



Sveriges lantbruksuniversitet

Fakulteten för veterinärmedicin och husdjursvetenskap

Institutionen för Kliniska Vetenskaper

The rabbit as an animal model in dental implant research – with special reference to bone augmenting materials

Lovisa Nalin

Uppsala

2013

Examensarbete inom veterinärprogrammet

ISSN 1652-8697

Examensarbete 2013:14

The rabbit as an animal model in dental implant research
- With special reference to bone augmenting materials
Kaninen som modelldjur inom forskning kring tandimplantat
- med fokus på material som stimulerar bentillväxt

Lovisa Nalin

Handledare: Patricia Hedenqvist, Institutionen för Kliniska Vetenskaper
Biträdande handledare: Marianne Jensen Waern, Institutionen för Kliniska Vetenskaper
Examinator: Ove Wattle, Institutionen för Kliniska Vetenskaper

Examensarbete inom veterinärprogrammet, Uppsala 2013
Fakulteten för veterinärmedicin och husdjursvetenskap
Institutionen för kliniska vetenskaper
Kurskod: EX0736, Nivå A2E, 30hp

Key words: rabbit, animal model, dental implant, bone augmenting material, bioceramic
Nyckelord: kanin, djurmodell, tandimplantat, benstimulerande material, biokeram

Online publication of this work: <http://epsilon.slu.se>
ISSN 1652-8697
Examensarbete 2013:14

CONTENTS

Summary	1
Sammanfattning	1
Introduction	2
Background	3
Dental implants	3
Endosseous alloplastic implants – short summary	3
Structure, formation and repair of bone tissue	4
Teeth and bone	7
Bone healing around dental implants	7
Implant failure	8
Materials used for bone augmentation	9
Bone grafts	10
Alloplastic bone substitutes.....	11
The rabbit as an animal model for implant research in bone	14
Materials and methods	15
Animals	15
Materials.....	16
Preparations	16
Pre-medication and anesthesia	16
Surgery	17
Post-operative care	18
Euthanasia	19
Results	19
Anesthesia, surgery and recovery.....	19
First week post-surgery	19
Second and third week post-surgery	19
Post-mortem examination	20
Discussion	23
Post-surgical monitoring	23
Tibial fractures	24
Implant size and number	24
Implant location.....	25
Housing considerations	27
The “Ångström” ceramic	30
Summary	30
Conclusion.....	30
Acknowledgement.....	30

SUMMARY

Dental implants that are integrated into the jaw bones are widely used to replace lost teeth in human beings. Tooth-loss is often related to loss of the surrounding alveolar bone. This can make implant placement difficult and a bone substituting material may be required. The standard method is to use a bone graft harvested from the patient which requires additional surgery than that needed for implant placement. Limitations in the amount of bone that can be harvested also pose a problem. In order to decrease the need for bone grafts, materials that can serve as substitutes and stimulate bone growth have been, and are being, developed.

In this pilot study the research protocol for an *in vivo* experiment with a new calcium phosphate bone augmenting ceramic material, was evaluated. Two titanium screw type dental implants were placed in each tibial diaphysis of three, 12 month old, female New Zealand White rabbits. The implants were either surrounded by the experimental material, Straumann®BoneCeramic (positive control) or blood (negative control).

For one week before as well as post-surgery the rabbits' body weights were recorded daily. Each rabbit's general condition, state of surgical wounds and the grade of limping was evaluated every day for as long as needed. Two weeks post-surgery an injection of a bone marker (calcein green) was planned. Three months post-surgery the rabbits were to be euthanized and the amount of newly formed bone evaluated with light microscopy. The osseointegration of the implants was to be measured histomorphometrically as the bone to implant contact in percent. However, the calcein green was never given and the bone formation and osseointegration never evaluated. All rabbits had to be euthanized within 24 days post-surgery because of fractures appearing in one or both tibia/e.

Apart from the pilot study, the master thesis includes a literature review of dental implants, bone augmenting materials and how the rabbit can be used as an animal model in dental implant research.

SAMMANFATTNING

Tandimplantat som integreras i käkbenet används flitigt inom humantandvården för att ersätta förlorade tänder. Förlust av tänder hänger ofta ihop med förlust av det omgivande alveolarbenet. I och med detta kan ett bensubstitut behövas för att implantatet ska kunna sättas in. Standardmetoden är att använda ett kroppseget bentransplantat. Detta medför att patienten behöver genomgå fler operationer än vad som krävs om implantatet kan sättas in direkt och det är också en begränsad mängd ben man får tillgång till på detta sätt. För att minska behovet av bentransplantat utvecklas material som kan ersätta ben och stimulera bentillväxt.

I pilotstudien som beskrivs här utvärderades ett försöksprotokoll inför en studie av ett nytt benstimulerande keram baserat på kalciumfosfat. I piloten användes 3 stycken 12 månader gamla New Zealand White kaniner av honligt kön. Två skruvformade tandimplantat av titan sattes in i vardera tibiadiaphys. Implantaten omgavs antingen av det experimentella materialet,

Straumann® BoneCeramic (positiv kontroll) eller blod (negativ kontroll).

Under en vecka före såväl som efter operationerna vägdes kaninerna dagligen. Deras allmäntillstånd, operationssår och grad av hälsa utvärderades varje dag så länge som det behövdes. Två veckor efter operationerna var planen att injicera en benmarkör, calcein grön, och tre månader efter operationen skulle kaninerna avlivas och bentillväxten utvärderas med ljusmikroskop. Osseointegrationen av implantaten skulle mätas histomorfometriskt som kontakten mellan implantat och ben i procent. Alla kaniner avlivades dock innan tre månader gått p.g.a. att de frakturerade en eller båda tibia/e inom 24 dagar efter operationen.

Utöver pilotstudien innehåller examensarbetet också en litteraturstudie om tandimplantat, material som främjar bentillväxt och om hur kanin kan användas som modelldjur vid forskning kring tandimplantat.

INTRODUCTION

Dental implants are used to replace lost teeth and to correct various types of defects (as reviewed by Xie et al., 2012). The method where titanium implants are integrated into the jaw bones has been used since the 1960's (Brånemark et al., 1977). However the traditional method of placement requires a long period of healing (as reviewed by Henry and Liddelow, 2008) and at least two surgical procedures since a two-step surgical protocol is used (as reviewed by Gapski et al., 2003). If the patient also needs an autogenous bone graft to the implantation site he or she must undergo additional surgery before the implant can function as a regular tooth. Current research aims to shorten the treatment time and minimize the surgical trauma to the patient (as reviewed by Henry and Liddelow, 2008). This can be achieved with materials that stimulate bone formation around implants (as reviewed by Xie et al., 2012).

This master thesis includes an *in vivo* experiment and a literature review.

A team from the Department of Chemistry at Ångström, Uppsala University and the Stockholm Craniofacial Centre at the Karolinska University Hospital has developed a new injectable calcium phosphate ceramic compound, the "Ångström ceramic", based mainly on monetite. The aim is to use the material for bone augmentation around dental implants in humans. The material has been tested *in vitro* and before it can be considered safe for human use it must also be evaluated in an animal model. Justifications for the use of experimental animals in the testing of biomedical devices are outlined in an International Standard regarding animal welfare requirements (International Standard, ISO 10993-2: 1996). The research team has an approval from the local ethical committee to use 12 rabbits in the experiment (Dnr C131/11). This master thesis describes a pilot study, using three of these rabbits, carried out to evaluate the experimental protocol. The pilot study was conducted in collaboration with the Department of Clinical Sciences at the Swedish University of Agricultural Sciences.

The purpose of the literature review is to provide a background for the pilot study and to point out pros and cons with the chosen animal model.

In order to understand how the rabbit can be used as an animal model in dental implant research it is important to know some facts about dental implants and common problems associated with implant placement. This is covered in the background, which also contains a short summary of materials used for bone augmentation. Furthermore, it is important to comprehend the structure and formation of bone tissue in order to understand the different materials that can be used to replace it.

BACKGROUND

Dental implants

In the year of 2000 there were almost 100 different dental implant designs on the market (Binon, 2000). Described here are the type of implants that are integrated into bone and different ways to augment the bone formation around them. Formation and maintenance of enough bone at the implantation site is important for the short and long term success of this type of dental implants (as reviewed by Masuda et al., 1998).

Endosseous alloplastic implants – short summary

Endosseous implants are implants placed within bone (as reviewed by Davies, 2003), and alloplastic means that they are made of non-biological material. Titanium is a material widely used for dental and orthopedic implants since it can be incorporated into bone with good biocompatibility (as reviewed by Marco et al., 2005). Standard dental implants have a diameter of 3-5 mm and a length of 6-15 mm (as reviewed by Huang et al., 2005).

An endosseous dental implant consists of three different parts: an implant body that is integrated into the bone, an abutment which is an anchor that connects the implanted body with the dental prosthesis, and the dental prosthesis itself (*see figure 1*). These can either be separate parts that are put together in certain steps when the implant is placed, or they can all be in one part from the beginning (as reviewed by Prasad et al., 2011). The implant bodies come in many different shapes (as reviewed by Binon, 2000), for example they can be fashioned like screws or flat cylinders.

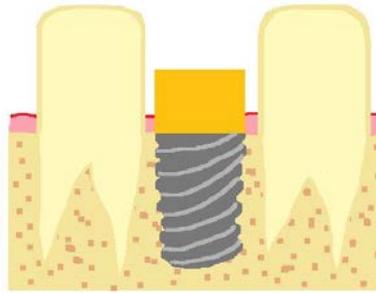


Figure 1. Schematic illustration of dental implant (grey) and abutment (yellow) between two regular teeth. By Lovisa Nalin based on the information from Prasad et al., 2011.

There are three different methods for how and when an endosseous implant can be placed; immediate, early and delayed loading (as reviewed by Gapski et al., 2003 and Prasad et al., 2011). Delayed loading is the traditional method used since the sixties (as reviewed by Henry and Liddelow, 2008). It means that the implant body is placed into the bone, left to be integrated and then loaded with the abutment and prosthesis several months later. After implantation, the gum is sutured over the area (as reviewed by Gapski et al., 2003). The implant is then said to be submerged (Prasad et al., 2011). With this method the tissue around the implant can heal without contact with the oral cavity and without being affected by the occlusal forces. This prevents movement of the implant and minimizes the risk for infection and ingrowth of epithelium (as reviewed by Gapski et al., 2003). The procedure requires two surgeries since the gum must be reopened to place the abutment (as reviewed by Gapski et al., 2003).

Immediate and early loading are newer techniques where the tooth prosthesis is put into place immediately or within weeks after the implantation (as reviewed by Gapski et al., 2003 and Henry and Liddelow, 2008). The advantages of immediate loading is that it only requires one surgical procedure, it shortens the treatment time and is more convenient for the patient (as reviewed by Gapski et al., 2003). The problem with this method is to get the implant properly integrated into the bone, but with new materials and methods this has become possible under the right circumstances (as reviewed by Gapski et al., 2003).

One very important factor that determines when an implant can be loaded is the amount of bone at the implantation site. Enough bone of the right quality is important to ensure an early stable fixation of the implant which shortens the time before the implant can be loaded (as reviewed by Marco et al., 2005) and therefore the treatment time.

Structure, formation and repair of bone tissue

Bone is a highly specialized connective tissue built up by intercellular calcified bone matrix, osteogenic cells, i.e. osteoblasts, osteocytes and osteoclasts, and vasculature.

Bone matrix

The bone matrix consists of both inorganic and organic matter. Calcium and phosphorus are the main components of the inorganic part (Junqueira and Carneiro, 2005). They form hydroxyapatite crystals and non-crystalline forms of calcium phosphate. The organic part is made of type 1 collagen and ground substance (proteoglycan aggregates and glycoproteins) (Junqueira and Carneiro, 2005). This composition makes bone both hard and flexible. If the minerals are removed, the shape of the bone is maintained but it becomes as flexible as tendon. If the organic part is removed instead, the bone becomes brittle and fragile (Junqueira and Carneiro, 2005).

Bone types

Macroscopically bone can be divided into compact (cortical) and cancellous (spongy, trabecular) bone. Microscopically the basic histological structure of the bone types is the same (Junqueira and Carneiro, 2005). The compact bone is dense whereas the cancellous bone consists of trabeculae and numerous interconnecting cavities (Junqueira and Carneiro, 2005). A long bone can be divided into different parts: two epiphyses, two metaphyses and a diaphysis (Ross and Pawlina, 2011). The epiphyses are the most proximal and distal parts of the bone, separated from the metaphyses by the epiphyseal lines which are rests of the epiphyseal growth plates. The metaphyses are the parts between the epiphyseal lines and the shaft of the bone which is called the diaphysis (Ross and Pawlina, 2011) see *figure 2*. The epiphysis of long bones (e.g. the femur and tibia) consists to the greatest part of cancellous bone (the medullae) covered only by a thin layer of compact bone (the cortex) at the surface (Junqueira and Carneiro, 2005). The diaphysis on the other hand, is built up mostly by compact bone with only a thin core of cancellous bone surrounding the bone marrow cavity.

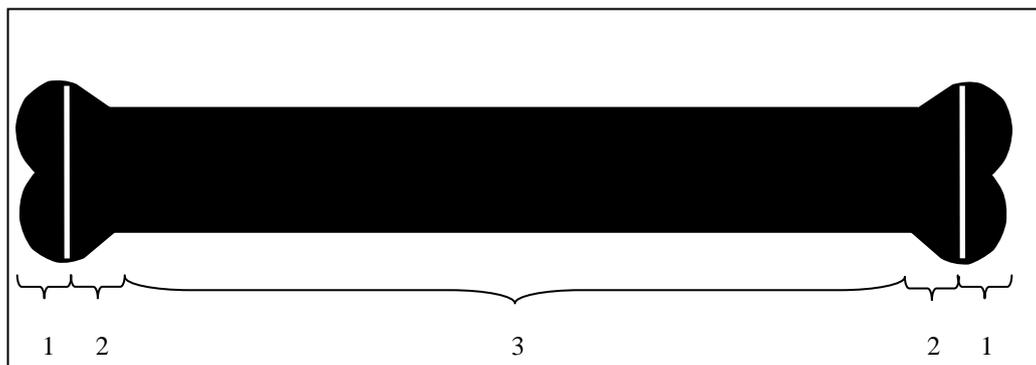


Figure 2. Schematic illustration of the different parts of a long bone. 1 = epiphysis, 2 = metaphysis, 3 = diaphysis. The white lines represent the epiphyseal lines. By Lovisa Nalin based on information from Ross and Pawlina, 2011.

Microscopically bone can be divided into primary (immature, woven) and secondary (mature, lamellar) bone tissue (Junqueira and Carneiro, 2005). Primary bone is characterized by a woven structure where the collagen fibers are un-organized. It also has a lower mineral density (darker on X-rays) and more osteocytes than mature bone and is the first bone to form in embryos and in bone repair (Junqueira and Carneiro, 2005). With a few exceptions (e.g. bone symphyses) this bone type is eventually replaced with mature bone which is characterized by a well-organized structure where the collagen fibers are arranged in lamellae,

see *figure 3* (Junqueira and Carneiro, 2005). A Haversian system (or osteon) is formed by multiple lamellae surrounding a canal with nerves, blood vessels and connective tissue (Junqueira and Carneiro, 2005). Cementing substance surrounds each haversian system (Junqueira and Carneiro, 2005) and can be seen as cement lines marking the border of the same (as reviewed by Davies, 2003). They form at the interface where old bone has been resorbed and new bone is being laid down (as reviewed by Marco et al., 2005).

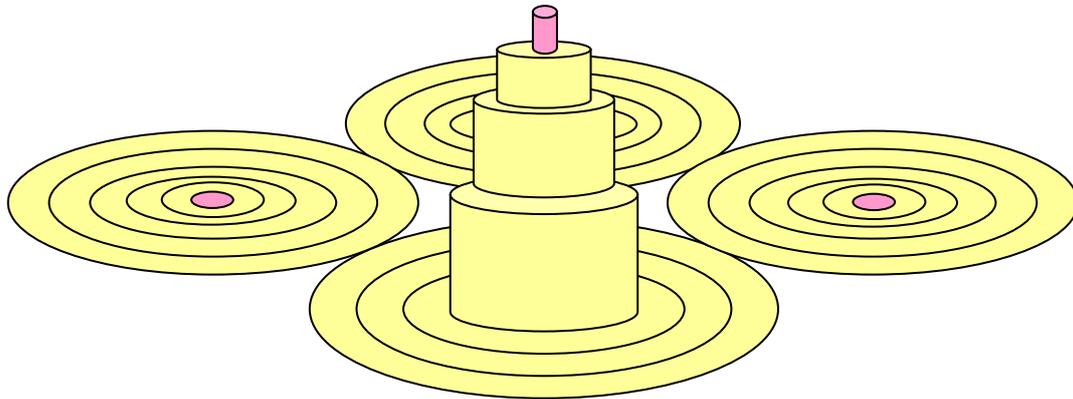


Figure 3. Schematic illustration of four Haversian systems. The middle one in the front is drawn elevated to illustrate how the lamellae surround the central canal (pink). By Lovisa Nalin based on information from Junqueira and Carneiro, 2005.

Bone formation and fracture repair

Bone can form in two different ways, intramembranous ossification and endochondral ossification. In the first case osteoid produced by osteoblasts is mineralized directly and in the second a hyaline cartilage template is first produced and then mineralized. In both cases primary bone is the first to appear and is later remodeled into secondary bone. The mandible and maxillae are formed through intramembranous ossification whereas the long bones are formed through endochondral ossification (Junqueira and Carneiro, 2005).

When bone is damaged cells at the site of injury dies and the matrix is destroyed. The bleeding from damaged vessels results in the formation of a blood clot (Junqueira and Carneiro, 2005). This initiates an inflammatory cascade. Important signaling factors during fracture healing are pro-inflammatory cytokines (interleukin-1 and 6, tumor necrosis factor- α) and growth factors (as reviewed by Hallman and Thor, 2008). The pro-inflammatory cytokines attract inflammatory cells, stimulate angiogenesis and accelerates matrix production (as reviewed by Hallman and Thor, 2008). Growth factors from the growth factor- β superfamily (bone morphogenic proteins, growth factor- β) stimulate new bone formation (the bone morphogenic proteins can even induce ectopic bone formation i.e. are osteoinductive) (as reviewed by Hallman and Thor, 2008). Other important growth factors during fracture healing are platelet derived growth factor, insulin like growth factor 1 and 2, fibroblast growth factor and growth factors involved in angiogenesis (as reviewed by Hallman and Thor, 2008). Mesenchymal stem cells in the area are activated and recruited by the released signaling factors and differentiate into bone forming cells (as reviewed by Albrektsson and Johansson, 2001). The periosteum and endostium proliferates and grow across the fracture ends to form a callus and during reparation the blood clot and the damaged tissue is removed by

macrophages (Junqueira and Carneiro, 2005). Primary bone is formed both through intramembranous and endochondral ossification (Junqueira and Carneiro, 2005). This bone is then gradually remodeled into secondary bone.

Cancellous bone is connected to the bone marrow which contains a rich vasculature and mesenchymal stem cells, this bone therefore heals and remodels faster than cortical bone (as reviewed by Davies, 2003)

Teeth and bone

The alveolar bone (also called alveolar ridge or process) is the part of the mandibula and maxillae that surrounds the teeth. In other words it constitutes the tooth sockets. The alveolar bone ends at the level of the tooth cervix and this border is called the alveolar crest. Together with the periodontal ligament and cementum of the teeth it forms the periodontium, the anchorage between teeth and bone. The bone lining the insides of the tooth sockets is called the alveolar bone proper and is made of compact bone (Bath-Balogh and Fehrenbach, 2011). The exterior of the tooth sockets are made of compact and cancellous bone and this is called the supporting alveolar bone (Bath-Balogh and Fehrenbach, 2011). The compact part of the supporting alveolar bone constitutes the facial and lingual part of the tooth sockets. Between this compact bone and the alveolar bone proper the cancellous part of the supportive alveolar bone is found. The bone between the roots of the teeth is composed of the alveolar bone proper and cancellous bone. The bone most apical to the roots is called the basal bone and is part of the mandibular and maxillar bodies (Bath-Balogh and Fehrenbach, 2011).

The alveolar bone requires functional stimulation from speech and mastication to be preserved, so if a tooth is lost the alveolar bone is resorbed all the way down to the basal bone (Bath-Balogh and Fehrenbach, 2011). This mechanism can be prevented by installing a dental implant (Bath-Balogh and Fehrenbach, 2011). Since the teeth are anchored to the alveolar bone the opposite mechanism is also a fact, if the bone is resorbed the teeth are lost.

Bone healing around dental implants

Brånemark et al. (1977) were the first to suggest that there could be a direct anchorage between bone and implant and introduced the term osseointegration.

Osseointegration

When an implant is inserted into the jawbone it can be integrated into the bone tissue with direct contact between implant and bone, or it can heal with a fibrous capsule around it (as reviewed by Esposito et al., 1998b, Gapski et al., 2003 and Marco et al., 2005). The latter happens when primary stability is not achieved and the implant moves during integration (as reviewed by Marco et al., 2005). An implant is properly osseointegrated if the clinically asymptomatic rigid fixation between the alloplastic material and bone can be maintained during the loading of the implant (as reviewed by Albrektsson and Johansson, 2001). If there is fibrous tissue around the implant, osseointegration is not possible and the anchorage is then not sufficient for a tooth prosthesis to function as a regular tooth (as reviewed by Gapski et al., 2003 and Marco et al., 2005). This can be put in contrast to the fact that natural teeth are

surrounded by soft tissue, the periodontal ligament. This ligament is however highly specialized and nothing like the unorganized soft tissue capsule, and so far no one has managed to reconstruct the periodontal ligament around an implant (Albrektsson, 1995). This means that a successfully integrated implant is rigidly fixated to the bone and therefore loaded immediately when a force is applied as opposed to the natural teeth which, since they are anchored to the bone by the periodontal ligament, are able to move slightly in the alveolar sockets and distribute the force (as reviewed by Rangert et al., 1997).

The amount and type of bone at the implantation site affects the primary stability (as reviewed by Esposito et al., 1998b, Gapski et al., 2003 and Marco et al., 2005). Bone is divided into 4 different categories in the dental implant field based on the proportions between compact and cancellous bone (as reviewed by Davies, 2003). Class 1 bone consists primarily of compact bone and class 4 mostly of cancellous bone. The bone in the anterior mandible is often class 1 bone whereas the bone in the posterior maxilla frequently is class 4. Compact bone provides a better anchorage than cancellous and therefore gives a better primary stability (as reviewed by Davies, 2003). Because of this Class 4 bone is often referred to as “poor quality bone” meaning that the quality is poor when it comes to the placement of implants and not that the bone is biologically impaired (as reviewed by Davies, 2003). As previously mentioned, cancellous bone actually remodels and heals faster than compact bone.

Mechanisms of bone healing around implants

The tissue in the drilled cavity into which the implant is put acts like the fracture repair in a common bone wound (as reviewed by Marco et al., 2005) but without endochondral ossification (as reviewed by Esposito et al., 1998b). The local tissue trauma caused by the drilling in the bone gives rise to an inflammation cascade. The bone implant interface is covered with blood, a clot forms and inflammatory cells migrate to the area (as reviewed by Davies, 2003). Damaged bone is broken down by osteoclasts and new bone is formed on top of it by osteoblasts. This new formation can occur in two different ways, distance osteogenesis or contact osteogenesis (as reviewed by Davies, 2003). In distance osteogenesis new bone is laid down on the peri-implant bone surfaces and the bone growth is directed towards the implant until it is surrounded and integrated into the bone. In contact osteogenesis bone forming cells colonize the implant surface and new bone is formed directly on it. This mimics the events where new bone is laid down on the surface of old resorbed bone and a cement line is formed between the implant and the new bone. Both types of bone formation occur at all implantation sites but the implant surface can be modified to enhance contact osteogenesis which provides a better primary stability. This is described in more detail in the section “Materials used for bone augmentation”.

Implant failure

There are many reasons why a dental implant may be considered to have failed. There can be something wrong with the implant, abutment or dental prosthesis (mechanical failure), the patient may not be satisfied with the result (patient related failure) or it might not have been put in properly (iatrogenic failure) (Esposito et al., 1998a). Apart from these factors implants fail because they are inadequately osseointegrated (biological failure) (Esposito et al., 1998a).

This can either be early in the process if the bone-implant contact is inadequate (as reviewed by Marco et al., 2005) or if the healing process is disturbed (as reviewed by Esposito et al., 1998a), or later when a so far successfully osseointegrated implant fails because osseointegration is lost. This can happen if the tissues around the implant become chronically inflamed (peri-implantitis) or if the bone quality and volume in the area are not sufficient to bear the occlusal load (as reviewed by Esposito et al., 1998b). The same authors discuss that if a dental implant is subjected to great loads, micro fractures can arise in the surrounding bone, resulting in stress fractures at the bone-implant interface if the breakdown is faster than the repairing processes. The load that an implant can withstand depends on the capacity of the bone, the implant and the prosthesis design, therefore the forces the implant will be subjected to need to be evaluated before the implant is placed so that appropriate implant designs and placement methods can be chosen (Ranger et al., 1997).

To ensure satisfactory osseointegration it is important to have a sufficient bone volume at the implantation site from the beginning (as reviewed by Chiapasco et al., 2009). As previously mentioned the alveolar bone atrophies when a tooth is lost and vice versa, the teeth are lost if the bone atrophies (Bath-Balogh and Fehrenbach, 2011). The latter is often the result of periodontal disease where the bone is broken down due to chronic inflammation in the structures surrounding the teeth. Old age and smoking are risk factors for severe disease and systemic metabolic diseases that affect bone can also lead to alveolar resorption (as reviewed by Bodic et al., 2005). Atrophy of the alveolar ridge both in height and width can make implant placement difficult (Hobkirk and Watson, 1995)

Bone augmenting procedures may therefore be needed before an implant can be placed or bone augmenting materials can be used when the implant is installed to speed up the bone formation.

Materials used for bone augmentation

Bone grafts or alloplastic bone substitutes are used to augment bone at implantation sites where there is not enough. They provide the structural base for new bone that can support the dental implant (as reviewed by Xie et al., 2012). One important characteristic is that the material can function as a “spacemaker” and maintain the area where new bone is meant to form, even if it is affected by compressing forces (as reviewed by Thomas and Puleo, 2009). An ideal material is degraded and completely replaced by bone, but a fast degradation time can cause problems in some clinical situations (as reviewed by Thomas and Puleo, 2009). For example the esthetic result may be compromised (as reviewed by Hallman and Thor, 2008).

Bone cells can be attracted to grow both by osteoinduction and osteoconduction. Osteoinduction is the mechanism in which mesenchymal stem cells are activated to differentiate into bone forming cells (as reviewed by Albrektsson and Johansson, 2001) and if an agent is truly osteoinductive it can induce bone formation at extra skeletal sites, for example in muscle. Osteoinduction naturally takes place when undifferentiated cells are activated by the signaling factors released due to bone injuries (e.g. fracture, introduction of an implant) (as reviewed by Albrektsson and Johansson, 2001) and a material that is in itself

osteoinductive can shorten the healing process around an installed implant (as reviewed by Hallman and Thor, 2008).

Osteoconduction is when bone is allowed to grow on a surface, for example on an implant or synthetic bone substitute (as reviewed by Albrektsson and Johansson, 2001). All materials are not osteoconductive, for example bone cannot grow on silver or copper, and if these materials are placed into bone they are permanently encapsulated by soft-tissue (Albrektsson, 1995). Osteoconduction is perhaps the most important quality of bone augmenting materials since osteoinduction, as previously described, always takes place at the site of a bone injury even if the material itself is not osteoinductive (Albrektsson and Johansson, 2001).

A substitute material must be biocompatible (not toxic to cells), not evoke an immunogenic response and not give rise to chronic inflammation (as reviewed by Rezwan et al., 2006) and when degraded it should be into products that the body can metabolize and/or excrete (as reviewed by Chaikof et al., 2002). Another requirement is that it must be easy to sterilize (as reviewed by Chaikof et al., 2002).

The ideal substitute would be able to release osteoinductive substances in a safe way, stimulate cell adhesion and proliferation and support the dental implant (as reviewed by Xie et al., 2012). It should also be resorbed and eventually fully replaced by bone (as reviewed by Tamimi et al., 2010), ultimately within a well-defined time frame (as reviewed by Thomas and Puleo, 2009). This is what research today aims to find.

Bone grafts

The standard method of bone augmentation is to use an autogenous bone graft (as reviewed by Xie et al., 2012). This type of graft is both osteoinductive, since it contains viable cells and proteins that stimulate osteogenesis, and osteoconductive as it works as a scaffold for new bone formation (as reviewed by Hallman and Thor, 2008). Autogenous bone grafts are harvested from the patient from intra oral sites, the iliac crest or the calvarium (as reviewed by Junker et al., 2009). If much bone is needed it is taken from the iliac crest (as reviewed by Hallman and Thor, 2008). The implant is either placed right away or several months after the graft have been put in place (as reviewed by Junker et al., 2009). Consequently this means that the patient has to go through one or two more operations, than the one or two needed for implant placement if a bone graft is not needed. All in all four operations might be necessary: one for harvesting the graft, one for inserting it, one for placing the implant and one for the attachment of the abutment and prosthesis.

Because of this patient inconvenience, limited availability of bone and also unpredictable graft resorption times, alternative methods have been invented (as reviewed by Hallman and Thor, 2008).

Bone grafts can also be allografts (derived from another individual of the same species) or xenografts (derived from another species). The risk with using grafts from another source than the own body are immunologic rejections and spreading of diseases.

In bone allografts there are no viable cells, but since they contain bone stimulating proteins they are still osteoinductive (as reviewed by Hallman and Thor, 2008). However, to reduce the immunogenicity of allografts they have to be processed in different ways, for example by freeze-drying, and this makes them less osteoinductive and osteoconductive than autografts (as reviewed by Habibovic and de Groot, 2007). Freeze-dried and demineralized freeze-dried allografts are the most commonly used allografts in implant dentistry and they are usually mixed with autogenous bone (as reviewed by Hallman and Thor, 2008).

Xenografts consist of bone minerals and are depleted of all proteins (to eradicate host immunologic reactions) (as reviewed by Hallman and Thor, 2008). Because of this they lack osteoinductive capacities and only function as osteoconductive scaffolds. Deproteinized bovine bone is the most researched material and since it resembles human bone it is often used in implant dentistry (as reviewed by Hallman and Thor, 2008).

Alloplastic bone substitutes

Calcium phosphates, calcium sulphate, bioactive glasses and glass ceramics are bioceramic materials used to replace and substitute bone (as reviewed by Eppley et al., 2005, Vallet-Regi, 2006 and Hallman and Thor, 2008). These materials are bioactive, which means that they can induce or modulate biological activity, and through this form a bond with living bone (as reviewed by Frayssinet et al, 1999). Calcium phosphates are very similar to the inorganic part of bone and have attracted a lot of attention in the field.

Ceramic materials come in different forms: dense blocks, porous solid pieces, granulates and injectable solutions (as reviewed by Vallet-Regi, 2006). Recent research shows that the bone substitutes should be porous with a certain size of the pores since this is important for their resemblance to real bone and bone healing properties (as reviewed by Hallman and Thor, 2008). Pore size is important for the vascularisation and oxygenation needed for new bone formation throughout the ceramic material. Without the right porous structure bone is only formed on the outer surface (as reviewed by Vallet-Regi, 2006). Despite this, many commercially available alloplastic bone substitutes are non-porous (as reviewed by Hallman and Thor, 2008). A problem in making a ceramic material porous is that its mechanical properties then can be impaired (as reviewed by Rezwan et al., 2006).

Bioceramics are osteoconductive. Calcium phosphates and glass ceramics can also, with the right chemical composition, micro- and macrostructure, be made osteoinductive although the complete mechanism for this is not understood (as reviewed by Habibovic and de Groot, 2007).

A recent trend in material chemistry is to also use the ceramic materials as delivery vehicles for drugs or growth factors (as reviewed by Vallet-Regi, 2006). The idea is that the delivered substances will be gradually released and then be able to act locally at the implant site, for example to prevent infections or stimulate bone growth.

Another category of materials that can be used to create scaffolds is biodegradable polymers (as reviewed by Eppley et al., 2005).

Many of the technologies described below can also be used to modify the surfaces of implants to stimulate the osseointegration process.

Calcium phosphates

Calcium phosphates include a wide variety of calcium phosphate based materials that have a composition that resembles real bone and can bind directly to it (as reviewed by Xie et al., 2012). Many different chemical compositions exist (as reviewed by Eppley et al., 2005) the most common consisting of biphasic calcium phosphate, hydroxyapatite, β -tricalcium phosphate or some type of nonsintered calcium (as reviewed by Hallman and Thor, 2008). Calcium phosphate ceramics can be made into solid blocks, granulates, cements that are self-setting and harden when inserted to the body and injectable preparations (as reviewed by Arisan et al., 2010 and Xie et al, 2012).

A drawback with calcium phosphate ceramics is that their resorption (and hence their complete replacement with natural bone) often is reduced *in vivo*. An exception is β -tricalcium phosphate that has a very rapid resorption rate, which actually limits its use (as reviewed by Hallman and Thor, 2008).

Calcium phosphate cements are made from calcium orthophosphates and depending on how they are formed they can be divided into apatite and brushite cements (as reviewed by Xie et al., 2012). Monetite, the material used in the *in vivo* experiment in this master thesis, can be made from dehydrated brushite cement (as reviewed by Tamimi et al., 2010). The two materials are similar but behave different *in vivo* because of differences in water solubility at physiological pH. Monetite does not form hydroxyapatite as brushite does, and this makes monetite more resorbable *in vivo* (as reviewed by Tamimi et al., 2010).

Calcium sulfate

The chemical structure of calcium sulfate is relatively simple and therefore the chemical composition cannot be varied as much as for calcium phosphates (as reviewed by Eppley et al., 2005). The material can be made into pellets, pastes, putty, chips and injectable forms and in oral dentistry paste and putty are the most commonly used forms (as reviewed by Thomas and Puleo, 2009). Calcium sulfate is highly biocompatible and bioresorbable (as reviewed by Thomas and Puleo, 2009) and generally the material is substituted with bone within 8 weeks (definitely within 6 months) (as reviewed by Eppley et al., 2005). This rapid resorption can cause problems in implant dentistry since the material is degraded too fast to maintain enough space for the new bone formation (as reviewed by Hallman and Thor, 2008 and Thomas and Puleo, 2009) .

Bioactive glass and glass ceramics

Bioactive glass has an amorphous structure whereas glass ceramics are crystallized. The standard bioactive glasses are silica based (as reviewed by Rahaman et al., 2011). They also contain sodium, calcium and phosphate. When the glass is incorporated into the body and comes in to contact with body fluids reactions take place that form a hydroxyapatite layer on

the surface of the glass (as reviewed by Rahaman et al., 2011). This layer is osteoconductive. The main drawbacks with the glass are the slow degradation rate and difficulties when it comes to producing a stable porous piece (as reviewed by Rahaman et al., 2011). The slow degradation rate does not always match the new tissue formation and sometimes the material is not completely substituted with hydroxyapatite as it is meant to be (as reviewed by Rahaman et al., 2011).

Biodegradable polymers

Biodegradable polymers can either be natural-based (e.g. polysaccharides like starch and hyaluronic acid derivatives, or proteins like collagen, fibrin and silk) or synthetic (the most commonly used for scaffolding are saturated aliphatic polyesters e.g. lactic acid and glycolic acid) (as reviewed by Rezwan et al., 2006). Synthetic polymers have been used for sutures and internal fixation devices for several decenniums (as reviewed by Eppley et al., 2005).

The synthetic polymers have strong mechanical properties and are highly biodegradable (the time varies with type of polymer) but have poor bioactivity (as reviewed by Rezwan et al., 2006).

Combinations of the materials

In order to develop the ideal material with the perfect absorption time, biocompatibility and mechanical properties combinations of the different materials exists.

Ceramics can be used together with biodegradable polymers to form composite materials that combine the osteoconductivity of the ceramic with the fast degradation time and strong mechanical properties of the polymer (as reviewed by Rezwan et al., 2006). Tamimi et al. (2012) mention that the enhancement of a ceramic with a polymer resembles the way the mineralized structure of bone is enhanced by proteins.

Different ceramics can be combined. For example a combination of calcium sulfate and calcium phosphates in an *in vivo* study on dogs showed an increased formation of stronger bone than when calcium sulfate was used alone (Urban et al., 2007).

Different compositions of the same type of material can also be combined. For example a porous calcium phosphate ceramic was combined with calcium phosphate cement in an *in vivo* study using sheep (Frayssinet et al., 2000). The cement was used to fill the pores in the ceramic and was progressively degraded so that new bone could grow into them. This enhanced the mechanical properties of the material but did not affect the amount of bone formed in the pores compared to a control, after 120 days.

To reduce the amount needed, bone grafts can also be mixed with alloplastic substitutes (as reviewed by Hallman and Thor, 2008).

The rabbit as an animal model for implant research in bone

In the development of new dental and orthopedic implants and associated biomaterials it is often necessary to use animal models before the products can be evaluated in human beings. *In vitro* methods with cell, tissue or organ cultures are initially used to test a new material but are not sufficient to determine if the material is biocompatible, mechanically functioning and safe in the human or animal body (as reviewed by Pearce et al., 2007 and Muschler et al., 2010). Experimental animals must be used for testing biomedical devices if the data cannot be obtained elsewhere and is essential to characterize the tested material for the intended use and if no validated methods not using animals are available (ISO 10993-2:2006). For example, animals are needed to evaluate the local effects of an implant after implantation (ISO 10993-6:2007). Relevant reduction and refinement strategies must also be identified and applied if animals are to be used (ISO 10993-2:2006). All implants sold in the European Union must be CE-marked. What the manufacturer must do to be allowed the mark on the product, is regulated in a directive (Council Directive 93/42/EEC).

Recommended animal species to use are rodents, rabbits, goats, sheep, dogs and pigs (ISO 10993-6:2007). They all have different advantages and disadvantages. Except for rodents the rabbit is the species with the least similarity to humans when it comes to histological bone structure and remodeling (as reviewed by Pearce et al., 2007). Rabbit long bones generally consist of primary bone tissue (as reviewed by Wang et al., 1998, Martiniaková et al., 2005) compared to the secondary of humans. Their bones also heal faster. Albrektsson et al. (1981) discuss that a dental implant placed in a rabbit long bone may only need 6 weeks to be osseointegrated whereas it takes 3-4 months in a human. This can make it difficult to extrapolate results from rabbit studies to humans (as reviewed by Pearce et al., 2007).

Despite that, rabbits are often used in skeletal research (Neyt et al., 1998) since they have certain advantages in other aspects.

Even though rabbits are small compared to goats, sheep and pigs, they are big enough for the placement of multiple implants. This is not possible in rats (as reviewed by Pearce et al., 2007). Placement of multiple implants limits the number of animals needed in an experiment and makes it possible to use them as their own controls (as reviewed by Muschler et al., 2010). However the size of a rabbit limits the amount of implants that can be placed (as reviewed by Pearce et al., 2007). The international standard for biological evaluation of medical devices (ISO 10993-6:2007) recommends a maximum of 6 implants/rabbit, the same recommendation for the larger animals are 12. The recommended size for screw-type implants placed in mid shaft (diaphysal) cortical rabbit bone is 2 – 4.5 mm (International Standard, ISO 10993-6:2007). The standard does not state if this is the diameter or the length. For cylindrical implants the recommended size is 2 mm in diameter and 6 mm in length.

Because of their size and temperament rabbits are easy to handle (as reviewed by Pearce et al., 2007) and it is possible to keep and observe many at the same time (as reviewed by Calasans-Maia et al., 2009). They are readily available and less expensive than larger animals (as reviewed by Mapara et al., 2012) and are more accepted as experimental animals to the

general public than dogs (as reviewed by Pearce et al., 2007). It is also convenient that rabbits develop and reach skeletal maturity fast. Skeletal growth in the New Zealand white rabbit is completed at 28 weeks of age (Masoud et al., 1986).

In an experiment where the aim is to test bioactivity and feasibility of a material it is sufficient that the tissue into which the implant is put matches the tissue at the intended implantation site. This means it does not necessarily have to be placed in the intended anatomical location (as reviewed by Muschler et al., 2010). However, if the aim is to test if the material is functional in the clinical setting it is intended for, it is important to mimic the clinic as much as possible (as reviewed by Muschler et al., 2010). In dental research the implants are generally put in rabbit long bones, the tibia and femur are recommended (ISO 10993-6:2007, Mapara et al., 2012). The rabbit jaw is too small for human standard size implants (as reviewed by Mapara et al., 2012). There are however studies where the first upper molars have been extracted and implants have been placed in rat maxillae, but these implants were smaller than standard, only 1,14 mm in diameter and 2,01 mm in length (Shirakura et al., 2003). Different locations can be chosen depending on what human anatomical site it should resemble. Sennerby et al. (1992) used the tibial metaphyses as sites resembling the more compact bone in the mandibula and the epiphysis of the femur in the knee-joint as a site resembling the more cancellous bone in the maxilla. The cortical thickness of the rabbit tibial diaphysis and metaphyses is about 1.2-1.5 mm (Lind et al., 1993, Ivanoff et al., 1997, as reviewed by Cho et al., 2010) and the length of the bone in a full-grown rabbit is about 11cm (Masoud et al., 1986).

A disadvantage with placing the implant in long bones instead of the jaws is that loading it with a dental prosthesis is not possible (as reviewed by Mapara et al., 2012). However they can be tested under different loads with the use of special devices (Roberts et al., 1984, Uysal et al., 2012).

In conclusion the rabbit is a good model to start with before testing implants and associated materials in a larger animal model (as reviewed by Pearce et al., 2007).

MATERIALS AND METHODS

Animals

Three female New Zealand White rabbits, 12 months old, were used in this study. The rabbits were bought from a licensed breeder (Lidköpings kaninfarm, Sweden). The rabbit colony was health monitored according to recommendations by FELASA (Nicklas et al., 2002) and found to be free from the following pathogens: rabbit hemorrhagic disease virus, rabbit rota virus/ rabbit corona virus, *Bordetella bronchiseptica*, *Clostridium piliforme*, dermatophytes, *Pasteurella multocida*, other *Pasteurellaceae*, *Salmonella spp.*, ectoparasites and pathogenic endoparasites.

The rabbits were numbered 2057, 2271 and 2277 and on arrival their body weights were 4.13 kg, 4.83 kg and 4.67 kg respectively. During acclimatization the rabbits were kept together in a floor pen of 3m², enabling them social contact and free movement. Aspen

shavings were used for bedding and the pen was cleaned weakly. Plastic boxes served as hiding places and look-outs.. The room temperature was $20 \pm 2^\circ\text{C}$ and humidity $55 \pm 10\%$ and the light-dark-cycle was 12:12 hours with lights on at 0600 hours.

The rabbits were fed autoclaved hay (*ad libitum*) and a standard pelleted rabbit diet (Lactamin K3, Lantmännen, Sweden). Access to water was *ad libitum*.

The experiment was approved by the local ethical committee for animal experiments (Dnr C131/11).

Materials

Each rabbit received 2 screw type titanium implants (Straumann® Bone Level, SLActive implant, Institut Straumann AG, Basel, Switzerland) per tibia. The screws were 8 mm long and 3.3 mm in diameter. Around 2 of the implants in each rabbit the experimental “Ångström ceramic” was applied whereas the other 2 implants were surrounded by either a bone ceramic (Straumann® BoneCeramic, Institut Straumann AG, Basel, Switzerland) as a positive control or blood as negative control, see *table 1*.

Preparations

The rabbits were acclimatized and accustomed to handling during 2.5 weeks prior to the study. Their body weight was monitored daily, for comparison with body weight post-surgery, as an aid to evaluate recovery. The rabbits were trained to eat from a syringe in case supplemented feeding would be necessary post-surgery. Three days before surgery the fur on the ears of the rabbits was clipped, to enable vessel catheterization at the time of surgery.

Pre-medication and anesthesia

On the day of surgery the rabbits were sedated in the pen with 0.1 mg/kg medetomidine (Domitor® 1 mg/ml, Orion Pharma AB, Animal Health, Sollentuna, Sweden) subcutaneously (SC), 20 minutes before being transported to the operating theater. On both ears a local anesthetic cream containing lidocaine and prilocaine (Emla®, 25 mg/g + 25 mg/g, AstraZeneca AB, Södertälje, Sweden) was applied.

In the preparation room 5 mg/kg enrofloxacin (Baytril® 25 mg/ml, Bayer A/S, Animal Health Division, Copenhagen, Denmark) and 2 mg/kg carprofen (Rimadyl® vet. 50 mg/ml. Orion Pharma AB, Animal Health, Sollentuna, Sweden), were administered SC. An arterial catheter was placed in one ear for monitoring of blood gases and blood pressure and a venous catheter in the other ear for infusion of anesthetic drugs and fluids. The fur on the tail was clipped and an O₂-saturation probe applied.

Anesthesia was induced and maintained by an intravenous infusion consisting of 2.3 µg/ml sufentanil (Sufenta®, 50 µg/ml, Janssen-Cilag AB, Sollentuna, Sweden) and 0.45 mg/ml midazolam (Midazolam Actavis, 1 mg/ml, Actavis AB, Stockholm, Sweden). After induction the larynx was sprayed with lidocaine (Xylocain®, 20 mg/ml, AstraZeneca AB, Södertälje, Sweden) and the trachea was intubated with a 3 or 3.5 Fr endotracheal tube using a special

laryngoscope (Flecknell™, Alstoe Ltd. Animal Health, York, UK). During induction oxygen was administered by face mask. When intubation was completed the endotracheal tube was attached to the anesthesia machine and a fresh gas flow of 1 l oxygen + 2 l air was administered. A ventilator was used as needed to maintain a physiological level of arterial CO₂ pressure.

Respiratory rate, heart rate, blood pressure, O₂-saturation, body temperature and EtCO₂ were monitored throughout anesthesia. Before induction of anesthesia, 2.5 ml of arterial blood was sampled for analysis of Serum Amyloid A and a white blood cell differential count. Additional arterial blood (0.1 ml) was drawn from the arterial catheter before induction and subsequently every 30 minutes throughout anaesthesia for blood gas analysis with a portable blood gas machine (i-STAT® system, Abbot Point of Care inc., Princeton, New Jersey). Intra-venous fluid was administered during anesthesia using either 10 ml/kg/h Ringer-Acetate or 15 ml/kg/h Voluven® (Fresenius Kabi AB, Uppsala, Sweden). The rabbit was kept on a heat pad during surgery.

Surgery

The surgical sites, medial part of tibia, were prepared by clipping the fur on both hind limbs from the ankles to above the knees. The skin was washed with soap and water followed by disinfection with Chlorhexidine-Alcohol. The rabbit was then transported from the preparation room to the operating theatre where it was placed on its back, a final antiseptic wash was performed and the rabbit draped was for sterile surgery.

Each incision line was subcutaneously infiltrated with a maximum of 1 mg/kg bupivacaine (Marcain® 2.5 mg/ml, AstraZeneca AB, Södertälje, Sweden). The skin was incised and the subcutaneous tissue dissected all the way down to the bone. Two implant beds, one in the proximal diaphysis and one in the central diaphysis, at a distance of 2.5 cm in between, were prepared in each tibia. By intermittent drilling with a 3.2 mm drill bit during irrigation with saline, 6 mm deep cavities reaching into the medulla were created.

The bone implants were screwed into each cavity, leaving 2 mm of the implant above the bone surface. Thereafter one of the bone augmenting materials was applied around the protruding implant, or blood was allowed to fill the space (see *table 1*). The “Ångström ceramic” was distributed subperiostally around the implants in two rabbits, on the left tibia (experimental side). In one rabbit the “Ångström ceramic” was injected into the cavities on the left tibia before installation of the implants. On the right tibia (control side) Straumann® BoneCeramic was applied around the implants on one rabbit, and on two rabbits the space was left to fill with blood.

Table 1. Distribution of materials and titanium implants in the three rabbits

Rabbit #	Left tibia implants (<i>n</i> = 2)	Right tibia implants (<i>n</i> = 2)
2057	”Ångström ceramic” subperiostally	Straumann® BoneCeramic
2271	”Ångström ceramic” subperiostally	Blood
2277	”Ångström ceramic” intra-medullary	Blood

The implant stability was measured with resonance frequency analysis (using apparatus from Osstell AB, Gothenburg, Sweden) before the periosteum and skin was sutured with resorbable sutures (Vicryl® 4-0, Ethicon inc., Somerville, New Jersey).

After closure of the skin 0.03 mg/kg buprenorphine (Temgesic® 0.3 mg/ml, RB Pharmaceuticals Limited, Slough, UK) was given intravenously (IV) and 0.02 mg/kg SC. After extubation of the airways, the rabbit was put in a cage under a heat lamp during recovery. The time to return of the righting reflex was noted and if it did not return within 15 minutes, 0.05 mg/kg flumazenil (Lanexat® 0.1 mg/ml, Roche AB, Stockholm, Sweden) was administered IV. The rabbit was monitored until sitting up, fully awake, after which it was returned to the animal department.

Post-operative care

Postoperatively the rabbits were kept individually in pens of 2-3 m². The pens allowed for the animals to see, hear and smell each other.

The appetite was closely monitored and if the rabbit did not eat within 12 hours post surgery it was fed with a syringe twice a day with Critical Care (Oxbow Animal Health, Murdock, Nebraska), in a dosage according to weight. If the rabbit did not drink properly, water was given by the mouth or Ringer-Acetate was administered SC.

Post-operative pain control was achieved with subcutaneous administration of 0.05 mg/kg buprenorphine every 12 hours and carprofen 2 mg/kg every 24 hours for 3-5 days. The rabbits were closely monitored for signs of pain (not eating, abnormal body posture, reluctance to move or aggression) and additional pain medication was given if necessary. Enrofloxacin (5 mg/kg) was administered subcutaneously every 12 hours for 3 days post-surgery for infection prophylaxis.

For one week post-surgery the rabbit's body weight was recorded daily. The rabbit's general condition, condition of the surgical wound and degree of limping was evaluated every day for as long as needed. The limping was graded using a 0-5 scale, with 0 being no limping and 5 being no usage of the leg.

According to the ethics permit, the humane end-points (reason for euthanasia) were; loss of 15% or more of the body weight compared to the day of surgery; signs of infection; poor general condition or severe diarrhea. If the rabbit only showed mild-moderate signs of illness, veterinary treatment could be attempted.

The plan was to keep the rabbits for 3 months after surgery, and record the body weight once a week, after the first week. Two weeks post surgery calcein green, a fluorescent bone marker that is deposited along the mineralization front of newly formed bone at the time of injection (as reviewed by Turner et al., 1995), was supposed to be administered through an intravenous ear catheter. After euthanasia, the implant stability was to be measured with resonance

frequency analysis, before taking out blocks of the bone containing the implants and surrounding tissues for histological preparation. The amount of newly formed bone was to be evaluated with a light microscope and the osseointegration measured histomorphometrically as bone to implant contact in percent.

Euthanasia

The rabbits were pre-medicated in the pen with 0.1 mg/kg medetomidine given subcutaneously. Local anesthetic cream (EMLA) was applied on the ear and 2 ml/kg of the euthanasia solution (Pentobarbital 100 mg/ml, Allfatal vet., Omnidea AB, Stockholm, Sweden) was administered into the ear vein.

RESULTS

Anesthesia, surgery and recovery

Anesthesia and surgery were uneventful. The airways of all rabbits needed to be mechanically ventilated. The body temperature was maintained over 36.8 °C during anesthesia.

All implants were satisfactorily placed and were stable when tested with resonance frequency analysis. The duration time of surgery varied between 90 and 120 minutes.

Rabbits # 2271 and 2277 regained the righting reflex within 10 minutes after ending anaesthesia. Rabbit # 2057 was given flumazenil post-surgery and the righting reflex then returned within 5 minutes. All rabbits started eating during the first 12 hours post-surgery.

First week post-surgery

The first two days post-surgery the rabbits recovered well. Rabbit # 2057 was limping slightly, with a score of 2 on the left hind limb. The operation wounds looked fine and the general condition was good. Rabbit # 2271 was not limping and did not show signs of illness the first day post-surgery, but on the second day it dragged its left leg behind. Closer examination revealed that both tibias were fractured and the rabbit was euthanized. Rabbit # 2277 moved well and was in good general condition. The left hind limb was slightly swollen laterally on the tibia.

Three days post-surgery the right tibia of rabbit # 2057 was fractured while the rabbit was struggling when being handled. The rabbit was euthanized. Rabbit # 2277 showed no signs of illness, was not limping and the operation wounds looked fine. Four days post-surgery Rabbit # 2277 stopped eating and was syringe fed. Five days post-surgery the rabbit did not show any signs of pain or infection and treatment with buprenorphine and enrofloxacin was discontinued. Treatment with carprofen was continued for two more days.

Second and third week post-surgery

During the second week post-surgery rabbit # 2277 was active and moved normally but continued to eat poorly and was supplementary fed with Critical Care. Ten days post-surgery

it showed a slight limping on the left hind limb (score 1). From post-surgery day 11-14 the rabbit was very energetic and struggled while being fed. On day 15 the rabbit had lost 14 % of its body weight. By day 21 post-surgery the limping had disappeared, the rabbit was active and had company by another rabbit in the opposite pen. A shallow wound, 3 mm long, could be seen on one tibia and there were traces of blood in the fur. Calcein green was not administered.

On day 24 post-surgery the rabbit was not using the left hind limb. The tibia was found to be fractured and the rabbit was euthanized. During the last four days the rabbit lost 35 g (8 %) in body weight. Representing a total loss of 13 % compared to the day of surgery. The pre and post-surgery weights for all three rabbits are displayed in *figure 4*.

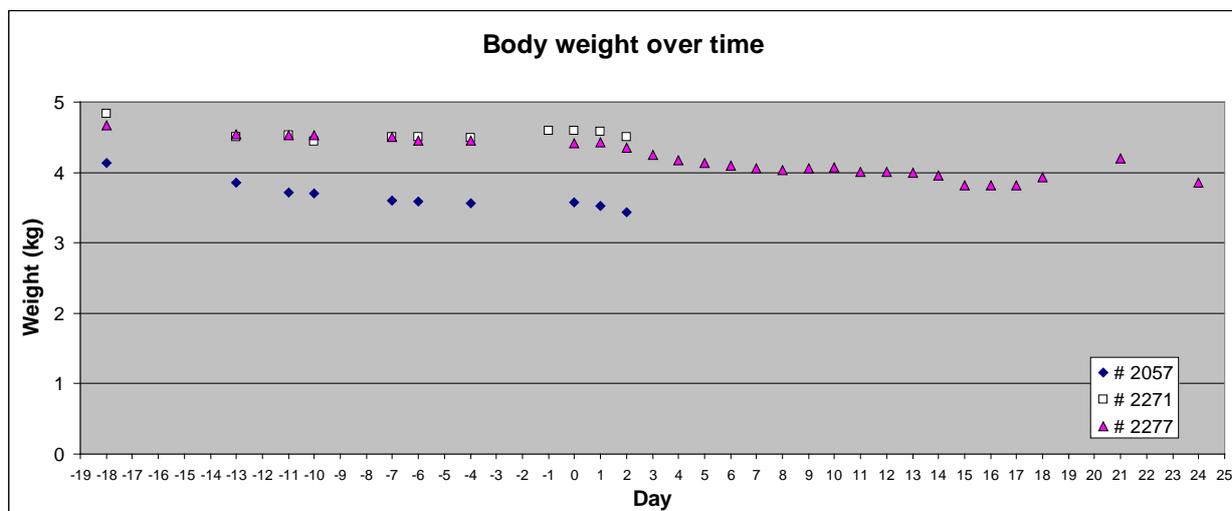


Figure 4. Body weight of three NZW rabbits before and after surgery with implantation of two bone screws per tibia. Day 0 represents the day of surgery.

Post-mortem examination

The right tibia of rabbit # 2057, for which Straumann® BoneCeramic was used, was fractured obliquely at the level of the distal implant (*figure 5*). The left tibia was not fractured but the examination showed that the “Ångström ceramic” was not in place but had spread in the surrounding tissues, (*figure 6*).

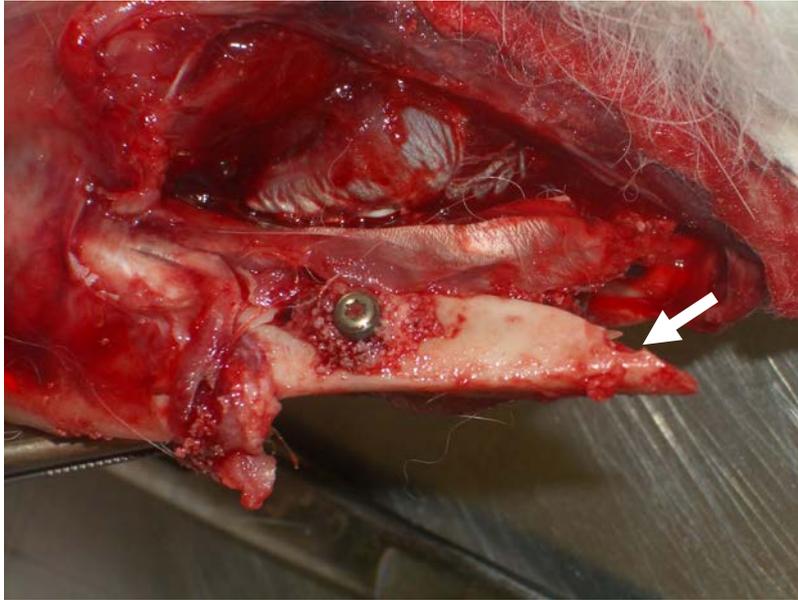


Figure 5. Right tibia of rabbit # 2057, fractured at the level of the distal implant (white arrow). Straumann® bone ceramic was used. With permission of the principle investigator.

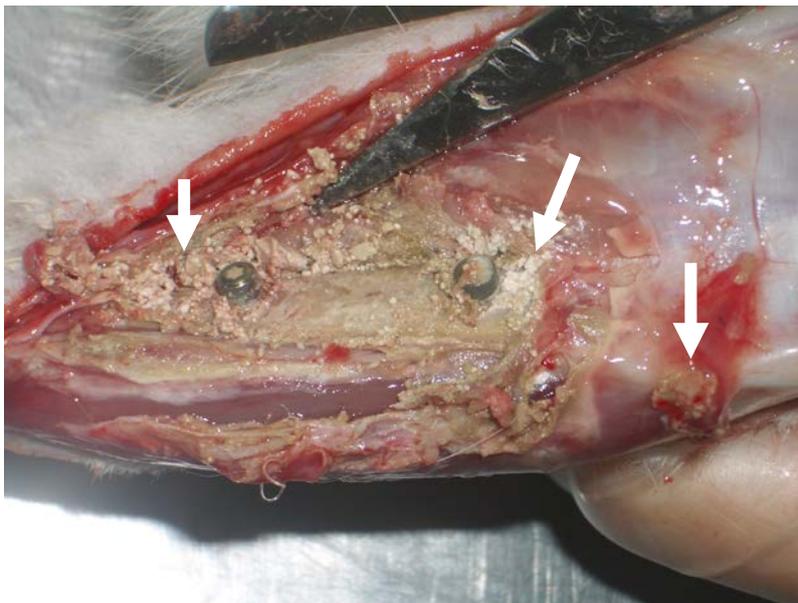


Figure 6. Left tibia of rabbit # 2057. The "Ångström ceramic" has spread out in the tissues surrounding the implants (white arrows). With permission of the principle investigator.

The left tibia of rabbit # 2271 was fractured just distally of the distal implant (*figure 7*) and the "Ångström ceramic" had spread out. The right tibia, in which no bone augmenting material had been used, was fractured at the level of the distal implant (*figure 8*). Both fractures were slightly oblique.

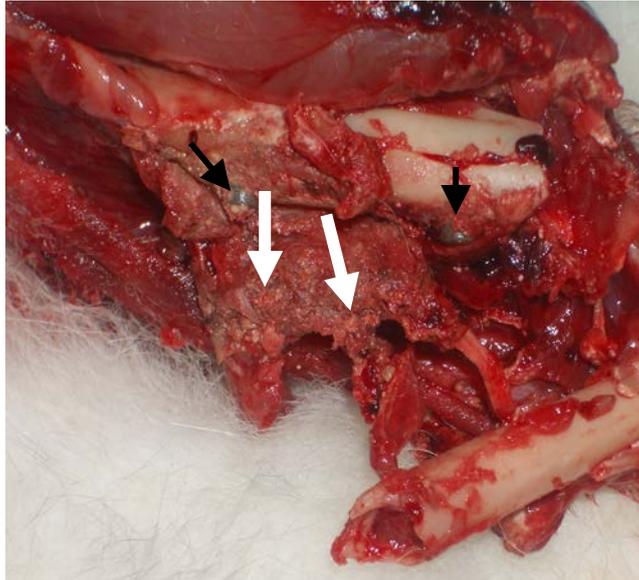


Figure 7. Left tibia of rabbit # 2271, fractured just distally of the distal implant. The “Ångström ceramic” has spread out in the tissues surrounding the implants (white arrows). Black arrows mark the implants. With permission of the principle investigator.



Figure 8. The right tibia of rabbit # 2271, fractured at the level of the distal implant. No bone augmenting material was used. With permission of the principle investigator.

Palpation of the left tibia in rabbit # 2277 showed that it was fractured at the level of the distal implant.

Table 2 shows the material that was used and the location of the fractures.

Table 2. Display of materials used and legs fractured in each rabbit

Rabbit #	Left tibia implants (n = 2)	Right tibia implants (n = 2)	Fractured leg
2057	"Ångström ceramic" subperiostally	Straumann® BoneCeramic	right
2271	"Ångström ceramic" subperiostally	Blood	both
2277	"Ångström ceramic" intra-medullary	Blood	left

DISCUSSION

The aim of the pilot study was to evaluate the feasibility and the result of the experimental protocol. The protocol was designed for evaluation of a new bone augmenting material. Two titanium implants were placed in each tibial diaphysis in three rabbits. The implants were surrounded by the experimental monetite-based calcium phosphate ceramic (the "Ångström ceramic") or the controls; Straumann® BoneCeramic or blood. The results were supposed to be evaluated three months after implantation, but the pilot study had to be terminated within 24 days since all three rabbits fractured one or two tibias.

Post-surgical monitoring

The rabbits' body weights were recorded pre- and post surgery and a limping score (0-5), was used post-surgery, to evaluate recovery.

Anorexia has been shown to be good a good indicator of pain in New Zealand White rabbits (Weaver et al., 2010). An indirect way of monitoring food intake is by recording body weight. By monitoring body weight pre- and post-surgery it is therefore possible to evaluate recovery. In experimental research, so called "humane end points" are used to minimize suffering of animals. The end points are suggested by the principal investigator and determined by the local animal ethical committee. One of the humane end-points for this experiment was a weight-loss of more than 15% compared with the body weight on the day of surgery (pre-surgery weight). The rabbits recovered well during the first days after surgery. Rabbits # 2271 and # 2057 only lived for two and three days post-surgery and their body weight and general condition were not close to any of the humane end-points until the tibias fractured, and the rabbits were euthanized. Rabbit # 2277 lived the longest and had lost 14 % of its body weight by day 15 post-surgery. After that the rabbit gained weight again so that it reached 95% of the pre-surgery weight. However, before it fractured its tibia, the rabbit lost 8 % of its current body weight in four days. This rapid weight-loss indicates that the rabbit was in pain. Four days before the tibia fractured, there was no obvious limping, but a small wound and blood in the fur was noted on one of the legs. This may have been a sign of pain, since rabbits can groom themselves excessively, up to the point of self-mutilation, in areas that have been exposed to trauma (Hess and Tater, 2012).

Rabbit #2277 stopped eating for a period after the other two rabbits had been euthanized and it was the only one left. Rabbits are known to show signs of depression, e.g. lethargy and

decreased appetite, when losing group members (Bradley Bays, 2012). Since rabbits are known to be social animals, a new rabbit was acquired to keep # 2277 company. After this the rabbit became more active and the appetite increased again.

Tibial fractures

A bone fractures when it is subject to forces exceeding its tensile strength. This can occur because of trauma or fatigue (as reviewed by Wang et al., 1998). The bone can also be weakened by pathological processes and therefore fracture when subjected to forces it normally tolerates.

Depending on what type of force is applied the bone fractures in different ways. Compressive forces that apply pressure on the bone along the longitudinal axis (*see fig. 9*), generally lead to oblique fractures (Johnson 2007a). Depending on the level of energy involved a bone fractures into many pieces (high energy) or it fractures into two pieces (low energy). In this study the bones fractured obliquely into two pieces suggesting a low energy compressive force.

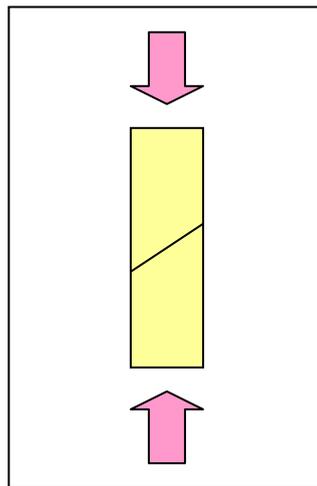


Fig 9. Schematic illustration of compressive forces on a long bone leading to an oblique fracture. Made by Lovisa Nalin based on the information by Johnson, 2007a.

Implant size and number

When an implant is placed in bone, the area is always damaged to some extent i.e. subject to trauma (as reviewed by Davies, 2003). The bone in that area is thus weakened, as in a pathological process, and might not withstand normal loading.

The recommended size for screw-type implants in the international standard (ISO 10993-6: 2007) probably concerns the diameter of the implant. It corresponds well with implant diameters used in these types of experiments (see *table 3*). Screw type implants can be inserted through a shorter extent of bone than the length of the implant. For example, in the present pilot study an 8 mm screw was inserted 6 mm into the bone. Maybe that is why no length recommendation is given in the international standard (ISO 10993-6: 2007).

The screw diameter in the present pilot study was 3.3 mm, which is within the recommended interval of 2-4.5 mm. In *table 3* the size and number of tibial implants in 20 experiments using New Zealand White rabbits are displayed. The diameter of the implants in all experiments but one (Ivanoff et al., 1997) was within the recommended ISO range, but the lengths vary quite a lot. Moreover the aim of the experiment by Ivanoff et al. (1997) was to evaluate implant designs that were wider than normal. All implants in the studies in *table 3* were not inserted into their full extent and in some of the experiments additional implants were placed in the femur, but none exceeded the 6 implants/rabbit recommended. None of the studies included in *table 3* report any post-surgical fractures in the rabbits.

Mapara et al. (2012) recommend that only one implant should be placed in each leg and that if two are placed, they should be as small as possible because of the fracture risk. Two implants/leg were placed in the present study but given the recommendations in the international standards and the use of more than one implant in 15 of the 20 experiments in *table 3* this is probably not the main reason why the tibias fractured.

Table 3. Display of implant size and number in 20 recent experiments using screw type implants placed in the tibia of New Zealand White rabbits. None reported post-surgery fractures. Search terms: rabbit AND tibia AND (screw OR thread) AND implant

Article	Implants/tibia (n)	Implant length (mm)	Implant diameter (mm)
Grandfield et al., 2012	2	5	3.75
Liu et al., 2012	1	18	2.5
Uysal et al., 2012	2	8	1.4
Hsu et al., 2011	1	8	3.6
Park, 2011	1	5.3	3.3
Poulos et al., 2011	2	7	3.75
Reigstad et al., 2011	2	8	3.75
Shin et al., 2011	2	6.6	3.3
Cho et al., 2010	2	8.3	3.8
Palmquist et al., 2010	2	5	3.75
Lee et al., 2009	2	7	3.75
Sul et al., 2009	3	7.8	3.75/4
Cordioli et al., 2000	2	4	3.75
Rasmusson et al., 1998	1	8	3.75
Ivanoff et al., 1997	2	6	3.0/ 3.75/ 5.0/ 6.0
Meredith et al., 1997	2	10	3.75
Larsson et al., 1996	2	4	3.75
Piattelli et al., 1996	1	Not mentioned	4.0
Sennerby et al., 1993	2	4	3.75
Sennerby et al., 1992	2	4	3.75

Implant location

In the present study, the implants were put into the proximal and middle part of the tibial diaphysis. This area seems to be sensitive for both traumatic and stress-induced fractures in rabbits. According to the experience of Zehnder and Kapatkin (2012) the most common sites for fractures in rabbits are the femur and the tibia/fibula. The combination of a fragile skeleton and powerful musculature makes the hind limb and back sensitive for fractures in

rabbits and they can easily injure themselves while struggling when being restrained (Graham, 2012).

Stress fractures arise when repetitive loading results in faster break-down than regeneration of the bone (Johnson 2007a) and in humans the most common site for stress fractures is the tibia (as reviewed by Burr et al., 1990). Two experimental studies have indicated that the middle part of the tibial diaphysis in rabbits is also sensitive for this type of lesion. Li et al. (1985) provoked rabbits to jump and run excessively by giving them electrical shocks in a controlled manner. Most signs of bone injury were seen in the middle third of the tibia. Burr et al. (1990) developed a rabbit animal model for studying the etiology of tibial stress fractures. In their model one hind limb of the animal was repetitively loaded in a special device and stress fractures developed in the middle and distal parts of the tibial diaphysis. The most common site was the midshaft of the bone where 89% of the fractures occurred.

Table 4 displays the anatomic locations of the implants in the same studies as in *table 3*. It is difficult to know the exact locations of the implants since most articles do not mention if it was laterally, medially, cranially or caudally in the bone. There also seems to be some confusion about the fact that a bone has two metaphyses, since some articles just state that the implant was placed in the metaphysis. But clear is that the tibial metaphysis is the most common site. The diaphysis was only used in one of the experiments in *table 4* (Shin et al., 2011) in which two implants were said to have been placed in that location. However, based on the description in the article, the proximal implant was placed 11mm distal of the patella, which is likely to be in the metaphysis.

Table 4. Display of implant locations and number in the 20 experiments in table 3

Article	Implant location	Implants/tibia (n)
Grandfield et al., 2012	Proximal and distal tibial metaphysis	2
Liu et al., 2012	Proximal tibial metaphysis	1
Uysal et al., 2012	Proximal anterior tibia	2
Hsu et al., 2011	Proximal tibial metaphysis	1
Park, 2011	Medial proximal tibia	1
Poulos et al., 2011	Tibial metaphysis (not mentioned which)	2
Reigstad et al., 2011	Medial proximal tibia near tibial tuberosity	2
Shin et al., 2011	Medial tibial diaphysis	2
Cho et al., 2010	Left and right side of proximal tibia	2
Palmquist et al., 2010	Proximal and distal tibial metaphysis	2
Lee et al., 2009	Lateral flat surface of proximal tibia	2
Sul et al., 2009	Implants placed on a longitudinal row from proximal to distal on the tibia but not described where or how far apart	3
Cordioli et al., 2000	Tibial metaphysis (not mentioned which)	2
Rasmusson et al., 1998	Proximal tibial metaphysis	1
Ivanoff et al., 1997	Cranial cortex, proximal and distal on the metaphysis (not mentioned which but looks like the proximal one in a figure)	2
Meredith et al., 1997	Proximal metaphysis of right tibia	2
Larsson et al., 1996	Proximal tibial bone	2
Piattelli et al., 1996	Tibial metaphysis (not mentioned which)	1
Sennerby et al., 1993	Tibial metaphysis (not mentioned which)	2
Sennerby et al., 1992	Tibial metaphysis (not mentioned which)	2

Housing considerations

The rabbits in this study had access to 2-3 m² of cage floor area each directly after surgery and despite the acclimatization period and handling during 2.5 weeks before the operations they were easily scared and “jumpy”. One rabbit fractured the leg when struggling while being handled and another one struggled vigorously whilst being syringe fed.

While researching for the background only two articles concerning post-operative care measures for rabbits used in experimental surgery were found (Calasans-Maia et al., 2009, Mapara et al., 2012). Both articles recommend that the rabbits are returned to their home cages after surgery and are closely monitored for signs of pain. Mapara et al. (2012) write that it is important to supervise the rabbit until it is fully awake since it can start moving excessively and uncontrollably and fracture its legs when it recovers from anesthesia.

The recommended home cages in both articles were smaller: (0.90m x 0.60m, giving a floor area of 0.54 m²) than the pens used in our experiment and the rabbits were kept individually pre- and post-surgery. Mapara et al. (2012) mention that movement should be restricted by placing boxes in the cage if the rabbit has already fractured its leg. The international standard describing suitable species and implant sizes for testing biomedical devices (ISO 10993-6:2007) do not mention how rabbits should be kept after surgery. The international standard describing animal welfare requirements in experiments testing biomedical devices (ISO

10993-2:2006) states that relevant post-surgical measures must be undertaken and analgesics administered responsibly and effectively.

In the current experiment the distal implant was placed in the midshaft of the tibia which was where they later on fractured. Giving that this area seems to be prone to fracturing by excessive running and jumping in the normal rabbit, the combination of the trauma from surgery, the space provided in the pens and the nervousness of the rabbits may explain why the tibias fractured. To prevent this from occurring, the post-operative care should probably be similar to that of a surgically fixated fracture. In dogs and cats that have undergone fracture fixation surgery the only movement allowed is controlled rehabilitation training and leash walks until the fracture has healed, which is continuously followed up with x-ray examinations (Johnson, 2007b). According to Richardson (2000) rabbits that have had external or internal fracture fixation should be confined in a stress-free environment and healing is normally completed within 6 weeks.

In the experimental setting it may be recommendable to restrain the movements of the rabbits by keeping them in individual cages initially after surgery. According to the European directive regarding the protection of research animals (Directive 2010/63/EU of the European parliament and of the council) the minimum floor area for one rabbit is 0.42 m² and the minimum cage height 45 cm. The enclosure must have a raised area covering no more than 40 % of the floor and the rabbit must be able to sit and lie under it. If a raised area cannot be used for medical reasons, the area of the enclosure must be increased with 33 %, which corresponds to a floor area of 0.56 m². This area is much smaller than the areas of the pens used in the current experiment.

None of the 20 articles in *table 5*, mention restriction of movement as a post-operative measure and the cage size is not stated in any of the 20 articles. Only in three of the articles is it mentioned that the rabbits were kept in cages or pens, in the others there is no information about housing. Of the 20 articles, 14 do not mention if animals were kept singly, in pairs or in groups. In 9 of the experiments male rabbits were used and they were most likely kept separately because of the risk of fighting. In two articles group housing is reported (Rasmusson et al, 1998. Palmqvist et al., 2010) and in one of them it was stated that the rabbits were kept individually post surgery (Palmqvist et al., 2010). Post-operative measures related to housing or movement was only mentioned in 6 of the 20 articles and the most common information was that the animals were kept separately and allowed immediate full weight-bearing on the legs.

To keep the rabbits from struggling and injuring themselves while being handled they should be carefully restrained, e.g. by placing them in a box or in the lap. They can also be wrapped in a towel or one person can firmly hold the rabbit and another person administer medications, food etc.

Table 5. Housing and post-operative movement of the rabbits in the articles from table 3

Article	Cage/Pen size (m2)	rabbits/cage	Rabbit sex	Post-operative housing and movement	Country
Grandfield et al., 2012	Not mentioned how kept	Not mentioned	Not mentioned	Not described	Sweden
Liu et al., 2012	According to university guidelines	Not mentioned	Male	Not described	China
Uysal et al., 2012	Standard cage	1	Male	Not described	Turkey
Hsu et al., 2011	According to university guidelines	Not mentioned	Male	Not described	China
Park, 2011	Not mentioned how kept	Not mentioned	Male	Not described	Korea
Poulos et al., 2011	Not mentioned how kept	1	Male	Not described	USA
Reigstad et al., 2011	Not mentioned how kept	1	Female	Not described	Norway
Shin et al., 2011	Not mentioned how kept	Not mentioned	Male	Not described	USA
Cho et al., 2010	Not mentioned how kept	Not mentioned	Male	Kept seperatly, allowed full weight-bearing immediatly	Korea
Palmquist et al., 2010	Not mentioned how kept	Group housing	Female	Kept seperatly for 7 days post-surgery	Sweden
Lee et al., 2009	Not mentioned how kept	1	Both	Kept seperatly, allowed full weight-bearing immediatly	Korea
Sul et al., 2009	Not mentioned how kept	Not mentioned	Male	Kept seperatly, allowed full weight-bearing immediatly	Sweden
Cordioli et al., 2000	Not mentioned how kept	Not mentioned	Not mentioned	Full weigh-bearing and movement immediatly	Italy
Rasmusson et al., 1998	Kept in purpose-designed room	10	Female	Not described	-
Ivanoff et al., 1997	Standard cage	Not mentioned	Both	Not described	Sweden
Meredith et al., 1997	Not mentioned how kept	Not mentioned	Female	Not described	-
Larsson et al., 1996	Not mentioned how kept	Not mentioned	Both	Not described	Sweden
Piattelli et al., 1996	Not mentioned how kept	Not mentioned	Male	Not described	Italy
Sennerby et al., 1993	Not mentioned how kept	Not mentioned	Both	Not described	-
Sennerby et al., 1992	Not mentioned how kept	Not mentioned	Both	The animals were allowed post-operative movement	-

The “Ångström” ceramic

Given that 2 right and 2 left tibiae fractured and the “Ångström” ceramic was only used on the left side, there are no indications that the new material caused the fractures. However the material needs to be modified for future experiments since it did not stay in place around the implant as intended but had spread in the surrounding tissues. This suggests that it did not set and harden as intended. The setting properties of the material should thus be altered.

Summary

The implants were placed in an area of the bone that is sensitive to fracturing in healthy rabbits. When the area was weakened by surgery and placement of the implants the compressive forces affecting the bone when the rabbits moved around became too much and the bone fractured.

CONCLUSION

The rabbit is a commonly used animal in this type of research and it is a good model to start with when testing bioactivity and feasibility (as reviewed by Pearce et al., 2007 and Muschler et al., 2010).

This experiment shows how important it is to start with a pilot study using few animals to evaluate an experimental design. This protocol failed, but only three rabbits had to be sacrificed which is more ethical and economical than a failed full scale experiment. The pilot makes it possible to modify the protocol for future experiments. The modifications should include selecting a different anatomic site for placing the implants, restricting the movement of the rabbits post-operatively and modifying the setting properties of the “Ångström” ceramic.

ACKNOWLEDGEMENT

Financial support was granted by the Swedish Medical Research Council.

- Albrektsson, T. (1995) Principles of osseointegration. In: Hobkirk, J. A. & Watson, R. M. (eds.) Color atlas and text of dental and maxillo-facial implantology. 9-19. London ; Baltimore: Mosby-Wolfe.
- Albrektsson, T., Brånemark, P. I., Hansson, H. A., Lindström, J. (1981) Osseointegrated titanium implants. Requirements for ensuring a long-lasting, direct bone- to- implant anchorage in man. *Acta Orthop Scand* 52(2), 155-70.
- Albrektsson, T. & Johansson, C. (2001) Osteoinduction, osteoconduction and osseointegration. *Eur Spine J* 10, Suppl 2, S96-101.
- Arisan, V., Anil, A., Wolke, J. G. & Ozer, K. (2010) The effect of injectable calcium phosphate cement on bone anchorage of titanium implants: an experimental feasibility study in dogs. *Int J Oral Maxillofac Surg* 39, 463-8.
- Bath-Balogh, M. & Fehrenbach, M. J. (2011) *Illustrated dental embryology, histology, and anatomy*, 3rd edition, St. Louis, Missouri: Elsevier Saunders.
- Binon, P. P. (2000) Implants and components: entering the new millennium. *Int J Oral Maxillofac Implants* 15, 76-94.
- Bodic, F., Hamel L., Lerouxel, E., Basle, M. F. & Chappard, D. (2005) Bone loss and teeth. *Joint Bone Spine* 72, 215-21.
- Bradley Bays, T. (2012) Behaviour of small mammals. In: Quesenberry, K. E. and Carpenter, J. W. (eds.) *Ferrets, rabbits and rodents: clinical medicine and surgery*. 3rd edition. 545-556. St. Luis, Missouri: Elsevier Saunders.
- Brånemark, P. I., Hansson, B. O., Adell, R., Breine, U., Lindstrom, J., Hallen, O. & Ohman, A. (1977) Osseointegrated implants in the treatment of the edentulous jaw. Experience from a 10-year period. *Scand J Plast Reconstr Surg Suppl* 16, 1-132.
- Burr, D. B., Milgrom, C., Boyd, R. D., Higgins, W. L., Robin, G. & Radin, E. L. (1990) Experimental stress fractures of the tibia. Biological and mechanical aetiology in rabbits. *J Bone Joint Surg Br* 72, 370-5.
- Calasans-Maia, M. D., Monteiro, M. L., Ascoli F. O. & Granjeiro, J. M. (2009) The rabbit as an animal model for experimental surgery. *Acta Cir Bras* 24, 325-8.
- Chaikof, E. L., Matthew, H., Kohn, J., Mikos, A. G., Prestwich, G. D. & Yip, C. M. (2002) Biomaterials and scaffolds in reparative medicine. *Ann N Y Acad Sci* 961, 96-105.
- Chiapasco, M., Casentini, P. & Zaniboni, M. (2009) Bone augmentation procedures in implant dentistry. *Int J Oral Maxillofac Implants* 24 Suppl, 237-59.
- Cho, L. R., Kim, D. G., Kim, J. H., Byon, E. S., Jeong, Y. S. & Park, C. J. (2010) Bone response of Mg ion-implanted clinical implants with the plasma source ion implantation method. *Clin Oral Implants Res* 21, 848-56.
- Cordioli, G., Majzoub, Z., Piattelli, A. & Scarano, A. (2000) Removal torque and histomorphometric investigation of 4 different titanium surfaces: an experimental study in the rabbit tibia. *Int J Oral Maxillofac Implants* 15, 668-74.
- Council Directive 93/42/EEC of 14 June 1993 concerning medical devices.
- Davies, J. E. (2003) Understanding peri-implant endosseous healing. *J Dent Educ* 67, 932-49.
- Directive 2010/63/EU of the European parliament and of the council of 22 september 2010 on the protection of animals used for scientific purposes.
- Eppley, B. L., Pietrzak, W. S. & Blanton, M. W. (2005) Allograft and alloplastic bone substitutes: a review of science and technology for the craniomaxillofacial surgeon. *J Craniofac Surg* 16, 981-9.
- Esposito, M., Hirsch, J. M., Lekholm, U. & Thomsen, P. (1998a) Biological factors contributing to failures of osseointegrated oral implants. (I). Success criteria and epidemiology. *Eur J Oral Sci* 106, 527-51.

- Esposito, M., Hirsch, J. M., Lekholm, U. & Thomsen, P. (1998b) Biological factors contributing to failures of osseointegrated oral implants. (II). Etiopathogenesis. *Eur J Oral Sci* 106, 721-64.
- Frayssinet, P., Mathon, D., Lerch, A., Autefage, A., Collard, P. & Rouquet, N. (2000) Osseointegration of composite calcium phosphate bioceramics. *J Biomed Mater Res* 50, 125-30.
- Gapski, R., Wang, H. L., Mascarenhas, P. & Lang, N. P. (2003) Critical review of immediate implant loading. *Clin Oral Implants Res* 14, 515-27.
- Graham, J. (2012) Basic approach to veterinary care. In: Quesenberry, K. E. and Carpenter, J. W. (eds.) *Ferrets, rabbits and rodents: clinical medicine and surgery*. 3rd edition. 174-182. St. Louis, Missouri: Elsevier Saunders.
- Grandfield, K., Palmquist, A. & Engqvist, H. (2012) Three-dimensional structure of laser-modified Ti6Al4V and bone interface revealed with STEM tomography. *Ultramicroscopy*. Epub ahead of print 2012-07-27. Available at: <http://dx.doi.org/10.1016/j.ultramic.2012.07.007>
- Habibovic, P., de Groot, K. (2007) Osteoinductive biomaterials- properties and relevance in bone repair. *J Tissue Eng Regen Med* 1(1), 25-32.
- Hallman, M. & Thor, A. (2008) Bone substitutes and growth factors as an alternative/complement to autogenous bone for grafting in implant dentistry. *Periodontol* 2000 47, 172-92.
- Henry, P. J. & Liddelow, G. J. (2008) Immediate loading of dental implants. *Aust Dent J* 53 Suppl 1, S69-81.
- Hess, L., Tater, K. (2012) Dermatologic diseases. In: Quesenberry, K. E. and Carpenter, J. W. (eds.) *Ferrets, rabbits and rodents: clinical medicine and surgery*. 3rd edition. 232-244. St. Louis, Missouri: Elsevier Saunders.
- Hobkirk, J. A. & Watson, R. M. (1995). *Color atlas and text of dental and maxillo-facial implantology*, London; Baltimore: Mosby-Wolfe.
- Hsu, S. K., Huang, W. T., Liu, B. S., Li, S. M., Chen, H. T. & Chang, C. J. (2011) Effects of near-field ultrasound stimulation on new bone formation and osseointegration of dental titanium implants in vitro and in vivo. *Ultrasound Med Biol* 37, 403-16.
- Huang, L. H., Shotwell, J. L. & Wang, H. L. (2005) Dental implants for orthodontic anchorage. *Am J Orthod Dentofacial Orthop* 127, 713-22.
- International Standard ISO 10993-2 (2006) Biological evaluation of medical devices - part 2: Animal welfare requirements.
- International Standard ISO 10993-6 (2007) Biological evaluation of medical devices - part 6: Tests for local effects after implantation.
- Ivanoff, C. J., Sennerby, L., Johansson, C., Rangert, B. & Lekholm, U. (1997) Influence of implant diameters on the integration of screw implants. An experimental study in rabbits. *Int J Oral Maxillofac Surg* 26, 141-8.
- Johnson, A. L. (2007a) Fundamentals of orthopedic surgery and fracture management. In: Welch Fossum, T. (ed.) *Small animal surgery*. 3rd edition. 930-1014. St. Louis, Missouri: Mosby Elsevier.
- Johnson, A. L. (2007b) Fundamentals of orthopedic surgery and fracture management. In: Welch Fossum, T. (ed.) *Small animal surgery*. 3rd edition. 1015-1142. St. Louis, Missouri: Mosby Elsevier.
- Junker, R., Dimakis, A., Thoneick, M. & Jansen, J. A. (2009) Effects of implant surface coatings and composition on bone integration: a systematic review. *Clin Oral Implants Res* 20 Suppl 4, 185-206.
- Junqueira, L. C. U. & Carneiro, J. (2005) *Basic histology : text & atlas*. 11th edition, New York: McGraw-Hill, Medical Pub. Division.

- Larsson, C., Thomsen, P., Aronsson, B. O., Rodahl, M., Lausmaa, J., Kasemo, B. & Ericson, L. E. (1996) Bone response to surface-modified titanium implants: studies on the early tissue response to machined and electropolished implants with different oxide thicknesses. *Biomaterials* 17, 605-16.
- Lee, J. E., Heo, S. J., Koak, J. Y., Kim, S. K., Han, C. H. & Lee, S. J. (2009) Healing response of cortical and cancellous bone around titanium implants. *Int J Oral Maxillofac Implants* 24, 655-62.
- Li, G. P., Zhang, S. D., Chen, G., Chen, H. & Wang, A. M. (1985) Radiographic and histologic analyses of stress fracture in rabbit tibias. *Am J Sports Med* 13, 285-94.
- Lind, M., Schumacker, B., Søballe, K., Keller, J., Melsen, F., Bünger, C. (1993) Transforming growth factor-beta enhances fracture healing in rabbit tibiae. *Acta Orthop Scand* 64(5), 553-6.
- Liu, Q., Liu, X., Liu, B. L., Hu, K. J., Zhou, X. D. & Ding, Y. X. (2012) The effect of low-intensity pulsed ultrasound on the osseointegration of titanium dental implants. *British Journal of Oral & Maxillofacial Surgery* 50, 244-250.
- Mapara, M., Thomas, B. S. & Bhat, K. M. (2012) Rabbit as an animal model for experimental research. *Dent Res J (Isfahan)* 9, 111-8.
- Marco, F., Milena, F., Gianluca, G. & Vittoria, O. (2005) Peri-implant osteogenesis in health and osteoporosis. *Micron* 36, 630-44.
- Martiniaková, M., Omelka, R., Chrenek, P., Ryban, L., Parkányi, V., Grosskopf, B., Vondráková, M., Bauerová, M. (2005) Changes in femoral bone tissue microstructure in transgenic rabbits. *Folia Biol (Praha)* 51(5), 140-4.
- Masoud, I., Shapiro, F., Kent, R. & Moses, A. (1986) A longitudinal study of the growth of the New Zealand white rabbit: cumulative and biweekly incremental growth rates for body length, body weight, femoral length, and tibial length. *J Orthop Res* 4, 221-31.
- Masuda, T., Yliheikkilä, P. K., Felton, D. A. & Cooper, L. F. (1998) Generalizations regarding the process and phenomenon of osseointegration. Part I. In vivo studies. *Int J Oral Maxillofac Implants* 13, 17-29.
- Meredith, N., Shagaldi, F., Alleyne, D., Sennerby, L. & Cawley, P. (1997) The application of resonance frequency measurements to study the stability of titanium implants during healing in the rabbit tibia. *Clin Oral Implants Res* 8, 234-43.
- Muschler, G. F., Raut, V. P., Patterson, T. E., Wenke, J. C. & Hollinger, J. O. (2010) The Design and Use of Animal Models for Translational Research in Bone Tissue Engineering and Regenerative Medicine. *Tissue Engineering Part B-Reviews* 16, 123-145.
- Neyt, J. G., Buckwalter, J. A. & Carroll, N. C. (1998) Use of animal models in musculoskeletal research. *Iowa Orthop J* 18, 118-23.
- Nicklas, W., Baneux, P., Boot, R., Decelle, T., Deeny, AA., Fumanelli, M., Illgen-Wilcke B; FELASA (Federation of European Animal Science Associations Working Group in Health Monitoring of Rodent and Rabbit Colonies) (2002) Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. *Laboratory animals* 36, 20-42.
- Palmquist, A., Lindberg, F., Emanuelsson, L., Branemark, R., Engqvist, H. & Thomsen, P. (2010) Biomechanical, histological, and ultrastructural analyses of laser micro- and nano-structured titanium alloy implants: a study in rabbit. *J Biomed Mater Res A* 92, 1476-86.
- Park, J. W. (2011) Increased bone apposition on a titanium oxide surface incorporating phosphate and strontium. *Clin Oral Implants Res* 22, 230-4.
- Pearce, A. I., Richards, R. G., Milz, S., Schneider, E. & Pearce, S. G. (2007) Animal models for implant biomaterial research in bone: a review. *Eur Cell Mater* 13, 1-10.
- Piattelli, A., Scarano, A., Corigliano, M. & Piattelli, M. (1996) Presence of multinucleated giant cells around machined, sandblasted and plasma-sprayed titanium implants: a histological and histochemical time-course study in rabbit. *Biomaterials* 17, 2053-8.

- Poulos, N. M., Rodriguez, N. A., Lee, J., Rueggeberg, F. A., Schupbach, P., Hall, J., Susin, C. & Wikesjo, U. M. (2011) Evaluation of a novel calcium phosphate-coated titanium porous oxide implant surface: a study in rabbits. *Int J Oral Maxillofac Implants* 26, 731-8.
- Prasad, D. K., Shetty, M., Bansal, N. & Hegde, C. (2011) Crestal bone preservation: a review of different approaches for successful implant therapy. *Indian J Dent Res* 22, 317-23.
- Rahaman, M. N., Day, D. E., Bal, B. S., Fu, Q., Jung, S. B., Bonewald, L. F. & Tomsia, A. P. (2011) Bioactive glass in tissue engineering. *Acta Biomater* 7, 2355-73.
- Rangert, B.R., Sullivan, R. M., Jemt, T. M. (1997) Load factor control for implants in the posterior partially edentulous segment. *Int J Oral Maxillofac Implants* 12 (3), 360-70.
- Rasmusson, L., Meredith, N., Kahnberg, K. E. & Sennerby, L. (1998) Stability assessments and histology of titanium implants placed simultaneously with autogenous onlay bone in the rabbit tibia. *Int J Oral Maxillofac Surg* 27, 229-35.
- Reigstad, O., Johansson, C., Stenport, V., Wennerberg, A., Reigstad, A. & Rokkum, M. (2011) Different patterns of bone fixation with hydroxyapatite and resorbable CaP coatings in the rabbit tibia at 6, 12, and 52 weeks. *J Biomed Mater Res B Appl Biomater* 99, 14-20.
- Rezwan, K., Chen, Q. Z., Blaker, J. J. & Boccaccini, A. R. (2006) Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. *Biomaterials* 27, 3413-31.
- Richardson, V. (2000) *Rabbits health, husbandry and diseases*. Oxford; London; Edinburgh; Malden; Carlton Victoria; Casimir Delavigne: Blackwell Science
- Roberts, W. E., Smith, R. K., Zilberman, Y., Mozsary, P. G., Smith, R. S. (1984) Osseous adaption to continuous loading of rigid endosseous implants. *Am J Orthod* 86(2), 95-111.
- Ross, M. H. & Pawlina, W. (2011) *Histology : a text and atlas : with correlated cell and molecular biology*. 6th edition, Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins Health.
- Sennerby, L., Thomsen, P. & Ericson, L. E. (1992) A morphometric and biomechanic comparison of titanium implants inserted in rabbit cortical and cancellous bone. *Int J Oral Maxillofac Implants* 7, 62-71.
- Sennerby, L., Thomsen, P. & Ericson, L. E. (1993) Early Tissue-Response to Titanium Implants Inserted in Rabbit Cortical Bone .1. Light-Microscopic Observations. *Journal of Materials Science-Materials in Medicine* 4, 240-250.
- Shin, D., Blanchard, S. B., Ito, M. & Chu, T. M. (2011) Peripheral quantitative computer tomographic, histomorphometric, and removal torque analyses of two different non-coated implants in a rabbit model. *Clin Oral Implants Res* 22, 242-50.
- Shirakura, M., Fujii, N., Ohnishi, H., Taguchi, Y., ohshima, H., Nomura, S. & Maeda, T. (2003) Tissue response to titanium implantation in the rat maxilla, with special reference to the effects of surface conditions on bone formation. *Clin Oral Implants Res* 14, 687-96.
- Sul, Y. T., Jonsson, J., Yoon, G. S. & Johansson, C. (2009) Resonance frequency measurements in vivo and related surface properties of magnesium-incorporated, micropatterned and magnesium-incorporated TiUnite, Osseotite, SLA and TiOblast implants. *Clin Oral Implants Res* 20, 1146-55.
- Tamimi, F., Sheikh, Z. & Barralet, J. (2012) Dicalcium phosphate cements: brushite and monetite. *Acta Biomater* 8, 474-87.
- Tamimi F., Torres, J., Bassett, D., Barraket, J. & Cabarcos, E. L. (2010) Resorption of monetite granules in alveolar bone defects in human patients. *Biomaterials* 31, 2762-9.
- Thomas, M. V. & Puleo, D. A. (2009) Calcium sulfate: Properties and clinical applications. *J Biomed Mater Res B Appl Biomater* 88, 597-610.
- Turner, CH., Owan, I., Takano, Y. (1995) Mechanotransduction in bone: role of strain rate. *Am J Physiol Endocrinol Metab* 269 (3), E438-42.

- Urban, R. M., Turner, T. M., Hall, D. J., Inoue, N. & Gitelis, S. (2007) Increased bone formation using calcium sulfate-calcium phosphate composite graft. *Clin Orthop Relat Res* 459, 110-7.
- Uysal, T., Ekizer, A., Akcay, H., Etoz, O. & Guray, E. (2012). Resonance frequency analysis of orthodontic miniscrews subjected to light-emitting diode photobiomodulation therapy. *Eur J Orthod* 34, 44-51.
- Vallet-Regi M. (2006) Revisiting ceramics for medical applications. *Dalton Trans* 5211-20.
- Wang, X., Mabrey, J. D. & Agrawal, C. M. (1998) An interspecies comparison of bone fracture properties. *Biomed Mater Eng* 8, 1-9.
- Weaver, L. A., Blaze, C. A., Linder, D. E., Andrutis, K. A. & Karas, A. Z. (2010) A model for clinical evaluation of perioperative analgesia in rabbits (*Oryctolagus cuniculus*). *J Am Assoc Lab Anim Sci* 49, 845-51.
- Xie, C., Lu, H., Li, W., Chen, F. M. & Zhao, Y. M. (2012) The use of calcium phosphate-based biomaterials in implant dentistry. *Journal of Materials Science-Materials in Medicine* 23, 853-862.
- Zehnder, A., Kapatkin, A. S. (2012) Orthopedics in small mammals. In: Quesenberry, K. E. and Carpenter, J. W. (eds.) *Ferrets, rabbits and rodents: clinical medicine and surgery*. 3rd edition. 472-484. St. Luis, Missouri: Elsevier Saunders.