Bone Allograft and Implant Fixation Tested Under Influence of Bio-burden Reduction, Periosteal Augmentation and Topical Antibiotics

Animal experimental studies

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THIS THESIS IS BASED ON THE FOLLOWING PAPERS:

1. Barckman J, Baas J, Sorensen M, Bechtold J, Soballe K. Rinsing allograft bone does not improve implant fixation - A study in 12 dogs. Acta Ortop. 2013 jun;84(3):307-13.

2. Barckman J, Baas J, Sorensen M, Bechtold J, Soballe K. Periosteal augmentation of allograft bone and its effect on implant fixation - An experimental study in 12 dogs. Open Orthop J. 2013;7:18-24.

3. Barckman J, Baas J, Sorensen M, Lange J, Bechtold J, Soballe K. Tobramycin impregnation of allograft bone and its effect on implant fixation - An experimental study in 12 dogs. J Biomed Mater Res Part B. 2013 Jul 30.

The papers will be referred in the text by their Roman numerals (I–III)

1. INTRODUCTION

Total hip replacement (THR) is a successful treatment of end-stage degenerative joint diseases, acute proximal femur fracture and fracture sequelae. According to the Danish Hip Arthroplasty Register, 8,787 primary THRs and 1,443 hip revisions were performed in the year 2012 [1]. The ten-year mean implant survival rate for patients between 60 and 75 years of age is 93% for the primary prosthesis, which declines to 82% for the first and to 72% for the second revision prosthesis [1]. The current trend is that younger and younger patient groups receive primary prostheses. These patients live longer and have a more active life, which increases the amount and duration of mechanical demands on the prosthesis. This may result in shorter implant survival and thus a higher risk of revision. More patients are therefore expected to need more than one revision during their lifetime [2].

Loosening of an artificial hip prosthesis is a serious and painful complication that causes severe disability, and the only treatment is replacement of the primary prosthesis. This is an effective treatment, but it is often complicated by inadequate proximal bone stock, which compromises its initial stability. In order to reconstruct these bony defects, the well-established method of impaction grafting is used. This method enables restoration of the bone-bed, which optimizes the initial mechanical support of the revision prosthesis [3-6] and potentially improves the scope for subsequent re-revision. Among bone graft options, autologous bone graft is the gold standard. It has a natural, good biocompatibility, but its use in revision surgery is curtailed by its limited volume and by considerable donor site morbidity. Allograft bone is readily available and is the most commonly used graft material in revision surgery. However, it has been shown that the incorporation of bone graft into the host bone is not always complete [7, 8], and considerable formation of fibrous tissue has been demonstrated within the bone graft [9, 10]. This may partly explain the higher failure rates and poorer functional outcomes among patients having undergone revision surgery than among patients who have received primary surgery [11]. In addition, several clinical trials have shown increased early implant subsidence when impaction grafting is used [12, 13]. This early implant subsidence has been found to be associated with an increased risk of later implant loosening [14]. Furthermore, when revising a potential infectious prosthesis, re-infection is of great concern.

AIM

The overall aim of this thesis was to evaluate different methods for improving the longevity of grafted revision implants. Autograft bone is the gold standard of bone grafts. It has osteoinductive, osteogenic and osteoconductive capacities. Whereas in autograft bone cells are vital, freezing largely destroys the cellular components of allograft bone and thereby diminishes its osteoinductive capacity [15]. Furthermore, allograft bone

can provoke a substantial immunological host response or can be colonized with bacteria. We evaluated the effect of lowering the immunogenic load by rinsing, periosteal augmentation and antibiotic protection of the allograft bone. Imitating clinically relevant conditions, we used an established experimental implant model. The implant fixation was evaluated by mechanical push-out test and histomorphometry.

HYPOTHESES

STUDY I

Rinsing of the allograft bone will enhance fixation of grafted implants.

Theory rationale: Rinsing will reduce the immunogenic load experienced by the host, which will lead to improved osseointegration.

Study II

Periosteal augmentation of allograft bone will enhance the fixation of grafted implants

Theory rationale: The periosteum contains pluripotent mesenchymal progenitor cells whose presence will aid initial bone healing.

Study III

Antibiotic impregnation of the allograft bone will impair fixation of grafted implants.

Theory rationale: Tobramycin is a cytotoxic drug, which may compromise bone graft incorporation.

The background for these hypotheses will be described in the following sections.

2. BACKGROUND

IMPLANT OSSEOINTEGRATION

Ensuring living rigid fixation of an implant by ongrowth of new bone is known as osseointegration and is the ultimate goal when inserting an uncemented orthopaedic implant [16]. Osseointegration was first described by Brånemark et al. in 1977 and later defined by Albrektsson et al. in 1981 as the direct contact at the light-microscope level between living bone and implant [17]. This histological definition of osseointegration does have some limitations as the clinical effect is not taken into consideration. For that reason, a more biomechanical definition has been suggested: Osseointegration is “a process whereby clinically asymptomatic rigid fixation of alloplastic materials is achieved, and maintained, in bone during functional loading” [18]. In clinical studies, radio-stereometric-analysis (RSA) can be used as a surrogate parameter for implant osseointegration. But in clinical practice, successful osseointegration of a cementless implant is evaluated on x-rays, where the implant is assessed for its direct contact with the surrounding bone at the macroscopic level [19]. In accordance with the functional definition of osseointegration, RSA analyses have described subsidence of the prostheses in conjunction with thigh pain, although x-ray findings showed bone in direct contact with the implant.

Implant osseointegration is dependent on a variety of factors. An important prerequisite for osseointegration is osteoinduction. This term refers to the capacity of chemicals in the body to stimulate primitive stem cells or immature bone cells to grow, mature and form healthy bone tissue. This stimulation is a basic biological mechanism that occurs regularly, e.g. in fracture healing and implant incorporation. It can be defined as the process by which osteogenesis is induced [20]. Most of these stimuli come from protein molecules, which collectively are called peptide growth factors or cytokines. Many of these growth factors are present in normal human bone and to some extent also in processed donor bone (allograft).

The induced osteogenesis is a process of bone formation in which new bone material is formed by osteoblasts. Only living cells can make new bone. The success of bone graft incorporation therefore depends on the presence of living bone-forming cells. If the grafting procedure is performed in healthy bone, the number of bone-forming cells may be sufficient. But in the clinical setting, the revision surgery is often performed in elderly patients with compromised bone quality. The autologous bone graft has an osteogenic capacity owing to its content of living bone-forming cells and is therefore the optimal choice of bone graft.

Another important factor in osseointegration is osteoconduction. The term osteoconduction refers to the ability of some materials to serve as a scaffold on which bone cells can attach, migrate, grow and divide. These cells may originate from either pre-existing preosteoblasts/osteoblasts that are activated by trauma or in cells recruited from primitive mesenchymal cells by osteoinduction. An osteoconductive material can serve as a spacer, which reduces the ability of tissue around the graft site to grow into the bone defect, thereby enabling bone cells to fill the entire gap between the two bone ends which will result in gap healing. Many materials are available as osteoconductive materials, and more are being developed. These materials include processed human bone (allograft bone), several calcium phosphate ceramics and synthetic polymers. Furthermore, the degree of osteoconduction is in part determined by the biocompatibility of the implant material, which has been illustrated in studies of the osseointegration of different metal surfaces [21].

BONE GRAFT

Bone graft is used for skeletal reconstruction in limb salvage surgery as a result of trauma, infection or tumour resection or in reconstruction of failed arthroplasties. The types of bone graft typically used in clinical practice are the non-vascularized autograft and allograft bone. The autograft bone is harvested from the patient him- or herself, whereas allograft bone is harvested from a donor patient. Xenograft bone, which is harvested from another species, is rarely used. Bone graft material can be divided into types by structure and histology as cortical, cancellous, corticocancellous or osteochondral. Cortical and corticocancellous grafts are used as structural grafts in reconstruction of large bone defects, while morselized cortical or cancellous grafts are used to impact closed defects.

The freshly harvested autologous bone graft is considered the golden standard of bone graft to which all other types of grafts must be compared. It contains living bone cells and is by the host recognized as “self”, whereas the allograft and xenograft may be recognized as “non-self” due to histoincompatibility between donor and host. Although being the gold standard, the use of autologous bone graft has consequences: 1) an additional surgical site is often needed; 2) donor site morbidity; 3) increased fracture risk at donor site due to weakening of the donor bone; and 4) limited volume available [22]. For these

reasons, allograft bone is the preferred graft material when larger volumes are needed. Allograft bone is usually modified or preserved to reduce immunogenicity before transplantation. These modifications include freezing, irradiation, rinsing or chemomodification [23]. These procedures cause bone cells in the allografts to undergo necrosis. Thus, allograft bone serves mainly as a construct providing structural support onto which new bone can be formed (osteoconduction).

History of bone grafting

The first described bone grafting procedure was performed in the year 1668 by the Dutch surgeon Job van Meekeren [24]. He successfully repaired a traumatic bone defect in a soldier's cranium with a bone graft derived from a dog's scull. Until the year 1915, published work on grafting procedures was sparse. But after the publication of F.H. Albee's work on bone graft surgery in 1915 [25], bone grafting procedures began to gain momentum. In the mid-1970s, the bone grafting technique was developed to a level where it became a common clinical practice. In 1975, D. E. Hasting described the use of bone graft in conjunction with bone cement for acetabular reconstruction of protrusio acetabuli [26]. T. J. Slooff introduced a modification of this technique in 1984, where he reconstructed the bony defects of the acetabular component with the use of a metal fibre mesh, which was tightly packed with morselized allograft bone chips. Pressurized cement was applied between the bone graft and the implant, a technique known as impaction bone grafting [27]. A femoral bone grafting technique was introduced in 1987 involving bone graft impaction in the circumferential part of the proximal femur. This procedure formed a neomedullary canal into which the revision femur prostheses were inserted [28]. However, success was limited due to high subsidence, and in 1994 the Slooff group introduced the cemented bone graft technique in which the stem-cement-graft construction provides the initial fixation [29].

Bone grafting in revision surgery

The effect of a bone grafting procedure performed in revision hip surgery is difficult to evaluate. Many factors influence the outcome of the prosthesis survival and function compared with the primary arthroplasty, e.g. increased patient age, comorbidity and the issue of compromised bone. Nevertheless, impaction grafting in revision THR has produced good medium- to long-term results on both the acetabular and the femoral side [5]. This has been evaluated by RSA-studies on subsidence, patient evaluation scores or the need of re-revision. On the femoral side, early subsidence of over 10 mm has been described in 10% of the patients and was associated with thigh pain [12]. On the acetabular side, the overall survival rate with aseptic loosening as the end point has been shown to be 94% at 11.8 years of follow-up. [30]. Although both the cemented and the uncemented grafting technique are being used today, there is no sharp definition as to when cement should be applied as illustrated in a study that compared impaction bone grafting in femoral revisions and found no difference in outcome between the two techniques [31]. However, it must be stressed that this study was not randomized, and the choice of technique was based on the perioperative evaluation of the revision cavity where patients with wide cavitary proximal defects underwent cemented procedures.

The outcome of a revision joint arthroplasty is considerably worse than the outcome of the primary arthroplasty. In Denmark, the 10-year survival of the primary arthroplasty is 93%, which declines to 82% at the first revision and 72% at the second revision [1]. The theoretically positive effect of grafting procedures on later re-revisions is not well described. However, experimental and retrieval studies indicate that bone grafting at least to some extent leads to regeneration of the lost bone stock [8, 29].

Aim of bone grafting

The primary aim of bone grafting performed in reconstructive joint surgery is to give structural support to the prosthesis. A second aim is to restore the bone loss with bone graft pending remodelling into living bone. The ideal course of a grafting procedure is for the bone graft to fill the bone defect around the prosthesis, which will provide optimal initial mechanical support. During remodelling, the mechanical properties should be maintained to avoid transient weakening. Thus, the osteoclastic bone graft resorption and simultaneous osteoblastic new bone formation have to be well-balanced.

RINSING OF ALLOGENEIC BONE GRAFT

As previously mentioned, contrary to autogenic bone, allograft bone is recognized as "non-self" due to histoincompatibility between donor and host. The transplanted bone tissue is believed to be subjected to a host vs. graft response of a magnitude proportional to the total antigen concentration and the total antigen dose [4]. This immunogenic response is thought to impair the bone graft incorporation and subsequently to lead to increased fibrous tissue formation [4]. The most antigenic cells of the allograft bone have been shown to originate from the bone marrow [32]; and by removing blood, marrow and fat from the bone graft, improved bone graft incorporation has been demonstrated in a bone chamber model in goats [7]. Furthermore, laboratory experiments have shown that rinsing improves the mechanical stability of impacted morselized allograft bone [33-35]. The authors concluded that the removal of fat, marrow and soft tissue optimized the contact between the bone chips which allowed for better particle interlock and tighter graft compaction.

Different methods have been used for rinsing the bone graft: Manual rinsing [36] and pulse-lavage washing [33, 37-39]. Manual rinsing preserves a broad range of bone graft particle sizes, which has been shown to be important to achieve optimal mechanical strength of the impacted bone graft [40]. Rinsing by pulse-lavage may be more efficient; but contrary to manual rinsing, it causes smaller bone graft particles to be lost, which narrows the particle-size distribution.

Other benefits of bone graft rinsing have been described. The risk of bacterial contamination is always an issue when fresh-frozen, non-irradiated femoral heads are being used [41, 42]. This risk has been found to be directly related to the amount of blood present in the bone graft [43] and rinsing has shown to significantly reduce the risk of bacterial transmission [44] as well as the transmission of blood-borne viruses such as human immunodeficiency virus [43].

CELLULAR AUGMENTATION OF ALLOGENEIC BONE GRAFT

Processing causes the cells of the allogenic bone to become necrotic, whereas the cells of the autogenic bone graft are living

cells. By adding living autologous cells with osteoblastic potential to the allogeneic bone graft, bone-healing properties like the one seen with autogenic bone graft may be obtained.

Periosteum, which covers nearly every bone in the body, plays a major role in promoting bone growth and bone repair [45, 46]. It consists of two distinct layers, an outer fibrous layer and an inner cambium layer that has significant osteoblastic potential [47]. The latter contains mesenchymal progenitor cells, osteoblasts and fibroblasts [48] and provides essential cellular and biological components necessary for fracture healing and bone repair [49, 50]. The periosteum can be described as an osteoprogenitor cell-containing bone envelope that can be activated to proliferate by trauma, inflammation and tumour growth [47]. The periosteum has also been shown to stimulate bone formation when transplanted as an autologous non-vascularized periosteal graft [51] or as cultured autologous periosteal cells seeded on a scaffold and implanted in vivo [52].

INFECTIOUS PROTECTION OF ALLOGENEIC BONE GRAFT

Systemically administered antibiotics are widely used as treatment and prophylaxis against infection. Since the impacted bone graft is initially an avascular area, these antibiotics cannot easily gain access to the graft site, which implies that the risk of post-operative infection is heightened [53]. For that reason, local antibiotic delivery has become a much researched area in recent years. In vitro studies have shown that morselized allogeneic bone graft have the ability to act as a delivery vehicle of antibiotics [54-58], and it has become practice to add antibiotics to the bone graft in order to decrease the risk of post-operative infection.

Periprosthetic infection

Infection after total hip arthroplasty is a serious complication. Revising an infectious arthroplasty is one of the most challenging procedures performed in revision surgery. Effective management demands control of both the infection and reconstruction of the often substantial loss of bone. The treatment commonly requires removal of the implant, radical debridement and long-term antimicrobial treatment. Radical debridement is required for curing the infection, but the operated site cannot be totally sterilized by the debridement alone. For that reason, the treatment of choice has been a two-stage procedure where the patient underwent long-term systemic antibiotic treatment after implant removal and debridement [59, 60]. When the signs of infection have declined and blood values for infection return to normal, a second operation is performed whereby a revision prosthesis is inserted. At this stage, the bony defects can be filled with either cement or bone graft. The cemented method is the most often used fixation method because it allows the surgeon to add antibiotics to the cement to obtain topical distribution. However, it has been reported that for aseptic loosening, the re-revision rate is higher in the cemented revisions than in the cement-less revisions (i.e., 15.1% versus 4.3% for the acetabular cup and 12.7% versus 5.5% for the femoral stem) [61]. The method of cement-less revisions relies on the use of bone grafts as a filler of the bony defects. However, the use of unvascularized bone grafts may introduce a risk of colonization and the risk of post-operative infection is believed to be heightened [53].

Carrier for topical antibiotics

Local administration of antibiotics makes it possible to achieve a local drug concentration that is much higher than the therapeutic level without reaching a toxic serum concentration [62]. Antibiotic-loaded cement, spacers or bead chains have been used [63], but they often require removal and grafting of defects in a second or third operative procedure. Furthermore, although concentrations of locally applied antibiotics initially exceed those achievable by systemic application, these high concentrations are short-lived, and the empty carriers can be problematic. Thus, colonization with surviving bacteria has been described [64]. A carrier should ideally not only deliver antibiotics locally, but should also restore bone-stock deficiencies. These characteristics may be found in morselized allograft bone which is widely used in reconstruction of bony defects in revisions surgery [65]. Local antibiotics delivery with bone graft as a carrier is possible as demonstrated by several laboratory studies [54-58]. Furthermore, several clinical trials have been conducted on one- and two-stage revisions on failed arthroplasties using antibiotic-impregnated bone graft. Promising in terms of bone graft incorporation and infection prophylaxis have been reported [53, 66, 67]

Antibiotic of choice

The choice of antibiotic depends upon the potential causative pathogen. A study examining biopsies from 291 patients with infected hip arthroplasties operated in the period from 1974 to 2005 shows predominantly Gram-positive bacteria where coagulase-negative staphylococci accounted for 67%, Staphylococcus aureus for 13%, E. Coli for 6% and streptococci for 9% [68]. Vancomycin and Tobramycin have been the antibiotics most studied in bone graft impregnation studies [54-58]. Vancomycin is a bacteriostatic glycopeptide antibiotic inhibiting proper cell wall synthesis in Gram-positive bacteria. It also has an effect on infections with MRSA [69] and Staphylococci, both coagulase-positive and -negative, which makes it ideal for treating infection with Gram-positive strains. Tobramycin is a widely used aminoglycoside [55]. It is a bactericidal antibiotic that is effective against many Gram-negative and Gram-positive pathogens, and it covers the majority of pathogens encountered in orthopaedic surgery [56, 70] and is for that reason the antibiotic of choice in the present thesis. As a cytotoxic drug, Tobramycin binds to the 30S subunit of the bacteria ribosome. It thereby hampers its translation of the mRNA, which causes an increased rate of error in the synthesis of proteins leading to cell death. It also has a low resistance rate and a low allergy rate [70].

3. MATERIALS AND METHODOLOGICAL CONSIDERATIONS

EXPERIMENTAL MODEL

Experimental animals

Different species are used for experimental studies exploring implant osseointegration. Dogs have many advantages and are regarded one of the best species for imitating the true nature of bone regeneration around implants in the human skeleton [71]. The dog is a large animal, and its bone quality and trabecular density reflects human bone better than that of most other animals [72]. The bone turnover time of the remodelling activity in dog is on average approximately 2.5 times faster than in

humans, which shortens the needed observation period [72, 73]. Furthermore, the metaphyseal parts of the long bones are large enough for bilateral implant insertion, which makes paired study designs possible. Our research group has much experience with dogs as an experimental animal owing to extensive research over the past three decades [74-88].

The dogs used the in present studies were all skeletally mature and bred for scientific purposes. Surgery and observation was performed at the Animal Care facilities of the Minneapolis Medical Research Foundation at the Hennepin County Medical Center (Minneapolis, MN, USA) according to the regulations of the National Institute of Health. The local Animal Care and Use Committee approved all experiments (MMRF, Minneapolis, NN, USA; Study I and II: submission ID 08-08-02, Study III: submission ID 10-11-02). Institutional guidelines for treatment and care of experimental animals were followed.

Study design

All experiments were conducted as paired, randomized, controlled studies where both the control and intervention implant were present in the same animal. The paired design eliminates the variation caused by any biological difference between the animals, which reduces the total variance and reduces the number of animals needed to detect a given difference.

Grafted gap implants were used in all three studies of this thesis. The implantation site was rotated with random start to avoid any systematic influence from potentially undetected minor site-dependent difference. Due to the small number of animals, this rotation was done systematically to secure a uniform distribution. This ensured that a site-dependent difference would add to data variance rather than bias the results.

- **Study I:** A three-armed study conducted in the distal femur with the control group in one femur and the two interventions implants in the other femur. The implantation site (medial lateral; left right) was alternated systematically.
- **Study II:** A two-armed study conducted in the distal femur. Both implants were inserted into the same femur (one in the medial epicondyle and one in the lateral epicondyle). The implantation site (medial lateral; left right) was alternated systematically.
- **Study III:** A two-armed study conducted in the proximal tibia with one implant in each tibia rotated systematically.

Blinding during surgery was not found practical, but specimen preparation, mechanical testing and histomorphometry were performed blindedly.

Multi-study animals

Surgery in large animals is time-consuming, and animal handling during and after surgery is expensive, which necessitates multi-study animals. The negative aspect of this is the theoretical contamination or bias from other intervention groups. However, all studies conducted simultaneously with the studies in this thesis were local surface treatment studies. Study I and II were conducted in the femoral bone of the same animal and the additional studies were performed in the proximal humerus and proximal tibia. The humerus study explored the impact on implant fixation of adding local Simvastatin (1.0 mg) to a poly-DL-lactide (PDLLA) surface coating; the tibia study explored the effect of topical bisphosphonate treatment of the bone cement interface. Study III was conducted in the proximal tibia of another group of animals; here additionally studies were also performed in the distal femur and proximal humerus. The femur study explored the benefits of washing the revision cavity with low-dose bisphosphonate before implant insertion, and the humerus study examined osseointegration of implants with different levels of surface-adherent endotoxin.

To minimize risk of contamination between study groups, each implant was inserted through separate skin incisions with separate instruments. The bisphosphonate studies were conducted as the last surgery after all other implants had been inserted and the skin closed in layers. Although systemic distribution between groups is possible, its impact is believed to be negligible owing to the fairly small concentrations and the paired comparison of the results.

Sample size

The number of animals included in each study was based on sample size estimation for the paired study group using the following formula:

$$N = \left(C_{2\alpha} + C_{\beta}\right)^2 x \frac{CV_{DIFF}^2}{\Delta^2}$$

The risk of type I error (α) was set to 0.05 and the risk of type II error (β) was set to 0.20. Based on previous studies using the same implant model, the coefficient of variance of the paired difference was set to 30%. The minimal, relevant difference to be detected was set to 30%. Assumptions for paired t-test were assumed fulfilled.

$$N = (2.262 + 0.883)^2 \, x \, \frac{(30\%)^2}{(30\%)^2} \; = \; 9.89$$

Two extra animals were added to the calculated sample size of ten to counteract a decrease in power if implants from one or two animals were lost to follow-up and subsequent analysis. Two additional animals not included in the studies were used as allograft donors.

N	=	Number of animals
$C_{2\alpha}$	=	The quantile in the t-distribution at two-sided testing (2.262; p=0.05; d.f.=9)
C_{β}	=	The quantile in the t-distribution at one-sided testing (0.883; p=0.20; d.f.=9).
CV^2_{DIFF}	=	Square of the coefficient of variance of the paired differences
Δ^2	=	Square of the minimal, relevant difference to be detected.

Implant model

The implant model used in this PhD thesis a well-proven model developed by Kjeld Søballe [87, 89]. It is designed to study early fixation and osseointegration of an uncemented implant by imitating the portion of a total joint replacement placed into a bed of impacted bone graft. The implants consisted of a cylindrical porous-coated titanium implant with a diameter of 6 mm

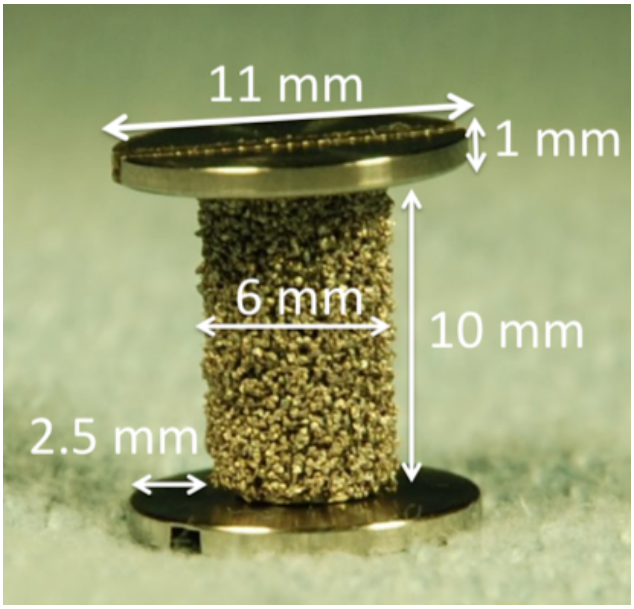

Figure 1 *Implant with measurements*

and a height of 10 mm. An 11-mm bottom screw was attached to each implant (*Figure 1*); and when inserted into an 11-mm drill hole, this centred the implant providing a uniform 2.5-mm peri-implanteric gap. The gap was filled with impacted morselized allograft bone (*Figure 3)*. For the three studies, the control group (morselized allograft bone) was tested against morselized allograft bone that was either rinsed, impregnated with antibiotic or augmented with fragmented periosteum. After impaction grafting, the gap was closed with an 11-mm top screw to ensure stability and bone-graft containment. The same implant model was used for all three studies in this thesis.

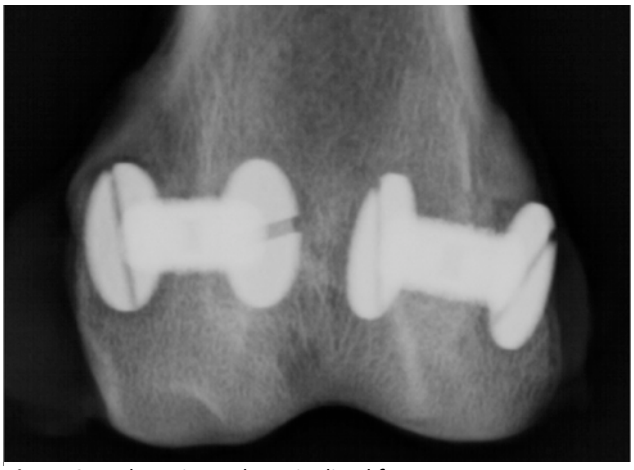

Figure 2 *Implants in Implants in distal femur*

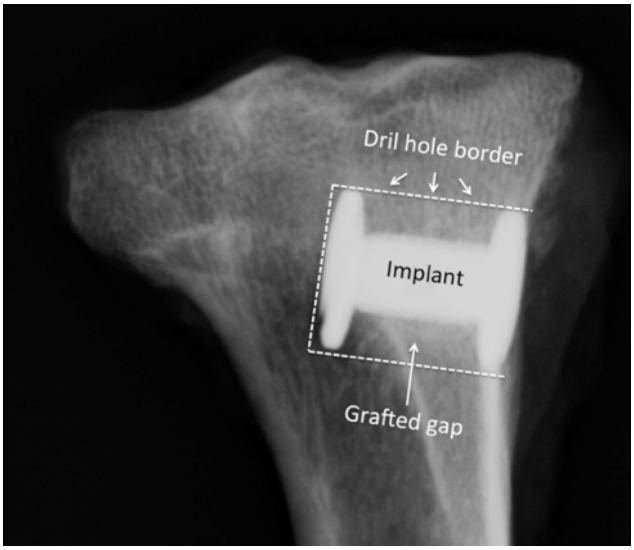

Figure 3 *Implants in proximal tibia*

All implant were inserted into the trabecular bone of the metaphyseal portion of long bones. Study I and II were conducted in the epicondyles of the distal femur (*Figure 2*) and Study III in the proximal tibia (*Figure 3*).

This implant model is simple and allows a high degree of control of study parameters [85] which makes it highly reproducible. Furthermore, it enables a paired study design and the opportunity to examine a bone defect around the implant that is large enough to simulate impacting grafting.

Implant characteristics

Implants for all studies were custom-made by the same manufacturer (Biomet Inc., Warsaw, IN, USA) and measured 10 mm in length and 6 mm in diameter. They were produced from a cylindrical titanium core (Ti-6A1-4V) with a diameter of 4.4 mm, onto which a porous Ti alloy coating was applied. The coating is the same as that which is used on the surface of clinical implants from the same manufacturer. All implants in a study were from the same batch. The cylindrical shape was advantageous in the subsequent specimen preparation and in the mechanical and histomorphometric analysis.

Bone graft preparation

Bone graft was harvested immediately post mortem under sterile conditions from two dogs not included in the study, and the graft was stored at -80°C. The proximal humerus, the proximal tibia and the distal femur were used. Prior to preparation, the bones were thawed and all soft tissue and cartilage was removed. The bones were morselized using a standard bone mill (Biomet®, Warsaw, IN, USA) on fine setting, creating bone chips of 1-3 mm in major diameter (*Figure 4*). The bone chips from the two dogs were mixed together. The preparation of the bone graft for the studies was undertaken in one session the day before surgery commenced.

SURGERY

All surgery was conducted under general anaesthesia and sterile conditions. The implantation site was exposed by a skin incision made with cautery and the periosteum elevated. A 2.5-mm K-wire was inserted perpendicular to the bone surface.

Figure 4 *Bone graft preparation*

With a cannulated drill bit of 11 mm in diameter, a 12-mm deep cylindrical bone defect was made. To avoid thermal damage to the bone, a drill speed of two rotations per second was used. The edges of the drill hole were trimmed with a scalpel prior to implantation to ensure that no soft tissue protruded into the gap. An end plate was attached to one end of the implant and inserted into the drill hole with a specially designed hollow cylinder impaction tool. The same tool, which fits into the defect between the implant and the drill hole border, was used to manually impact the bone graft into the gap. To ensure a homogeneous distribution of the graft in the defect, one third of the graft was impacted into the bottom third of the gap, then one third of the graft was impacted into the middle third of the gap, and, finally, the remaining graft was impacted into the most superficial third of the gap.

To ensure uniform implant placement and minimal impaction-technique variance, all surgeries were undertaken by the same surgeon (JBA). Finally, an end plate was mounted onto the implant to maintain its concentric location within the gap, and the soft tissues including the periosteum were closed in layers (*Figure* 5).

Bupivacaine, 0.5% was administered locally following closure of the skin. Prophylactic antibiotics were administered as follows: A 1-g dose of Ceftriaxone was given intravenously to each animal before surgery. Post-operatively, the animals were given Ceftriaxone 1 g intramuscularly for three days. A Fentanyl transdermal patch (75 µg/h) lasting three days was given for post-operative analgesia. The animals were allowed unlimited activity.

Implantation site – Tibia (Study I)

A skin incision was made on the anteromedial part of the tibia just distal to the joint line. The periosteum was elevated and a 2.5-mm Kirschner wire was inserted perpendicular to the anterior medial plane of the proximal tibial surface and 12 mm distal to the tibiofemoral joint line. A hole of 12 mm in depth and 11 mm in diameter was drilled with a cannulated drill bit.

Figure 6 *Bone graft of the two treatment groups*

The gap volume of this model is 0.67cm^3. From prior studies performed by the same surgeon, we know that approximately 1.38g of morselized bone can be impacted into this gap. Twelve portions and four reserve portions of bone allograft were needed in each treatment group. Two portions of morselized allograft bone each weighing 22g (1.38g x 16) were therefore placed in separate glass containers under sterile conditions. In the intervention group, 220ml of 37°C saline (10ml saline per 1g

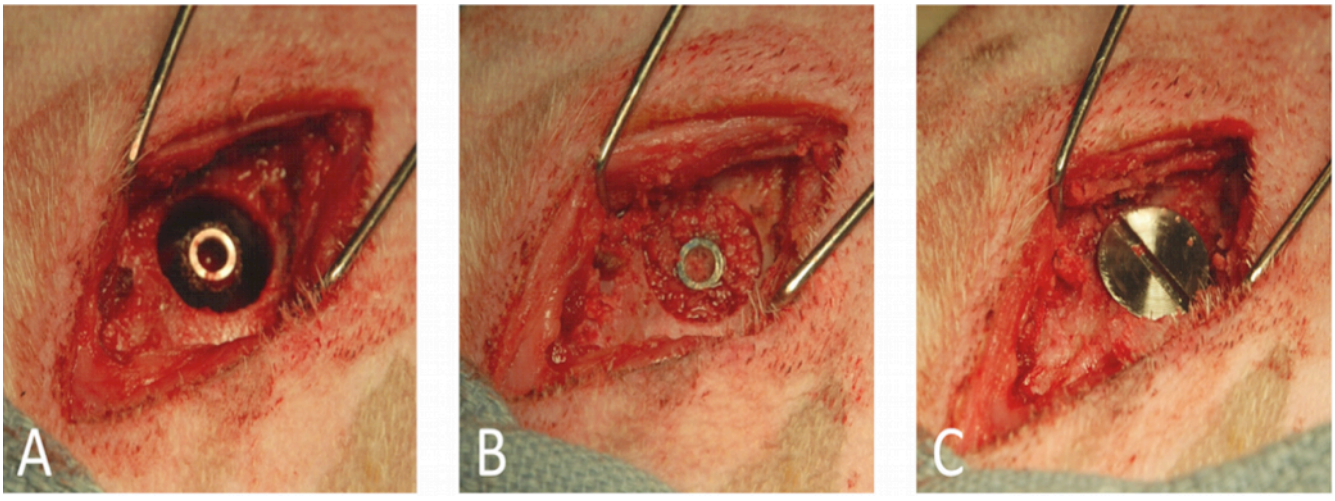

Figure 5 *Surgery: A: Implant inserted and the peri-implanteric gap of 2.5 mm is empty. B: The gap is impacted with morselized allograft bone. C: The gap is closed with a top screw*

bone graft) was added and gentle digital stirring was performed. After one minute, the water was poured off and the bone graft drained by compression. This procedure was repeated three times. Finally, the rinsing water was poured through a fine sieve. No bone remnants were found, which indicates that the amount of bone graft was unaltered by this rinsing procedure. The final volume of rinsed bone graft was measured in 10-ml syringes where maximum manual force was applied. This squeezed the water from the bone graft and rendered it as dry, compressed bone graft. An identical compression procedure was conducted to measure the volume of the non-rinsed control bone graft (*Table 1*). The bone graft in each group (*Figure 6*) was finally divided into 16 equal portions and stored in sterile double-containers at –80°C pending surgery.

Table 1 *Allograft wash*

	Weight (g)	Volume (cm^3)	Volume/impl. (cm^3)
Control	22.08	19.62	1.23
Wash	22.44	17.82	1.11
Wash/ control	-1.63%	9.17%	

Volume and weight was measured after water was drain by maximum manual force.

Implantation site – Femur (Study II and III)

The femoral epicondyles were exposed by a medial and a lateral incision. The joint capsule was opened and the collateral ligament identified. The 2.5-mm guide-wire was placed in the centre of the epicondyle perpendicular to the surface, and a 12-mm deep drill hole was made with the 11-mm cannulated drill bit.

Study II: At surgery, a circular periosteal flap of 1 cm in diameter was elevated from the anterior medial surface of the proximal tibia and fragmented with a scalpel (*Figure 7*). This created small (< $1mm^2$) fragments of periosteum, which were mixed together with the bone graft prior to impaction.

Study III: The bone graft was impregnated with a tobramycin solution prior to impaction. We used a modified Witso method [58] that enabled us to know the precise quantity of Tobramycin added. Tobramycin powder was dissolved in sterile water at two concentrations: 200 mg/ml (low-dose) and 800 mg/ml (high-dose). At surgery, three portions of 1-ml bone graft were thawed for 10 min and placed in separate small sterile metal containers. To each portion, we added as follows: To the control group: 0.25 ml of sterile water; low-dose group: 0.25 ml of 200 mg/ml Tobramycin solution; high-dose group: 0.25 ml of 800 mg/ml Tobramycin solution. The mixtures were stored at room temperature for a minimum of five minutes before implantation.

Post-operative management

Post-operatively, the dogs were housed individually, but socialized in groups with two hours of daily exercise. Our veterinarian inspected the animals daily regarding wound healing, diet and general condition. All animals were allowed unrestricted post-operative weight bearing.

Observation time

The choice of a four-week observation time was based on previous studies conducted in the same animal model [78, 83, 87, 90]. The time point is essential as the evaluation of an intervention is highly determined by the stage of bone graft incorporation. If the observation period is too short, there is a risk that the potential effect of an intervention has not yet manifested itself. On the other hand, if the observation period is too long, there is a risk that a potential effect is not detected at all, since the control implant may be able to reach same level of fixation even if this happens at a slower pace.

Specimen preparations

After the observation period of four weeks, the animals were sedated with Acepromazine, 0.5 mg/kg, anaesthetized using Propofol, 4 mg/kg, and euthanized with an overdose of hypersaturated barbiturate. The bones with the incorporated implants were dissected free and immediately frozen and stored at -80°C. All further preparation of the specimens was performed blindedly. Prior to dividing the bone into the mechanical

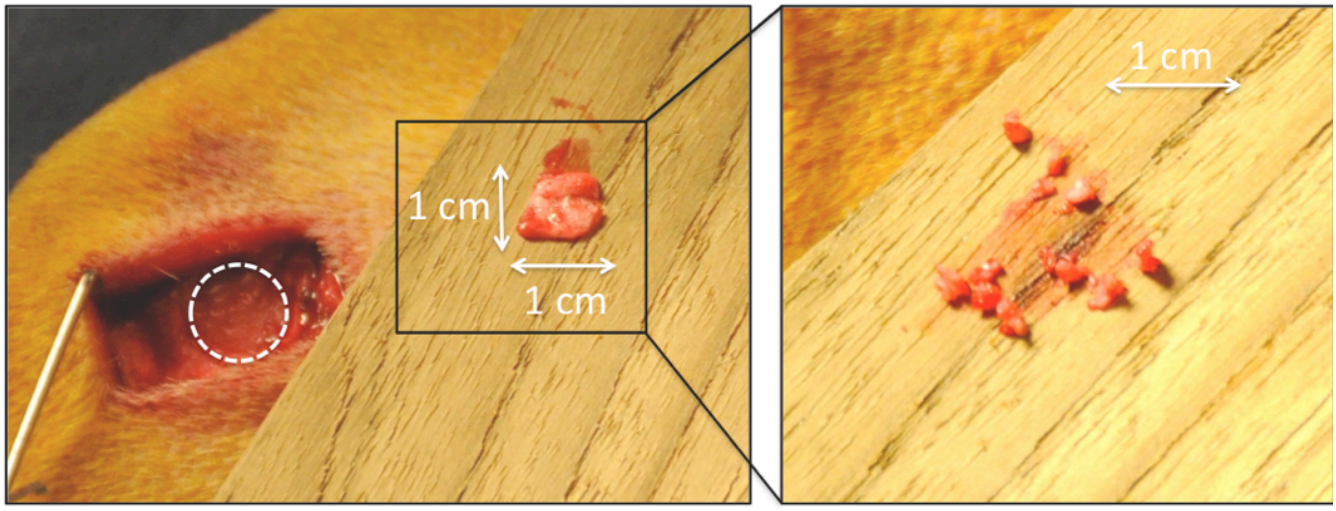

Figure 7 *Left: Periosteum harvest from the anterior medial surface of the proximal tibia. Right: Enlarged picture of the fragmented periosteum*

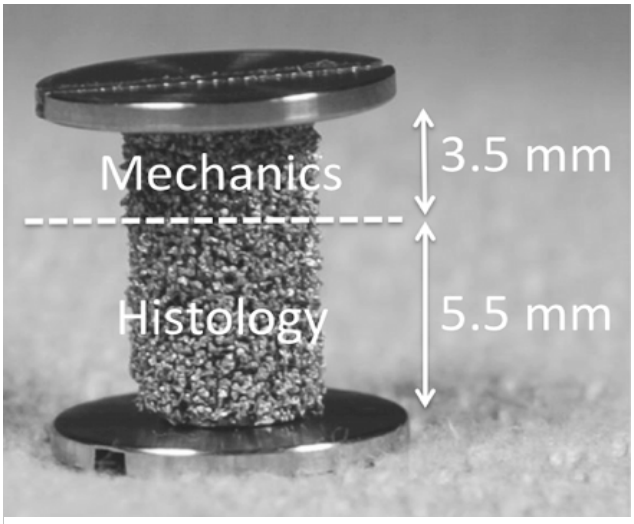

Figure 8 *Transverse sectioning*

and histomorphometric sections, it was thawed and the outermost 1 mm of the implant-bone sample below the top-screw was cut off and discarded. The remaining 9 mm of the implant with surrounding bone was divided into two sections perpendicular to its long axis (*Figure 8*). This procedure was performed using a water-cooled diamond band saw (Exakt, Exakt Apparatebau, Norderstedt, Germany). The first section was cut to a thickness of 3.5 mm and stored at –20°C and used for mechanical push-out testing. The second section with the inner 5.5 mm of the bone implant compound was fixed in 70% ethanol and processed for histomorphometric evaluation.

MECHANICAL TESTING

The thawed specimens were tested to failure by an axial push-out test on an MTS Bionics Test Machine (MTS 858 Mini Bionix, MN, USA, Software: MTS Test Star 790.00 Version 4.0C).

Testing was performed blindedly and in one session. The specimens were placed with the cortical side facing upwards on a metal support jig with the 6-mm diameter implant centred over a 7.4-mm opening, assuring a 0,7-mm distance between the implant and the support jig as recommended [91]. A cylindrical test probe with a diameter of 5 mm was mounted, and a preload of 2 N defined the contact position at the start of the test. The implant was pushed from the surrounding bone in the direction of the implant axis at a velocity of 5 mm/min. Load (N) versus implant displacement (mm) data were continuously recorded for every 10µm of implant displacement (*Figure 9*). To normalize for differences in thickness and diameter of the implants, the load data were converted to strength (*Eq 2,Figure 10*) by an approximation of the implant surface area (*Eq 1, Table 2*).

Eq 1

$$Implant\ surface(m^2) = d \times h\ x\ \pi$$

Were, d was the mean diameter of the implant (m) and h the mean height of the implant (m).

Table 2 *Implant measurements (mean (SD))*

Study	Height (mm)	Diameter (mm)
I	3.30 (0.29)	5.96 (0.17)
II	3.29 (0.19)	6.04 (0.17)
III	3.32 (0.24)	5.94 (0.09)

TEST PARAMETERS

Three parameters were calculated from the strength displacement curves [87]:

- Ultimate shear strength (MPa)
- Maximum shear stiffness (MPa/mm)
- Total energy absorption (KJ/m2)

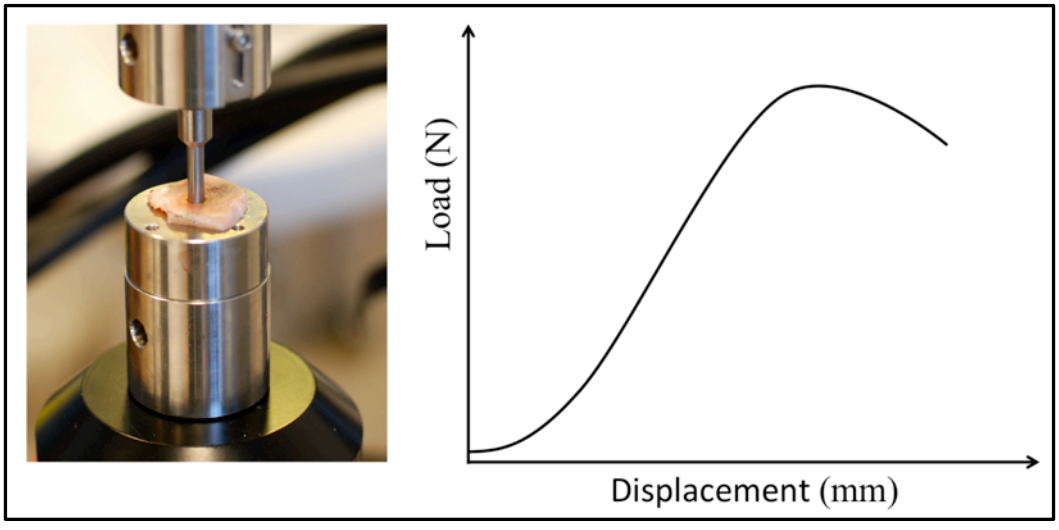

Figure 9 *Mechanical testing by axial push-out. Left: Specimen placed on metal platform with central opening. Specimen thickness 3.5 mm, implant diameter 6 mm, support hole 7.4 mm, preload 2N, displacement velocity 5 mm/min. Right: Load (N) displacement (mm) curve*

The ultimate shear strength (MPa)

This parameter was determined from the maximum load applied until failure of the bone-implant interface (represented as the first peak on the load displacement curve) and calculated as

Eq 2

$$Shear\ strength\ (MPa) = \frac{Load(N)}{Implant\ surface(m^2)}$$

This parameter was determined from a single load, which displaced the implant in the direction of the longitudinal axis. This may not be fully clinically relevant as the force applied to an arthroplasty is more complex than the one-dimensional factor tested here. Arthroplasties are influenced by a complex pattern of simultaneous, non-destructive shearing and bending forces. Nevertheless, due to the destructive nature of the mechanical push-out test, we were only able to do the test on one parameter. We choose the one-dimensional axial push-out due to the nature of the hip replacement prosthesis, which is mainly subject to an axial load during the gait cycle. The force applied until failure reflects the upper limit for the implant fixation and is a relevant parameter for evaluating different interventions comparing fixation of implants in a standardized setting.

Maximum shear stiffness (MPa/mm)

The maximum shear stiffness is defined as the maximum slope (between 10 successively sampled points at testing) of the load displacement curve before failure. This parameter reflects the rigidity of the tissue at the bone-implant interface and is calculated as

Eq 3

$$Shear\ stiffness(MPa/mm) = \frac{\Delta\ shear\ strength(MPa)}{\Delta\ displacement(mm)}$$

This parameter is highly susceptible to the tissue characteristic in which the implant is fixated. Whereas calcified tissue is rigid yielding a high stiffness value, fibrous tissue is more elastic resulting in a lower value.

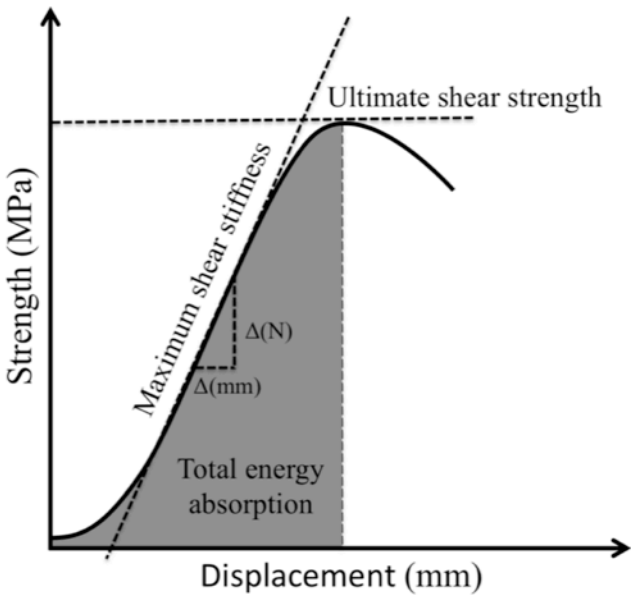

Figure 10 *Load displacement curve, normalized (strength = load/surface) enables calculation of ultimate shear strength (MPa), apparent shear stiffness (MPa/mm) and total energy absorption (KJ/m^2).*

Total energy absorption (KJ/m2)

The total energy absorption was calculated as the area under the load-displacement curve until failure and it represents the total energy absorbed in the tissue at failure. The evaluation of this parameter is complex as two bone-implant samples with identical energy absorption may have very different ultimate shear strengths and shear stiffness values (*Figure 11*). This parameter should therefore be evaluated with care and is most meaningful if evaluated along with the two other parameters.

As described, the three mechanical parameters illustrate different aspects of the mechanical fixation and have been shown to correlate well with the histological evaluation of a well-osseointegrated implant [87]. The preparation of the specimens as well as the test itself was standardized and conducted

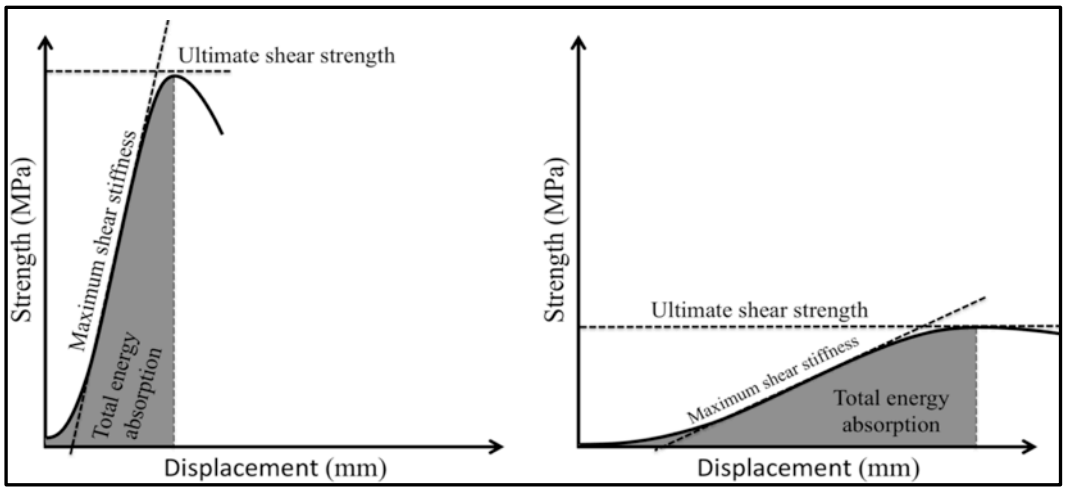

Figure 11 *Equal energy absorption at two different fixation scenarios. Left: rigid fixation; Right: elastic fixation.*

in one session. The reproducibility of a push-out test cannot be determined due to its destructive nature, which implies that the sections that have been tested by push-out cannot subsequently be tested by another mechanical test or by histomorphometry.

HISTOMORPHOMETRY

Histomorphometry was used to evaluate the quantity of tissue in contact with the implant surface and in the peri-implanteric gap. The tissue of interest was new bone, allograft bone, fibrous tissue and marrow tissue. Prior to the histomorphometric analysis, the 5.5-mm histological specimens (*Figure 8*) were dehydrated in graded ethanol (70– 100%) containing 0.4% basic fuchsin (Study I) or nothing (Study II and III) and embedded in methylmethacrylate (Art. 800590, Merck, Darmstadt, Germany).

Using Vertical Uniform Random (VUR) sectioning technique [92], four 30-µm-thick central histological sections were cut parallel to the implant axis with a hard-tissue microtome (KDG-95; MeProTech, Heerhugowaard, The Netherlands) (*Figure 12*). About 400 µm of tissue was lost to the saw blade during each section [93]. This technique has been described to provide highly reliable histomorphometric results with negligible bias [94]. In Study II and III, surface staining was performed with 0.1% Toluidine Blue (pH=7) (Fluka, Sigma-Aldrich, St. Louis, MO, USA) for 10 min and the specimens were rinsed and mounted on glass. This preparation resulted in blue staining of non-calcified tissue and light blue staining of calcified tissue, but only at the cutting surface, which thereby provided a reliable plane of focus in the light microscope regardless of minor differences in the thickness of the sections. In Study I, the section surfaces were stained with 2% light green (Light Green SF, BDH Laboratory Supplies, Poole, England) for 2 min, rinsed and mounted on glass. This preparation provided red staining of the non-calcified tissue and green staining for calcified tissue.

The different types of calcified tissues, such as newly formed bone (woven bone) and bone graft (lamellar bone), were categorized on the basis of their morphological characteristics for both surface stainings [95]. New bone was recognized as disorganized bone substances with embedded cells, while allograft bone was a more dense substance with well-organized

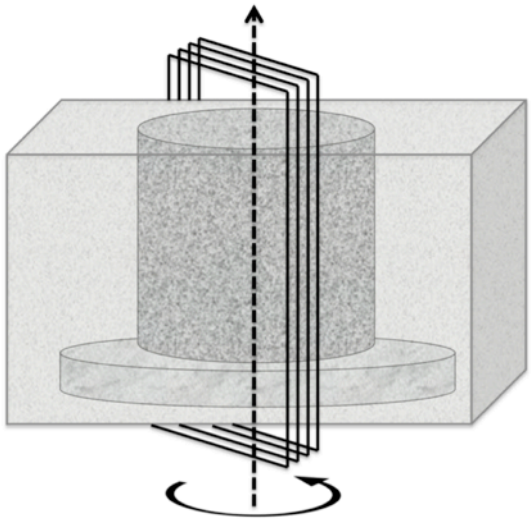

Figure 12 *Principle of random sectioning. The embedded bone-implant blocks were randomly rotated around the vertical axis before being sectioned.*

empty cell lacunae. The bone marrow was characterized as a less dense, but cell rich, disorganized conglomerate with empty areas from dissolved fat. The fibrous tissue was seen as dense, well-organized bundles of fibres with spindle-shaped cells (*Figure 13*).

We performed blinded quantitative histomorphometry using an Olympus light microscope (Olympus, Ballerup, Denmark) with Visiopharm Integrator System software (newCAST-version 3.4.1.0; Visiopharm A/S, Horsholm, Denmark). The region of interest (ROI) was defined as the area spanning from a reference line (defined as a line between the innermost porous depth and the outermost top of the porous coating) and 2.0 mm into the circumferential gap with a 0.5-mm free margin to the cutting edge and the end plate (*Figure 14*).

Stereology

Stereological methods are tools for obtaining quantitative information about three-dimensional (3-D) structures based on

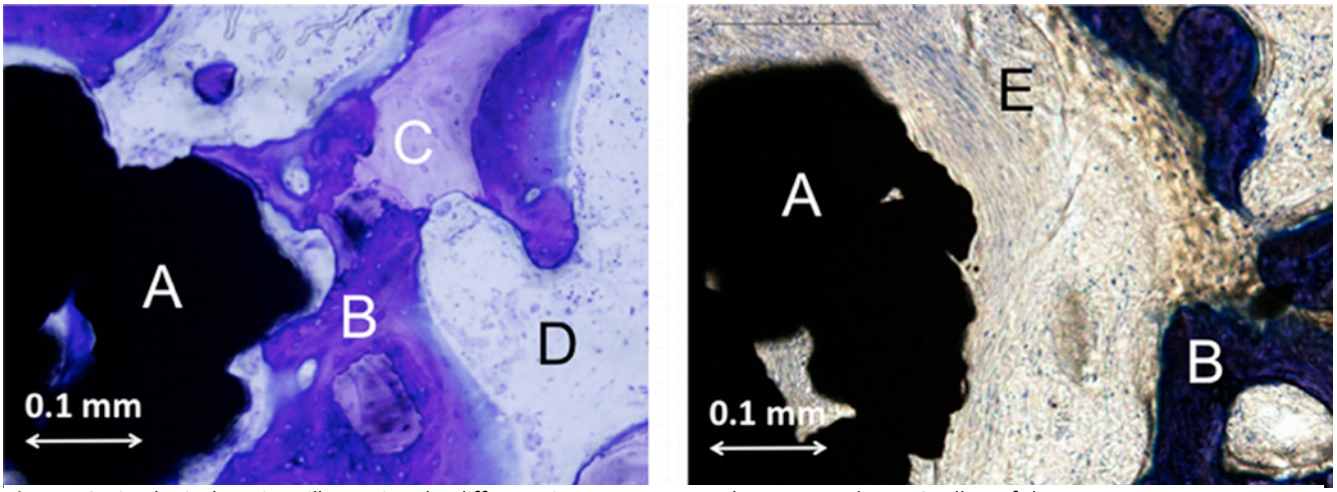

Figure 13 *Histological sections illustrating the different tissue types. A: Implant, B: New bone, C: Allograft bone, D: Marrow space, E: Fibrous tissue*

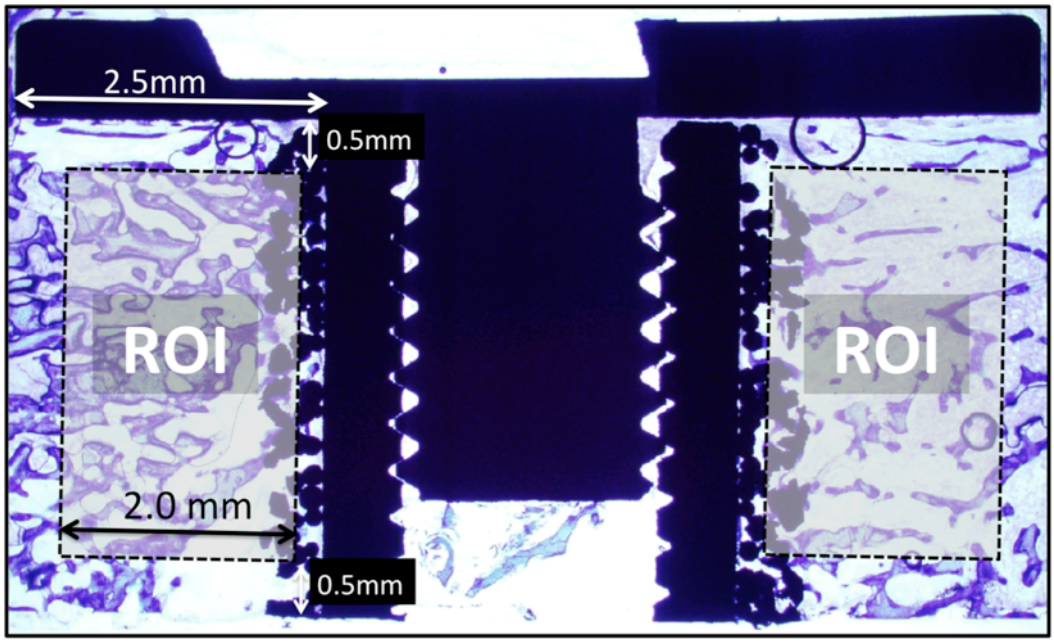

Figure 14 *Histomorphometry. Regions of interest (ROI).*

observation done in two-dimensional sections. The evaluated tissues can be quantified with the use of a test probe superimposed on the microscope object. The dimension of the probe plus the dimension of the geometric characteristic of the object must equal three so that the geometric characteristic, which is being estimated, is meaningful in 3-D space. Thus, volume estimates can be obtained by the use of a zero-dimensional point probe (volume is 3-D) [96], and surface fractions (ongrowth) can be estimated with a one-dimensional line probe (surface cover is two-dimensional) [92] (*Figure 15*).

To obtain unbiased estimates, some requirements must be fulfilled regarding the sectioning and stereology procedure. Isotropy must be accomplished, which means that the probability of interception between the test probe and the structure must be independent of their orientation. When evaluating the volume fraction, the test probe is a zero-dimensional point with no orientation, and the requirement of isotropy is therefore fulfilled. But regarding the ongrowth estimates, the test probe is a one-dimensional line. This line probe has an orientation and the assumption of isotropy is therefore not fulfilled.

In order to estimate tissue ongrowth without assuming isotropic orientation, the following requirements must be met: (1) The sections must possess an identifiable directional axis. This is secured by sectioning the bone implant specimens in the direction of the long axis of the implant. This makes the vertical direction identifiable in all sections. (2) The orientation of the sections around the vertical axis must be random. This is obtained by random rotation of the specimens before sectioning (*Figure 12*). (3) To obtain isotropic, uniformly random intercepts, either the line probe or the structure must be isotropic. This is achieved with the use of sine-weighted line probes which make the line probe isotropic [97].

The four vertical sections representative of each implant were analysed and sampled, and the percentage of ingrowth and ongrowth of each tissue was calculated. As the biological variation has been proven to be of far greater magnitude than the variation in repeated sampling, three to four sections per specimen have been shown to yield adequate precision [98].

Sampling

The histomorphometry was performed using a light microscope linked to a computer. Fields of vision were captured on the computer monitor, and the ROI was defined at low magnification (*Figure 14*). In order to quantify the tissue fraction, systematic uniform random sampling in 2D space was used, a method

Table 3 *Sampling intensity*

	Points	Lines	Fraction
Study I	5x5	20	100%
Study II	3x3	10	100%
Study III	3x3	10	100%

called meander sampling. Meander sampling is the systematic sampling of the ROI with random start of the first screen field (*Figure 15*). To achieve adequate precision, a minimum of 100 hits per tissue of interest is recommended [98, 99]. The sampling intensity can be adjusted and is based on the variables

- Numbers of probes (points and lines)
- Sampling fraction

The relation can be regarded as an equation

Eq 4

$$Hits\ needed = number\ of\ hists \times sampling\ fraction$$

The number of probes or the sampling fraction can therefore be adjusted so that a sufficient number of hits or intercepts may be obtained. The sampling fraction is a ratio of the sampled area to the total ROI. If the sampling fraction is 100%, then a

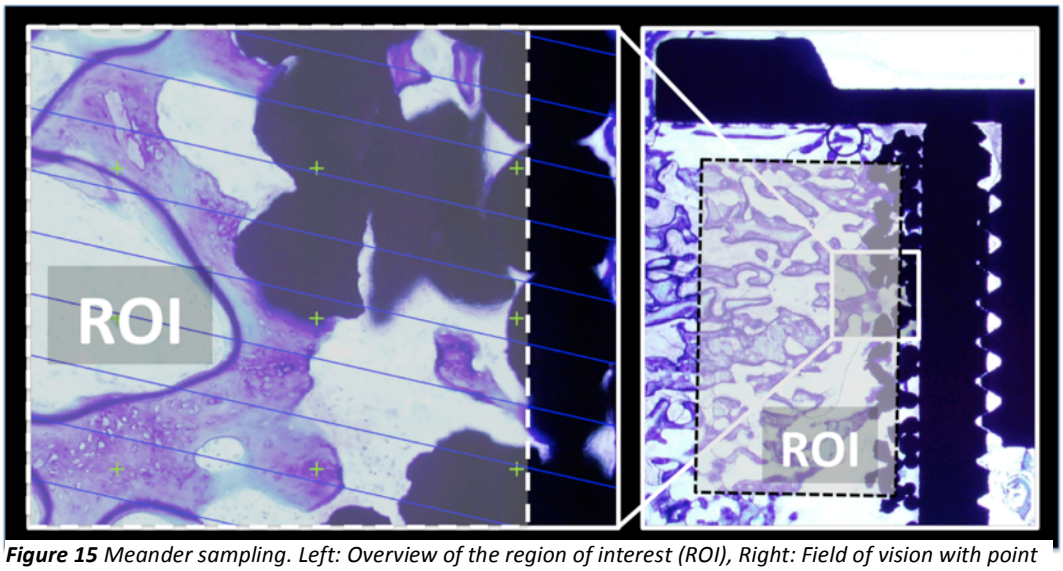

Figure 15 *Meander sampling. Left: Overview of the region of interest (ROI), Right: Field of vision with point (3x3) and line probes (10)*

complete sampling of the ROI has been made as opposed to a sample fraction of 75% where 25% of the ROI is left out of the sampling. This should, however, not influence the precision of the estimates as the sampling is randomised and the sections have been prepared isotropically. The number of point probes can be adjusted in a grid of 3x3 or 4x4 and should not exceed 5x5, as this will complicate the sampling procedure. If this results in an insufficient number of hits then it is better to adjust the sampling fraction than to increase the number of pints. The line probes should be kept under 20 per field of vision for the same reason.

The studies described in this thesis have been sampled with a 100% sampling fraction, but with some difference in number of points and line probes (*Table 3*).

For all three studies, new bone was a tissue of interest regarding volume ingrowth (point counts) and surface ongrowth (line intercepts). The ingrowth and ongrowth of fibrous tissue was of interest in Study I; and, furthermore, the remaining volume of graft bone was of interest in all studies (*Table 4*)

Reproducibility of histomorphometry

The histomorphometric reproducibility was estimated from double measurements performed by the same person (intra-observer variation). An identical equipment and setup was used. One specimen (a total of four sections) was re-counted from the control group and from each intervention group. Reproducibility can be expressed as the within-subject coefficient of variation (CV)

E

Eq5

$$CV = \frac{s}{\bar{x}} \;;\; s = \sqrt{\frac{1}{2 \times k} \times \sum_{1}^{k} d^2}$$

Where, $\bar{x}$ are the mean value of the first and the second estimate, k the number of double estimates and d the difference between the first and the second estimate

We aimed at keeping the CVs under 20%, and a CV under 10% was considered as a high reproducibility. As illustrated (*Table 5*) in the intra-observer reproducibility was sufficient in most cases, although the CV of fibrous tissue was above the acceptable level in Study I. This was due to a problematic staining procedure where the fibrous tissue became difficult to distinguish from the surrounding marrow tissue. The CVs were in accordance with those reported in other studies using the same models [94].

The CV is not only dependent on the intra-observer variation, but also on the meander sampling intensity and the fraction of the particular tissues. If the tissue fraction is low, the relative impact of a misclassification increases. This is evident when evaluating the very high CV value of the bone graft ongrowth (line intercepts), where only few hits were sampled per specimen.

Table 4 *Average point counts and line intercepts for each specimen (mean (SD))*

	New bone	Graft bone	Fibrous tissue
Study I (wash)			
Points	**316 (124)**	**478 (183)**	**137 (147)**
Line intercepts	**96 (48)**	3 (3)	**245 (291)**
Study II (periost)			
Points	**260 (75)**	**99 (43)**	36 (75)
Line intercepts	**122 (49)**	0 (1)	18 (21)
Study III (Tobramycin)			
Points	**255 (88)**	**103 (47)**	6 (10)
Line intercepts	**129 (44)**	0 (1)	25 (38)

The highlighted numbers were tissues of interest

Table 5 *Reproducibility, CVs are shown in %*

	New bone	Bone graft	Fibrous tissue
Study I			
Points	**14**	**5**	**38**
Lines	**14**	78	23
Study II			
Points	**3**	**5**	7
Lines	**5**	N/a	18
Study III			
Points	**8**	**13**	7
Lines	**8**	N/a	23

N/a: Not available due to zero measurements. The highlighted numbers were tissues of interest

Statistical analysis

All mechanical and histomorphometric data were tested for normality by evaluating QQ plots of the residuals. For the parametric data, the assumptions for repeated measures of ANOVA were fulfilled, and paired t-test was used to evaluate differences between the treatment groups. The estimates are presented as means with standard deviation and the absolute differences between treatment groups as means with 95% confidence intervals (CIs). The nonparametrically data were evaluated with Friedman repeated measures analysis of variance by ranks followed by Wilcoxon signed-rank test. Estimates are given as medians and inter-quartile ranges (IQR). Differences between medians or means were considered statistically significant for p-values less than 0.05.

The statistical analysis was performed using STATA statistical software (Stata 11.2, StataCorp, College Station, TX, USA).

4. RESULTS

STUDY I

Hypothesis: Rinsing of the allograft bone will enhance fixation of grafted implants.

Theory rationale: Rinsing will reduce the immunogenic load experienced by the host which will lead to improved osseointegration as evaluated by improved mechanical implant fixation, increased new bone formation and decreased fibrous tissue formation.

Hypothesis disproved: Yes

Comments: We found no statistically significant difference in the histomorphometric (*Table 6 and Figure 16*) or the mechanical evaluation (*Figure 17*) between the two groups. Although a 61% reduction in fibrous tissue ongrowth and a 50% reduction in ingrowth were found in the intervention group, these differences were not statistically significant.

Table 4 *Study I: Histomorphometric analysis: Fraction of new bone, bone graft and fibrous tissues at implant surface (surface fraction) and in the concentric peri-implanteric gap (ingrowth volume fraction).*

	New bone mean% (SD)		Bone graft mean% (SD)		Fibrous tissue median% (IQR)	
Implant surface						
Control	6	(3.7)	0	(0.2)	18	(3-31)
Wash	7	(3.0)	0	(0.2)	7	(1-18)
Absolut difference						
Wash - Control	0	(-1.1; 3.0)	0	(-0.1; 0.3)		
T-test	p = 0.34		p = 0.37			
Wilcoxon					p = 0.24	
Ingrowth						
Control	9	(2.7)	13	(4.1)	4	(1-6)
Wash	9	(3.3)	13	(4.4)	2	(1-5)
Absolut difference						
Wash - Control	0	(-2.1; 3.0)	0	(-1.5; 2.7)		
T-test	p = 0.71		p = 0.54			
Wilcoxon					p = 0.27	

New bone and bone graft are presented as mean with standard deviation (SD) and the absolute difference between the treatment groups as mean difference with 95% confidence interval (CI). Fibrous tissue is presented as median with interquartile range (IQR)

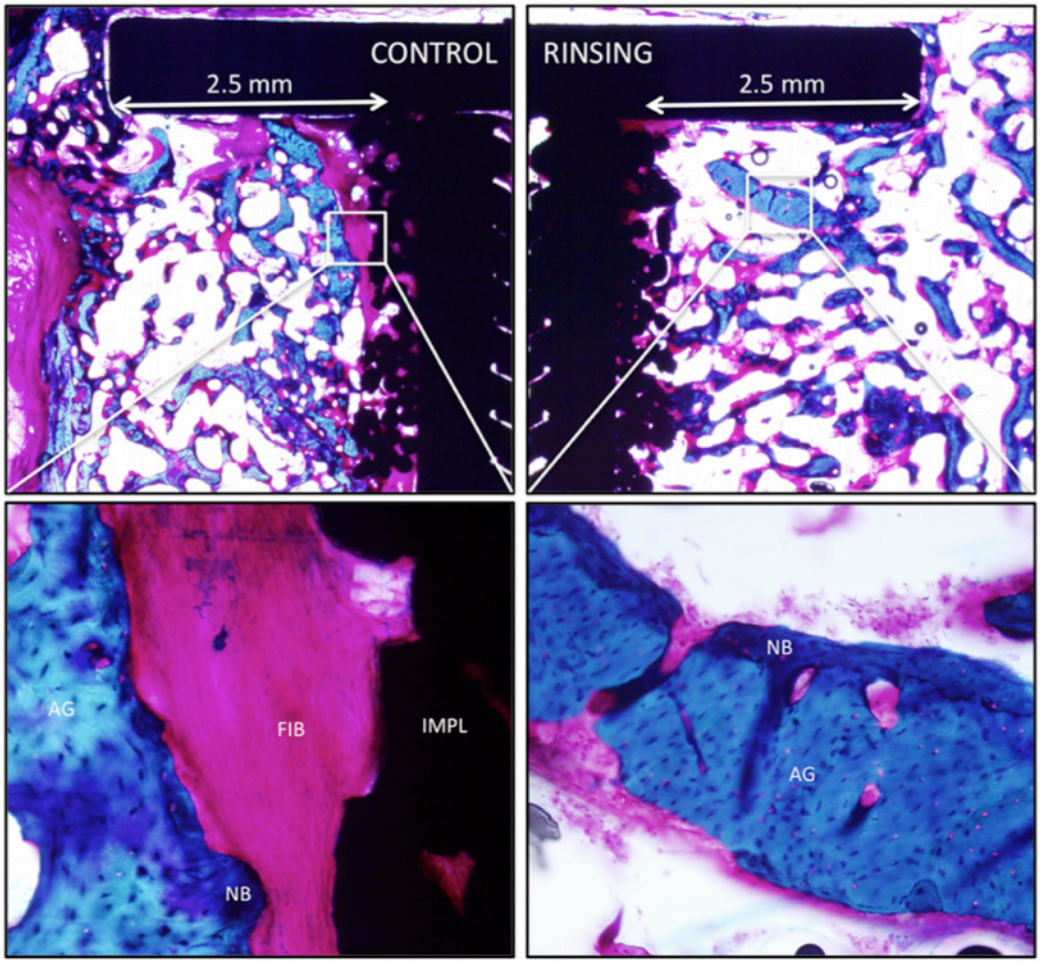

Figure 1 *Study I: Histology: Representative histological sections of the two groups. The displayed sections are from the two implants inserted into the same animal; Porous-coated Ti implants surrounded by a 2.5-mm concentric defect. The sections are cut parallel to the long axis of the implant. CONTROL (left): Fibrous (FIB) tissue at the implant (IMPL) surface. RINSING (right): New bone (NB) on graft (AG) remnants. Basic fuchsine/ Light Green stain.*

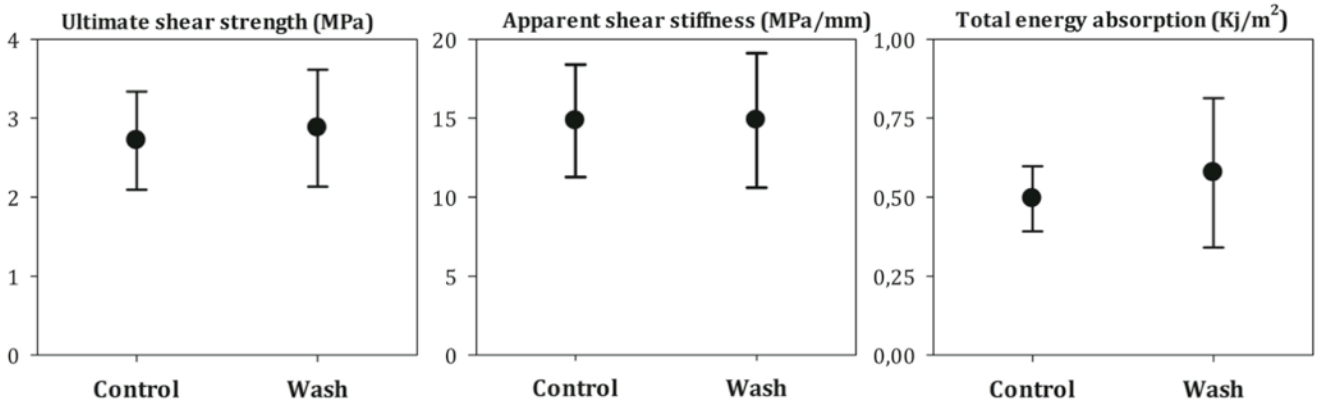

Figure 17 *Study I: Mechanical test. T-test. Mean (95% CI). No significant difference between the groups*

Study II

Hypothesis: Periosteal augmentation of allograft bone will enhance the fixation of grafted implants.

Theory rationale: The periosteum contains pluripotent mesenchymal progenitor cells whose presence will aid initial bone healing.

Hypothesis disproved: Yes

Comments: We found no statistically significant difference in the mechanical evaluation between the two groups (*Figure 19*).

Histomorphometrically (*Table 7*), we found the periosteum-augmented implants to have an 18% (p=0.05) reduction in ongrowth of new bone. No difference was observed in new bone formation in the gap around the implant (p=0.11), but enhanced new-bone formation was seen around the periosteum fragments (*Figure 18*). The volume fraction of fibrous tissue was increased (p<0.01). Furthermore, the intervention group showed more fibrous tissue on the implant surface than the control group, but the amounts were small and the difference was not statistically significant (p=0.12).

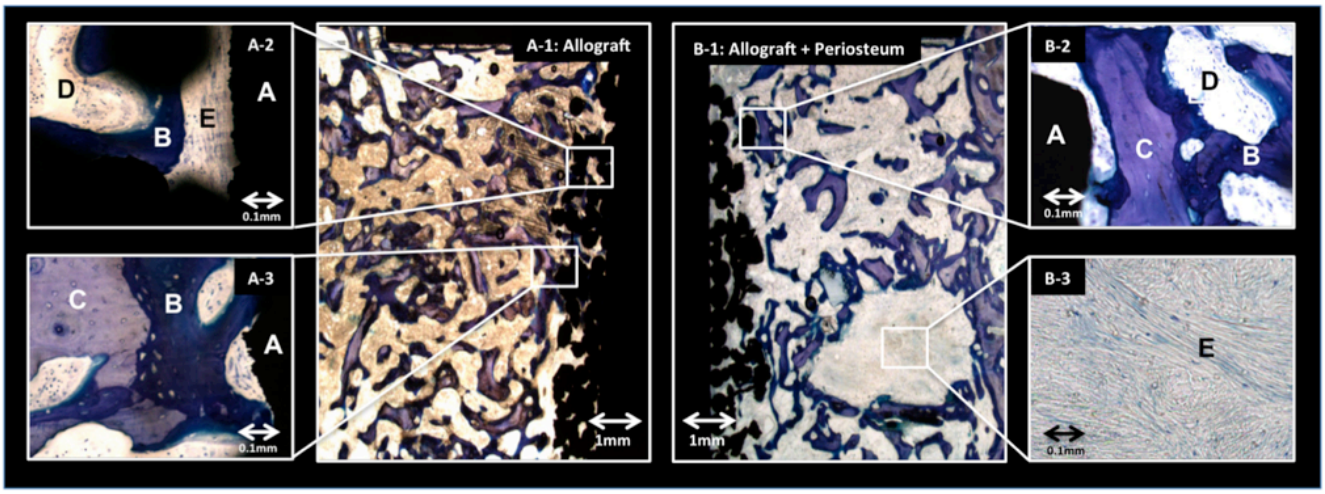

Figure 18 *Study II: Histology: Representative histologic sections of the control group (A: Allograft) and the intervention group (B: Allograft + periosteum). The sections are from two implants inserted into the same animal and stained with Toluidine Blue (A: Implant; B: Woven bone; C: Graft bone; D: Marrow; E: Fibrous tissue). Left side (allograft only): A-1: Overview of implant and bone graft; A-2: Thin fibrous tissue membrane in contact with implant; A-3: Allograft bone chip with ongrowth of new bone in contact with implant. Right side (allograft + periosteum): B-1: Overview of implant and bone graft. Large islands of fibrous tissue are seen in the gap with activity of new bone formation in its vicinity. B-2: Allograft bone chip with ongrowth of new bone in contact with implant; B-3: Magnification of the island revealing solid fibrous tissue.*

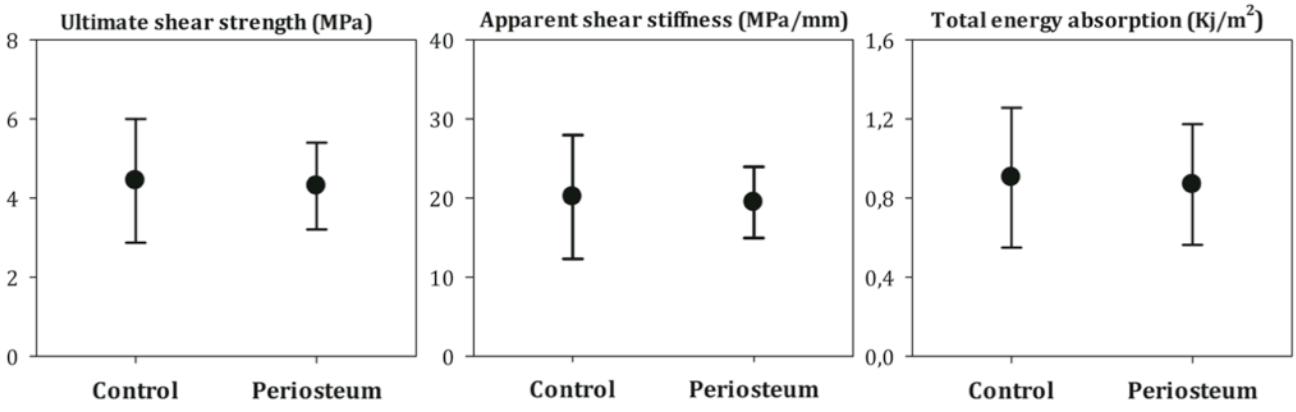

Figure 19 *Study II: Mechanical test. T-test. Mean (95% CI). No significant difference between the groups*

Table 5 *Study II: Histomorphometric analysis: Fraction of new bone, bone graft and fibrous tissues at implant surface (surface fraction) and in the concentric peri-implanteric gap (ingrowth volume fraction).*

	New bone mean% (SD)		Bone graft mean% (SD)		Fibrous tissue median% (IQR)	
Implant surface						
Control	17	(6.3)	0.0	(0.0 - 0.1)	0.7	(0.0 - 1.9)
Periosteum	14	(3.6)	0.0	(0-0 - 0.0)	3.8	(0.5 - 5.0)
Absolut difference						
Periosteum - Control	-3	(-6.0; 0.0)				
T-test	p = 0.05					
Wilcoxon			p = 0.13		p = 0.12	
Ingrowth						
Control	21	(3.7)	8.4	(3.3)	0.1	(0.0 - 0.2)
Periosteum	18	(4.0)	6.7	(2.5)	4.4	(0.3 - 6.4)
Absolut difference						
Periosteum - Control	-2.3	(-5.2; 0.6)	-1.7	(-3.6; 0.2)		
T-test	p = 0.11		p = 0.07			
Wilcoxon					p = <0.01	

New bone and bone graft are presented as mean with standard deviation (SD) and the absolute difference between the treatment groups as mean difference with 95% confidence interval (CI). Fibrous tissue is presented as median with interquartile range (IQR)

Study III

Hypothesis: Antibiotic impregnation of the allograft bone will impair fixation of grafted implants.

Theory rationale: Tobramycin is a cytotoxic drug, which may compromise bone graft incorporation.

Hypothesis disproved: Yes

Comments: We found no statistically significant difference in the histomorphometric (*Figure 21, Table 8 and Table 9*) or the mechanical evaluation (*Figure 20*) between the two groups. Tobramycin impregnation of allograft bone did not appear to impair implant fixation or tissue in-growth. However, surgeons should weigh the potentially cytotoxic effect of Tobramycin against its beneficial anti-microbial effect in joint arthroplasty revisions until further basic data on toxicity are available.

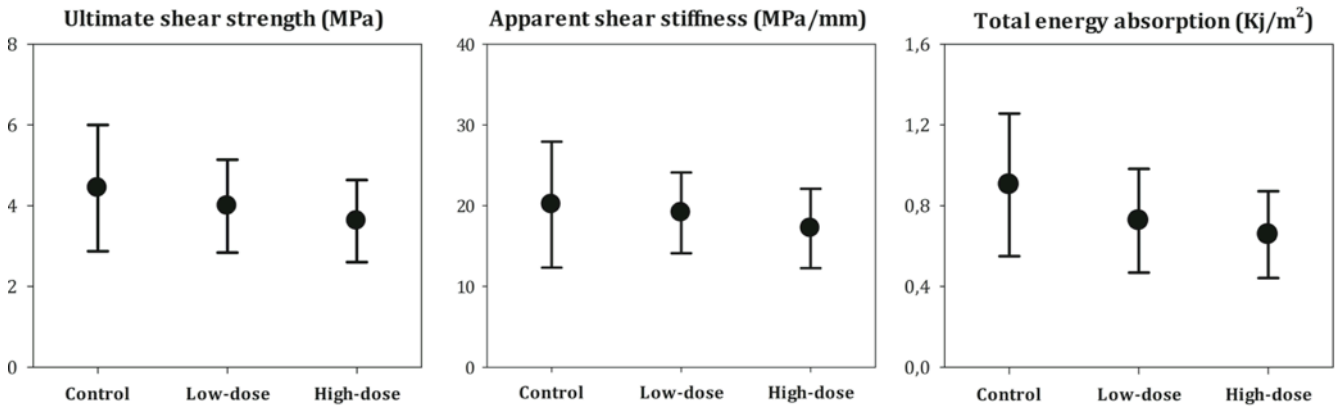

Figure 20 *Study III: Scatter-plot. T-test. Mean (95% CI). No significant difference between the groups*

Table 8 *Study III: Histomorphometric ongrowth: tissue area fractions on implant surface*

Surface fraction	New bone mean% (SD)		Fibrous tissue median% (IQR)		Bone graft, median% (IQR)	
Control	17	(6)	1	(0-2)	0	(0-0)
Low-dose	16	(4)	1	(0-7)	0	(0-0)
High-dose	17	(5)	2	(0-8)	0	(0-0)
Absolut difference						
Low dose - Control	-0.7	(-5.4; 4.0)				
High dose - Control	0.2	(-5.1; 5.4)				
High dose - Low dose	0.9	(-3.8; 5.6)				
p-value (ANOVA)		p = 0.92				
p-value (Friedman)				p = 0.57		p = 0.47

New bone is presented as mean with standard deviation (SD) and the difference between the treatment groups is presented as mean difference with 95% CI. Fibrous tissue and bone graft are presented as median with interquartile range (IQR)

Table 9 *Study III: Histomorphometric ingrowth: tissue area fractions in the concentric peri-implanteric gap*

Volume fraction	New bone Mean% (SD)		Fibrous tissue median% (IQR)		Bone graft, mean% (SD)	
Control	21	(4)	0	(0-0)	8.4	(3.3)
Low-dose	20	(6)	0	(0-0)	8.0	(3.5)
High-dose	19	(6)	0	(0-1)	7.4	(3.8)
Absolut difference						
Low dose - Control	-0.6	(-3.4; 2.4)			-0.4	(-2.0; 1.3)
High dose - Control	-2.1	(-5.4; 1.2)			-0.9	(-3.2; 1.4)
High dose - Low dose	-1.5	(-5.4; 2.5)			-0.6	(-2.5; 1.4)
p-value (ANOVA)		p = 0.40				p = 0.60
p-value (Friedman)				p = 0.57		

New bone is presented as mean with standard deviation (SD) and the difference between the treatment groups is presented as mean difference with 95% CI. Fibrous tissue and bone graft are presented as median with interquartile range (IQR)

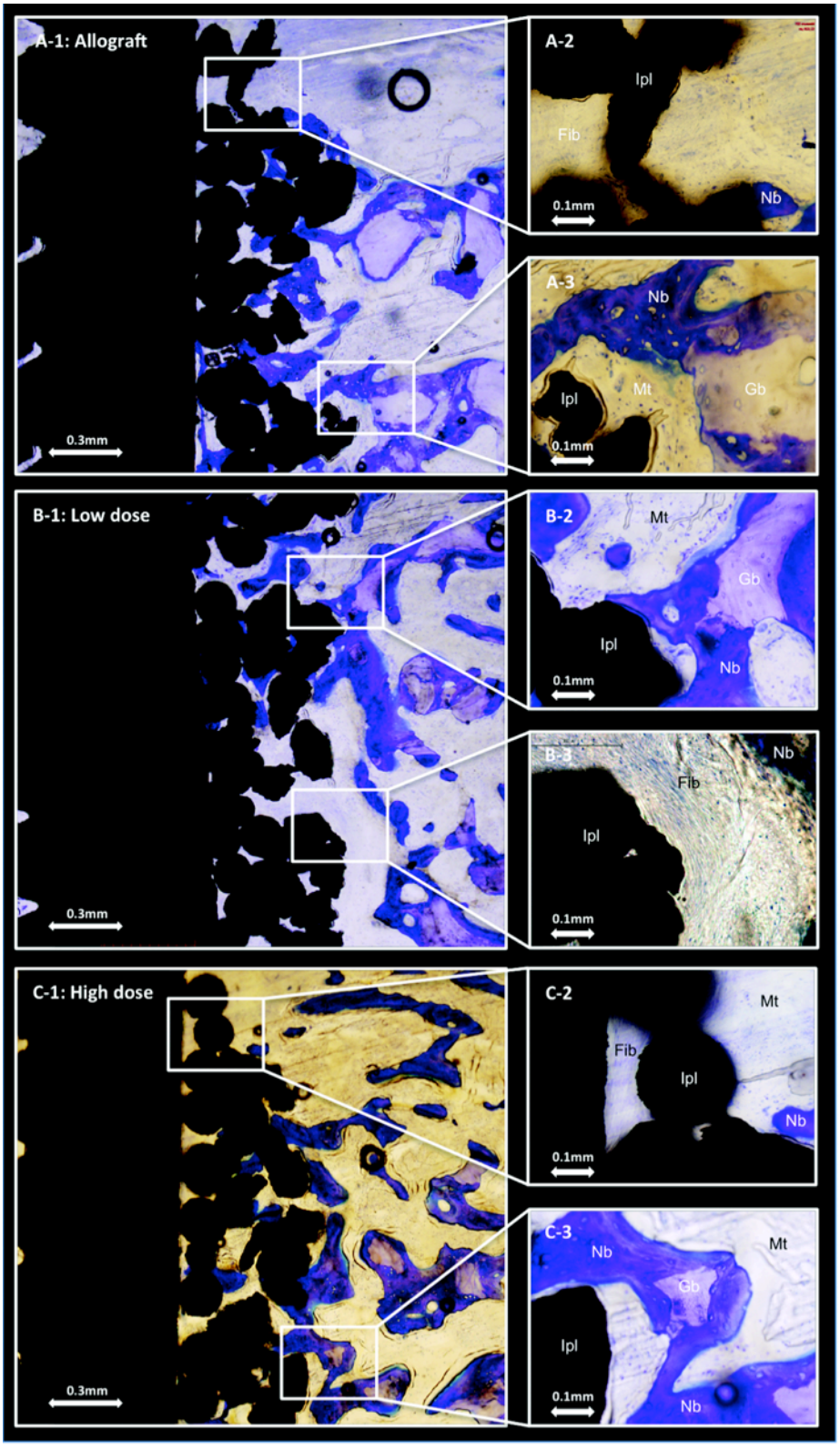

Figure 21 *Histology: Representative histologic sections of the control group (A: Allograft) and intervention groups (B: Allograft + low dose tobramycin; C: Allograft + high dose tobramycin) form the same animal. Control: A-1: Overview of implant and bone graft; A-2: Thin fibrous tissue membrane in contact with implant; A-3: Allograft bone chip with ongrowth of new bone. Low dose: B-1: Overview of implant and bone graft; B-2: Allograft bone chip with ongrowth of new bone in contact with implant; B-3: Fibrous tissue membrane in contact with implant. High dose: C-1: Overview of implant and bone graft; C-2: Thin fibrous tissue membrane in contact with implant; C-3: Allograft bone chip with ongrowth of new bone. A = Implant, B = New bone, C = Allograft bone, D = Marrow space, E = Fibrous tissue. (Stain, toluidine blue; magnification, x28 (left), x230 (right).*

5. COMPARISON OF MEDIAL AND LATERAL FEMUR IMPLANT

In Study II and III, the implant was inserted into the condyles of the distal femur. The implantation sites was systematically rotated between the medial and the lateral femoral condyles (*Figure 2*) and evaluated in a paired design. This systematic rotation addresses the potential difference in bone-healing properties between the condyles. The assumption that the medial and the lateral femoral condyle has identical bone healing capacity has, however, not yet been examined.

Different aspects may influence the healing potential of the two condyles. The load-bearing conditioning may be different, which will cause mechanical stimulation of the two sides to be different. The surgical procedure for getting access to the bone surface of the two condyles is fairly simple, but the posture of the surgeon while grafting the defect is quite different. The animal in lying on its back and the surgeon is bending over the leg while grafting the medial bone defect, which is almost horizontally orientated. Where grafting is performed on the lateral site, the positioning of the leg makes the procedure more difficult which results in a more vertically orientated bone defect. A simple, standardized three-step grafting procedure (see surgery section on page 7) was used in order to minimize the risk of surgeon-related differences between the medial and lateral implant. Differences (biological and non-biological) between the medial and the lateral condyle were diminished by the systematic rotation. But, if a difference in healing properties were present, this rotation of the implantation sites would have increased data variation and subsequently led to decreased data precision.

EXPERIMENT

An additional experimental animal study was conducted to examine the above issues. The animal and implant model as well as the evaluation method used were the same as those used in the other three studies of this thesis. Twelve mature American Hounds with a mean age of 11 month (10-15) and a mean weight of 32.1 kg (28.7-36.2) were used. Two control implants were inserted into the same femur of all 12 animals. In the "control 1" group, implants were inserted into the lateral

Table 10 *Biomechanical results [mean (SD)]*

Groups	Shear strength (MPa)		Shear stiffness (MPa/mm)		Energy absorption (KJ/m2)	
Medial	5.0	(2.1)	25	(10)	1.0	(0.6)
Lateral	5.0	(2.3)	22	(10)	1.0	(0.6)
Absolut difference						
Medial - Lateral	0.0	(-1.9; 2.0)	2.2	(-6.7; 11)	0.0	(-0.4; 0.5)
T-test	p = 0.97		p = 0.60		p = 0.96	

Absolute difference between the treatment groups [mean (95% CI)]

Table 11: *Histomorphometric analysis: Fraction of new bone, bone graft and fibrous tissues at implant surface (surface fraction) and in the concentric peri-implanteric gap (ingrowth volume fraction)*

	New bone mean% (SD)		Bone graft mean% (SD)		Fibrous tissue mean% (SD)	
Implant surface						
Medial	18	(6.5)	1	(1.1)	5.4	(8.8)
Lateral	22	(9.9)	1	(0.4)	5.7	(8.6)
Absolut difference						
Medial - Lateral	-3.2	(-8.9; 2.6)	0.1	(-0.1; 0.1)	-0.3	(-3.5; 3.0)
T-test	p = 0.25		p = 0.69		p = 0.86	
Ingrowth						
Medial	26	(7.4)	15	(3.2)	1.5	(3.1)
Lateral	30	(6.7)	13	(4.5)	1.1	(1.5)
Absolut difference						
Medial - Lateral	-3.8	(-8.7; 10)	1.8	(-1.4; 4.9)	0.4	(-0.2; 2.7)
T-test	p = 0.11		p = 0.24		p = 0.75	

Estimates are presented as mean with standard deviation (SD) and the absolute difference between the treatment groups as mean difference with 95% confidence interval (CI).

condyle and in the "control 2" group, the implants were inserted into the medial condyle (*Figure 2*). These control groups were paired with two interventions groups where implants were inserted into the opposite femur to evaluate the effect of adding nano-hydroxyapatite to the bone graft (an issue not addressed in the present thesis). The two control implants were grafted with the same volume of rinsed morselized allograft bone (1.2 cm3). The rinsing procedure was identical to the one described in the Methods section (page 7).

We hypothesised that unevenness of bone healing in the medial and the lateral femoral epicondyle would be revealed by mechanical push-out tests and histomorphometry.

Hypothesis disproved: Yes

Comments: We found no statistically significant difference in the histomorphometric (*Table 11*) or the mechanical evaluation (*Table 10*) of the two groups.

DISCUSSION

The objective of this study was to evaluate the difference in bone healing properties between the medial and the lateral femoral condyle. We hypothesized that the implant fixation would be different at these two sites for biological reasons and because the two surgical approaches would differ. As seen in the results, the difference between the two groups was not statistically significant although a slightly increased level of new bone formation was seen in the lateral control group.

CONCLUSION

The findings of this study allow the conclusion that there is no significant difference between medial and lateral femoral condyles in terms of bone healing in the present animal model. Future studies conducted in the condyles of the distal femur may therefore be designed as four-armed studies with one control group and three intervention groups systematically rotated between medial/lateral and left/right.

6. DISCUSSION

Due to insufficient bone stock, extensive bone grafting is often required in revision of a failed arthroplasties. Autograft bone is the gold standard among bone grafts. It is naturally biocompatible, but its use in revision surgery is hampered by its limited volume and considerable donor site morbidity. Allograft bone is readily available and it is the most commonly used graft material. However, as opposed to the autologous bone graft, it consists of dead and foreign-bone tissue. The purpose of Study I and II was to explore methods for optimizing the allograft bone by making it more "autologous". This was done by lowering its immunogenicity (Study I) and by adding living bone-forming cells from the periosteum (Study II). Finally, Study III evaluated the effect of antibiotic protection of the bone graft. The experimental aim was to increase the short-term implant fixation (Study I and II) or to maintain an unchanged implant fixation (Study III) as assessed from mechanical push-out test and histomorphometry. The clinical aim was to prolong implant longevity.

RINSING OF THE BONE GRAFT (STUDY I)

The objective of this study was to evaluate the mechanical and histologic effects of rinsing the morselized allograft bone in saline before impaction grafting. Previous studies have shown that simple rinsing procedures can lower the immunogenic load of the allograft bone and thereby render it less "foreign". We hypothesized that this procedure would lead to improved osseointegration of a grafted implant as evaluated by increased new bone formation, decreased fibrous tissue formation and improved mechanical implant fixation. The results of the present study did not confirm our hypothesis, and we found no statistically significant difference in the histomorphometric or the mechanical evaluation between the two groups. One reason for this could be that the presence of immunogenic factors does not matter. The initial inflammatory process is comparable between the fresh autograft bone and the fresh allograft bone [4]. Both graft types elicit an inflammatory response involving formation of a haematoma as well as recruitment and activation of immune cells which results in the release of osteoinductive factors like cytokines and bone mineral peptide, which stimulates bone regeneration [100, 101]. Rinsing may lower the overall immunogenic load, but this might not influence the bone regeneration.

Another possible explanation could be that the presence of immunogenic factors does matter, but that our rinsing method was insufficient. Different methods have been used for rinsing the bone graft, including pulse-lavage washing [33, 37-39] and manual rinsing [36]. The manual rinsing method used in this present study was chosen because of its simplicity and its ability to preserve a broad range of bone graft particle sizes, which has been shown to be important for achieving an optimal mechanical strength of the impacted bone graft [40]. Rinsing by pulse-lavage may, in contrast, cause smaller bone graft particles to be lost, thus narrowing the particle-size distribution. By our method, the bone graft was macroscopically cleared of obvious fat, blood and marrow tissue (*Figure 3*), as was also the case in other studies [7, 44]. As described previously, the most antigenic cells of the bone graft are found in the bone marrow [4, 32]; and although our rinsed bone graft appeared clean, the method may not have been sufficiently effective to reduce a foreign-body reaction. It could be that we only removed the erythrocytes. This would imply that we left the majority of the immunogenic factors; we can neither confirm nor deny this possibility.

The lack of a statistically significant difference between the two groups could also be caused by insufficient power. However; since the data variation was low, the magnitude of a true difference missed by type 2 error is low.

Although it was not evident in this study, rinsing of the allograft bone may be advantageous in the clinical setting as demon-

strated by the previously documented positive influence on mechanical shear strength of this procedure. Additional studies may be conducted to address different rinsing methods and their influence on osseointegration and to evaluate the potentially improved mechanical implant fixation owing to tighter bone graft impaction.

PERIOSTEAL AUGMENTATION (STUDY II)

The objective of this study was to evaluate the effect of adding fragmented periosteum to morselized allograft bone. We hypothesized that adding periosteum to the bone graft would provide a signal for improved osteogenic differentiation owing to its large content of pluripotent mesenchymal progenitor cells. Contrary to our hypothesis, we found no benefit of adding periosteum prepared by mincing to the allograft bone. Overall, we found a reduced level of osseointegration, but unaltered mechanical properties. Histologically, several implants in the periosteum group showed large fibrous tissue islands in the peri-implanteric gap, which we believe represent the transplanted periosteum. High activity of new bone formation in the vicinity of these islands was a common finding. This may indicate that the osteoinductive properties of the transplanted minced periosteum are maintained, which is also in accordance with previous studies [51, 102]. The method used for periosteum harvest could have been incomplete in some animals, which would cause the harvest of the cell-rich cambium layer to be incomplete. This may explain why some specimens only showed limited new bone activity in the vicinity of the fibrous tissue islands. The surgeon's ability to conduct a complete periosteal harvest was not evaluated.

The osteoinductive mechanism of the fragmented periosteum may be multifactorial. By fragmenting the periosteum, the cambium layer maintains its relation to the original periosteum tissue. This may prolong its window for influencing new-bone formation in the non-vascularized, impacted environment. An additional mechanism could be the potential liberation of essential bone-stimulating substances once the cambium cells necrotize. Augmenting the bone graft by smaller fragments of periosteum, isolated cambium layer tissue or cultured periosteal cells would therefore be interesting approaches for future research in grafted implants.

ANTIBIOTIC PROTECTION OF THE BONE GRAFT (STUDY III)

In-vitro studies have shown that morselized bone graft has the ability to act as a delivery vehicle of antibiotics [54-58]. However, toxicity studies have shown that a high local antibiotic concentration can have a negative effect on osteogenesis [62, 103]. Despite this concern, small clinical trials using Tobramycin or Vancomycin-impregnated bone graft in infectious revision surgery have been conducted with promising results regarding infection prophylaxis [53, 66, 67]. The purpose of the present study was to bridge the gap between in vitro studies and clinical trials by investigating biological differences in osseointegration between antibiotic- and non-antibiotic-impregnated bone graft in the presence of an implant. We hypothesized that Tobramycin impregnation would impair implant fixation.

We added 0.25 ml of a Tobramycin solution to 1 ml packed morselized bone. In the low-dose group, this equals an addition of 50 mg Tobramycin, and in the high-dose group 200 mg Tobramycin was added. The low-dose level was comparable with concentrations used in clinical trials and considered as a clinically relevant dose level [67]. The high dose group was included to reflect the upper boundary of a clinically relevant concentration and to increase the detectability of potentially adverse effects. The mechanical test revealed no statistically significant difference although there was a trend towards decreased mechanical properties in all parameters with both doses of Tobramycin. Histologically, the treatment groups were comparable with a similar distribution of bone graft and new bone in the gap. The histomorphometric analysis revealed no trend or any statistically significant difference between the groups. No adverse effects were seen in the high-dose group.

This study did not examine the potential anti-bacteriological benefits of adding Tobramycin to the bone graft; it solely examined the influence on implant fixation. Furthermore, the antibiotic concentration within the gap was not measured. A method for obtaining this information would be to use a microdialysis catheter placed within the grafted gap. However, its presence within the gap would critically influence the fixation of the implant due to its relative large size compared with size of the gap. We therefore chose not to use microdialysis catheters as our focus in this study was mainly on implant fixation. Further studies may evaluate the local release of Tobramycin through the use of microdialysis catheters in order to evaluate the anti-bacteriological effect of this method.

LIMITATION

Our experimental implant model is simple and affords a high degree of variance control. However, the model also has limitations: First, the implant is not influenced by a clinically relevant direct load transmission and the results are therefore limited because the full effects of weight-bearing conditions are not addressed. Second, the model has no access to the joint space. The lack of joint fluid in the bone-implant interface does, however, make the model less complex and the results easier to interpret. Third, the grafted defect was created in bone of young healthy animals and not in the compromised bone typically surrounding a loose implant in humans. Fourth, dogs were chosen as experimental animals because their bone quality and trabecular density reflect human bone well; however, it is noted that canine bone remodels three times more quickly than human bone [72, 73]. Fifth, the observation period of four weeks was chosen based on previous experience with graft resorption and new bone formation in the same animal model enabling comparison of the data with those of previous studies [94]. Sixth, the detectable, clinically relevant threshold was set to 30% and was chosen based on the consideration that the bone quality in revision surgery is highly variable and an experimental study therefore has to demonstrate substantial effect before being clinically relevant. Seventh, the data are limited to the portion of a cement-less joint replacement that is surrounded by impacted morselized allograft bone and placed in cancellous bone. Caution is therefore advisable when extrapolating the data to the structural and biological setting of clinical cemented revision arthroplasties.

The design of Study I also has limitations with regard to graft volume. Equal volumes of un-treated morselized allograft

bone were measured in both groups before the rinsing procedure. The 9% volume reduction seen in the rinsed group (*Table 1*) resulted in a mean impacted volume in the control group of 1.23cm^3 compared with 1.11cm^3 in the intervention group. We found no bone tissue in the rinsing water and therefore consider the volume reduction to be caused mainly by the removal of blood, fat and bone marrow tissue. Thus, the impacted volumes of bone graft were considered equal in the two groups even if the actual volumes of impacted tissue were different. This design enabled us to isolate the effect of lowering the immunogenic factors on osseointegration. An alternative study design would be to impact an equal volume of graft material regardless of preparation. If this design was used, the total bone graft volume would have been larger in the intervention groups because there would be less blood, fat and marrow tissue [36]. This would potentially improve early implant fixation due to a denser bone graft impaction [33-35]. This may have been a more clinically relevant approach, but the results would have been more difficult to interpret, as both reductions of the immunogenic load as well as denser impaction would have influenced the fixation of the implant.

7. CONCLUSION

Within the limits of the used experimental model, allograft bone did not benefit from rinsing out immunogenic material or adding of minced autologous periosteum. Tobramycin impregnation of allograft bone appears not to impair implant fixation or tissue in-growth; however, surgeons should weigh the potential cytotoxic effect of Tobramycin against its beneficial antimicrobial effect in joint arthroplasty revisions until further basic data on toxicity are available.

8. SUMMARY

Loosening of an artificial joint prosthesis is a painful and debilitating condition that can be treated only by re-operation. Re-operations accounted for approximately 15% of all hip replacement operations performed in Denmark between the year 1995 and 2010. The process of loosening is often accompanied by destructive inflammation and osteolysis, which leads to insufficient bone stock that often requires extensive bone grafting. Impacted morselized bone graft is a well-established method for improving the amount and quality of bone stock that ensures sufficient stability and anchorage of the revision implants. Among bone graft options, the autologous bone graft is considered the gold standard. It is naturally biocompatible, but its use in revision surgery is curtailed by its limited volume and by considerable donor site morbidity. Allograft bone is readily available and is the most commonly used graft material. However, it has been shown that the incorporation of allograft bone into the host bone is not always complete, and substantial fibrous tissue formation has been described. A reason for this may be that allograft bone is a foreign tissue, which, contrary to autogenic bone, may induce an immunogenic response that leads to increased fibrous tissue formation. Furthermore, the fresh-frozen allograft has minimal osteoinductive and no osteogenic capacity. The studies in this thesis have investigated ways of improving the incorporation of allograft bone by adding osteoinductive cells from the periosteum and reducing the immunogenic load of the allograft bone by rinsing. Furthermore, the impact of antibiotic protection of the bone graft has been evaluated.

The same experimental implant model was used in all three studies. This model enables evaluation of early implant fixation and osseointegration of an uncemented implant surrounded by impacted morselized bone graft. Unloaded gap implants were inserted into the metaphysis of the proximal tibia (Study I) and distal femur (Study II and III) in dogs. The observation period was four weeks and the bone-implant specimens were evaluated by mechanical tests and histomorphometry.

Study I compared the fixation of grafted implants where the morselized allograft bone was either rinsed in saline or not. Since the majority of immunogenic factors in allograft bone are present in the blood, the marrow and fat, the objective of this study was to investigate whether rinsing of the allograft bone would lower the immunogenic load and thereby improve osseointegration and bone graft incorporation. We found no statistically significant difference in the histomorphometric or the mechanical evaluations between the two groups.

Study II investigated the addition of minced periosteal tissue to the allograft bone. The objective of this study was to investigate whether adding autologous tissue containing bone-forming cells could augment the bioactivity of allograft bone and thereby improve bone graft incorporation. Contrary to our hypothesis, we found no benefit of adding autologous periosteum to the allograft bone. No differences in mechanical fixation were observed, and the periosteum-treated implants had reduced new-bone ongrowth and increased amounts of fibrous tissue.

Study III evaluated the impact of antibiotic impregnation of the allograft bone prior to impaction. Antibiotic-impregnated bone graft has been used in one-stage septic revisions and in cases where potential infection is suspected, but its potentially harmful effect on bone graft incorporation has not been studied. The aim of this study was to evaluate the impact of Tobramycin impregnation of bone-grafted implants by mechanical testing and histomorphometric assessment. We hypothesized that Tobramycin impregnation would impair implant fixation. Under the conditions of the present study, Tobramycin impregnation of allograft bone did not appear to impair implant fixation or tissue in-growth.

In conclusion, under the premises of the present studies, no benefits of periosteal augmentation or rinsing of the bone graft prior to impaction were found. Antibiotic impregnation seems safe in terms of osseointegration, but surgeons should weigh the potentially cytotoxic effect of Tobramycin against its beneficial anti-microbial effect in joint arthroplasty revisions until further basic data on toxicity are available

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