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REINDEER-DERIVED BONE PROTEIN EXTRACT IN THE HEALING OF BONE DEFECTS

EVALUATION OF VARIOUS CARRIER MATERIALS AND DELIVERY SYSTEMS

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Academic dissertation to be presented with the assent of the Faculty of Medicine of the University of Oulu for public defence in Auditorium 2 of Oulu University Hospital, on 9 December 2011, at 12 noon

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Oulu, Finland

Abstract
Various bone proteins and growth factors are needed during the bone healing cascade. If the body cannot produce sufficient quantities of these factors, bone trauma healing can be improved with an implant that contains the required growth factors. However, an added bone protein extract needs a suitable delivery system to protect the proteins from degradation and to release them gradually, promoting new bone formation. This study focused on evaluating and optimization of the bone forming capacity of various scaffold systems of reindeer bone protein extract formulations using different experimental models. The tested carrier systems for various reindeer bone protein extract doses were collagen sponge and bioactive glass granules in critical-size defect model of rat femur. Calcium salt compositions (beta-tricalcium phosphate (β-TCP), hydroxyapatite or calcium sulphate) in disc and compressed pellet forms were tested in the thigh muscle pouch model of mouse. Various β-TCP granules combined with polyethylene/glycerol and stearic acid gel were tested in a hole defect model of sheep femur and humerus. Control groups involved carrier materials with no protein extract or untreated defects. In the sheep study, reference materials also included autograft and demineralized bone matrix (DBM). New bone formation, bone healing, and carrier resorption were evaluated based on radiographs, peripheral computerized tomography (pQCT), mechanical tests, histological examination, and micro-CT. New bone formation and bone union were markedly better in groups receiving higher doses of the extract and with follow-ups of six or more weeks, compared to empty defect or carrier without extract. Resorptions of carrier materials in active groups were faster and more active than in the control groups. The greatest bone formation occurred in the groups that had the bone protein extract readily available, which indicated that bone forming factors are required in sufficient concentrations at an early stage. The micro-CT analysis showed that bone formation in the groups with the extract was comparable to autograft, while the least bone formation was observed in the DBM and untreated groups. The present study indicated that the tested reindeer bone protein extract can be used to improve bone formation with various carriers. The study suggests that an inorganic carrier material together with stearic acid is the one of most suitable carrier alternatives for this extract. The developed medical device in paste form can be an alternative for autograft use.

Keywords: bone defects, bone extracts, calcium salts, carriers, fracture healing, growth factors
Tiivistelmä


Erilaisia kantaja-aine- ja implantointimahdollisuuksia

Oulun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Lääketieteen teknikka, Kliinisen lääketieteen laitos, Kirurgia, Ortopedian ja traumatologian osastoryhmä, PL 5000, 90014 Oulun yliopisto


Oulu
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>CS</td>
<td>Calcium sulphate</td>
</tr>
<tr>
<td>CSD</td>
<td>Critical-size defect</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DBM</td>
<td>Demineralized bone matrix</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GDF</td>
<td>Growth and differentiation factor</td>
</tr>
<tr>
<td>GuHCl</td>
<td>Guanidine hydrochloride</td>
</tr>
<tr>
<td>HA (HAP)</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>μCT</td>
<td>Micro computerized tomography</td>
</tr>
<tr>
<td>OP-1</td>
<td>Osteogenic protein-1</td>
</tr>
<tr>
<td>ORIF</td>
<td>Open reduction and internal fixation</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PGA</td>
<td>Polyglycolic acid</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>Polylactic-co-glycolic acid</td>
</tr>
<tr>
<td>PMMA</td>
<td>Polymethyl methacrylate</td>
</tr>
<tr>
<td>pQCT</td>
<td>Peripheral quantitative computerized tomography</td>
</tr>
<tr>
<td>rhBMP</td>
<td>Recombinant human bone morphogenetic protein</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>TCP</td>
<td>Tricalcium phosphate</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular-endothelial growth factor</td>
</tr>
</tbody>
</table>
List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals (I-IV).


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1 Introduction

Native bone contains growth and differentiation factors and signalling molecules such as bone morphogenetic proteins (BMPs) and other proteins that are important in regeneration of bone and cartilage (Cho et al. 2002, Cheng et al. 2003, Xiao et al. 2007, Dimitriou et al. 2005). These factors and molecules and their different concentrations are also needed in different phases during the whole fracture healing process (Cho et al. 2002, Dimitriou et al. 2005). If the body cannot produce sufficient quantities of these factors, bone trauma healing can be improved with an implant that contains the required growth factors (Dimitriou et al. 2005, Pekkarinen et al. 2006, Baas et al. 2008). However, as a treatment of bone fracture, added bone protein extract needs a suitable delivery system or carrier to guarantee a gradual release during new bone formation (King et al. 1998).

The techniques used to repair bone defects are important, and when an area of damaged bone is too large for self-repair, the repairing must be done by using alternative materials. Autograft is the traditional method of bone repair enhancement, but harvest site bleeding, pain, and infection are known issues (Arrington et al. 1996, Finkemeier 2002). Autografts also have limited availability, and many inorganic materials are used as an alternative (Betz 2002). The quality of autograft in old people has been discussed. Calcium phosphates such as hydroxyapatite and tricalcium phosphates and their variations are commonly known materials (Moore et al. 1987, Li & Wozney 2001, Niedhart et al. 2003, Barrack 2005, Douglas et al. 2009). These materials provide an osteoconductive scaffold to new bone formation (Carey et al. 2005, Peters et al. 2006, Baas et al. 2008). Bioactivity of inorganic materials can be increased by adding osteogenic stimulus to the bone graft extender (Alam et al. 2001, Barrack 2005, Kim et al. 2005, Baas et al. 2008, Reichert et al. 2011).

A mixture of BMPs, growth factors and other bone proteins and molecules has been extracted from the bone materials of a variety of animal species, humans and bone tumours. Reindeer bone protein extract has been shown to be an effective stimulator of new bone formation in a muscle pouch model in mice (Jortikka et al. 1993a, Pekkarinen et al. 2003, 2004, 2005a,b,c). Furthermore, the good healing capacity of reindeer bone extract in a segmental bone defect was previously demonstrated in the rabbit (Pekkarinen et al. 2006).

Reindeer bone protein extract has high bone formation activity. However, in a real bone healing situation, the extract cannot work without an appropriate carrier
that retains bone proteins and growth factors at the defect site for a prolonged time frame, providing an initial support to which cells attach and form new bone (Seeherman & Wozney 2005). The knowledge about the optimal formulation system for bone protein extracts is limited. Despite modern surgical techniques, segmental long bone defects are often difficult to manage. For this reason, animals with segmental long bone defects similar to clinical defects have been used as models for assaying the efficacy of bone protein extract and carrier materials. The bone healing properties of reindeer bone extract have been previously evaluated in the rabbit radius (Pekkarinen et al. 2006). However, the time and dose-dependent effects of reindeer bone extract have not been evaluated previously.

This study focused on evaluation and optimization of the bone forming capacity of various organic and inorganic scaffold systems of reindeer bone protein extract formulations. Bone healing capacity and new bone formation were studied in various experimental models. The critical-size defect model of rat was used to show trauma healing capacity of reindeer bone protein extract with collagen sponge and bioactive glass granule carriers. Furthermore, various doses of extract and follow-ups were tested in rat model studies. The muscle pouch model in mice and the hole defect model in sheep were the methods used when evaluating the suitability of calcium-based carrier materials with different formulations of reindeer bone protein extract.
2 Review of the literature

2.1 The principles of the fracture healing

Fracture healing is a complex, but well orchestrated, physiological process. During the repair process, injured bone can be reconstituted almost identically to its original shape. The bone marrow, the cortex, the periosteum, and the external soft tissues contribute in the healing process, but their role and activity depends on the type of fracture, its location and multiple other parameters, such as growth and signalling factors, pH, hormones as well as the way the fracture is treated (Einhorn 1998, Dimitrou et al. 2005, Giannoudis et al. 2007).

The process of fracture healing can be divided into primary and secondary healing. Primary cortical healing is typical when rigid internal fixation is used to stabilize a fracture. This healing process means that bone-resorbing cells on one side of the fracture provide tunnelling to the other side of the cortex, whereby they re-establish new haversian systems by the formation of demodelling units, known as cutting cones. New blood vessels are accompanied when osteoprogenitor cells become osteoblasts. Little or no response of the bone marrow, periosteum or external soft tissue is noted and thus, no callus formation is seen. (Schatzker et al. 1989, Einhorn 1998, 2005). The majority of fractures heal by secondary or indirect bone healing. This process involves responses in the periosteum and external soft tissue, and it is thus a combination of intramembranous and endochondral ossification that follows a specific time sequence (Einhorn 1998, 2005).

A haemorrhage is typically seen in the first phase in the injured area because of the rupture of blood vessels, which is soon followed by the formation of a clot. This phase is also called inflammation stage as inflammatory cells invade the injured area. These cells together with the haematoma are a source of signalling molecules and cytokines that regulate the early events of the healing process (McKibbin 1978, Leeson et al. 1988: 168–189, Einhorn 1998, 2005, Dimitrou et al. 2005). Clinically, this phase is associated with pain, swelling and heat and lasts about two to three weeks (Slutsky & Herman 2005). A chondrogenesis process is seen during the first 7 to 10 days of fracture healing. This leads to cartilage formation or hard callus formation where intramembranous ossification is taking place. The cells produce mainly type III collagen in this phase (Aro & Kettunen 2010: 218-221). Two weeks after fracture, cell proliferation declines.
and the mass of cartilage calcifies. The chondrocytes release phosphatases and proteases enzymes. The phosphatases precipitate delivering of the calcium from the mitochondria of the hypertrophic chondrocytes and form calcified cartilage. The proteases control the rate of the mineralization process. Histologically, type II collagen is mainly seen in this phase (Aro & Kettunen 2010: 281-221). This phase corresponds to the time when clinical union is achieved (Slutsky & Herman 2005). It is called as soft callus stage and lasts until four to eight weeks after the injury. Between four and eight weeks, the new bone begins to bridge the fracture. This bony bridge can be also seen on x-rays. The resorption cells of the cartilage, chondroclasts, become more active and degrade the calcified cartilage. This is also a signal for the ingrowth of blood vessels that bring with them perivascular mesenchymal stem cells (MSCs). These cells differentiate into osteoprogenitor cells and then into osteoblast cells to form new bone. This phase can be seen as the expression of type I collagen (Aro & Kettunen 2010: 218-221). The creation and mineralization of callus takes several weeks within new bone fills the fracture. (Slutsky & Herman 2005). Beginning about 8 to 12 weeks after the injury, osteoclast cells remodel and replace the callus with functionally competent lamellar bone structure. The fracture site remodels itself, correcting any deformities that may remain as a result of the injury. This final phase of the fracture healing can last up to several years. (Einhorn 1998, 2005, Slutsky & Herman 2005).

2.2 Growth factors and signalling molecules in the bone healing cascade

Native bone contains pro-inflammatory cytokines, the transforming growth factor beta (TGF-β) superfamily and other growth factors, and angiogenic factors (Cho et al. 2002, Gerstenfeld et al. 2003). These factors and molecules and their specific concentrations are required during the various phases of skeletal tissue formation and the entire fracture healing process (Cho et al. 2002, Dimitriou et al. 2005, Tsiridis et al. 2007) (Table 1).
Table 1. The summary of essential signaling molecules and growth factors during the fracture healing cascade; their source, time of expression, and major functions.

<table>
<thead>
<tr>
<th>Essential signalling molecules and growth factors</th>
<th>Source</th>
<th>Time of expression</th>
<th>Major functions during the fracture healing</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines: IL-1, IL-6, TNF-α</td>
<td>Inflammatory cells, MSCs</td>
<td>Days 1 to 3 and during bone remodelling</td>
<td>Hematoma formation, inflammation, angiogenesis</td>
<td>Kon et al. 2001, Ai-Aql et al. 2008</td>
</tr>
<tr>
<td>PDGF</td>
<td>Degranulating platelets, Macrophages, Osteoblasts</td>
<td>Days 1 to 3</td>
<td>Stimulator for inflammatory cells, MSCs and osteoblasts</td>
<td>Solheim 1998, Cho et al. 2002, Dimitriou et al. 2005</td>
</tr>
<tr>
<td>BMPs: BMPs 2-8, GDF-1, -5, -8, -10</td>
<td>Extracellular matrix, Osteoblasts, Osteoprogenitors, MSCs, Chondrocytes</td>
<td>Days 1 to 21 (Table 2)</td>
<td>Differentiation of MSCs into chondrocytes and osteoblasts and osteoprogenitors into osteoblasts</td>
<td>Cho et al. 2002, Cheng et al. 2003, Dimitriou et al. 2005, He 2005</td>
</tr>
<tr>
<td>FGFs</td>
<td>Monocytes, Macrophages, MSCs, Chondrocytes</td>
<td>Days 1 to 21</td>
<td>Angiogenesis, Mesenchymal cell mitogenesis</td>
<td>Solheim 1998, Hughes-Fulford &amp; Li 2011</td>
</tr>
<tr>
<td>Essential signalling molecules and growth factors</td>
<td>Source</td>
<td>Time of expression</td>
<td>Major functions during the fracture healing</td>
<td>Reference</td>
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<td>------------------------------------------------</td>
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<td>---------------------------------------------</td>
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<tr>
<td>IGFs: IGF-I, IGF-II</td>
<td>Bone matrix, MSCs, Osteoblasts Chondrocytes</td>
<td>IGF-I: Days 1 to 21  IGF-II: Days 7 to 14</td>
<td>Bone matrix formation  Endochondral ossification</td>
<td>Pesell et al. 1993, Lieberman et al. 2002, Dimitriou et al. 2005,</td>
</tr>
<tr>
<td>Metalloproteinases</td>
<td>Extracellular matrix</td>
<td>Days 3 to 21</td>
<td>The invasion of blood vessels</td>
<td>Gerstenfeld et al. 2003, Ai-Aql et al. 2008</td>
</tr>
<tr>
<td>VEGFs</td>
<td>MSCs</td>
<td>Days 14 to 21</td>
<td>Endothelial cell proliferation</td>
<td>Street et al. 2002, Willems et al. 2011</td>
</tr>
<tr>
<td>Angiopoietin-1, Angiopoietin-2</td>
<td>MSCs</td>
<td>Days 3 to 21</td>
<td>Larger vessel structures formation</td>
<td>Suri et al. 1998, Gerstenfeld et al. 2003, Dimitriou et al. 2005</td>
</tr>
</tbody>
</table>

IL = Interleukin, TNF = Tumour necrosis factor, TGF = Transforming growth factor, PDGF = Platelet-derived growth factor, MSC = Mesenchymal stem cell, BMP = Bone morphogenetic protein, GDF = Growth and differentiation factor, FGF = Fibroblast growth factor, IGF = Insulin-like growth factor, VEGF = Vascular-endothelial growth factor
Cytokines such as Interleukin-1 (IL-1), Interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) play a role especially in initiating the bone repair cascade. Inflammatory cells, macrophages and cells of mesenchymal origin secret cytokines during the first three days of the bone healing cascade as well as during the bone remodelling (Kon et al. 2001, Dimitriou et al. 2005, Ai-Aql et al. 2008). The most important tasks of the cytokines are chemotactic effect on other inflammatory cells, stimulation of extracellular matrix synthesis, and angiogenesis (Kon et al. 2001, Cho et al. 2002, Ai-Aql et al. 2008).

The TGF-β superfamily is a large group of various growth and differentiation factors including bone morphogenetic proteins (BMPs), TGF-β, growth differentiation factors (GDFs), activins and inhibins (Burt & Law 1994, Cho et al. 2002). These factors promote fracture healing in many ways. TGF-β has various roles from the inflammatory phase throughout the whole fracture healing process. Its main sources are degranulating platelets, inflammatory cells, extracellular matrix, chondrocytes and osteoblasts, and its main role is to work during chondrogenesis and endochondral bone formation (Bonewald & Mundy 1990, Solheim 1998, Cho et al. 2002). It induces production of extracellular proteins such as collagen and alkaline phosphatase and enhances proliferation of MSCs, chondrocytes and osteoblasts (Bonewald & Mundy 1990, Erlebacher et al. 1998, Solheim 1998, Lieberman et al. 2002).

Platelet-derived growth factor (PDGF) is a dimer of two peptides and released by degranulating platelets, macrophages, monocytes and osteoblasts at the very early stages of fracture healing (Andrew et al. 1995, Solheim 1998). PDGF is a stimulator for inflammatory cells and MSCs and osteoblasts (Lieberman et al. 2002). During the early phases of bone healing, until osteoblast formation, fibroblast growth factor (FGF) polypeptides play a critical role in angiogenesis and mesenchymal cell mitogenesis (Hughes-Fulford & Li 2011, Willems et al. 2011). Monocytes, macrophages, mesenchymal cells, osteoblasts and chondrocytes synthesize these factors (Solheim 1998, Lieberman et al. 2002). Furthermore, insulin-like growth factors (IGFs) are important factors having their source in the bone matrix, endothelial and mesenchymal cells, osteoblasts and non-hyperthrophic chondrocytes (Solheim 1998). IGF-I is regulated by the growth hormone and it promotes bone matrix formation throughout fracture healing (Lieberman et al. 2002, Dimitriou et al. 2005). IGF-II stimulates type I collagen production, cartilage matrix synthesis and cellular proliferation in the later stage of endochondral bone formation (Prisell et al. 1993).
Optimal bone healing and regeneration requires adequate blood flow. Specific metalloproteinases from the extracellular matrix degrade cartilage and bone, allowing the invasion of blood vessels during the final stages of endochondral ossification and bone remodelling (Gerstenfeld et al. 2003, Ai-Aql et al. 2008). Vascular-endothelial growth factors (VEGFs) are essential stimulators of endothelial cell proliferation and are expressed during endochondral formation and bone formation (Street et al. 2002, Willems et al. 2011). Angiopoietin 1 and 2 are needed in formation of larger vessel structures and development of co-lateral branches from existing vessels during the early stages throughout fracture healing (Suri et al. 1998, Gerstenfeld et al. 2003, Dimitriou et al. 2005).

2.2.1 Bone morphogenetic proteins

In an osteoinduction process some osteoinductive agents or signals (bone graft substitutes, generally proteins) stimulate primitive, undifferentiated and pluripotent cells to develop a bone-forming cell lineage and to enable the remodelling of new bone tissue (Parikh 2002). In 1938, Levander found that acid alcohol-extracted bone induced heterotopic cartilage and bone growth when injected intramuscularly. Decades later, in 1965, Urist confirmed that demineralized bone matrix (DBM) has good osteoinductive capacity because this matrix consists among others of bone morphogenetic proteins (BMPs) that are growth factors and belong to the TGF-β superfamily. The pure form of BMPs is not immunogenic and species-specific (Parikh 2002). BMPs enable skeletal tissue formation during embryogenesis, growth, adulthood, and trauma healing (Kirker-Head 2000). They constitute a large family of non-collagenous proteins, which are osteoinductive but can also have effects outside of bone tissue (Jaatinen et al. 2002, Reddi 2005, Barrientos et al. 2008, Corradini et al. 2010). Therefore, BMPs are also called body morphogenetic proteins (Reddi 2005).

More than twenty BMPs have now been described and about ten of them have functions in different phases of bone healing (Wozney & Rosen 1998, Cheng et al. 2003) (Table 2). Members of the BMP family are divided into different subgroups depending on their primary amino acid sequence. Group one includes BMP-2 and BMP-4, and group two consists of BMP-5, -6, and -7 (Celeste et al. 1990, Wozney 1992). The third group includes BMP-12 (or GDF-7), BMP-13 (GDF-6) and BMP-14 (GDF-5). Group four includes BMP-3 (or osteogenin) and BMP-3b (GDF-10) (Sakou 1998, Dimitriou et al. 2005). BMP-1 is a member of the astacin family and it plays a role in modulating BMP actions by binding and
antagonizing the actions of BMP-2 and BMP-4 (Wozney et al. 1990, Sarras 1996).

### Table 2. Major functions of important BMPs during the fracture healing.

<table>
<thead>
<tr>
<th>BMP</th>
<th>Time of expression</th>
<th>Major functions during the fracture healing</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDF-8</td>
<td>Day 1</td>
<td>Negative regulator of skeletal muscle growth</td>
<td>Cho et al. 2002</td>
</tr>
<tr>
<td>BMP-2</td>
<td>Days 1 to 21</td>
<td>Initiation of the healing cascade</td>
<td>Cheng et al. 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Regulation of the expression of other BMPs</td>
<td>He 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Osteogenesis</td>
<td>Tsuji et al. 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mineralization</td>
<td>Hughes-Fulford &amp; Li 2011</td>
</tr>
<tr>
<td>BMP-3, -8</td>
<td>Days 14 to 21</td>
<td>Regulation of osteogenesis</td>
<td>Cho et al. 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A second higher concentration of BMP-2 is seen during</td>
<td>Cheng et al. 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>osteogenesis or the phase of mineralization</td>
<td></td>
</tr>
<tr>
<td>BMP-4</td>
<td>6 h to day 5</td>
<td>Formation of callus</td>
<td>Bostrom et al. 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lammens et al. 2009</td>
</tr>
<tr>
<td>BMP-7</td>
<td>Days 14 to 21</td>
<td>Ossification</td>
<td>Cho et al. 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cheng et al. 2003</td>
</tr>
<tr>
<td>GDF-10, BMP-5</td>
<td>Days 3 to 21</td>
<td>Ossification</td>
<td>Cho et al. 2002</td>
</tr>
<tr>
<td>BMP-6, BMP-9</td>
<td>Days 3 to 21</td>
<td>Ossification</td>
<td>Cho et al. 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chondrocyte maturation</td>
</tr>
<tr>
<td>GDF-5</td>
<td>Days 7 to 14</td>
<td>Chondrogenesis</td>
<td>Cho et al. 2002</td>
</tr>
</tbody>
</table>

The bone extracellular matrix, osteoblasts, osteoprogenitors, mesenchymal cells, and chondrocytes are the main sources of BMPs. Even though BMPs are structurally and functionally closely related, each of them has a unique role during the fracture repair process. BMP-2 initiates fracture healing and regulates the expression of other BMPs. It has a role in chondrogenesis and together with BMP-6 and BMP-9 it induces osteoblast differentiation of MSCs (Cheng et al. 2003, He 2005). A second higher concentration of BMP-2 is seen during osteogenesis or the phase of mineralization (Cheng et al. 2003, Tsuji et al. 2006, Hughes-Fulford & Li 2011). The main difference between BMP-2 and BMP-9 is seen in heterotopic bone formation: BMP-9 induces new bone only in damaged
soft tissue, whereas BMP-2 promotes bone formation in skeletal muscle (Leblanc et al. 2011). BMP-3 and BMP-8 have a role in the regulation of osteogenesis at the end of fracture healing (Cho et al. 2002, Cheng et al. 2003). BMP-4 has a main role in the formation of callus at a very early stage in the bone healing process (Bostrom et al. 1996, Lammens 2009). BMP-7 has a regulatory role both in direct and indirect ossification, especially towards the end of these processes (Cho et al. 2002, Cheng et al. 2003). BMP-5, BMP-6 and GDF-10 have the same role as BMP-7 but during the entire fracture healing process (Cho et al. 2002). BMP-6 can initiate chondrocyte maturation (Cho et al. 2002, Huang et al. 2005). Expressions of GDF-5 and GDF-1 are seen during the chondrogenic phase because they induce angiogenesis through chemotaxis of endothelial cells and degrade matrix proteins (Cho et al. 2002). Furthermore, some BMPs can stimulate the synthesis of other bone and angiogenic growth factors (Deckers et al. 2002).

2.2.2 Recombinant BMPs

The development in molecular biotechnology allowed the purification of bone-inducing proteins (Sampath & Reddi 1981). The first three successfully isolated and characterized polypeptides were named BMP-1, BMP-2 and BMP-3 (Wang et al. 1988). Transfection of the full-length complementary DNAs led to the production of the first recombinant human bone morphogenetic proteins (rhBMPs) (Wozney et al. 1988). Today, recombinant BMPs are usually produced by two expression systems that are mammalian cells or bacteria (Bessa et al. 2008a). rhBMPs have been used to induce bone in many animal and clinical experiments, and some commercial products have also been manufactured (de Biase & Capanna 2005, McKay et al. 2007, White et al. 2007). Especially, the bone healing capacity of rhBMP-2 has been well documented, and it has been given approval by the European Union and the United States Food and Drug Administration (FDA) (Govender et al. 2002, Burkus et al. 2002, Einhorn et al. 2003, Kim et al. 2005, Seeherman et al. 2006, Chu et al. 2007, McKay et al. 2007, Issa et al. 2008). Furthermore, rhBMP-7 (or osteogenic protein, OP-1) is commercially available and both the European Union and FDA-approved for repairing tibia fractures (White et al. 2007).
2.3 Bone grafting and bone-graft substitute

In the reconstruction of skeletal defects, it is common to transplant cancellous or cortical bone to restore skeletal integrity and to enhance bone healing. Especially, segmental bone defects due to infection or trauma are difficult to manage without invoking the fracture healing cascade by transplanting bone to the defect. The materials used in bone grafting can be broadly divided into autologous or allogeneic cancellous or cortical bone, DBM, autologous bone marrow and synthetic materials, and combinations of these (Bauer & Muschler 2000, Finkemeier 2002, Van der Stok et al. 2011).

The differences between various bone grafts lie in their properties. Grafts can be osteoinductive, osteoconductive, osteogenetic, or have two or more properties. BMPs and other growth factors are primarily osteoinductive because they have the capacity to stimulate development of new bone. The concept of osteoconduction means that bone grows on a surface. Osteoconductive material itself has a weak capacity to form new bone; it is more like a support scaffold. When this kind of material is placed in an osseous environment, living cells of the host tissue can migrate into the scaffold, resulting in new bone formation and promoting bone bridge formation in the defect. Synthetic materials are typically osteoconductive. Combination of osteoconductive and osteoinductive is typical for DBMs with carrier. DBMs have a weak osteoinduced capacity to new bone formation alone but together with an osteoconductive carrier they are more applicable for bone defect healing. Autografts and bone marrow cells are both osteoconductive and osteogenetic materials with surviving pre-osteoblasts and osteoblasts that have capacity to form new bone. (Bauer & Muschler 2000, Finkemeier 2002, Schroeder & Mosheiff 2011).

However, there is no single method available to treat all kinds of bone healing problems. All three abovementioned components (osteogenesis, osteoinduction and osteoconduction) are needed in fracture healing (Schroeder & Mosheiff 2011). Furthermore, clinically, it is very important to take into account the injured site in the skeleton (such as the spine, cranium, pelvis, ribs, limbs), fracture type, form and size, stability of the fracture (Giannoudis et al. 2007), possible infections in the trauma site, age of the patient, and patient-related factors (smoking history, medications) when selecting the most suitable bone void filler (Salo 2011).
2.3.1 Autograft and allograft

Bone-grafting with autologous cortical and cancellous bone harvested from the iliac crest is the standard and traditional way to enhance bone repair (Pape et al. 2010). Autogenous grafts have been shown to be superior to allografts in many studies, as in studies on bone remodelling and bone healing rate (Friedlaender 1987, Goldberg & Stevenson 1987, Gross et al. 1991, Kienapfel et al. 1992, Johnson & Stein 1988, Virolainen et al. 1993, Athanasiou et al. 2010). Other advantages of autografts are histocompatibility and low risk of transmitting diseases. However, harvesting of bone grafts can lead to complications, such as bleeding, pain, and infection (Arrington et al. 1996, Finkemeier 2002). Furthermore, autografts have limited availability, especially in massive segmental bone loss.

Limited availability of autografts has resulted in widespread use of allografts with variable biologic properties. DBMs, morselized and cancellous chips, cortical grafts, osteochondral and whole-bone segments are typical preparations of allogeneic bone (Finkemeier 2002, Athanasiou et al. 2010). Bone banking allows allograft bone to be widely used in clinical orthopaedics (Tomford & Mankin 1999, Cornu et al. 2010). Allografts, DBMs and other bone extracts have been shown to increase bone-healing capacity and to enhance integration in many different studies (Einhorn et al. 1984, Peters et al. 2006, Pekkarinen et al. 2006, Huffer et al. 2007, Baas et al. 2008, Freilich et al. 2009, Athanasiou et al. 2010).

Morselized and cancellous allografts are often preserved by freeze-drying and vacuum-packing, which are associated with reduced immunogenicity, and in the latter case the mechanical strength of the graft is decreased (Friedlaender 1983, Pelker et al. 1984, Wolfe & Cook 1994, Cornu et al. 2010). Morselized and cancellous allograft is typically used to fill larger defects and it provides some mechanical support (Finkemeier 2002). Osteochondral and cortical allografts are harvested from various regions of the skeleton and are available as whole-bone or joint segments for limb salvage procedures and for filling bone defects (Finkemeier 2002). These grafts are osteoconductive and provide good structural support.

Allograft bone is also not without problems. It is associated with the risk of viral diseases, and it may otherwise cause immunological reactions that interfere with the bone healing process (Burchardt 1983, Stevenson 1987, Kinney et al. 2010). Thus, deep-frozen and sterilized allografts are more optimal than fresh allografts (Finkemeier 2002, Cornu et al. 2010).
2.3.2 Demineralized bone matrix

Demineralized bone matrix (DBM) is prepared by an acid extraction process, resulting in loss of most of mineralized component but retention of collagen, non-collagenous proteins and other growth factors present in the extracellular matrix (Urist & Dawson 1981, Reddi & Huggins 1972). Thus, DBM acts as an osteoconductive material but it does not offer structural support. DBMs are widely used to enhance fusion of the spine (Thalgott et al. 2001, Vaccaro et al. 2007, Weinzapfel et al. 2008). Furthermore, applications of DBM are grafting of nonunion, ostelytic lesions around total joint implants, and benign bone cysts (Whiteman et al. 1993, Tiedeman et al. 1995, Hass et al. 2007, Feng et al. 2011). DBM is also typically used together with autologous bone as a volume extender (Finkemeier 2002, Feng et al. 2011).

Various human DBM products are commercially available, both in Europe and in the USA, as a bone graft substitute, bone graft extender, and bone void filler in bony voids or gaps of the skeletal system (Finkemeier 2002, Barrack 2005, Engelhardt 2008). However, there are no studies on DBM use alone in humans to show DBM as an excellent bone grafting material (Kinney et al. 2010). Typical forms of human DBM products are a freeze-dried powder, crushed granules or chips, gel and paste, and combined with other components such as sodium hyaluronate, glycerol, or calcium phosphate (Peterson et al. 2004, Reynolds et al. 2007, Athanasiou et al. 2010). There are some studies in the literature in which bone-healing differences between various commercially available DBM products have been found (Peterson et al. 2004, Wildemann et al. 2007). Furthermore, DBM has much lower bone forming effect compared with the recombinant product (Bomback et al. 2004).

Animal DBMs have been also used in many animal and clinical experiments (Einhorn et al. 1984, Wang et al. 1988, Solheim et al. 1992, Feighan et al. 1995, Kloss et al. 2004, Kujala et al. 2004, Walboomers & Jansen 2005, Li et al. 2006, Wang et al. 2007, Huffer et al. 2007, Baas et al. 2008, Zou et al. 2008, Murugan et al. 2008, Athanasiou et al. 2010). Bovine DBM is the most frequently used in experimental studies because of its good availability and bone forming activity (Wang et al. 1988, Murugan et al. 2008, Athanasiou et al. 2010). Collos® DBM products may be the most well-known bovine-derived DBMs (Kloss et al. 2004, Walboomers et al. 2005, Li et al. 2005, Li et al. 2006). However, the risk of disease transmission of bovine materials is an issue of discussion in clinical applications. To overcome this problem, a similar other animal-derived DBMs are
also prepared such as equine DBM (Collos® E, by Ossacur AG) (Huffer et al. 2007, Baas et al. 2008, Nienhuijs et al. 2009) or a porcine DBM product (Altis OBM) from Altis Biologics Ltd.

### 2.3.3 Reindeer-derived bone protein extract

The fracture healing cascade needs a mixture of different BMPs, growth and signalling factors and other proteins (Dimitriou et al. 2005). This kind of natural cocktail can be obtained from the bone by a specific extraction method. The extraction process has many steps involving demineralization of bone material in acid (typically HCl), extraction of non-collagenous proteins, filtration, concentration, dialysis, freeze-drying and sterilization (Jortikka et al. 1993a). The natural bone proteins have been extracted from the demineralized bone materials of a variety of animal species as cow, pig, moose, sheep and reindeer, from humans and from bone tumours and dentin matrix (Sampath & Reddi 1983, Nilsson et al. 1986, Ko et al. 1990, Bessho et al. 1991a, Jortikka et al. 1993a,b, Viljanen et al. 1996, Oksanen et al. 1998, Laitinen et al. 1997). Reindeer bone protein extract has been shown to be an effective stimulator of new bone formation in a muscle pouch model in mice (Jortikka et al. 1993a, Pekkarinen et al. 2003, 2004, 2005a,b,c). Furthermore, the good healing capacity of reindeer bone extract in a segmental bone defect was previously demonstrated in the rabbit (Pekkarinen et al. 2006).

The ability of reindeer bone extract to heal various bone traumas has been observed to be good, better than that of for example bovine or ostrich extract, which has been suggested as being due to the fact that reindeer renew their antlers annually (Jortikka et al. 1993b, Kapanen et al. 2002, Ulmanen et al. 2005). Furthermore, it has been suggested that more of the protein material extracted from reindeer bone is in monocomponent form compared with other species such as cow, sheep or pig (Jortikka et al. 1993b). The manufacturing methods and applications of reindeer bone protein extract are comparable to the other animal-derived tissue extracts. The closest products are demineralized bone extracts made from bovine and equine bone (Huffer et al. 2007, Murray et al. 2007, Baas et al. 2008, Nienhuijs et al. 2009). However, reindeer bone protein extract is more purified compared to DBM, and bone proteins and growth factors may thus be more available in the bone healing process than when using DBMs.
2.3.4 Synthetic materials

Numerous products including calcium salts, bioactive glasses, synthetic polymers and their combinations are currently available in trauma and orthopaedic surgery. For example, there are eighteen various bone substitutes in clinical use in the Netherlands today (Van der Stok et al. 2011). Their biological, biomechanical and structural properties determine their clinical application (Van der Stok et al. 2011). Common for synthetic materials is that they form an osteoconductive scaffold to new bone formation (Carey et al. 2005, Peters et al. 2006, Baas et al. 2008, Alves et al. 2008). Thus, for repairing bone tissues, osteointegration means the capability to make direct contact with living bone after implantation, and bioactivity is a measure of the osteointegration rate (Moore et al. 2001, Carson & Bostrom 2007, Ohtsuki et al. 2009). Calcium phosphates and their variations are the most commonly known inorganic bone substitute materials (Moore et al. 1987, Li et al. 2001, Niedhart et al. 2003, Barrack 2005, Carey et al. 2005, Alves et al. 2008, Douglas et al. 2009, Guda et al. 2011). Synthetic, biodegradable, polymers have been also widely used in tissue-engineering. Polylactide (PLA), polyglycolide (PGA) and their copolymers poly(lactic-co-glycolic acid) (PLGA) are traditionally known as materials in bone fracture fixation such as self-reinforcing screws and rods but also as carriers for antibiotics and rhBMPs (Pyhältö et al. 2004, Carson & Bostrom 2007, Bessa et al. 2008b, Koort et al. 2008). Fiber technology has increased synthetic polymers and composition materials use in bone tissue-engineering (Huttunen et al. 2008, Wang et al. 2010). Especially, nanofibrous materials produced by electrospinning processes are under developing (Jang et al. 2009). Furthermore, various bone cements as glass ionomer cements and polymethyl methacrylate (PMMA) bone cements have long history as orthopaedic applications (Moore et al. 2001, Provenzano et al. 2004, Carson & Bostrom 2007).

Bioactivity of synthetic materials can be increased by adding osteogenic stimulus to the bone graft extender (Moore et al. 1987, Alam et al. 2001, Barrack 2005, Kim et al. 2005, Baas et al. 2008, Reichert et al. 2011). Combinations of bovine bone-derived growth factors in collagen and DBM or coralline-HA carriers have been shown to be as good as iliac crest autografts when studying fusion rates in spinal arthrodesis in rabbits and monkeys (Damien et al. 2002, Yee et al. 2003, Urrutia et al. 2008, Dodds et al. 2010) and humans (Boden et al. 2004).
2.4 Carriers of bone protein extract

A mixture of bone proteins and growth factors extracted from long bones is defined as bone extract. A preparation of this mixture with the carrier material is named as an implant that can be used as a bone void filler or implanted into the site of bone trauma (Pekkarinen et al. 2003, Dimitrou et al. 2005). The bone proteins and other growth factors are soluble within biologic fluids, thus it must be mixed with a carrier substance (Forslund & Aspenberg 1998). Otherwise, a morphogenetic signal is needed in bone regeneration together with a respondent cell population, as well as a matrix that delivers the signal and acts as a scaffold for the new bone formation (cell recruitment, attachment, proliferation and differentiation) (Ripamonti & Duneas 1998, King 2001).

2.4.1 Criteria of ideal carrier

An optimal carrier matrix should fulfil several criteria. The matrix should be biocompatible, bioabsorbable, malleable, and sterilizable (Kirker-Head 2000, Li & Wozney 2001, Lugienbuehl et al. 2004, Seeherman & Wozney 2005).

An ideal bone implant would be osteoconductive, osteoinductive, resorbable, and radiolucent to allow easy radiographic evaluation of bone formation and healing (Winn et al. 1999, Kirker-Head 2000, Saito & Takaoka 2003, Lugienbuehl et al. 2004, Babis & Suocacos 2005). The bone graft substitute should also avoid being rejected or inducing a foreign body response (Sciadini et al. 1997) and should retain growth and signalling factors at the implant site for a period of time sufficient to induce bone formation (Takita et al. 2004). Porosity of carrier is also essential because it affects the mechanical properties of bone implant together with graft macro and micro architecture (Li et al. 2003, Zyman et al. 2008). Thus, in the scaffold design it should be considered that it exceeds the strength of the natural bone that it replaces and that adequate strength is maintained throughout the bone remodelling phase (King 2001, Babis & Suocacos 2005). DBMs, collagenous materials, synthetic polymers, calcium phosphate materials (bioceramics), calcium sulphate materials, bioactive glasses and metals are typical carrier devices (Kirker-Head 2000).

inorganic materials have been tested as suitable carriers in this study; the review is thus focused on them.

**2.4.2 Collagen**

Collagen is the most well known of natural polymers that are widely used and studied as carriers for bone treatment. Collagenous materials are superior in compatibility, because collagen is the major protein component of hard and soft tissues (Wozney & Rosen 1998). The major component of bone collagen is type I collagen with traces of type III and V collagens (Keene et al. 1991, Gelse et al. 2003). Type I collagen defines various biomechanical properties in the bone such as load bearing, tensile strength and torsional stiffness, particularly after calcification (Ikada 2002: 2–3, Gelse et al. 2003). Collagen type II is the most typical protein of cartilage together with types IX and X (Seyer et al. 1974, Kandel et al. 1997, Gelse et al. 2003). Type IV collagen is found in basement membranes, where it is the major structural component, providing a framework for the binding of other basement membrane components and a substratum for cells (Herbst et al. 1988, Olivero & Furcht 1993, Gelse et al. 2003, Khoshnoodi et al. 2008). In bone, this collagen type plays a role in embryonic cartilage and bone induction (Paralkar et al. 1990, Gelse et al. 2003).

Collagen can be prepared in solution, membrane film, thread, sponge, gel or tubed form for implantation (Kirker-Head 2000, Ikada 2002: 2–3, Geiger et al. 2003, Wallace & Rosenblatt 2003, Bessa et al. 2008b). Collagen sponges have the advantage of being very versatile, easily manipulated and wettable. The manufacture of collagen sponge carrier depends on several factors, including sponge mass, sterilization methods, soaking time, protein concentration and buffer composition (Geiger et al. 2003). They are the most common of bovine-tendon-or skin-derived and commercially available products (Uludag et al. 1999). Collagens (typically bovine-derived type I or IV collagens) have been tested and evaluated in several animal models and clinical trials over the decades (Gao et al. 1993, Hollinger et al. 1998, Bessho et al. 1999, Kirker-Head 2000, Chen et al. 2002, Seeherman et al. 2002, Geiger et al. 2003, Barboza et al. 2004, Pekkarinen et al. 2006, McKay et al. 2007, White et al. 2007, Miyazaki et al. 2009, Moghadam et al. 2010). Although a large variety of collagen and other carrier formulations have been developed, BMPs and bone extracts have previously been successfully used in the healing of fractures with absorbable collagen carrier of type I or IV collagens alone (Hollinger et al. 1998, Bax et al. 1999, Bessho et al. 2002).
Collagen as a carrier is not structurally strong but easy to shape. Therefore, collagen-coated frames have typically been used as composite carriers of BMPs and DBMs. rhBMPs are reconstituted with water prior to injection and impregnated the collagen sponge for several minutes before implantation (Geiger et al. 2003, McKay et al. 2007). DBM is usually mixed together with collagen to a form gel or putty (Coulson et al. 1999, Kirker-Head 2000). A typical composite implant contains collagen together with some inorganic material, such as HA or natural coral (Gao et al. 1996, 1997, Tuominen et al. 2000, Kujala et al. 2004, Pekkarinen et al. 2005a, Kim et al. 2005, Yunoki et al. 2011, Wen et al. 2011).

2.4.3 Bioactive glass

Bioactive glasses are inorganic materials that have been used in granular form to fill the bone defects. They meet many criteria of optimal carrier matrices including biocompatibility, osteoconductivity, and biodegradability (Cao & Hench 1996). Some bioactive glasses have already been used to repair bone defects because their bioactivity allows them to achieve tight fixation resulting from direct bonding to living bone. Thus, they are called surface-bioactive ceramics (Oonishi et al. 1999, Silver et al. 2001, Pyhältö et al. 2004, Hench 2006, Ghosh et al. 2008).

In the traditional bioactive glass (Na₂O–CaO–SiO₂–P₂O₅), SiO₂ works as a network former, with slow and incomplete resorption. Na₂O, CaO, and P₂O₅ are compounds found in bioglass granules, and the variation in their ratios can be changed to adjust the dissolution rate (Hall et al. 1999, Griffon et al. 2001, Livingston et al. 2002, Karageorgiou & Kaplan 2005). This material was the first bioactive ceramic developed by Hench et al., and it is known as Bioglass (or 45S5 Bioglass®) and is FDA-approved (Hench et al. 1971, Cao & Hench 1996, Chen et al. 2006). This composition has been in clinical use since 1985. Today, there are many variations on the original composition, such as sol-gel derived 58S and S70C30 (Silver et al. 2001, Xia & Chang 2006, Chen et al. 2010, Mortazavi et al. 2010).

There is also a long research history with bioactive glasses in Finland. Bioactive glass S53P4 was developed in Turku, Finland (Andersson & Karlsson 1991). In 1991, clinical tests started with this bioactive glass, especially on craniomaxillary but also in spinal surgery (Aitasalo & Peltola 2004, Peltola et al. 2010).
This kind of bioactive glass is a unique clinically used bone graft, because it is also antibacterial (Zhang et al. 2010). It has been successfully used in the treatment of osteomyelitis (Lindfors et al. 2010). Bioactive glass S53P4 received European approval for orthopaedic use in 2006 (Frantzén et al. 2011).

The mechanisms that enable bioactive glasses to act as materials for bone repair have been investigated and various inorganic reaction stages are commonly thought to occur when bioactive glass is immersed in a physiological environment (Ohtsuki et al. 2009). There is the ion exchange stage in which modifier cations (mostly Na+) in the glass exchange with hydronium ions in the external solution. In the hydrolysis Si-O-Si bridges are broken, forming Si-OH silanol groups, and the glass network is disrupted. Then, in condensation of silanols, the disrupted glass network changes its morphology to form a gel-like surface layer, depleted in sodium and calcium ions. Precipitation is the reaction in which an amorphous calcium phosphate layer is deposited on the gel, and in mineralization the calcium phosphate layer gradually transforms into crystalline hydroxyapatite, which mimics the mineral phase naturally contained with vertebrate bones. (Cao & Hench 1996, Hench 2006, Ohtsuki et al. 2009). Furthermore, it has been discovered that the morphology of the gel surface layer is a key component in determining the bioactive response (Cao & Hench 1996, Zhong & Greenspan 2000, Xia & Chang 2006). These kinds of glasses contain significantly higher concentrations of SiO2 than traditional melt-derived bioactive glasses and still maintain bioactivity (Saravanapavan et al. 2004).

Bioactive glasses have been used as carriers of BMPs, especially rhBMP (Mahmood et al. 2001, Takita et al. 2004, Välimäki et al. 2005, Bergeron et al. 2007). There are many systems to prepare a BMP involved bioactive glass implant: rhBMP was infused to the manually molded glass fiber balls (Mahmood et al. 2001), the carrier and used dose of rhBMP had been mixed together before implantation (Takita et al. 2004), and the defect site had been filled with bioactive glass granules and growth factors have been injected there (Välimäki et al. 2005). There is also studies where DBM cylinders contain bioactive glass rods to accelerate bone formation (Pajamäki et al. 1993a,b). Some properties of bioactive glass granules can affect the bone healing capacity of bone extracts, such as porosity of granules, granule size, and degradability of glasses (Jones & Hench 2004, Jones et al. 2006). Bone bonding capacity is a significant advantage when using bioglass as bone void filler (Hench 2006, Ohtsuki et al. 2009). The calcium phosphate formation at the surface of bioactive glass occurs this strong bonding.
Bioactive glass bonds into bone when the surface between material and tissue is mineralized (Andersson & Kangasniemi 1991). Variations in local pH levels also strongly influence the osteoclastic activity and reactions of bioactive glass in bone trauma (Cerruti et al. 2005).

2.4.4 Calcium salts

Inorganic materials fulfil many of ideal carrier requirements. Most of them are structurally strong, immunologically inert, highly osteoconductive and variably biodegradable (Kirker-Head 2000, Blom et al. 2002). Calcium salts have been used for years in various variations because their composition is close to natural bone composition and structure (Alam et al. 2001, Blom et al. 2002, Carey et al. 2005, Kroese-Deutman et al. 2005, Peters et al. 2006, dos Santos et al. 2008).

Calcium phosphate materials, including hydroxyapatite (HA), tricalcium phosphate (TCP) and their composites, have been proposed as potential carrier materials for BMPs and other bone protein extracts. Stoichiometric TCP [Ca$_3$(PO$_4$)$_2$] has Ca/P ratio of 1.5. There are both alpha and beta crystal forms of TCP in available. The alpha state is formed at high temperatures and is more soluble than a beta form (Bohner 2001, Yamada et al. 2007). Especially, β-TCP is shown to be useful carrier for rhBMPs or DBM (Gao et al. 1996, Ohura et al. 1999, Blom et al. 2001, Niedhart et al. 2003, Kim et al. 2005, Huffer et al. 2007, Baas et al. 2008). β-TCP has many positive features as carrier material. Its resorption rate closely matches the course of normal cancellous bone remodelling, it can bond directly to bone, and it is primarily osteoconductive in nature. β-TCP is also more soluble than HA (Koerten & Meulen 1999, Bodde et al. 2007). According to Moore et al. 2001, commercially available β-TCP granules dissolve in 6-18 months whereas a commercially available HA granules reabsorb 1-2% per year (Moore et al. 2001). β-TCP as hardenable calcium phosphate can be prepared in a paste form that can be molded to a desired shape prior to setting (Carey et al. 2005, Xu et al. 2006, Urban et al. 2007). Thus, an injectable form of β-TCP combined with rhBMP-2 has been shown to successfully heal femoral defects in rabbits (Seeherman et al. 2006).

HA [Ca$_{10}$(PO$_4$)$_6$(OH)$_2$] is fairly osteoconductive and has high protein-binding capacity. High porosity and high surface implanted area are achieved because of the continuous structure of HA-based material (den Boer et al. 2002, Itoh et al. 2006, Douglas et al. 2009, Schnettler et al. 2009). This property has been made use of in surface coating of carrier with rhBMP-2 (Schnettler et al. 2009). It has
been suggested that the structural configuration of HA may be an important factor in osteogenesis, and typical applications forms are powder, granule, disk, and block (Magan & Ripamonti 1996, Tsuruga et al. 1997, Kuboki et al. 1998). A porous form of HA is more susceptible to osteogenesis than non-porous particles or solids (Rohanizadeh & Chung 2010, Roohani-Esfahani et al. 2010). HA has been used as a carrier for BMPs in many studies (Takaoka et al. 1988, Horisaka et al. 1991, Kuboki et al. 1998, Kirker-Head 2000, Aebli et al. 2005, Rohanizadeh & Chung 2010).

Porosity and particle size affect the degradation rate of the compound and the biomechanical properties of the implant (Li et al. 2003, Galois & Mainard 2004, Zyman et al. 2008, Ioku 2010, Roohani-Esfahani et al. 2010). There has been a lot of discussion about optimal pore size and the role of micro- and macroporosity. It has been found that the range of optimal size is 150–600 microns (Pratt et al. 2002, Roohani-Esfahani et al. 2010). HA is often combined with β-TCP to form a more resorbable and porous carrier with a greater degree of bone formation than HA alone. Thus, resorption kinetics of composite materials can be adjusted by varying the ratios of HA and β-TCP. This combination of calcium phosphates has also been used as a carrier for rhBMPs and DBM. The most typical methods have been that carrier granules have been mixed with DBM and molded to form an implant or a used carrier has been coated or loaded with the rhBMP-solution. (Alam et al. 2001, Pratt et al. 2002, Ruhé et al. 2004, Jung et al. 2008, Schopper et al. 2008).

Calcium sulphate (CS) [CaSO₄] has been studied as a bone void filler for more than a hundred years and it has many functions as part of a bone graft composite. It acts as a binder to improve total bone contact and bone volume around the implant (Turner et al. 2001, Ricci 2001: 1-10, Peters et al. 2006, Schneiders et al. 2009). The hemihydrate form of CS [CaSO₄·0.5H₂O] is better known as plaster of Paris, while the dehydrate form [CaSO₄·2H₂O] occurs naturally as gypsum (Singh & Middendorf 2007, Thomas & Puleo 2009). Depending on the method of dehydration of calcium, specific hemihydrates are distinguished according to their crystal size as alpha-hemihydrate and beta-hemihydrate (Singh & Middendorf 2007, Thomas & Puleo 2009). Alpha-hemihydrate crystals are more prismatic than beta-hemihydrate crystals and, when mixed with water, form a much stronger and harder superstructure (Thomas et al. 2005, Thomas & Puleo 2009). Pore size has an important role in bone ingrowth, and increasing of pore size improves the bone healing effect of inorganic materials such as calcium sulphates (Xu et al. 2006, Thomas & Puleo 2009).
disadvantage of CS is that it resorbs fast, within a couple of weeks after implantation. Therefore, it does not provide long-term mechanical support for the healed bone, but it can work as carrier in the initial stage of the healing cascade (Moore et al. 2001). Clinically, CS-based grafts are mainly used to fill bone defects after tumor resection surgery (Van der Stok et al. 2011). CS has for many years been used as carrier for rhBMPs and DBMs, also in clinical studies, showing excellent biocompatibility. Preparation method of CS-based implant depends on a form use. rhBMP-2 has been impregnated into the CS powder and then formed by applying high pressure (Cui et al. 2008). Turner et al. 2001 filled the defect site with DBM material and CS tablets (Turner et al. 2001). In the study of Reynolds et al. 2007, the defects were filled with DBM and CS graft binder (Reynolds et al. 2007).

2.4.5 Carriers of reindeer bone protein extract

A mixture of bone proteins and growth factors extracted from long bones is defined as bone extract. A preparation of this mixture with the carrier material is named as an implant that can be used as a bone void filler or implanted into the site of bone trauma (Pekkarinen et al. 2003, Dimitrou et al. 2005). Reindeer bone protein extract has high bone-forming activity, as seen in bioactivity and previous tests, but in a real bone healing situation it cannot work without a carrier system (Jortikka et al. 1993a, Pekkarinen et al. 2003, Ulmanen et al. 2005). The reindeer bone protein extract sets its own limitations for the selection of carrier because of its characteristics. The main limitation is that the extract is not water-soluble. Thus, there are at least three different possibilities for implant preparations:

- The formulated bone extract suspension is impregnated into a porous matrix (Fig. 1a).
- The extract and carrier are moulded together to form putty or compressed into the discs (Fig. 1b).
- The carrier discs or granules are surface-coated with the bone extract (Fig. 1c).
Fig. 1. There are three possibilities of preparing implants of reindeer bone protein extract: Impregnation into a porous matrix (a), moulding to form a paste (b), and surface coating of granules (c). (H Tölli, unpublished).

Pure collagen as a carrier has been tested in some of our previous studies (Pekkarinen et al. 2003, 2005c,b, 2006). Lyophilized extract was mixed in water and pipetted onto the collagen sponge, or the collagen sponge soaked in water and then extract was bundled up to form an implant. The combinations of TCP, HA and coral together with reindeer bone protein extract and collagen sponge have been tested in mouse model (Pekkarinen et al. 2005a). Table 3 presents the used carrier systems and evaluation methods of reindeer bone protein extract.

Table 3. Carrier systems and experimental models of reindeer bone protein extract.

<table>
<thead>
<tr>
<th>Carrier (Source of collagen)</th>
<th>Implant form</th>
<th>Experimental model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen (gelatine, type A, porcine skin)</td>
<td>Injectable</td>
<td>Mice muscle pouch model</td>
<td>Pekkarinen et al. 2003, 2005c</td>
</tr>
<tr>
<td>Collagen (type IV, bovine)</td>
<td>Sponge</td>
<td>Mice muscle pouch model</td>
<td>Pekkarinen et al. 2004, 2005a</td>
</tr>
<tr>
<td>TCP/Collagen</td>
<td>Composite implants in form of frames</td>
<td>Mice muscle pouch model</td>
<td>Pekkarinen et al. 2005b</td>
</tr>
<tr>
<td>Collagen (type IV, bovine)</td>
<td>Sponge</td>
<td>Rabbit radius segmental defect</td>
<td>Pekkarinen et al. 2006</td>
</tr>
</tbody>
</table>
2.5 Experimental models

The development of new biomaterials or treatment techniques requires experimental and clinical testing to ensure biocompatibility, bioactivity and other ideal system criteria (Liebschner 2004). Various animal species from mouse to sheep, as well as various implantation sites and methods have been used in biocompatibility and functional tests. Due to species-specific differences in bone physiology, it is important to be able to choose a model that resembles the human system as much as possible. (Nunamaker 1998).

Animal models with various fixation models are used to evaluate repair materials of long bone fractures. Typical animals are sheep, rabbit, dog, primate species and rat. The bones used as long bone defect models are typically ulna, tibia, radius, femur or mandible (Liebschner 2004). A drill hole defect is one of the model systems to test new methods and concepts. This model allows the intraosseous implantation of various samples per animal without the need of an extra fixation system (Nuss et al. 2006, Obenaus & Hayes 2011).

Other important study models are the non-union fracture or osteotomy model, skull or cranial defect model and spinal fusion model. They have been widely used in the evaluation of bone substitutes and trauma treatment (Nunamaker 1998, Burkus et al. 2003, Drespe et al. 2005, Seeherman et al. 2006, Cooper et al. 2010, Szpalski et al. 2010).

Muscle and subcutaneous tissues are the most common implantation sites for studying osteoinductive properties of implants and bioassays. Mouse is the typically used animal, but rats have also been used in osteoinductive bioassays (Jortikka et al. 1993a, Pajamäki et al. 1994, Barr et al. 2010, Lee et al. 2011).

Segmental defect, hole defect, and muscle pouch models are used in the present study, which is why the review focuses on them.

2.5.1 Rat segmental defect model

Rats have for a long time been popular animals in the study of fracture healing. Animal costs are quite reasonable even in large group studies compared to rabbits or some bigger animals (Nunamaker 1998). Solheim et al. (1992) have tested how well the composite of demineralized bone mixed with polyorthoester can heal the diaphyseal defect in the rat radius. Fracture healing has also been studied by Chakkalakal et al. (1999) who evaluated the rigidity of the rat fibula, the mineral content of the repair site, and the histological changes in the defect site.
A critical-size defect (CSD) is frequently used in the studies of segmental bone defects models. It means the smallest size of the bone defect that does not heal spontaneously or within the lifetime of the animal if it is left untreated for a certain period of time (Schmitz & Hollinger 1986). The length of the CSD depends on animal species and the defect place in the bone. It is typically twice the diameter of long bones (Nilsson et al. 1986, Yasko et al. 1992, Hunt et al. 1996, Pekkarinen et al. 2006).

The femur is one of the longest bones and thus it is used in many segmental bone defect studies as an experimental model for bone fractures and their repairing methods. Additionally, the fractures of the femur are relatively common and often associated with a major trauma, especially in humans. The most widely used defect size in the rat has been 8 mm (Zart et al. 1993, Wolff et al. 1994, Stevenson et al. 1994, 1997, Feighan et al. 1995). Einhorn et al. (1984) used the criterion that the segment was about 20 per cent of the length of the femur and so their defect size was 6 mm. The greatest defect size of rat femur used in experiments has been 1 cm (Takagi & Urist 1982, Vögelin et al. 2005).

Segmental bone defect in the rat femur has been used as a model in both biodegradable and non-biodegradable material studies. In the study of Yasko et al. (1992) the osteoinductive activity of rhBMP-2 in the osseous location of the rat femur was tested with good results. Other rhBMP-2 studies also confirm the good bone healing capacity of this protein together with inorganic and organic carrier (Ohura et al. 1999, Vögelin et al. 2005). A load-bearing scaffold system was manufactured from polypropylene fumarate and TCP. rhBMP-2 together with dicalcium phosphate dehydrate was added to the scaffold prior to implantation to the defect in rat femur. This did not increase bone mineral density, but the scaffold system maintained bone length and allowed regeneration (Chu et al. 2007). BMP-3 and BMP-7 have been tested in the rat femur model (Stevenson et al. 1994, Chen et al. 2002, Little et al. 2005). The main finding was that BMP-7 can maintain its osteoinductive capability in the presence of infection, and a sufficient dose of BMP-7 is essential for callus formation in the infected defects. (Chen et al. 2002).

In 1982, Takagi and Urist used the rat femur defect model to show that bovine extract could be used to heal large femoral diaphyseal defects, especially together with autologous marrow (Takagi & Urist 1982). Bone marrow cells in ceramic blocks or fibre metal implants were also effective in enhancing osteogenesis and osteoconduction (Ohgushi et al. 1989, Wolff et al. 1994). However, significantly more new bone was found in ceramic implants (Wolff et
The effect of DBMs or allografts with or without extra carriers as a suitable alternative to autologous grafts has been studied for decades (Einhorn et al. 1984, Zart et al. 1993, Feighan et al. 1995, Stevenson et al. 1997). Hunt et al. (1996) have studied whether an extract from human osteosarcoma cells has the ability to simulate the healing of the defect in the femoral diaphyses in rats. The materials used in comparison have been collagen and autogenous bone graft. The finding was that the cell extract was the most effective in bone forming, promoting bone growth in major fracture. (Hunt et al. 1996).

Conservative fracture treatment includes the use of casts and splints. When surgery must be used, internal or external fixation methods are commonly used to treat the fractured bone (Park et al. 2003: 174–179). Finding the right bone fixation system depends on the place and size of the defect and test animal species. If internal fixation is selected, the skin is slit and muscles and periosteal tissues are separated circumferentially from the diaphysis to expose the bone. Next, a pre-drilled plate or other system is fixed to the bone with some fastening techniques, after which the segmental defect is created using some burr-system and saline solution (Yasko et al. 1992, Little et al. 2005). This shares the basic elements of ORIF (open reduction and internal fixation) used in surgical treatment of fractures (Nunamaker 1985, Giannoudis et al. 2007). An intramedullary nail is also one alternative that can be used as a bone-internal fixation method. This fixation system provides accurate fracture reduction and stabilization without soft tissue dissection. (Hietaniemi et al. 1995, den Boer et al. 1999, 2003, Chu et al. 2007). A less rigid fixation system has been shown to promote endochondral osteogenesis, while a more rigid fixation enhances intramembranous ossification (Cullinane et al. 2002).

### 2.5.2 The model of ectopic bone formation

Subcutaneous or intramuscular implantation of scaffold-including osteogenic material invokes bone formation at ectopic location (Reddi 1981). Embryonic endochondral bone formation is the typical way of bone formation. This method is also called the bioassay method (Pekkarinen et al. 2003). Mouse and rat are the most typically used animal models.

Bioassay in the mouse hindquarter muscle is currently used as a standard method. Ectopic new bone formation has been estimated after 10-21 days, usually by radiology and histology (Jortikka et al. 1993a, Viljanen et al. 1993, Oksanen et al. 1998, Pekkarinen et al. 2004, Barr et al. 2010).

### 2.5.3 Sheep hole defect model

Sheep, goats, and pigs are defined as big animal models because they are more closely related to humans in size as well as bone and joint characteristics (Nunamaker 1998). Sheep have typically been used to study fixation devices or the healing effect of biomaterials with or without growth factors in segmental defect model (Gerhart et al. 1993, Gao et al. 1996, 1997, Kirker-Head et al. 1998, den Boer et al. 2003, Pluhar et al. 2006, Donati et al. 2008,) but due to the large size of sheep there is often a risk of second fracture during the study (Gao et al. 1996). Therefore, the cancellous bone defect model or hole defect model in sheep has less risk of a second fracture, but works well in testing the biocompatibility of biodegradable materials and gives answers about material resorption and substitution with new bone (Nuss et al. 2006).

The size of the drilled hole in sheep has varied, which is why the CSD criteria in this model are not very clear. Typically, the diameter has been more than 5 mm and depth more than 8 mm (Nuss et al. 2006, Bodde et al. 2007, Maus et al. 2008). Huffer et al. 2007 used the size 5 mm x 5 mm, but they found that this size did not fulfill the CSD criteria (Huffer et al. 2007). Proximal and distal femur, tibia and humerus are the most typical bones (Kessler et al. 2004, Worth et al. 2005, Nuss et al. 2006, Bodde et al. 2007, Huffer et al. 2007, Maus et al. 2008). Nuss et al. (2006) presented a method for sheep hole defect surgery with eight holes. They discovered that an important thing in the operation is to place the holes correctly. Thus, spontaneous fracture is avoided and reliability of the study
results increases (Nuss et al. 2006). Various radiographical, histological methods as well as mechanical testing are the most used methods when analysing bone regeneration and material biocompatibility. Histological methods typically give a better view of the situation in the bone defect after follow-up than radiography alone. (Aebli et al. 2005, Worth et al. 2005, Bodde et al. 2007, Huffer et al. 2007, Maus et al. 2008).

The sheep hole defect model has been used in many material studies. Two commercially available phosphate ceramics were found to be biocompatible and osteoconductive in the study of Bodde et al. 2007. They used a model of two holes drilled on the medial surface of the femur on both sides. The location of defects was marked with three small titanium pins at each side of the drill holes. (Bodde et al. 2007). Various growth factors have been used as composite of bone replacement materials. Nuss et al. 2006 used various bone replacement materials with or without parathyroid hormone, BMP-2, TGF-β and IGF-1 when they developed and tested a sheep hole defect model with 8 holes (Nuss et al. 2006). BMP-2 had been added with HA-coated implants or solvent-dehydrated bone transplants. The result in both experiments was that BMP-2 accelerates bone regeneration in the defects (Aebli et al. 2005, Kessler et al. 2004), whereas Maus et al. 2008 found that an injection of hyaluronic acid-augmented BMP-2 did not increase new bone formation compared to autologous bone or pure hyaluronic acid (Maus et al. 2008). Xenoimplant, autograft and DBMs have been used in the studies of Worth et al. 2005 and Huffer et al. 2007. The latter group found that bovine or equine derived DBMs gave equivalent bone regeneration result as autograft and better than sintered β-TCP ceramic (Huffer et al. 2007).
3 Aims of the study

The present study consists of experiments designed to evaluate the effects of reindeer bone protein extract and its various formulation systems in the healing of various animal bone defects and in bone forming in the ectopic muscle model. The specific aims of this study were:

- to investigate the bone healing capacity of reindeer bone extract dosing with bovine collagen sponge using a critical-size defect model of rat femur.
- to evaluate the bone healing effect of composite implants containing reindeer bone extract combined with bioactive glass using a critical-size defect model of rat femur.
- to evaluate the bone forming effect of reindeer bone extract formulations in calcium salt carriers using a mouse muscle pouch model.
- to compare the bone formation performance of reindeer bone extract formulation in inorganic carriers to autograft and commercial demineralized bone matrix using a hole-defect model of sheep femur and humerus.
4 Materials and methods

The materials and methods used in the study series are summarized in Table 4.
Table 4. Summary of the materials and methods used in the studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Methods</th>
<th>Carrier (Type)</th>
<th>Dose of bone protein extract</th>
<th>N</th>
<th>Follow-up (weeks)</th>
<th>Analysis methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>CSD, left femur Rat</td>
<td>Collagen (type IV, bovine)</td>
<td>2 mg</td>
<td>14</td>
<td>6</td>
<td>Radiography</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collagen (type IV, bovine)</td>
<td>5 mg</td>
<td>14</td>
<td>6</td>
<td>Radiography</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collagen (type IV, bovine)</td>
<td>15 mg</td>
<td>14</td>
<td>6</td>
<td>Radiography</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collagen (type IV, bovine)</td>
<td>20 mg</td>
<td>13</td>
<td>6</td>
<td>Radiography</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collagen (type IV, bovine)</td>
<td>50 mg</td>
<td>13</td>
<td>6</td>
<td>Radiography</td>
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<td></td>
<td></td>
<td>Collagen (type IV, bovine)</td>
<td>30 μg rhBMP-2</td>
<td>13</td>
<td>6</td>
<td>Radiography</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Untreated defect</td>
<td>-</td>
<td>14</td>
<td>6</td>
<td>Radiography</td>
</tr>
<tr>
<td>II</td>
<td>CSD, left femur Rat</td>
<td>Bioactive glass (S53P4)</td>
<td>5 mg</td>
<td>9</td>
<td>8</td>
<td>Radiography</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bioactive glass (S53P4)</td>
<td>10 mg</td>
<td>9</td>
<td>8</td>
<td>Radiography</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bioactive glass (S53P4)</td>
<td>15 mg</td>
<td>8</td>
<td>8</td>
<td>Radiography</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bioactive glass (S53P4)</td>
<td>40 mg</td>
<td>8</td>
<td>8</td>
<td>Radiography</td>
</tr>
<tr>
<td></td>
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<td>Bioactive glass (S53P4)</td>
<td>-</td>
<td>8</td>
<td>8</td>
<td>Radiography</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Untreated defect</td>
<td>-</td>
<td>8</td>
<td>8</td>
<td>Radiography</td>
</tr>
<tr>
<td>III</td>
<td>Muscle pouch model, hind legs Mouse</td>
<td>HAβ-TCP/CS 60:30:10</td>
<td>3 mg (impregnated)</td>
<td>8</td>
<td>3</td>
<td>Radiography</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HAβ-TCP/CS 30:60:10</td>
<td>3 mg (impregnated)</td>
<td>8</td>
<td>3</td>
<td>Radiography</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β-TCP/CS 90:10</td>
<td>3 mg (impregnated)</td>
<td>8</td>
<td>3</td>
<td>Radiography</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cem-Ostetic</td>
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<td>3</td>
<td>Radiography</td>
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<tr>
<td></td>
<td></td>
<td>CS hemihydrate,</td>
<td>3 mg (mixed)</td>
<td>8</td>
<td>3</td>
<td>Radiography</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CS dihydrate with SA,</td>
<td>3 mg (dry mixed)</td>
<td>8</td>
<td>3</td>
<td>Radiography</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Corresponding controls</td>
<td>-</td>
<td>8</td>
<td>3</td>
<td>Radiography</td>
</tr>
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</table>

pQCT = pQCT, histology = histology
<table>
<thead>
<tr>
<th>Study Methods</th>
<th>Carrier (Type)</th>
<th>Dose of bone protein extract</th>
<th>N</th>
<th>Follow-up (weeks)</th>
<th>Analysis methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV Hole defect (0.283 cc), Humerus and femurs, Sheep</td>
<td>Cam β-TCP granules, with PEG-GLY and SA</td>
<td>60 mg / 3 cc syringe</td>
<td>8</td>
<td>8 μCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cam β-TCP granules, with PEG-GLY and SA</td>
<td>30 mg / 3 cc syringe</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cam β-TCP granules, with PEG-GLY and SA</td>
<td>-</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cur β-TCP morsels, with PEG-GLY and SA</td>
<td>60 mg / 3 cc syringe</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cur β-TCP morsels, with PEG-GLY and SA</td>
<td>-</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cam β-TCP granules</td>
<td>-</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autograft</td>
<td>-</td>
<td>8</td>
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</tr>
<tr>
<td></td>
<td>DBM (human)</td>
<td>-</td>
<td>8</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Untreated defect</td>
<td>-</td>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n = number of study cases, CSD = critical-size defect, pQCT = peripheral quantitative computerized tomography, rhBMP-2 = recombinant human bone morphogenetic protein-2, HA = hydroxyapatite, β-TCP = beta-tricalcium phosphate, CS = calcium sulphate, SA = stearic acid, Cam = Cambioceramics, PEG = polyethylene glycol, GLY = glycerol, Cur = Curasan, DBM = demineralized bone matrix, μCT = micro-computerized tomography
4.1 Animals

All study protocols were carried out according to Finnish laws on animal welfare and were approved by the institutional animal experiment and ethical committee. The animals were supplied by the Laboratory Animal Centre, University of Oulu. The numbers of test animals in the different research categories were as follows:

- Study I: One hundred and nine 2.5-month-old male Sprague-Dawley rats.
- Study II: Fifty 2.5-month-old male Sprague-Dawley rats.
- Study III: Forty-eight 7- to 12-week-old BALB/c mice.
- Study IV: Ten 3-year-old Finnish archipelago sheep.

4.2 The reindeer bone protein extract

The bone protein extract was extracted and purified from diaphyseal bone of the reindeer as described previously (Jortikka et al. 1993a). In studies III and IV, the obtained bone protein extract was freeze-dried at -20°C degrees using excipients (surfactant (Polysorbat 20, Fluka, Sigma-Aldrich), lyoprotectant (D-(+)-Trehalose Dihydrate, Fluka, Sigma-Aldrich), bulking agent (Glycine, Riedel-de Haën, Sigma-Aldrich) and buffer (D-Mannitol, Fluka, Sigma-Aldrich)).

The protein profile and the bioactivity of the freeze-dried bone protein extract were evaluated by SDS-page (Ulmanen et al. 2005) and mouse muscle pouch model study as described in study III (Fig. 2).
4.3 Reference materials

The reference materials used in this study were chosen according to known and used materials in bone trauma surgery.

A commercial product of recombinant human bone morphogenetic protein-2 (InductOs™ kit, Wyeth Europa, Maidenhead/Berkshire, UK) was used as comparison material in study I.

A commercial product of demineralized bone matrix (Grafton Plus® Demineralized Bone Matrix 1cc (DBM) Paste, Osteotech Inc., Eatontown, New Jersey, USA) was used as reference material in study IV.

Autograft is the most typical treatment device in difficult bone traumas. It was used as reference material in study IV.

4.4 Carriers

Various carrier systems for reindeer bone protein extract were tested in this study. Collagen was tested as an organic carrier. Bioactive glass and calcium salts were tested as inorganic alternatives. The materials used were prepared for different implant forms.
A commercially available, absorbable collagen sponge (Lyostypt®, compress from a native collagen of bovine origin, mainly consisting of type IV collagen, B. Braun, Tutlingen, Germany) was used as a collagen carrier.

The distribution size of the bioactive glass granules used (Bioactive glass, S53P4, Vivoxid, Turku, Finland) was 500-800 μm.

The carrier materials used and the study groups in study III were:

a) Porous discs that were 5 mm x 3 mm in size (Berkeley Advanced Biomaterials Inc, USA), with a composition of 30% hydroxyapatite (HA), 60% beta-tricalcium phosphate (β-TCP), and 10% calcium sulfate (CS);

b) Cem-Ostetic porous discs that were 5 mm x 3 mm in size, with a composition of 90% β-TCP and 10% CS;

c) Cem-Ostetic® (Berkeley Advanced Biomaterial Inc., USA) powder for putty;

d) CS hemihydrate (97%, Sigma-Aldrich) powder for putty;

e) Non-porous discs with a composition of 60% HA, 30% β-TCP and 10% CS; and

f) CS dihydrate granules with stearic acid with a composition of stearic acid 50, a mixture of fatty acids that consisted mainly of stearic acid and 40-60% palmitic acid (Fluka, Sigma-Aldrich).

In the Study IV, the carrier materials used were:

1. Custom-made Cambioceramics β-TCP (Cambioceramics, Cam Bioceramics, Leiden, The Netherlands), 300-500 μm of 1.24 g/cm³ density combined with Polyethylene Glycol/Glycerol (PEG/GLY) (Clariant, Kemi Intressen, and Croda, Kemi Intressen) matrix modified with stearic acid (Stearic acid 50, mixture of fatty acids, consisting mainly of stearic acid and 40–60% palmitic acid, Fluka, Sigma-Aldrich) (Study groups named: Paste 1, Paste 2, Paste 3, and Granule);

2. Curasan β-TCP Cerasorb M (Cerasorb® M Ortho, Curasan AG, Frankfurt, Germany), 500-1000 μm of 0.61 g/cm³ density combined with PEG-GLY matrix modified with stearic acid (Study groups named: Paste 4, and Paste 5);

3. Curasan β-TCP Cerasorb (Cerasorb®, Curasan AG, Frankfurt, Germany), 500-1000 μm of 1.21 g/cm³ density combined with PEG-GLY matrix modified with stearic acid (Study group named: Paste 6).
4.5 Preparation of implants

4.5.1 Reconstitution of collagen implants (I)

2 mg, 5 mg, 15 mg, 20 mg or 50 mg of the lyophilized reindeer bone extract was sprinkled onto a collagen sponge (35 x 8 mm for small amounts and 25 x 25 mm for large amounts, Lyostypt®). The collagen sponge was wetted in 200 μl sterile water and the sponge with extract was bundled up to form an implant.

InductOs™ kit for implant contained 12 mg of rhBMP-2 and solvent. The solvent was made according to manufacturer’s instructions just before the operation. 20 μl of solvent, containing 30 μg of rhBMP-2, was pipetted on the collagen sponge (Lyostypt®) to form the implant.

4.5.2 Reconstitution of bioactive glass implants (II)

A dose of 5 mg, 10 mg, 15 mg, or 40 mg of the reindeer bone extract was added to carboxymethyl cellulose (CMC, Sigma-Aldrich) to obtain a 3.2% (water/weight, w/w) gel. Bioactive glass granules (S53P4) were combined with extract-CMC-gel, and the mixture was shaped into a rod (diameter 5 mm) and lyophilized. Before drying, one 8-mm-wide implant contained 40% w/w of CMC-gel with a propriety amount of bone extract, and 60% (w/w) bioactive glass granules. The amount of CMC in the dried product was 2% (w/w).

4.5.3 Reconstitution of calcium salt implants (III)

The amount of 3 mg of the lyophilized reindeer bone extract was reconstituted in 0.9% physiological saline solution (Sodium chloride, Fagron, Tamro, Finland) and impregnated into the porous discs (30% HA, 60% β-TCP, 10% CS and 90% β-TCP, 10% CS) and the non-porous disc (60% HA, 30% β-TCP, 10% CS), or mixed with the Cem-Ostetic powder (Cem-Ostetic®) or CS hemihydrate to form a moulded disc, or dry-mixed with CS dihydrate granules and stearic acid to form a compressed disc (Table 4).

4.5.4 Reconstitution of β-TCP implants (IV)

Polyethylene glycol 2000 (PEG), glycerol and stearic acid were heated until a clear mixture was formed. The mixture was cooled under continuous mixing to
form an opalescent paste, after which the required amounts (60 mg or 30 mg) of the lyophilized bone extract and \(\beta\)-TCP granules were added (Table 4). The formulated paste was packed in syringes (including about 5.66 mg of reindeer bone protein extract) and closed in aluminium foil pouches. All samples were manufactured in a laminar flow cabin to reduce the bioburden, and then terminally gamma-sterilized (15 kGy).

4.5.5 Reconstitution of autograft implants (IV)

In the autograft group the bone material removed from the test hole-sites of the same sheep using chisel and trephane drill. The autograft material was preserved in 0.9% physiological saline solution (Sodium chloride, Fagron, Tamro, Finland) until implanting.

4.5.6 Control implants

The control implants were constructed in an identical fashion, but they contained only carrier. In the mouse study (Study III) the right leg was used as a control containing the respective carrier and excipients, excluding the bone extract. Furthermore, studies I, II and IV involved the untreated group without any implant.

4.6 Surgical methods

Bone healing capacity and new bone formation were studied in the various experimental models, including the critical-size defect model in rat, the muscle pouch model in mouse and the hole defect model in sheep.

4.6.1 Critical-size rat femur defect study (I, II)

Surgery was performed under general anaesthesia with a blend of fentanyl citrate (80 µg/kg) - fluanisone (2.5 mg/kg) (Hypnorm®) and midazolam (1.25 mg/kg) (Dormicum®). Preoperatively, the animals were administered cefuroxime (Zinacef®, 20 mg/kg) subcutaneously. The pain medication after the operation consisted of buprenorfin (Temgesic®) at 0.01-0.05 mg/kg subcutaneously. In cases with respiratory problems, animals were treated with 1 mg of furosemid.
(Furesis®). Eye gel (Viscotears®) was applied to avoid dehydration of eyes during anaesthesia. A transverse skin incision was made over the posterior aspect of the thigh after shaving the hair round the left hind limb. The muscles were elevated circumferentially from the femoral diaphysis. A 23 mm x 4 mm x 2.5 mm polyacetyl plaster plate was placed on the surface of the femur. The plate was temporarily secured in place with a stainless steel holder, which was exclusively designed and manufactured for this operation (Technical Services Unit, University of Oulu) (Fig. 3). The plate was fixed using 0.8-mm threaded Kirschner wires (K-wires), which have a 5-mm long threaded distal end (Synthes Oy). The canal for the threaded K-wire was predrilled through the plate and through both cortices of the femur using a 0.7 mm drill. The threaded K-wire was bent so as to easily screw the threaded end of the wire through the plate and into the bone. The correct depth for inserting the wire was determined by carefully dissecting the other side of the bone with a probe to confirm that the wire penetrated the distal cortex of the bone. A total of 6 threaded K-wires were used for each plate, three on each side of the bone defect, each screwed through two cortices. The excess wire was removed to the level of the plate using side-cutting pliers.

An 8-mm diaphyseal critical-sized defect was created using a stainless steel mini bur. After a thorough wash with saline, the implant was applied to the defect, or the defect was left empty for controls. The muscles and skin were closed in two layers using absorbable 4.0 sutures (Dexon®). The pain medication after the operation consisted of buprenorfin (Temgesic®) at 0.01-0.05 mg/kg administered subcutaneously. In case of respiratory problems during anaesthesia, animals were treated with 1 mg of furosemid (Furesis®) subcutaneously. Eye gel (Viscotears®) was applied to avoid eye dehydration during anaesthesia. The animals were allowed full activity in their cages postoperatively. After the follow-ups, the rats were killed in a chamber with carbon dioxide (CO2). The left leg of each rat was dissected from the body, wrapped in a saline-wetted serviette, and frozen at -20 ºC until biomechanical analysis.
4.6.2 Mouse muscle pouch model (III)

Surgery was performed under general anaesthesia with a blend of fentanyl citrate (80 µg/kg) - fluanisone (2.5 mg/kg) (Hypnorm®) and midazolam (1.25 mg/kg) (Dormicum®). Both legs were cleaned and the eyes were treated with eye gel against drying. The mouse was operated on the thermal mattress. Transverse skin incisions were made near the spine at the site of the femurs. Then the implants were introduced into both thigh muscle pouches in the bilateral hind legs (Fig. 4). After the implantation, the muscles were closed with two sutures and the skin with one suture.

The pain medication after the operation consisted of buprenorfin (Temgesic®) at 0.01-0.05 mg/kg subcutaneously. The animals were allowed full activity in their cages postoperatively. All animals were euthanized 21 days later, and the hind legs were harvested.
The mouse muscle pouch model was used to show ectopic bone formation of various calcium salt implants. An active implant is on the left side and a control on the right side.

4.6.3 Sheep model study (IV)

The operation was performed under general inhalation anaesthesia, induced by an intravenous injection of propofol (5-7 mg/kg i.v.) and maintained with isoflurane in 1-1.5% oxygen-air mixture. Before the anaesthesia the sheep were premedicated with medetomidine by an intramuscular injection (0.015 ml/kg i.m.) and intubated. The sheep were controlled with a heart monitor during the operation.

A fentanyl (2 μg/kg/hour, Durogesic®) depot plaster was given preoperatively for 72-h pain relief. Additionally, 2 ml of fentanyl (50 μg/ml i.m.) was injected intramuscularly during the first 72 h after the operation. Then buprenorfin (0.3 mg/dose i.m.) was injected twice a day continuously for two days or more after the operation when the depot plaster was removed.

Amoxycillin (15 mg/kg i.m., Betamox® Vet, 150 mg/ml) was injected intramuscularly as antibiotic prophylaxis into the anterior half of the neck 24 h preoperatively and once per day for two days postoperatively.

Hole defects were induced to the femoral and humeral distal and proximal condyles of the sheep hind and front legs with a drill as described by Nuss et al. (2006). A hole (size 0.283 cc) with a diameter of 6 mm and a depth of 10 mm was drilled (cordless drill, Bosch PSR12-2). Meanwhile, the location of the defect was marked, using 1.0 mm dental, radiopaque, glass-fibre root canal posts. The posts were cut to suitable length with a diamond blade. The drilled holes were filled
according to the randomization table with the test materials or left empty (untreated controls) (Fig. 5). Finally, the subcutaneous tissues were closed in layers with resorbable continuous 3-0 Polysorb sutures and skin with non-resorbable 2-0 Monosof sutures.

After a predetermined time period of 8 weeks the animals were euthanized and bone samples were taken for analysis. Euthanasia was performed with pentobarbital (60mg/kg i.v. Mebunat®). Before this, the sheep were anaesthetized intramuscularly with medetomidine (0.015 ml/kg Domitor® Vet, and 0.04 ml/kg Ketalar®).

After euthanasia the bones were excised and preserved in ice. Then the bone blocks were preserved in 4% buffered formalin for the first seven days and then in 70% ethanol. Some samples were broken in the excision phase and they were removed from the analysis.

![Fig. 5. Hole defects were induced to the femoral and humeral distal and proximal condyles of the sheep hind and front legs with a drill as described by Nuss et al. (2006). The drilled holes were marked with dental posts and filled with the test materials.](image)

**4.7 Analysis methods**

New bone formation, bone healing, and carrier material resorption were evaluated based on radiographs, peripheral computerized tomography (pQCT), mechanical tests, histological examination, and micro-CT.
4.7.1 Radiographic evaluation (I, II, III)

In studies I and II, radiographs of the left femur (26 kV, 9.00 mAs, 0.09 s/exp, Mamex dc® ami, Orion Ltd., Soredex) were taken postoperatively, three weeks and six weeks after the operation under neuroleptic analgesia (Hypnorm-Dormicum®, 0.7 ml) and after sacrificing. Radiographs were evaluated with Osiris 4.19 (Digital Imaging Unit, Geneva) software. The percentage of orthopic new bone formation (BF) and the development of union or non-union (BU) of the bone were estimated according to the scoring system presented in the study of Sciadini et al. 1997.

In study III, radiographic evaluation (20 kV, 8.00 mAs, 0.32 s/exp, Mamex dc® ami, Orion Ltd., Soredex) was used to evaluate formation of new bone and resorption of the implant. New bone formation and resorption of implant was evaluated by measuring the opalescent area in mm² (Osiris 4.19 Digital Imaging Unit, Geneva, software).

4.7.2 Computed tomography (I, II)

In studies I and II, all left femurs were scanned using a peripheral quantitative computerized tomography (pQCT) device (Stratec XCT 960A, 5.21 software version, Norland Stratec Medizintechnik GmbH, Birkenfeld, Germany). A voxel size of 0.148 mm x 0.148 mm x 1 mm was used. One cross-sectional slice from the middle of the defect in each sample was scanned in two directions: with fixation system above the bone, and turned 90° axially. Average values of the two scans were used in the statistical analysis. Cross-sectional bone areas (mm²) from the scanned slices of the samples were recorded using the pQCT software, with a threshold of 169 mg/cm³ to distinguish bone from surrounding soft tissue and a threshold of 464 mg/cm³ for the inner surface of the bone.

4.7.3 Micro-CT (IV)

Samples were scanned by using micro-computerized tomography (micro-CT) device (Magnification 15.54, Source 72 kV/138 μA, Rotation 180°, Rotation step 0.45°, Exposure 3.9 sec, Gain 1.0, Frame aceragin OFF, Flat field correction ON, Random movement 20, Filter Al 0.25 mm, SkyScan 1072, x-ray microtomograph, SkyScan, Belgium). Scanned samples were analysed using CTAn (SkyScan) software. Resorption of carrier, the amount and quality of new bone and general
bone healing were visually evaluated from the images. Furthermore, new bone volume (BV, mm$^3$), bone surface to bone volume ratio (BS/BV, mm$^{-1}$), trabecular thickness (Tb.Th, mm), open porosity (Po(op), %), and closed porosity (Po(cl), %) were measured from the defects.

### 4.7.4 Biomechanical testing (I, II)

In studies I and II, after pQCT imaging, the fixation system was removed from the samples. The bone ends were embedded into the moulds of the sleeves with dental stone (GC Fujirock, Improved Dental Stone). The torsional shaft was adjusted to 2 cm. After the cast was hardened, the samples were placed in the torque machine (Lepola et al. 1993), and torsionally loaded at a constant angular speed of 6° per second until failure (Jämsä & Jalovaara 1996) (Fig. 6). Maximum breaking load (Nm) and torsional stiffness (Nm°) were recorded. Mechanically unstable bones were not tested, and their values were considered to be zero in the statistical analysis.

![Mechanically stable rat femurs were torsionally loaded after pQCT imaging by the torque machine of Lepola et al. 1993.](image)

### 4.7.5 Histological examination (I, III)

In studies I and III, two samples from every group were prepared for histology. The specimens were fixed in 10% neutral-buffered formalin, decalcified in EDTA-formalin solution (pH 7), processed in a tissue processor, and finally embedded in paraffin. 4.5-μm-thick slices were prepared using a microtome and stained with haematoxylin-eosin. The quality of new bone and inflammatory
response on the defect site were evaluated in histological analysis by light microscopy (Nikon Eclipse, E200, Japan).

4.7.6 Statistics

Statistical analysis was performed using the SPSS for Windows statistical package (SPSS Inc., version 15.0). The independent-samples t-test (study IV) or the non-parametric Kruskall-Wallis test (study I–IV) was used to evaluate the statistical differences between the groups. The Mann-Whitney test was used for pairwise comparison between the reindeer bone protein extract-treated and the control groups (study I-IV). The Benjamini-Hochberg procedure was used to correct $p$-values for multiple comparisons (Study II). Values of $p < 0.05$ were considered statistically significant.
5 Results

In study I, twelve rats died on the night after the operation, and two died later. Seventeen rats had to be killed due to the failure of fixation before the study endpoint. In study II, ten rats died within 24 hours of the operation. In study III, five mice died or were sacrificed before the follow-up. No obvious cause was found for the deaths of the rats. With mice, some deaths or sacrificing were caused by walking or breathing problems. No direct relation to the reindeer bone extract was found as some deaths occurred in the control groups as well.

All surviving animals were analysed after follow-ups. Reindeer bone protein extract was able to heal the critical-size segmental bone defect in six weeks, and significant bone formation was already observed after three weeks. However, the healing effect depends on the doses and concentration of extract in the carrier. The minimum dose for segmental defect healing in rat seems to be 5 mg. Furthermore, study II showed for the first time that bioactive glass is a suitable carrier for reindeer bone protein extract. In study III, inorganic carrier material together with stearic acid had the best ectopic bone formation capacity. This kind of carrier in paste form was used in study IV. It showed to be comparable to autograft, and much better in bone formation than DBM.

5.1 Dosing of the reindeer bone protein extract (I, II)

Radiographical evaluation

In study I, bone formation (BF), measured by roentgenography, at the defect site was greater at three and six weeks in all bone extract groups \( (p < 0.05) \) when compared to the untreated and collagen groups, except for the group of 2 mg bone extract in comparison with the untreated controls at three weeks. In this study bone formation showed a clear dose-dependency, but only the difference between the groups with 2 mg and 20 mg of bone extract reached the level of significance \( (p = 0.030) \) (Table 5). Bone union (BU) at three weeks was better in the groups receiving 5 mg, 20 mg and 50 mg of the extract than in the untreated or collagen groups \( (p < 0.05) \). At six weeks, BU was better in the groups with 5 mg and 50 mg of the bone extract than in the untreated group \( (p < 0.05) \), and also better in the groups with 5 mg, 15 mg and 50 mg of bone extract than in the collagen group \( (p < 0.05) \). There were no significant differences in BU between the groups.
receiving various amounts of the bone extract (Table 6). BF and BU were better in the commercial rhBMP-2 group than in any of the other groups both three and six weeks after the operation \((p < 0.001)\) (Table 5 and 6).

In study II, all doses of the extract with bioactive glass increased BF observed on radiographs compared with the bioactive glass alone and untreated groups \((p < 0.002\) to \(p < 0.03\)). With the extract doses of 10-40 mg + bioactive glass, bone formation was superior as soon as 3 weeks postoperatively when compared to the plain bioactive glass and untreated groups \((p < 0.03)\) (Table 5). BU at 6 weeks was better in the groups receiving bioactive glass + 10-40 mg of the extract compared with the plain bioactive glass and untreated groups \((p < 0.009\) to \(p < 0.03\), respectively) (Table 6).
Table 5. Bone formation (BF) in the defect area of rat femur three to eight weeks after the operation evaluated by radiographic analysis using the scoring system presented in the study of Sciadini et al. (1997).

<table>
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*p < 0.005, *p < 0.001 vs. untreated, *p < 0.001 vs. collagen, *p < 0.05 vs. 2 mg of bone extract, *p < 0.01 vs. all other groups in study I.

*p < 0.05, *p < 0.01, *p < 0.005 vs. untreated after Benjamini-Hochberg corrections

*p < 0.05, /p < 0.01, *p < 0.005 vs. bioactive glass after Benjamini-Hochberg corrections
Table 6. Bone union (BU) in the defect area of rat femur three to eight weeks after the operation evaluated by radiographic analysis using the scoring system presented in the study of Sciadini et al. (1997).

<table>
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<td></td>
<td>6 6 0 0 0</td>
<td>5 0 0 0</td>
<td>6 0 0 0</td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.03 vs. untreated, † p < 0.05 vs. collagen, ‡ p < 0.001 vs. all other groups in the study I.

₅p < 0.05, ‧ p < 0.01, ‧ ‧ p < 0.005 vs. untreated and bioactive glass after Benjamini-Hochberg corrections.
**Biomechanical evaluation**

In study I, maximum torsional load of the bones (Nm) was significantly higher in the groups receiving 2 mg, 20 mg, or 50 mg of the bone extract than in the collagen group \((p < 0.05)\). Stiffness of the bones (Nm/º) was significantly higher in the groups receiving 2 mg, 15 mg, 20 mg, or 50 mg of the bone extract than in the collagen group \((p = 0.046 \text{ to } p = 0.004)\). Maximum torsional load in the rhBMP-2 group was the highest of all groups \((p < 0.05)\). Stiffness of the bones in the rhBMP-2 group was higher than in the control groups and in most bone extract groups \((p < 0.05)\).

In study II, torsional stiffness of the bone was marginally higher in the group receiving bioactive glass + 15 mg of the extract compared with the untreated group \((p = 0.066)\).

**Histological evaluation**

In study I, descriptive histology examination showed no new bone formation, only fibrotic tissue, in the specimens of the negative control groups. New bone formation was seen in the specimens of the groups containing the reindeer bone extract or rhBMP. The most abundant bone formation and bone union was seen in the specimens of 20 mg to 50 mg extract and in rhBMP groups, which showed hypertrophied and calcified chondrocytes, remodelling ectopic trabecular woven bone and bone marrow. No inflammatory reaction was seen.

**5.2 Collagen and bioactive glass as carrier materials (I, II)**

Collagen sponge was used as carrier material for the reindeer bone protein extract and as reference material (rhBMP-2) in study I. Computed tomography (pQCT) showed cross-sectional bone areas to be greater in the groups that received 15 mg to 50 mg extract with collagen than in the control groups without extract (Table 4) after six weeks \((p = 0.019 \text{ to } p < 0.001)\). Cross-sectional bone area in the rhBMP-2 group was the greatest compared to other groups \((p < 0.001)\).

Bioactive glass material was used as a scaffold in study II. In this study, pQCT showed cross-sectional bone areas to be greater in the bioactive glass + 15 mg of the extract group and in the plain bioactive glass group than in the untreated group \((p < 0.03)\). Bone density at the defect site was greater in all groups that received the bone protein extract with bioactive glass and in the

65
bioactive glass alone group than in the untreated group ($p < 0.004$). There were no significant differences in bone density between the plain bioactive glass group and any of the extract groups.

5.3 Bone formation capacity with calcium salt carrier materials (III)

In study III, the radiographic evaluation of the HA/β-TCP/CS 30:60:10 discs demonstrated some bone formation outside the active implant; however, the control implants remained intact. The measurement area was significantly higher for the active implants compared to the controls ($p < 0.01$). For the group β-TCP/CS 90:10 discs, the radiographic evaluation displayed some bone formation outside of the active implants; however, the control implants were nearly intact, and there were no statistically significant differences between the active implants and the controls. For the Cem-Ostetic group, the radiographic analysis displayed no new visual bone formation; however, the measurement area was larger in the active group than in the control group ($p < 0.01$). For the group CS hemihydrate discs, the radiographic analysis revealed some new bone formation, and significant differences ($p < 0.01$) were apparent between the active and control groups (the control group had visibly resorbed). For the group HA/β-TCP/CS 60:30:10 discs, the radiographic evaluation showed some bone formation outside of the implant on the active side and some in the control implants. Furthermore, the active group had a larger measurement area than the control group ($p = 0.001$). For the group CS dihydrate-stearic acid compressed discs, the radiographic analysis and harvesting analysis revealed new bone formation in the active implant group. The difference between the active implants and the controls was statistically significant ($p < 0.01$). (Fig. 7).

The comparison between all active groups revealed that the group CS dihydrate-stearic acid compressed discs had the largest measurement area, as determined by the radiographic analysis ($p < 0.05$). Furthermore, the groups Cem-Ostetic and CS hemihydrate had significantly larger areas than the groups HA/β-TCP/CS 30:60:10, β-TCP/CS 90:10 and HA/β-TCP/CS 60:30:10 ($p < 0.01$). There was also a statistically significant difference between the active groups HA/β-TCP/CS 30:60:10 and HA/β-TCP/CS 60:30:10 ($p < 0.05$). (Fig. 7).
Fig. 7. Radiographic analysis of active implant, containing the bone extract, and controls after three weeks of follow-up (average opalescent area in mm\(^2\)). Active group is marked as dark grey and its control as light grey. The standard deviation is shown. *\( p < 0.01 \) vs. control, *\( p < 0.05 \) vs. other active groups, *\( p < 0.01 \) vs. HA/\( \beta \)-TCP/CS 30:60:10, \( \beta \)-TCP/CS 90:10 and HA/\( \beta \)-TCP/CS 60:30:10, *\( p < 0.01 \) vs. HA/\( \beta \)-TCP/CS 30:60:10.

In study III, for group a, the harvesting analysis indicated that this new formation was bone-like. The histological evaluation demonstrated that endochondral bone formation occurred in the active sample, but not in the control sample. For the group \( \beta \)-TCP/CS 90:10, visual inspection during harvesting indicated bone-like formations. The histological evaluation showed endochondral bone formation in the active sample, but not in the control sample. For the groups Cem-Ostetic and CS hemihydrate, harvesting and histological analysis confirmed that no new bone was found in the samples. For the group HA/\( \beta \)-TCP/CS 60:30:10, the harvesting analysis indicated that this new formation was bone-like. The histological evaluation revealed endochondral bone formation and mature cartilage cells in the active sample; however, none were found in the control sample. For the group CS dihydrate and stearic acid, histological analysis revealed clear bone formation and mature and calcified cartilage cells in the active sample (Fig. 8a). No visual bone formation was apparent in the control sample (Fig. 8b).
Fig. 8. Histological analysis revealed clear bone formation, and mature and calcified cartilage cells in the CS dihydrate + stearic acid carrier with the reindeer bone protein extract (a), but no visual bone formation was apparent in the control sample without the extract (b). C = calcified cartilage cells, B = bone, M = muscle, F = fibrotic tissue, and I = implant carrier. (Original magnification 10x). (Reprinted with permission from MDPI Publishing).

5.4 Bone formation of the formulated bone protein extract in inorganic carrier (IV)

In study IV, micro-CT analysis was used to show new bone formation, carrier resorption, and to measure new bone volume (BV), bone surface to bone volume ratio (BS/BV), trabecular thickness (Tb.Th), open porosity (Po(op)), and closed porosity (Po(cl)) in the defect site. For the groups Paste 1 and Paste 2 (the bone protein extract and Cambioceramics β-TCP in PEG-GLY matrix with stearic acid) micro-CT evaluations showed good bone formation, bone union, and bone remodelling, especially in the group Paste 1 (60 mg) in the defect area, even though there were cortical areas without new bone or remnants of β-TCP granules. Almost all β-TCP granules had resorbed during the follow-up. Paste 2 had half of the amount of bone protein extract (30 mg) and there were still some remnants of β-TCP granules in the defects. In micro-CT analysis, Paste 1 and Paste 2 had significantly greater BV and Po(cl) than the groups DBM or Untreated ($p < 0.05$) (Fig. 9). They also had higher BS/BV than Autograft and Untreated (Fig. 10). The Tb.Th was smaller than in the groups Autograft, DBM or Untreated, but greater than in the groups including granules without the bone protein extract ($p < 0.05$) (Table 7).
For the group Paste 4 (the bone protein extract and Cerasorb® M β-TCP in PEG-GLY matrix with stearic acid) micro-CT evaluations showed good new bone formation, bone union and bone remodelling in the defect area, although there were cortical areas without new bone or remnants of β-TCP granules. β-TCP granules had resorbed during the follow-up. Paste 4 had significantly greater BV and Po(cl) than DBM or Untreated \( (p < 0.05) \) (Fig. 9). It also had higher BS/BV than Autograft, DBM and Untreated (Fig. 10). The Tb.Th was smaller than in the groups Autograft, DBM or Untreated, but greater than in the groups including β-TCP granules without the bone protein extract \( (p < 0.05) \) (Table 7).

For the group Paste 6 (the bone protein extract and Cerasorb® β-TCP in PEG-GLY matrix with stearic acid) micro-CT evaluations showed good new bone formation and bone union in the defect area. Most of the β-TCP granules had resorbed during the follow-up, but there were still some separate, unresorbed granules in the defects. There was still the same problem as in the other groups with PEG-GLY matrix and stearic acid that the implant (granules) had not filled the whole defect and there were some empty areas, especially in the cortical site. Similarly to other groups with the reindeer bone protein extract, also Paste 6 had significantly greater BV than the groups DBM and Untreated \( (p < 0.05) \) (Fig. 9). It also had higher BS/BV than autograft, DBM and Untreated (Fig. 10). Measured Tb.Th was smaller than in the groups Autograft or Untreated, but greater than in the groups including granules without the bone extract (Table 7). Although new bone formation and bone volume were good, Paste 6 had the greatest proportional Po(cl) amount \( (p < 0.05) \) (Table 7).

For the control groups Paste 3 and Paste 5 (Cambioceramics in PEG-GLY matrix with stearic acid, without bone protein extract), micro-CT evaluations showed that most β-TCP granules had not yet resorbed and they had packed as a thick mass into the bottom of the defect. Unresorbed β-TCP granules could clearly be seen, and they had packed as a thick mass at the bottom of the defect. There was new bone formation in this granule mass, but only around the granules. Bone union was not seen. Paste 3 and Paste 5 had statistically significantly greater BV and Po(cl) than DBM and Untreated \( (p < 0.05) \) (Fig. 9). They also had higher BS/BV than Autograft, Untreated and Paste 1 \( (p < 0.05) \) (Fig. 10). Measured Tb.Th was smaller in these groups compared to the other control groups (except the group Granule) or the groups with the reindeer bone protein extract \( (p < 0.05) \) (Table 7).

For the other control group, Granule (pure Cambioceramics β-TCP), micro-CT evaluations showed that implanted granules had filled the whole defect area.
but the granules had not resorbed during the follow-up. There was some new bone formation around the granules but no bone union. The Granule group also had significantly greater BV and Po(cl) than DBM or Untreated ($p < 0.05$) (Fig. 9). It also had greater Po(cl) than Autograft and higher BS/BV than Autograft, Untreated and the groups including the reindeer bone extract ($p < 0.05$) (Fig. 10). Measured Tb.Th was smaller in this group compared to the other control groups (except Paste 5) and the groups with the bone protein extract ($p < 0.05$) (Table 7). Furthermore, Autograft, DBM and Untreated defects were used as controls. For the group Autograft, micro-CT evaluation showed clear new bone formation and bone remodelling in the defect area. Empty areas in the cortical sites were not seen, except in a couple of defects. In the DBM group (Grafton Plus® DBM) and the Untreated group micro-CT evaluations showed no new bone formation in the defect sites during the follow-up.

![Fig. 9. Bone volumes (BV, mm$^3$) were measured from the defect areas using micro-CT. Mean value and standard deviation are shown. In the statistic comparisons all other groups had significantly greater BV than DBM and untreated defects ($p < 0.05$). The control groups have been marked as brick pattern.](image)
Fig. 10. Bone surface to bone volume ratio (BS/BV, mm\(^{-1}\)) was measured using micro-CT analysis. It shows the amount of unresorbed granules after follow-up. Median (quartiles, min and max values) is shown in box-plot figure. ´*´ \(p < 0.05\) vs. autograft, DBM and untreated, ´´*´ \(p < 0.05\) vs. autograft, DBM, untreated or the groups with the reindeer bone extract.

Table 7. Trabecular thickness (Tb.Th), open porosity (Po(op)), and closed porosity (Po(cl)) were measured from the defect areas in imaging analysis of micro-CT results. Mean and median are given. Values of \(p < 0.05\) are considered statistically significant.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Tb.Th (mm) median (quartiles)</th>
<th>Po(op) (%) mean (std. dev)</th>
<th>Po(cl) (%) median (quartiles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone protein extract and (\beta)-TCP granules in PEG-GLY matrix</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paste 1</td>
<td>8</td>
<td>0.16 (0.15, 0.17)(^{a,b,c})</td>
<td>60.60 (11.73)(^{a,b})</td>
<td>3.67 (1.37, 5.46)(^{a,b})</td>
</tr>
<tr>
<td>Paste 2</td>
<td>8</td>
<td>0.16 (0.15, 0.17)(^{a,c})</td>
<td>59.38 (11.27)(^{a,b})</td>
<td>4.93 (2.77, 5.90)(^{a,b,c})</td>
</tr>
<tr>
<td>Paste 4</td>
<td>7</td>
<td>0.15 (0.14, 0.17)(^{a,c})</td>
<td>61.46 (16.30)(^{a,b})</td>
<td>3.51 (1.77, 5.71)(^{a,b})</td>
</tr>
<tr>
<td>Paste 6</td>
<td>8</td>
<td>0.15 (0.14, 0.18)(^{a,c})</td>
<td>52.77 (6.37)(^{a,b})</td>
<td>9.09 (4.84, 10.93)(^{c})</td>
</tr>
<tr>
<td>(\beta)-TCP granules in PEG-GLY matrix</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paste 3</td>
<td>8</td>
<td>0.13 (0.12, 0.14)(^{a,c,f})</td>
<td>57.05 (18.61)(^{a,b})</td>
<td>2.95 (2.39, 4.45)(^{a,b})</td>
</tr>
<tr>
<td>Paste 5</td>
<td>8</td>
<td>0.12 (0.11, 0.15)(^{a,c,f})</td>
<td>62.47 (18.49)(^{a,b})</td>
<td>3.06 (1.76, 5.00)(^{a,b})</td>
</tr>
<tr>
<td>(\beta)-TCP granules</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granule</td>
<td>7</td>
<td>0.12 (0.11, 0.13)(^{a,b,c,d,g})</td>
<td>56.00 (7.54)(^{a,b})</td>
<td>4.14 (2.70, 4.33)(^{a,b,c})</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autograft</td>
<td>8</td>
<td>0.18 (0.17, 0.18)</td>
<td>50.78 (10.80)(^{a,b})</td>
<td>2.04 (1.19, 3.33)(^{a,b})</td>
</tr>
<tr>
<td>DBM</td>
<td>7</td>
<td>0.18 (0.17, 0.18)</td>
<td>89.75 (5.20)</td>
<td>0.54 (0.33, 0.85)</td>
</tr>
<tr>
<td>Untreated</td>
<td>8</td>
<td>0.20 (0.17, 0.22)</td>
<td>79.40 (8.28)</td>
<td>0.75 (0.32, 1.25)</td>
</tr>
</tbody>
</table>

\(^{a}\) \(p < 0.05\) vs. DBM. \(^{b}\) \(p < 0.05\) vs. Untreated, \(^{c}\) \(p < 0.05\) vs. Autograft, \(^{d}\) \(p < 0.05\) vs. Paste 3, \(^{e}\) \(p < 0.05\) vs. all other groups, \(^{f}\) \(p < 0.05\) vs. groups including the reindeer bone extract.
6 Discussion

6.1 Time and dose-dependent effects of the reindeer bone protein extract

The current results confirm previous results, which showed that the reindeer bone extract has good osteoconductivity both in the mouse muscle pouch model and the rabbit radius defect model (Pekkarinen et al. 2003, 2005c,b, 2006). It is known that animal and fracture models, delivery systems and route of administration influence the dose response in the bone healing studies (Nunamaker 1998). Above a certain threshold, bone formation cannot be further enhanced, as was seen in this study. The minimally effective doses of the reindeer bone extract appear to be between 2 mg and 5 mg in this study and with an organic scaffold model. Pekkarinen et al. (2006) used 5 mg and 10 mg of non-sterilized reindeer bone extract in the rabbit radius. These amounts healed the defect much better than the controls after six weeks of follow-up in the study with collagen carrier and after eight weeks in the study with bioactive glass carrier. The 10 mg bone extract group had significantly higher mean stiffness than the collagen group (Pekkarinen et al. 2006).

New bone formation was seen in all specimens containing the reindeer bone extract or rhBMP, but the most abundant bone formation and bone union was seen in the specimens with 15 mg to 50 mg extract. Furthermore, the area of healed new bone at the defect site increased at the doses of 15 mg of the extract or more, which might support the use of the higher doses. On the other hand, in studies III and IV, the formulated bone protein extract together with inorganic carrier gave good bone formation with extract doses of 3 mg or lower. In study I, a larger collagen sponge carrier was used with a higher amount of extract, and the concentration was different compared to lower amounts. Perhaps the larger sponge remained longer in the defect and gave better support for growth factors and proteins to improve the bone-healing cascade. The bone protein extract together with bioactive glass granules was moulded to the same implant size. The concentration was thus greater and bone healing results were better with the higher doses. This result was ensured with the ectopic bone formation model. When bone proteins were not very well absorbed in the carrier or if the carrier with extract had broken, new bone formation was greater. Thus, a minimum effective dosage of extract is dependent on optimization of the extract.
formulation, carrier material and form of implant. The greatest bone formation occurred with the formulations that had the bone protein extract readily available. This indicates that bone forming factors are required in sufficient concentrations at the early stage.

More than twenty BMPs have now been described, and about ten of them have functions in different phases of bone healing (Wozney & Rosen 1998, Cheng et al. 2003). In study I, rhBMP-2 was included in the comparisons because the bone healing capacity of rhBMP-2 has been well documented and it has been approved by the EU and FDA for lower back spinal fusion and tibial fractures in clinical use (Govender et al. 2002, Burkus et al. 2002, Einhorn et al. 2003, Kim et al. 2005, Seeherman et al. 2006, Chu et al. 2007, McKay et al. 2007, Issa et al. 2008). The bone formation capacity of rhBMP-2 in the rat femur segmental defect model was superior compared to the negative control groups and to the reindeer bone extract groups. However, in most cases of bone treated by 30 µg rhBMP-2, the dose was too high, causing new bone area to overgrow. The optimal effective dosage of rhBMP-2 is difficult to find because the amounts of rhBMP-2 used in rat studies with collagen as a carrier have ranged from 0.5 µg to 200 µg (Yasko et al. 1992, Fujimura et al. 1995, King et al. 1998, Hyun et al. 2005, Kim et al. 2005). The used doses of bone extracts are milligrams compared to recombinant products that are in micrograms. This is because the bone extracts and DBM products involve a wide spectrum of all bone proteins and collagens while recombinants have only one specific protein with a specific task in bone regeneration (Sampath & Reddi 1983, Baas et al. 2006). Therefore, DBM products are closer reference material for reindeer-derived extract than rhBMPs (Huffer et al. 2007).

The resorption rate of carrier material is a factor that is important to consider not only in planning a follow-up of an experimental study but also in choosing clinical treatment (Van der Stok et al. 2011). Resorption times of synthetic bone grafts vary from weeks to years (Van der Stok et al. 2011). Calcium sulphate has the shortest reabsorption time (weeks) and bioactive glass the longest (years) (Moore et al. 2001). The resorption rate of calcium phosphate is typically between 6 and 24 months (Bohner 2001, Ioku 2010). In study II, we decided to extend the follow-up time of the Bioactive glass + 40 mg extract group from 8 to 10 weeks to assess longer-term healing. The results of bone formation and bone union in this group were significantly better after 10 weeks compared with all the other groups after 8 weeks of follow-up. However, there was plenty of bioactive glass material remaining even after 10 weeks’ follow-up when granule sizes of
500-800 µm were used. It is known that bioglass is radiopaque and can have influence on the pQCT results, the density of bioactive glass being slightly higher than that of bone (Griffon et al. 2001). This may explain why there were no statistically significant differences in bone density between any of the implant groups, although the bone extract groups displayed more bone formation than the bioactive glass group (which displayed the highest density value). Thus, bioactive glass may overcome the density of new bone. On the other hand, plain radiographs showed signs of increased dissolution of bioactive glass in the bone extract groups, which could decrease the density values.

6.2 Experimental models

For more than twenty years, our research group has been using the muscle pouch model as a standard assay in evaluating the bone formation activity of reindeer bone protein extract (Jortikka et al. 1993a, Pekkarinen et al. 2003). Furthermore, our study group has experience of sheep long bone defect model and both dog and rabbit ulnar defect models (Gao et al. 1996, 1997, Tuominen et al. 2000, 2001, Pekkarinen et al. 2006). The rat femur defect model is also widely used in the literature (Takagi & Urist 1982, Ohgushi et al. 1989, Yasko et al. 1992, Stevenson et al. 1994, Wolff et al. 1994, Hunt et al. 1996, Chen et al. 2002, Vögelin et al. 2005). Thus, it was a surprise that the effect of reindeer bone extract in the rat femur defect model was not as good as in the rabbit ulnar defect model owing to differences in animal species and experimental design (Pekkarinen et al. 2006). Nienhuijs et al. 2011 reported about same problem with their product (Collos® E) when they used a rat subcutaneous implantation model (Nienhuijs et al. 2011). It could imply that animal-derived extracts can be immunogenic in the rat model (Sampath & Reddi 1983, Viljanen & Lindholm 1993, Bessho et al. 1999). In some studies nude rats have been used to obviate problems of immunogenic reactions to bone protein extracts (Ripamonti et al. 1991, Edwards et al. 1998, Bomback et al. 2004, Wang et al. 2007, Qiu et al. 2009, Lee et al. 2011). It was also surprising that DBM did not work in study IV although there were no infections or other problems with the sheep. It could be that sheep as a model is not suitable for Grafton® or other human DBM products compared to DBM products of animal origin, such as Collos®, which has given superior healing results in sheep and dog models (Huffer et al. 2007, Baas et al. 2008). However, the Grafton® product had also been tested in the muscle pouch model of our study group, but no signs of bone formation were seen within 21 days either
roentgenographically or histologically (data not shown). Furthermore, there are some studies in the literature in which bone healing differences have been found between different commercially available DBM products (Peterson et al. 2004, Wildemann et al. 2007, Wang et al. 2007, Athanasiou et al. 2010). There are not many animal-derived products that used in the clinical trials but not also reported adverse effects (Bai et al. 1996, Kujala et al. 2004, 2008).

6.3 From carrier of the reindeer bone protein extract to an ideal implant

Ideally, bone formation and scaffold degradation follow one another until the defect area has been completely replaced by new bone. As long as bone formation is not extensive enough to supply mechanical strength, the scaffold material should degrade so slowly that support characteristics are not exposed (Kroese-Deutman et al. 2005). Reindeer bone protein extract has high bone formation activity, as seen in bioactivity and previous tests (Jortikka et al. 1993b, Pekkarinen et al. 2003, Ulmanen et al. 2005); however, in a real bone-healing situation, the extract cannot work without a scaffold system. Pure collagen as carrier has been tested in some of our previous studies (Pekkarinen et al. 2004, 2005b,c, 2006) and in study I. Lyophilized extract was mixed in water and pipetted onto the collagen sponge, or the collagen sponge, soaked in water and then in extract, was bundled up to form an implant. The results showed good bone formation in the mouse pouch model and also in the segmental defect model. However, it seems that collagen alone is not an adequate scaffold and carrier system for bone proteins. Kim et al. 2005 found in their study that β-TCP with rhBMP-2 was more effective in ectopic bone formation than collagen with rhBMP-2. They supposed that β-TCP provided more space and support for bone formation (Kim et al. 2005). Thus, an inorganic scaffold would be a better choice to support bone healing with extract as well.

Study II showed for the first time that bioactive glass is a candidate carrier for the reindeer bone protein extract when used in a rat femur defect model. The results suggest that the bone extract enhances the healing of bone, compared with bioactive glass alone. Although bioactive glass is used alone in bone surgery, its healing effect is increased when it is combined with bone extract or BMP products (Kotani et al. 1992, Pajamäki et al. 1993a,b, Mahmood et al. 2001, Takita et al. 2004, Välimäki et al. 2005, Bergeron et al. 2007).
Granulometry is influenced by the biological properties of bioactive glass (Wheeler et al. 1998, Oonishi et al. 1999, Mahmood et al. 2001, Granito et al. 2009). The size of the bioactive glass granules used in this study was 500-800 μm, which appeared to be a good size because the granules could be impacted firmly in the bone defect and because granules of this size are not resorbed too quickly. In our preliminary bioactive glass study (results not shown) we used smaller bioactive glass granule size (< 300 μm), but after 3 weeks the granules had resorbed, and no bone formation could be seen even after 8 weeks. Different ratios of the compounds of bioactive glass granules could have been used to adjust the degradation process, allowing more time for osteoconduction and a larger pore size would also have yielded slower resorption of the bioactive glass; thus pore sizes > 300 μm are recommended (Wheeler 1998, Hall et al. 1999, Lindfors & Aho 2003, Karageorgiou & Kaplan 2005, Granito et al. 2009).

6.4 Calcium salts as potential carrier candidates

Study III was aimed to find a suitable inorganic carrier candidate for the reindeer bone protein extract. The lyophilized extract was absorbed into pores in the β-TCP/CS 90:10 group, partly surface-coated and partly absorbed in the HA/β-TCP/CS 30:60:10 group, surface-coated in the HA/β-TCP/CS 60:30:10 group, blended into the carrier material in the CS hemihydrate and the Cem-Ostetic groups, and dry-blended without re-suspending the lyophilized extract in the CS dihydrate-stearic acid group. Because the combination of the lyophilized formulation and the carrier was different in each of the study groups, the distribution and availability of the extract was also different and statistical comparison between carrier groups is not very valid. The greatest bone formation was seen in the groups that had the bone extract readily available, which indicates that the bone forming factors are needed at the early stage at sufficient concentration. This was seen especially in the groups HA/β-TCP/CS 60:30:10 and CS dihydrate-stearic acid. In the groups β-TCP/CS 90:10, Cem-Ostetic putty and CS hemihydrate a difference was seen between active implants and controls, and they worked as implant with bone protein mixture coating. The lowest bone formation was found in the group HA/β-TCP/CS 30:60:10. This may indicate that the bone extract was absorbed deep into the scaffold during implant preparation, and the releasing amount of bone proteins was too low to induce new bone formation. Our results support previous studies that formation of new bone depends on a ceramic content with high HA/TCP ratio and high dose of bone.
proteins (Alam et al. 2001, Baas et al. 2008). Furthermore, this study confirms that the presence of bioactive components reduces fibrous tissue formation and increases bone formation around inorganic scaffolds (Wolff et al. 1994, Chen et al. 2007, Baas et al. 2008, Ioku 2010). However, the amount and availability of bone proteins should be in balance with the bone healing and forming cascade (Dimitriou et al. 2005).

In study III, it was found that stearic acid had a positive effect, enhancing bone ingrowth and formation in the environment of calcium sulphate carrier. Stearic acid has been widely used as excipient in tablet manufacturing because adding stearic acid decreases the viscosity of ceramic suspension while increasing the microstructural uniformity of particle packing (Tseng et al. 1999). Wright Medical Technology Inc. has used stearic acid as a tableting aid in their calcium sulphate products, such as Osteoset®, and noticed good bone healing capacity, as we found in this study (Urban et al. 2003, Turner et al. 2003). Therefore, we think that calcium stearate has not only tableting aid properties but also supports bone formation, like carboxymethyl cellulose does (Reynolds et al. 2007).

Study IV supports earlier findings showing that the presence of bioactive components reduces fibrous tissue formation and increases bone formation around inorganic scaffolds (Baas et al. 2008). The implantation in study IV was easy to perform and no extra mixing of product was needed on the operation table, which is a recommendable criterion for implants in trauma surgery. However, the analysis showed that the amount of granules used was not enough to fill the whole defect area after the matrix was dissolved, so that the cortical site of the defect was usually empty without new bone or traces of granules. This can have deleterious effects on bone healing (Lindfors et al. 2010). This was compared to the pure β-TCP group in which double the amount of granules was used and the whole defect area was full of granules after the follow-up as well. Thus, the effect of bone healing cannot be compared only by the difference in bone formation between the study groups; optimizing of the PEG-GLY matrix with stearic acid and granules is needed.

Ideally, bone formation and scaffold degradation follow one another until the defect area has been completely replaced by new bone. As long as bone formation is not extensive enough to supply mechanical strength, the scaffold material should degrade so slowly that support characteristics are not exposed (Kroese-Deutman et al. 2006). Evaluating of micro-CT images showed that granule resorption was the fastest in the group with the bone protein extract, but there was also good new bone formation seen and bone remodelling was ongoing. The
resorption of granules was not observed in the group that involved only granules or granules with PEG-GLY matrix and stearic acid. Bone formation was seen, but usually only around the granules. Analysis of micro-CT images confirmed the evaluation. The highest bone surface to bone volume ratio value but the smallest trabecular thickness was seen in the control groups with no reindeer bone protein extract. This might mean that the granules block normal bone formation or that the granules give enough load. Therefore, thicker new bone lamellas are not needed before the granules are resorbed in the defect. However, the follow-up time used was too short to confirm these consequences. Traditional micro-CT analysis is not the most optimal analysis method to show details in bone healing because of $\beta$-TCP granule remnants in the defects. However, all results of micro-CT supported each other. In future, histology and scanned electron microscopy (SEM) imaging could confirm our micro-CT results.

It has been found that the form, shape and micro- and nanostructures of the scaffold affect both bone formation and scaffold resorption properties (Li et al. 2003, Zyman et al. 2008, Roohani-Esfahani et al. 2010). In the group involving morsel form of $\beta$-TCP and the bone protein extract, the granules had resorbed during the follow-up. New bone formation and bone union was seen and the healing of the defect was very similar with that of autograft. The highest bone volume was seen in the group that involved a smaller and spherical $\beta$-TCP granule form. However, the measured closed porosity was also greatest in this group, while open porosity was almost lowest. Thus, the quality of new bone may not be similar to the quality of old bone in this group. This was also seen in trabecular thickness. Autograft, DBM and even empty defects had the thickest new trabeculars. The groups that had been treated with the reindeer bone protein extract had very similar trabecular thickness. The smallest thickness was seen in the groups with granules with no bone extract. This means that synthetic osteoconductive scaffold provides adequate support to new bone formation in the injury site, but lack of biological activity slows down resorption of granules, and new bone lamellae have no space to increase their thickness (Moore et al. 2001, Kim et al. 2005). Autograft, DBM and reindeer-derived bone protein extract have osteoinductive stimuli that improve the bone-healing cascade, increase carrier resorption and form space for new bone in the defect.

In study IV, one aim was to compare ceramic implants containing the reindeer bone protein extract with autograft. Micro-CT evaluation showed that the results of new bone formation and trabecular thickness were similar in the autograft group and the groups with the reindeer bone extract. This was an encouraging
result in terms of finding a substitute for autograft use. Previous results with DBM materials support our results (Huffer et al. 2007). Although the paste form implant containing the reindeer bone protein extract with β-TCP-carrier needs still minor optimization, clinical application has been reached and studies on the reindeer bone protein extract can be focused on clinical trials.
7 Conclusions

The present study indicates that the tested reindeer bone protein extract can be used to improve bone formation with various carriers. The study suggests that an inorganic carrier material together with stearic acid is the one of most suitable carrier alternatives for the reindeer bone protein extract. The developed medical device in a paste form can be an alternative for autograft use. Based on the current findings it can be concluded that

- Reindeer bone extract improved bone formation in critical-size defect model in a six-week follow-up. The needed dose of bone protein extract depends on the formulation and carrier systems and the trauma being treated.
- Bioactive glass used in this study can be used as a carrier for reindeer protein extract. The long-term stability of the selected form of bioactive glass can interfere with results.
- The greatest bone formation and carrier resorption were seen in the groups that had the bone extract readily available near the surface of the implant. The combination of β-TCP or CS and stearic acid appears to be the most ideal carrier alternative for reindeer bone protein extract.
- β-TCP granules in the PEG-GLY matrix with stearic acid provide a functional scaffold system for reindeer bone protein extract, but the proportional amount of granules in the matrix must be still optimized. Bone formation effect was significantly better with the reindeer-derived bone extract than with a commercially available DBM product. The tested medical device containing the reindeer bone protein extract and β-TCP formulation can be a suitable alternative for autograft use.
References


Original articles


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<td></td>
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<tr>
<td>1116</td>
<td>TP53 as clinical marker in head and neck cancer</td>
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<tr>
<td>1117</td>
<td>Magnetic resonance imaging-guided percutaneous abdominal interventions</td>
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<tr>
<td>1118</td>
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<td>autonomic dysregulation in subjects with chronic epilepsy</td>
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<tr>
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<tr>
<td>1120</td>
<td>Vesicoureteral reflux in children</td>
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<td>2011</td>
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<tr>
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<td>&amp; Enb24 in polarity of kidney epithelium</td>
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<tr>
<td>1122</td>
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<td>human tissues</td>
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<tr>
<td>1123</td>
<td>Genetic background of HDL-cholesterol and atherosclerosis : Linkage</td>
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<td>2011</td>
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<td>and case-control studies in the Northern Finnish population</td>
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<tr>
<td>1124</td>
<td>Relationship of physical activity, unacylated ghrelin and gene</td>
<td>Cederberg, Henna</td>
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<td>variation with changes in cardiovascular risk factors during military</td>
<td></td>
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<tr>
<td>1125</td>
<td>Paternal age, psychosis, and mortality : The Northern Finland</td>
<td>Miller, Brian</td>
<td>2011</td>
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<td>1966 Birth Cohort, Helsinki 1951–1960 Schizophrenia Cohort, and</td>
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<td>Finnish Nonaffective Psychosis Cohort</td>
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<tr>
<td>1126</td>
<td>Changes in adolescents’ oral health-related knowledge, attitudes</td>
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<tr>
<td>1127</td>
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<td>element models to predict fractures</td>
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<tr>
<td>1129</td>
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<td>Moilanen, Kristina</td>
<td>2011</td>
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<tr>
<td></td>
<td>1966 Birth Cohort Study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1130</td>
<td>Low-dose aspirin therapy in IVF and ICSI patients</td>
<td>Haapsamo, Mervi</td>
<td>2011</td>
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