oral maxillofac implants (2002),17, p.820-828

145. Trisi P, lazzara R, Rao W, Rebaudi A. Bone-implant contact and bone quality: Evaluation of expected and actual bone contact on machined an osseotite implant surfaces. Int J Periodontics Restorative Dent (2002), 22, p.535-545

146. Trisi P, Lazzara R , Rebaudi A, Rao W, Testori T, Porter SS. Bone-implant contact on machined and dual acid-etched surface after 2 months of healing in the human maxilla.J Periodontal (2003), 74, p.945-956

147. Rahnem O .The influence of hydroxyapatite coating on the peri-implant migration of polyethylene particles. PhD dissertation, Aarhus, Denmark (2002)

148. Rost FWD Fluorescence Microscopy, Vol. II. Cambridge: Cambridge University Press, (1995)

149. Plenk H jr, Lederer J. Histomorphology of bone regeneration after sinus floor elevation with two types of TCP granulate – a case report, Zeitschrift für Orale Implantologie (2005) p.32-38

150. Klawitter, J.J. und S.F. Hulbert Application of porous ceramics for the Attachment of load bearing internal orthopedic applications. J. Biomed. Mater. Res. Symposium(1971), 2, p.161 – 229

151. Eggli, P.S., W. Mueller und R.K. Schenk The role of pore size on bone ingrowth and implant substitution in hydroxyapatite and tricalcium phosphate ceramics; a histologic and morphometric study in rabbits. In: Pizzoferrato, A. et al.: Biomaterials and Clinical Applications, Elsevier Science Publishers B.V., Amsterdam (1987), p.53-56

152. Cornell, C.N. Osteoconductive materials and their role as substitutes for autogenously bone grafts. Bone Grafting and Bone Graft Substitutes (1999),30, p.591-598

153. Foitzik, C. und H.-A. Merten ,Restitutio ad integrum. Vom Knochenersatz zum Knochenaufbau. Spitta Verlag, Balingen, (1999)

154. Shimizu, S.-I. Subcutaneous tissue responses in rats to injection of fine particles ofsynthetic hydroxyapatite ceramic. Biomedical Res.(1988), 9, p.95-111

155. LeGeros RZ.Biodegradation and bioresorption of calcium phosphate ceramics.Clin Mater. (1993), 14(1), p.65-88

156. Goran Knežević, Marijana Rinčić, Dinko Knežević. Radiological Evaluation of the Healing of Bone Defects Filled with Tricalcium Phosphate (Bioresorb) after Cystectomy of the Mandible.Acta Stomatol Croat. (2007), 41(1), p.66-73

157. Crubezy E, Murail P, Girard L, Bernadou JP.False teeth of the Roman world.Nature (1998), 391, p.29

158. Strock AE .Experimental work on a method for the replacement of missing teeth by direct implantation of a metal support into the alveolus. Am J Orthod (1939). 25. p.467-472

159. Müller R In.Tagung der Gesellschaft für Zahn-, Mund- und Kieferheilkunde.Düsseldorf ,(1937),Bericht der 74, p.45

160. Marziani L Dental implants and implant dentures. Their theory, history and practice. Dental Implants (1954), 4, p.459-481

161. Brånemark PI Osteointegration and its experimental background. J Prosthet Dent(1983) ,50, p.399-410

162. Brånemark PI, Hansson BO, Adell R, Breine U, Lindstrom J, Hallen O, Ohman A Osseointegrated implants in the treatment of the edentulous jaw. Experience from a 10-year Period.Scand J Plast Reconstr Surg Suppl (1977), 16, p.1-132

163. Linkow LI, Endosseous blade-vent implants: a two-year report. J Prosthet Dent (1970). 23, p.441-448.

164. Hodosch M, Shklar G, Povar M, Current status of the polymer tooth implant concept.Dent Clin North Am (1970), 14, p.103-115

165. Schulte W, The intra-osseous Al2O3 (Frialit) Tuebingen implant.Developmental status after eight years (I). Quintessence Int (1984), 1, p.9-26

166. Schroeder A, van der Zypen E, Stich H, Sutter F The reaction of bone, connective tissue and epithelium to endosteal implants with sprayed titanium surfaces. J Oral Maxillofac Surg (1981), 9, p.15-25

167. Hansson Stig, Michael Norton. The relation between surface roughness and interfacial shear strength for bone-anchored implants. A mathematical model. Journal of Biomechanics,(1999), 32, p.829-836

168. Branemark PI, Adell R, Breine U, Hansson BO, Lindstrom J, Ohlsson A: Intra-osseous anchorage of dental prostheses. I. Experimental studies. Scand J Plast Reconstr Surg (1969), 3, p.81-100.

169. Tallgren A. The continuing reduction of the residual alveolar ridges in complete denture wearers: a mixed longitudinal study covering 25 years. J Prosthet Dent (1972), 27, p.120-32

170. Atwood DA. Some clinical factors related to the rate of resorption of residual ridges. J Prosthet Dent (1962) 12, p.441-50

171. Esposito M, Hirsch JM, Lekholm U, Thomsen P .Biological factors contributing to failures of osseointegrated oral implants.(I).Success criteria and epidemiology. Eur J Oral Sci (1998), 106, p.527-551

172. Albrektsson T, Zarb G, Worthington P, Eriksson AR ,The long-term efficacy of currently used dental implants: a review and proposed criteria of success. Int J Oral Maxillofac Implants (1986), 1, p.11-25

173. Albrektsson T, Sennerby L, State of the art in oral implants. J Clin Periodontol (1991), 18, p.474-481

174. Shulman LB, Rogoff GS, Savitt ED, Kent RL Evaluation in recosntructive implantology.Dent Clin North Am (1986), 30, p.327-349

175. Schmitt A, Zarb GA, The longitudinal clinical effectiveness of osseointegrated dental implants for single-tooth replacement. Int J Prosthodont (1993), 6, p.197-202

176. D.F. Williams Biocompatibility of clinical implant materials, CRC Press, New York (1971)

177. B. Kasemo, Biocompatibility of titanium implants: surface science aspects. J Prosthet Dent (1983), 49, p.832–837

178. Steinemann S. Werkstoff Titan. In: Schroeder A, Sutter F, Krekeler G, editors. Orale Implantologie. Stuttgart: Thieme, (1988)

179. T.Albrektsson and H.-A. Hansson, “An ultrastructural characterization of the interface between bone and sputtered titanium or stainless steel surfaces,”Biomaterials (1986), 7, p.201-205

180. L. Linder, K. Obrant, and G. Boivin, “Osteointegration of metallic implants. 2. Transmission electron microscopy in the rabbit,”Acta.Orthop. Scand., (1989), 60, p.135-139

181. T. Albrektsson, The response of bone to titanium implants. CRC Crit Rev Biocompat (1985), 1, p.53

182. Chacon G.E, Bower D.L, Larsen P.E, McGlumphy E.A, Beck F.M: Heat production by 3 implant drill systems after repeated drilling and sterilization, Journal of Oral and Maxillofacial surgery(2006), 64 (2), p.265-269

183. McCracken M, Dental implant materials: commercially pure titanium and titanium alloys. J Prosthodont (1999), 8, p.40-43

184. Lautenschlager EP, Monaghan P, Titanium and titanium alloys as dental materials. Int Dent J (1993), 43, p.245-253

185. Wataha JC, Materials for endosseous dental implants. J Oral Rehabil (1996), 23, p.79-90

186. Schmalz G, Garhammer P, Biological interactions of dental cast alloys with oral tissues.Dent Mater (2002), 18, p.396-406

187. Tschernitschek H, Borchers L, Geurtsen W Nonalloyed titanium as a bioinert metal—a review. Quintessence Int (2005), 36, p.523-530

188. Hösch A, Strietzel R Korrosion von Titan in thiocyanat-, chlorid- und fluoridhaltigen künstlichen Speicheln. Dtsch Zahnärztl Z (1994), 49, p.767-769

189. Lenz E, Der Einfluß von Fluoriden auf das Korrosionsverhalten von Titan. Dtsch Zahnärztl Z (1997), 52, p.351-354

190. Patyk AJ, Ohm H Die Wirkung von fluoridhaltigen Prophylaxemitteln auf Titanoberflächen.Dtsch Zahnärztl Z (1997), 52, p.364-367

191. Koike M, Fujii H In vitro assessment of corrosive properties of titanium as a biomaterial.J Oral Rehabil (2001), 28, p.540-548

192. Parr GR, Gardner LK, Toth RW Titanium: the mystery metal of implant dentistry.Dental materials aspects. J Prosthet Dent (1985), 54, p.410-414

193. B.D. Boyan, T.W. Hummert, D.D. Dean and Z. Schwartz.Biomatherials (1996), 17, p.137

194. P.F.Chauvy, C. Madore and D. Landolt.Surf.Coat.Technol. (1998), 110, p.48

195. Ban. S Maruno S, Arimoto N, Harada A, Hasegawa J. Effect of electrochemically deposited apatite coating on bonding of bone to the HA-G-Ti composit and Titanium. J Biomed Mat Res (1997), 36, p.9-15

196. Yang CY, Wang BC, Lee TM, Chang E, Chang GL. Intramedullary implant of plasma-sprayed hydroxyapatite coating: An interface study. J Biomed Mat Res (1997), 36, p.39-48

197. Massimiliano Baleani, Marco Viceconti, Aldo Toni. The effect of sandblasting treatment on endurance properties of titanium alloy hip prostheses. Artificial Organs, Blackwell science, Inc.(2000), 24(4), p.296-299

198. O. Moztarzadeh, D. Hrusak, P. Andrle, L. Hauer, L. Hosticka: Ectopic Chondroosteoid tissue formed by tricalcium phosphate. Journal of Cranio-Maxillofacial Surgery, Vol. 36, Suplement 1, September 2008, P. 272 (IF: 1.171)

199. André Schroeder, Franz Sutter, Daniel Buser, and gisbert Krekeler: Oral Implantology, (1996), p.102-110

200. L. Dluhos, J. Petruzelka, R. Valiev, O. Moztarzadeh: Biological Properties of Nanostructured Titanium made by SPD. Journal of Cranio-Maxillofacial Surgery, Vol. 36, Suplement 1, September 2008, P. 260 (IF: 1.171)

201. D. Hrusak, L. Dluhos, C. Arnold, O Moztarzadeh: Immediate load of nanostructured Ti Implants. Journal of Cranio-Maxillofacial Surgery, Vol. 36, Suplement 1, September 2008, P. 262 (IF: 1.171)