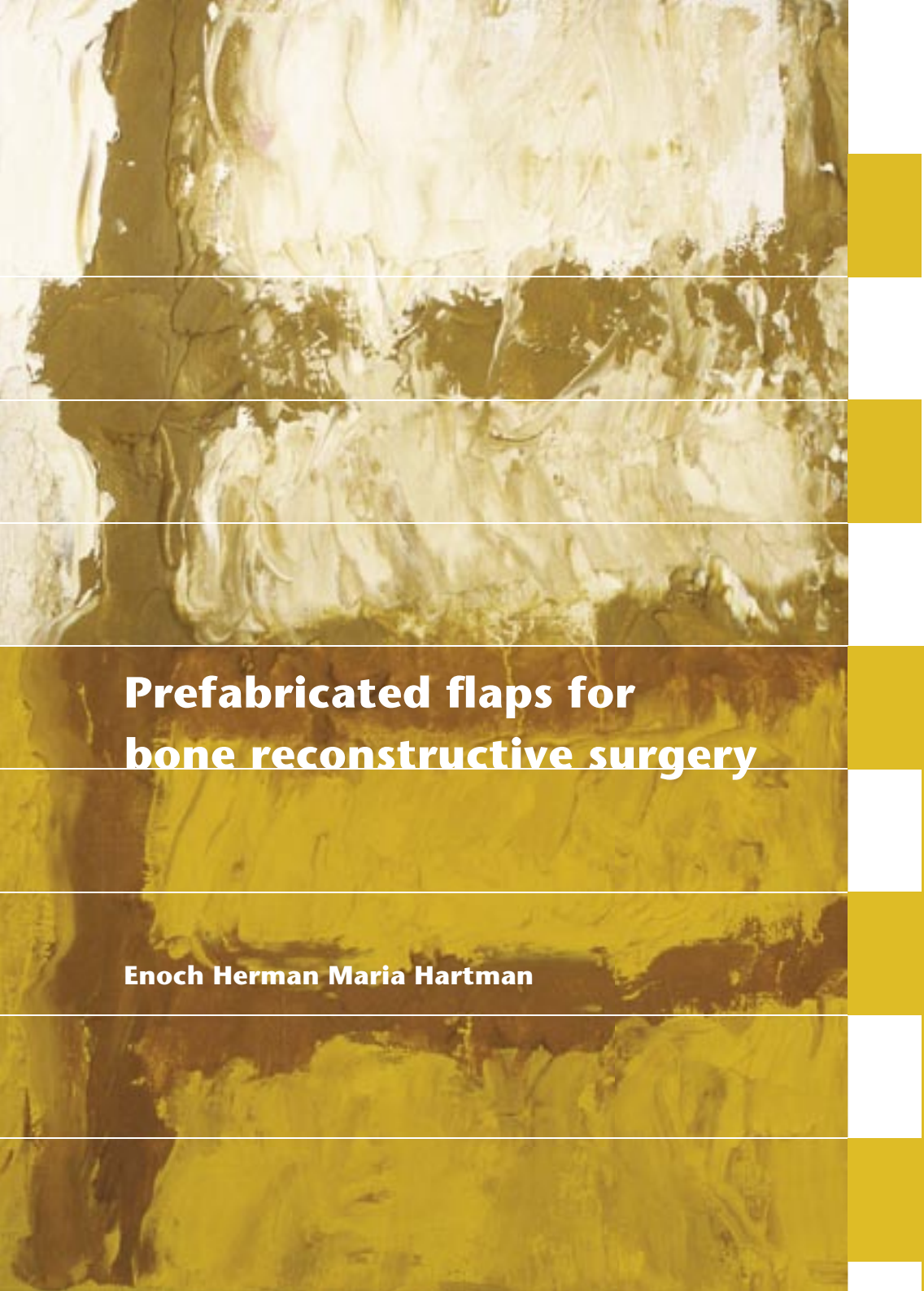

Prefabricated flaps for bone reconstructive surgery

Enoch Herman Maria Hartman

Prefabricated flaps for bone reconstructive surgery,
a scientific proof in the field of Medical Sciences

Thesis

submitted to fulfil the requirements
of the Ph.D. Degree in Medical Sciences
on the authority of the rector magnificus
prof. dr. C.W.P.M. Blom
of the Radboud University Nijmegen,
according to the decision of the Board of Deans
to be defended in public
on Thursday October 7, 2004 at 1.30 p.m.

by

Enoch Herman Maria Hartman

born February 6, 1959 in Amsterdam

Prefabricated flaps for bone reconstructive surgery

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Prefabricated flaps for bone reconstructive surgery

Thesis Radboud Universiteit Nijmegen, the Netherlands
with summary in Dutch - 176 p. © 2004

ISBN 90-9018433-3

Cover illustration 'die Leiter', 17-1-1992, 30x24 cm,
used by kind permission of Armando

Graphic Design Arjan Biel grafisch ontwerp, www.helderontwerp.nl

Print drukkerij Zalsman Kampen, www.zalsman.nl

Paper cover: Curious Touch, 300 gr/m^2, ArjoWiggins
pages: Hello Silk, 160 gr/m^2, BuhrmannUbbens

Fonts ITC Stone Serif and ITC Stone Sans

Zij die de behoefte voelen om de hoger gelegen delen van de stam te beroeren, zijn op de vingers van een hand te tellen. Voor hen is er, onder voorbehoud, een ladder beschikbaar.

Armando

'De Ladder'

uitgave Amersfoortse Culturele Raad / De Zonnehof (1994), pagina 29

met dank aan mevrouw A.S. de Meijere

Prefabricated flaps for bone reconstructive surgery

Een wetenschappelijke proeve op het gebied
van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de Rector Magnificus prof. dr. C.W.P.M. Blom,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op
donderdag 7 oktober 2004
des namiddags om 1.30 uur precies

door

Enoch Herman Maria Hartman

geboren op 6 februari 1959 te Amsterdam

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Introduction / review of donor site complications in vascularised bone flap surgery

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Journal of Investigative Surgery, 2002; 15(4): 185-197

Introduction

In general a high morbidity is associated with ablative surgery for cancer in the head and neck. The aim of reconstructive surgery is restoration of function and form, thereby allowing aggressive oncological resections. Reconstructions in head and neck surgery are performed by tissue transfer. Local flaps have been used extensively but are limited in their application. Microvascular free flaps give the opportunity to transfer a wide variety of flaps, which can be tailor-made to the missing tissue to be reconstructed. Almost every defect can thus be treated in one operation, using microvascular composite flaps. One of the major challenges in head and neck reconstructive surgery is reconstruction of the mandible. Reconstructions with non-vascularised bone grafts have been used in the past mainly as secondary reconstructions. Primary reconstruction with free bone grafts is difficult to achieve because of a high risk of infection and leads to a high failure rate. The non-vascularized bone has little resistance against bacterial invasion. Microsurgically revascularised bone flaps have sufficient vascularity, which produces higher success rates. Using microvascular flaps, a revolution in reconstructive surgery has been established and this new and powerful method of reconstruction even makes reconstructions in radiated patients possible[1].

Unfortunately, the inevitable consequence of these reconstructions is donor site morbidity. In contrast to the abundance of articles describing the advantages of vascularised bone flap reconstructions, little attention has been paid to donor site morbidity. Our study of the literature on donor site morbidity in vascularised bone flap surgery serves as the basis of this article, in which we will, 1) present a short introduction of free flap surgery in head and neck reconstructions in general and in mandibular reconstruction in particular, 2) discuss specific flaps for bone reconstruction, and 3) review the donor site morbidity of these bone flaps. Further, we will take a look at the future when tissue engineering may minimize donor site morbidity by creating prefabricated free flaps[1].

Free flap surgery in head and neck reconstructions

Shan R. Baker and William R. Panje at the University of Iowa College of Medicine performed the first successful reconstruction of the oral cavity with a microsurgical skin flap[2]. Since then a wealth of microvascular free flaps has been used: myocutaneous, osteocutaneus, and osteomusculocutaneous flaps as well as portions of the stomach, jejunum, and large bowel have been applied for a wide variety of defects in the head and neck region[3, 4].

Oral cavity reconstruction

Defects of the oral cavity can be analyzed as to their component parts: deficits of internal lining, loss of skeletal support (mandible or maxilla), and defects of external soft tissue or any combination of these[5]. For intra-oral lining the radial forearm flap is the flap of choice. This is a thin and pliable flap, which is highly reliable. As an alternative, the lateral upper arm flap is used[6]. By anastomosing the lateral cutaneous nerve, it is possible to provide sensation to the radial forearm flap, used for oral reconstruction[7, 8].

Hypopharyngeal reconstruction

The aim of hypopharyngeal reconstruction is to obtain a successful conduit and to allow swallowing. Hypopharyngeal reconstruction for partial circumferential defects can be achieved by a patch graft of the radial forearm flap. In case of a complete circumferential defect, the hypopharynx can be reconstructed by a tubed radial forearm flap[9] or a free jejunal graft[10]. Fistula formation remains a common, but usually self-limiting, complication.

Skull base surgery

The resection of tumors of the infratemporal fossa extending to the skull base is now accessible by an intracranial-extracranial approach. Resection of these tumors often involves a sacrifice of the nasopharyngeal and paranasal sinus structures. The resultant defects place the cranial cavity

in communication with the oral cavity. An essential requirement of the reconstructive procedure in this situation is to ensure a reliable separation of these two areas to prevent meningitis. The creation of a functional separation of intracranial and extracranial cavities can be extremely difficult to accomplish, especially when multiple cavities (nasal, oral, and pharyngeal) are violated. In select cases free flaps provide a solution to this problem[11, 12].

Facial palsy

Facial palsy can be a congenital[13] or an acquired palsy of the mimic musculature. Many reconstruction methods have been used, ranging from static reconstructions with tendon slings where only symmetry at rest is obtained, to dynamic reconstructions with transposition or transplantation of functional muscle. Emphasis has been placed on the latter: dynamic reconstruction for restoration of active smiling[14]. For this purpose different functional free muscle transfers are used, like the latissimus dorsi[15], the pectoralis minor[16-18], the gracilis[19, 20] and the rectus femoris[21].

Mandibular reconstruction

The goals of mandibular reconstruction are to reconstitute the mandibular arch and to allow for prosthetic restoration. Conventional bone-grafting techniques have been applied, but the widespread use of radiotherapy in head and neck cancer patients limits its usefulness. In vascularised bone reconstructions, healing by callus replaces the creeping substitution of conventional bone grafts[22]. In addition, the vascularity of the attached soft tissues promotes primary healing and decreases the chance of fistula and sinus formation. When recipient beds are hostile due to irradiation, scarring, or chronic infection, these flaps can survive and even improve the blood supply of the sites into which they are placed by their vascular independence. Large segments of bone can be transferred with the same success rate as smaller ones because of their autonomy. Furthermore, free osteocutaneous transfer is achieved in a single stage. This permits the patient to resume his or her regular social activities in the shortest possible time. The immediate restoration of appearance and function and absence of

long-term sequelae are particularly important in the older patient whose life span is limited because of age, general health, or the disease process itself. In these patients, preservation of the quality of life is paramount even if survival is barely improved. In case of younger patients, the reliability and durability of vascularised bone grafts and their ability to accomodate oral implants for oral rehabilitation make them the procedures of choice, even when the host bed is not hostile[23].

Microvascular free flaps can provide radiotherapy resistant reconstructions, while at the same time providing oral lining or external lining, or both. A variety of osteo (musculo-) cutaneous flaps is applicable. The most often used are the iliac crest or deep circumflex iliac artery flap, fibula flap, scapular flap and radial forearm flap (fig. 1). The donor site morbidity of these free bone flaps will be discussed.

Figure 1. This is a combination of a osteocutaneous fibular flap and a fasciocutaneous radial forearm flap to treat a large defect of the mandible and soft tissues. The fibula has already been osteotomized in shape and linked to the radial forearm flap. The donor defect is extensive.

Donor site morbidity in free bone flaps

Radial forearm free flap

Introduction

The radial forearm flap was introduced in the English literature by Song in 1982[24] but originally developed in the Shenyang Military Hospital in China by Yang et al[25, 26]. This popular free flap is based on the radial artery and its concomitant veins or subcutaneous forearm veins. It can be used as a fasciocutaneous flap; vascularised tendons, nerves[7], and bone[27] may be included. Vascularised bone as a component of this flap can be removed

from the distal lateral portion of the radius extending from the insertion of the pronator teres tendon to the insertion of the brachioradialis tendon. This bone is supplied by perforating branches of the radial artery and venae comitantes by means of direct fascio-osseous perforators proximally and small perforators through the flexor pollicis longus muscle distally. Approximately 10 to 12 cm of vascularised radius may be elevated in this fashion[28]. Usually a split skin graft[26] or a full thickness graft[29, 30] is used to close the resulting skin donor site defect. Specific donor site complications comprise: fracture of the radius, delayed wound healing, cold intolerance, neuroma formation of the superficial radial nerve, impaired strength of the donor hand, and restricted wrist mobility.

Delayed wound healing

The most frequent problem is delayed wound healing of the grafted donor site. This is observed in approximately 30 to 50 percent of cases[31]. Although most donor wounds eventually heal, this sometimes requires more than 6 months. Especially in the group of patients with an osteocutaneous flap this is a great problem[32]. After delayed healing, adhesions of skin to the flexor tendons occur. To prevent these adhesions, the tendons can be buried under the adjacent flexor muscle bellies[33-35]. In this way a better wound bed for skin grafting is obtained. Another solution is to prevent a skin graft by using tissue-expansion[36], a pursestring closure[37], or a skin stretching device[38, 39]. In small flaps, the donor site may be closed by a pedicled ulnar forearm flap[32, 40]; then no adhesions will occur[32]. To cover the osteotomized radius the pronator quadratus muscle with a split skin graft can be used[41]. Although the donor defect is visible and unsightly, only few patients complain about this cosmetic problem[42].

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Radial forearm flap

In the forearm the radial artery and comitant veins supply perforating branches to the skin via the intermuscular septum. Other perforating branches that travel through the muscle belly of the flexor pollicis longus muscle, supply vascularity to the radial bone. Due to this anatomy a flap consisting of the skin, subcutis of the volar side of the forearm and part of the radial bone, can be transplanted to any part of the body, provided this pedicle, consisting of the radial artery and comitant veins is included. The pedicle can be anastomosed by microsurgical techniques to vessels in any part of the body. These so called 'free flaps' give the reconstructive surgeon the opportunity to use distant tissue for reconstructions in difficult area's, e.g. radiated tissue.

Cold intolerance

Cold intolerance can develop after accidental as well as surgical trauma to the hand. It has been described after replantation of digits and after repair of nerve injury[43]. The incidence of cold intolerance ranges from 0 to 27 percent[44]. Little is known of the cause of this intolerance. One study in which ultrasound was used, reveals that there is no vascular basis[42]. Another retrospective study with thermography revealed that the width of the donor site influences the rewarming of the donor hand. This is probably due to a change in vasoregulation as a result of sympathetic nerve damage[45].
Out of concern of postoperative hand ischemia[46], the radial artery was routinely reconstructed by a vein graft in the past, but graft patency was a disappointing 50 percent. The unreconstructed cases showed no signs of alteration attributable to vascular insufficiency in function of the hand[32]. Therefore, routine reconstruction of the radial artery has been abandoned[47].

Neuroma formation

Absence of pinprick sensation is a common finding in grafted donor sites and an early finding in transposition flaps. Sensory recovery in transposition flaps usually occurs.
After harvesting a radial forearm flap, in a majority of the patients reduced sensation in the distribution of the superficial radial nerve occurs. In time, sensation often returns[48]. When raising the flap, the superficial radial nerve is easily identified and should be preserved intact. The presence of hyperesthetic areas has been reported in 3 percent of the cases, while neuromata in branches of the superficial radial nerve have been observed in 2-10 percent of the patients[32, 48].

Fracture of the radius and grip strength

Fracture of the radius is a serious complication of the composite (skin and bone) radial forearm flap. Fracture can occur at any time during or after surgery, but usually within 6 weeks of operation (fig. 2). Females are affected more than males, possibly because the radius is smaller and osteoporosis is more common in females. If the fracture is recognized immediately during the operation and treated properly, no loss of function will result.

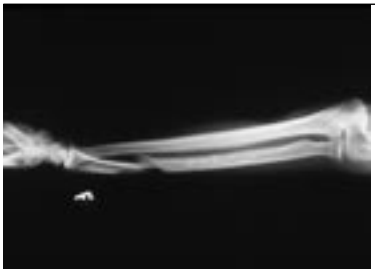

Figure 2. A radiograph of a fracture of the radius. This patient underwent a mandibular reconstruction by an osteocutaneous radial forearm flap. In the post operative period the patient fell, while riding horse-back.

Otherwise, loss of supination and of pinch power between the thumb and index finger will occur due to loss of flexor pollicis longus function. Several measures can be taken to prevent fracture of the radius: removal of a smaller bone segment (about one third the circumference of the radius); use of a boat-shaped osteotomy to prevent overcutting, as can occur in a traditional right angled osteotomy; adequate immobilization for 6 to 8 weeks; or even prophylactic plating of the radius[30, 38, 49]. Soutar and McGregor have stated in early reports utilizing this osseocutaneous vascularised flap in head and neck reconstruction that no more than 40 percent of the cross-sectional area of the radius should be removed in the clinical situation, stating that this should not jeopardize donor radius strength[50]. A one-third-thickness osteotomy results in a 76 percent decrease in breaking strength[31]. Despite limited bone removal, the current literature reports a donor site fracture rate of 8 to 43 percent[26, 32, 48,50-52].

Reduction in wrist mobility and grip strength are a complication of fracture of the radius. In a prospective study, small reductions in wrist mobility were noted in fasciocutaneous and composite no-fracture groups, but large reductions were noted in the composite fracture group. Reductions in flexion (58 percent), extension (50 percent), pronation (31 percent), supination (31 percent), and adduction (55 percent) were found48. Still, even without a fracture of the radius, grip strength may be diminished. The cause for this lies in the harvest of the flexor pollicis longus muscle in composite flaps. This muscle is needed to ensure perfusion of the bone. Surprisingly, few patients complain of symptoms relating to joint motion, and very few complain of any functional deficit. In fasciocutaneous flaps without bone harvesting, grip strength is 11.9 percent less than on the contralateral hand, but since the operated hand is usually the non-

dominant hand, this is not clinically significant[42, 53]. An unsuspected but significant correlation was found between sensory defect of the radial nerve and weak grip strength in the operated hand. Reduction in circumference reflects muscle atrophy. Remarkably, when this was measured, reduction in circumference was found on the control arm as well as on the donor arm. This means that at least part of the atrophy is due to systemic factors, and not to raising of the flap itself. In addition to this, Richardson reported more reduction in grip strength in the control arm than in the donor arm in composite flaps. No explanation for this phenomenon could be found[48]. As it is clear that patients with a fracture of the radius develop serious impairment of hand function, some authors recommend not to use this osteo-cutaneous flap anymore, and to use other donor sites.

Fibula free flap

Introduction

In 1975 Ian Taylor described for the first time the free vascularised fibula flap for reconstruction of the lower extremity[54]. Later, Hidalgo adapted this flap for mandibular reconstruction[55]. Since then, it has become one of the most popular flaps in mandibular reconstruction[56]. About 25 cm of bone can be harvested, while preserving 6 to 7 cm of bone both distally and proximally to maintain the integrity of the knee and ankle joints. The bone is perfused by segmental branches of the peroneal artery. A skin flap can be included in the dissection, based on perforators from the peroneal artery[57]. A composite flap can be obtained by including the soleus muscle or flexor hallucis longus muscle[58-60]. The fibula has numerous advantages to other bone flaps in mandibular reconstruction; i.e. virtually no length limitations, consistent bone shape, adequate bone height for oral implants, segmental blood supply and a flexible skin island. Most authors report a low rate of morbidity[61], although when objectively measured, some authors do find problems[62].

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Fibula flap

In the lower leg the peroneal artery and comitant veins supply perforating branches to the skin via the intermuscular septum. Other branches supply the fibula. Just as with the radial forearm flap, this fibula flap can be harvested as a free flap consisting of bone and skin, provided the peroneal vessels are included as the pedicle.

Delayed wound healing

Usually the donor site can be closed primarily, which leads to minor postoperative donor site morbidity: cellulitis and small wound breakdown[63]. If a skin graft is needed, more than 50 percent of the patients will experience delayed wound healing[64] (fig. 3). Still, if there is tension at the suture line, a skin graft can minimize the possibility of wound complications[65].

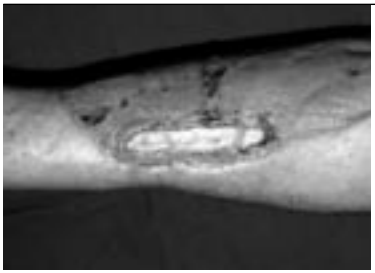

Figure 3. Delayed healing in a patient with a fibular free flap for a mandibular reconstruction. The donor site was skin grafted. The Achilles tendon is uncovered.

Nerve damage

The peroneal nerve is the nerve at risk in using the fibula flap. This includes sensory and motor disturbances. Sensory disturbances can occur in 24 percent of the cases[63]. Hypesthesia involves either the superficial or deep peroneal nerve sensory distribution. Motor disturbances lead to weakness in dorsiflexion, which can occur in 7 percent of cases due to damage of peroneal nerve branches[66]. All these nerve deficits were completely resolved within 3 months.

Ambulation

Although changes in gait, paresis of the flexors of the toes and hallux, and sensibility changes have been described, the majority of patients are able to ambulate normally. Some patients experience limitations with running. An average of 5.1 weeks after surgery, people are able to ambulate without pain, and only a minority reports mild occasional discomfort. Gait analysis shows only minimal joint motion effects in patients with follow-up times longer than 10 months, but muscle strengths are significantly impaired, especially

foot inversion and eversion. An inverse relationship existed between the length of resected fibula and muscle strength in ankle eversion[67]. The loss of the fibula and the interosseus membrane, which form the origin of the deep muscles, leads to muscle disturbance with consequent loss of function. This loss of function is greater if a musculo-osteo-cutaneous flap is used. Patients may complain of pain and inability to run. Pain and edema usually occur after prolonged standing or extensive ambulation. X-rays may reveal osteoporosis of the distal remnant of the fibula, which is likely the result of the decrease in load transmission. Longer segments are less osteoporotic because there is more available interosseous membrane to connect and stabilize the fibula to the tibia[68]. Occasional ankle instability and ankle stiffness is reported. Isokinetic testing reveals a significant decrease in ankle mobility and a significant decrease in strength during all four ankle motions. No decrease in knee mobility is seen, but there is a significant decrease in strength during knee flexion and extension. Almost all patients have limited flexion capability of the hallux consistent with inclusion of the flexor hallucis longus muscle with the flap. This does not interfere with ambulation, however[64]. The most likely explanation for the apparent discrepancy between the actual measured decreases in leg joint range of motion and muscle strength and the patient's perception is that the leg and ankle have a large physiologic reserve, which is only partially compromised by fibula flap harvest. Pre- and early post operative physiotherapy are recommended, emphasizing the maintenance of ankle range of motion. When the integrity of the ankle joint in adults is in doubt, fixation of the distal fibula just proximal to the syndesmosis by means of a screw is indicated[69]. In children distal tibio-fibular arthrodesis is always advocated to avoid elevation of the lateral malleolus and valgus deformity of the ankle[70].

Iliac crest free flap

Introduction

The iliac crest flap is based on the deep circumflex iliac vessels[71, 72]. These vessels originate from the external iliac vessels, after which they travel on the deep surface of the inguinal ligament, give off a branch to the internal oblique muscle, and then lie in the groove between the attachment of the iliac and transverse abdominal muscles. From here, perforators go through

the abdominal muscles to supply the skin. The deep circumflex iliac vessels anastomose posteriorly with branches from the lumbar vessels[73, 74]. This flap can be harvested as bone only (bicortical or monocortical[75]), or in combination with skin and/or external oblique muscle[76]. The donor site of the skin can be closed primarily, which leaves a scar that can easily be hidden under the clothes[23]. Postoperatively, patients are encouraged to walk within the first week, usually walking unassisted after 2 weeks.

Nerve damage

Advantages of this large bone flap are more highlighted than the donor site complications[77, 78]. If the lateral femoral nerve is transected, the sensation of the lateral side of the thigh is impaired[79]. Injury to the lateral cutaneous and ilioinguinal nerves can occur, and thigh pain and anesthesia are the unpleasant postoperative sequelae.

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Iliac crest flap

The iliac crest is another osteocutaneous free flap, supplied by the Deep Circumflex Iliac Artery. The external iliac artery gives off this branch just above the inguinal canal. The DCIA runs at the inside of the pelvis, between the insertion of the iliac muscle and the origin of the internal oblique muscle of the abdominal wall at the iliac crest. An ascending branch of the DCIA supplies this internal oblique muscle. Multiple branches from the DCIA perforate the abdominal wall, just above the iliac crest, and supply the overlying skin. With this pedicle bone, muscle and skin can be harvested in one flap.

Contour and herniation

Removal of bicortical bone produces significant donor site deformity (fig. 4) and asymmetry. Herniation of the abdomen is a risk, which can be worsened by denervation of the rectus muscle. Meticulous dissection of the nerves, which lie between the internal oblique and transverse abdominal muscles, can prevent this denervation[80]. Sometimes a mesh repair is necessary. Herniations occur from 3[23] to 9 percent[81].

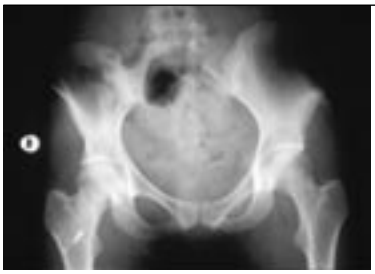

Figure 4. Left iliac crest was used as a donor site for mandibular reconstruction.

Ambulation

Commonly, one finds an initial weakness of the hip, resulting in an antalgic limp. Usually, but not always[23], this resolves by 6 months. Sometimes a paresis of the femoral nerve occurs, with subsequent loss of knee extension, but in the majority of cases this is not permanent. In 5 percent of the patients, permanent loss is caused by either excessively vigorous retraction at the time of surgery or pressure from a tight closure[23].

In conclusion, the iliac crest free flap offers abundant bone and has the possibility of including skin and muscle. Some serious problems in walking can occur.

Scapular free flap

Introduction

The subscapular system of flaps depends on branches emanating from the vascular tree of the subscapular artery. Arising from the axillary artery, this vessel bifurcates in the circumflex scapular artery and the thoraco-dorsal artery. The circumflex scapular artery gives off an ascending, transverse, and descending branch. From the descending branch, multiple periosteal branches supply the lateral border of the scapula. The thoraco-dorsal artery bifurcates in the serratus anterior branch and in two muscular branches in the latissimus dorsi muscle, a lateral and a medial branch. The angular branch to the inferior pole of the scapula arises either just proximal to the serratus branch, or from the serratus branch itself. This independent nutrient vessel allows a second means of obtaining a vascularised scapular bone graft[82].

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Scapula flap

In the back the subscapular artery, a branch of the axillary artery, supplies part of the scapular bone, the latissimus dorsi and serratus anterior muscles and overlying skin. Combined flaps can be harvested from this region and used for reconstructions in any part of the body.

This vascular system gives the microsurgeon the opportunity to use multiple independent flaps with just one common vascular source. Especially the generous latitude in the placement of the bone relative to the skin or muscle portions of the flap is a clear advantage. The latissimus dorsi and serratus anterior muscles, together with the ascending scapular, scapular and parascapular fasciocutaneous flaps, and a portion of the scapular bone can be used in a variety of combinations[83]. A bone graft up to 14 cm can be obtained from the lateral border of the scapula.

Shoulder function

The weakness of the rotator cuff muscles resulting from division of the teres major and minor muscles in the lateral scapular flap can be a serious disability when added to the shoulder dysfunction of an ipsilateral radical neck dissection. If possible, the ipsilateral scapular flap donor site should be avoided if the 11th nerve is sacrificed in the neck dissection[84]. Two patients out of 26 developed restricted elevation of the arm, probably because of insufficient cooperation in shoulder exercises[85]. Although patients consider their shoulder motion functionally adequate 6 months postoperatively, half of the patients have mild to moderate limitations of shoulder abduction and external rotation[86]. To prevent problems in shoulder function, vigorous postoperative physical therapy is necessary[87, 88]. To put these donor site problems in perspective, a Japanese questionnaire investigation did not find any problems that would indicate that the lateral border of the scapula should not be used as a donor site[89]. Thoma describes a modification of the scapular free flap, in which he uses the medial part of the scapula. The method of harvesting the medial scapular flap requires some rhomboid muscle dissection. The disability from this division was inconsequential in his patients' postoperative shoulder dysfunction. The shoulder weakness which his patients experienced was attributed to the neck dissections. This reduction in donor site morbidity is a major advantage over the lateral scapular flap which can impose significant rotator cuff dysfunction.

Concluding, this versatile flap is a very important one in modern reconstructive surgery. The donor site morbidity consists mainly of a mild impairment of shoulder function[90], which may become important when an ipsilateral neck dissection with sacrifice of the eleventh cranial nerve has been carried out.

Rationale

Considering the relative importance of donor site morbidity (Table 1), the most important complication of the radial forearm flap is fracture of the radial bone, which can lead to serious loss of function. Partly because of this serious complication the radial forearm flap is used less frequently for mandibular reconstruction. Currently, the fibula flap is the flap most widely used for mandibular reconstruction. Although delayed wound healing is a frequent complication in this flap[91], it usually has no serious long term consequences. Gait however, seems to be a potentially more serious problem. Nevertheless, patients complain relatively infrequently about this, probably because of the large physiologic reserve people have. The iliac crest is less popular than the fibula flap in mandibular reconstruction., Patients frequently complain of the contour deformity, although this can be avoided if a monocortical flap is used and a meticulous repair of the inguinal ligament is performed. The scapula flap is used less frequently; despite the fact that it appears to have little donor site morbidity.

flap	number	freq. used (%)	morbidity	freq. complications (%)
radial forearm flap	6	18	fracture	33
fibula	22	65	delayed wound healing; gait	32; 9
iliac crest	5	15	gait	20
scapula	1	3	shoulder function	0
total	34	100		

Table 1. Frequencies of use and complications of osteocutaneous flaps (own experience 1992-2000)

Although microsurgical techniques have made reconstructions possible, which three decades ago were considered impossible, the donor site morbidity inherent to these reconstructions is underexposed. Autografting (transplantation of bone from one site to another site in the same person) leaves the patient with inherent donor site morbidity. Another problem with the use of autogenic bone can be a mere lack of sufficient bone tissue.

Therefore, other approaches have to be explored that can solve these problems. Evidently, allografts (transplantation of bone from a donor to a recipient of the same species) are not the solution, because this material shows a very high resorbtion rate. In addition, disease transmission (AIDS, HIV) from donor to recipient is possible and reported, even though the tissue undergoes extensive processing and testing[92]. The same is true for the use of pure synthetic materials as grafting material, like calcium phosphate ceramics and bioglass. Although these materials support the bone repair process, they do not actively influence the bone regeneration process. Another strategy to stimulate bone growth and to create bone graft substitutes (BGS) is the use of tissue engineering methods, either by use of bone morphogenic proteins with an appropriate carrier, or by the use of cultured cells with an appropriate carrier[93, 94]. In both methods, a suitable carrier material is needed, which until now, has not been defined[95, 96]. Various studies have shown that CaP ceramics[97], biodegradable polymeric materials[98-101] (polylactic acid, polyglycolic acid) as well as metals[102] can be used as scaffold material. Therefore, it can be suggested that developments for bone graft substitutes will proceed with as final goal the manufacturing of engineered, preshaped, vascularised bone grafts. These preshaped, vascularised bone grafts can then be designed at a convenient donor site. New advancements in this process of tissue engineering can perhaps solve the current problems with donor site morbidity.

Objective and hypothesis

In view of the above, the objective of this thesis is to develop a method for microsurgical reconstruction of bone defects with the help of tissue engineering techniques. First, we will evaluate the usefulness of a non-invasive technique in the evaluation of bone formation like Magnetic Resonance Imaging (MRI). Standardized invasive evaluation techniques like histology and histomorphometry are widely used for this purpose, but they have an important disadvantage: to avoid interanimal differences large numbers of experimental animals are necessary. MRI is a tool that can be used to evaluate bone formation in vivo. With this technique, less animals would be necessary in experiments. Demineralized Bone Matrix

(DBM), which consists of a scaffold with Bone Morphogenic Proteins will be used for our first experiments and a comparison will be made to another non-invasive technique: Dual Energy X-ray Absorptiometry (DEXA). Also Magnetic Resonance Angiography (MRA) will be used to evaluate the blood flow in the pedicle of the flap.
Then, the next step is the induction of bone formation. In our strategy, bone marrow is the source for the bone forming cells, which are subsequently added to an appropriate carrier or scaffold material. The optimal carrier, as well as the optimal site for this bone forming process still has to be determined. One step further is to transplant this newly formed bone to the appropriate defect site, e.g. a segmental mandibular defect. This can be done by using the prefabricated flap as a pedicled flap or as a microsurgical free flap.

In order to develop a method for microsurgical reconstruction of bone defects with the help of tissue engineering techniques, we will address the following questions.

1. Can MRI be used in a longitudinal study to noninvasively detect bone formation in a rat model and can it give quantitative as well as morphological information about the construct?
2. What is the best method for noninvasive monitoring of bone formation, MRI or DEXA? And can MRA be used to demonstrate the vascularity of the pedicle of a prefabricated flap?
3. What is the influence of the surface area of the carrier for the bone forming capacity?
4. What is the most useful carrier for a cell-based construct, titanium fibre mesh or a ceramic implant?
5. What is the most useful acceptor site for a prefabricated flap, muscle or subcutaneous tissue?
6. What is the influence of a microsurgical anastomosis of the vascular pedicle of a prefabricated muscle flap on the tissue engineered bone in this flap?

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chapter two

In vivo magnetic resonance imaging explorative study of ectopic bone formation in the rat

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Tissue Engineering 2002; 8(6): 1029-1036

Introduction

In reconstructive surgery donor site morbidity is a consequence of the use of free revascularized flaps[1]. Tissue engineering is a promising new tool which could help to minimize this donor site morbidity. Since the pioneering work on ectopic bone formation by Urist 1965[2], animal models have been commonly and successfully used in the study of tissue engineering of bone[3, 4]. In animal research histology remains the gold standard for assessing bone formation. A disadvantage associated with this analysis method, is that longitudinal studies are difficult to perform. For example, we know that the final tissue or healing response can be affected by specific characteristics of an individual animal. To avoid such interanimal differences, longitudinal study designs have always to be based on large numbers of experimental animals. In view of this, other techniques have to be explored to study and follow wound healing phenomena in animals. Alternative methods for histology as already have been used in the past; include radiographs and Dual Energy X-ray Absorptiometry (DEXA). DEXA is based on the principle that X-rays pass through various body tissues with different attenuation. Therefore, they can be differentiated. By using X-rays at two different energy levels, better tissue differentiation is possible compared with single energy systems[5]. Although radiographs and DEXA measurements can provide some additional information, they still suffer from significant disadvantages. For example, quantification of bone formation with X-ray imaging is not possible. The DEXA technique is very well suited for the overall monitoring of bone behavior around implants[6, 7]. However, quantification of the bone density in a narrow zone close to an implant is less predictable[8]. This makes DEXA inappropriate for determining the bone healing sequence in porous scaffolds, with pore sizes between 100-300 µm, as frequently used in bone tissue engineering.

On the other hand, during the last decennium, large progress has been made with another diagnostic approach, i.e. Magnetic Resonance Imaging (MRI). Although MRI, just as DEXA scan, doesn't have the same high resolution as histology, this imaging modality allows the three-dimensional visualization of body tissues. Diagnostic applications for this technology include the brain, chest, abdomen and skeletal system. Further, MRI is

used clinically in humans and in animal research. For this last application, separate equipment has been developed. Despite these developments and innovations, MRI is still not an accepted or well-documented technique to follow wound healing dealing with bone reconstruction.
In this report we explore the possibilities of using in vivo MRI methods for studying bone formation. Consequently, the objective of the present study was: to investigate whether it is possible to use MRI to demonstrate ectopic bone formation in the rat. This investigation was done by comparing MRI to the conventional techniques currently in use for demonstrating ectopic bone formation, i.e. soft X-ray images and histology. In this pilot study, histology was obtained after sacrifice only.

Materials and methods

Implants

Demineralized bone matrix (DBM)

Two 10 week male Wistar (W.U.) donor rats were sacrificed by cervical dislocation. From the diaphysis of the resected femora, pieces with a length of 1 cm were cut and, after flushing with saline to remove the bone marrow, immediately chilled to 0°C. The fragments were decalcified in 0.6 N HCl (1g bone per 100 ml 0.6 N HCl) under constant stirring during 48 hours at a temperature of 2°C, the hydrochloric acid being changed after 24 hrs. After elution of the acid in 0.15 N NaCl, the fragments were lyophilized and stored at -20°C. The average weight of the DBM implants was 31.3 mg and the average diameter of the implants was 5 millimeter.

Inactivated demineralized bone matrix (iDBM)

Appropriate pieces of rat demineralized bone matrix were extracted for 24 hrs. at 4°C with 4 M guanidine hydrochloride in 50mM TRIS-HCl, pH 7.4 (4 pieces DBM in 10 ml), containing protease inhibitors (5 mM benzamidine hydrochloride, 5 mM N-ethylmaleimide, 100 mM 6-aminocaproic acid, and 0.5 mM phenylmethylsulphonyl fluoride). The insoluble rat organic matrix remaining after the 4M guanidine hydrochloride extraction was washed five times with an excess of demineralized water during 24 hrs, lyophilized and

stored at -20 °C. The average weight of the iDBM implants was 19.8 mg and the average diameter of the implants was 5 millimeter.

Experimental design and surgical procedure

Surgery

One male Wistar rat (W.U., 10 weeks of age, weight 280 grams) was anaesthetized using a mixture of fifty percent oxygen with fifty percent nitric oxide and 1.2% Isoflurane (Forene® (1-chloro-2,2,2-trifluorethyl-difluoromethyl-ether).

Four implants were placed subcutaneously in the back of the animal as schematically shown in Figure 1: two DBM (DBM_prox and DBM_dist) and two iDBM (iDBM_prox and iDBM_dist). At 1, 3, 5 and 7 weeks after surgery, soft X-ray images and MRI were obtained. After seven weeks, the animal was sacrificed using an overdose of diethyl ether anesthesia. Following euthanasia, the implants with their surrounding tissue were retrieved and prepared for histological and histomorphometrical evaluation.

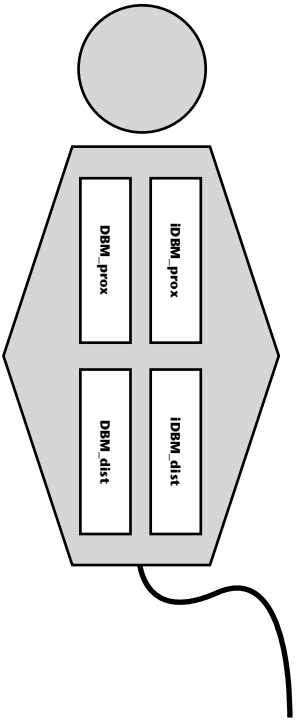


Figure 1. Schematic illustration of the subcutaneous placement of the DBM implants. Two DBM implants (DBM_prox and DBM_dist) were placed on the left side, two iDBM implants (iDBM_prox and iDBM_dist) were placed at the right side.

Figure 2. The rat is lying on his back in the MRI with his tail on the right. He is seen from the undersurface through a transparent plate. The four subcutaneous implants are located within the diameter of the 40 mm circular RF surface coil.

MRI

The implants were studied by MRI at weeks 1, 3, 5, and 7 post operatively. During the MRI investigation the rat was anesthetized with isoflurane (4% initially and 1.3% for maintenance) and a 1:1 (v/v) nitrous oxide and oxygen gas mixture. The animal was placed into the magnet in a supine position, on top of a 40 mm circular RF surface coil (Figure 2) covering all four implanted pieces of (i-)DBM. During the MRI experiments the respiration rate was continuously monitored and the core temperature was registered with a rectal probe and maintained with a variable-temperature circulating-water bed. Gradient-echo MRI experiments (TE = 6 ms; TR = 1000 ms; flip angle = 60°; field of view = 55 mm; matrix size = 256 x 256 data points) were carried out on an S.M.I.S. 7T/200mm horizontal-bore MR spectrometer equipped with a Magnex Scientific 150mT/m gradient set. With these experimental parameters, the MRI shows mainly proton-density weighting. T2* weighting, however, cannot be completely excluded since tissue T2* values are generally very short at the high magnetic field strength used (7.0 Tesla). After a pilot scan to establish the orientation of the DBM implants, twenty parallel transverse slices (thickness 1.5 mm; separation 2.0 mm) were acquired. As the length of the DBM implants is 10 mm, each DBM is visible in 5 contiguous slices. The total duration of the scanning was 4.3 minutes.

X-ray images

Standardized whole body soft X-ray images were obtained at weeks 1, 3, 5 and 7. A working distance of 30 cm, an exposure time of 0.8 sec and an accelerating voltage of 45 kV were used. At 7 weeks, the animal

was euthanized and all implants with surrounding tissue were retrieved. Subsequently, these specimens were fixed and stored in phosphate-buffered 4% formalin solution (pH = 7.4). Then, the tissue blocks were dehydrated in a graded series of ethanol and embedded in methyl-methacrylate. After polymerization, a final soft X-ray image was made of all implants (6 cm; 0.05 sec; 60 kV).

Light microscopic evaluation

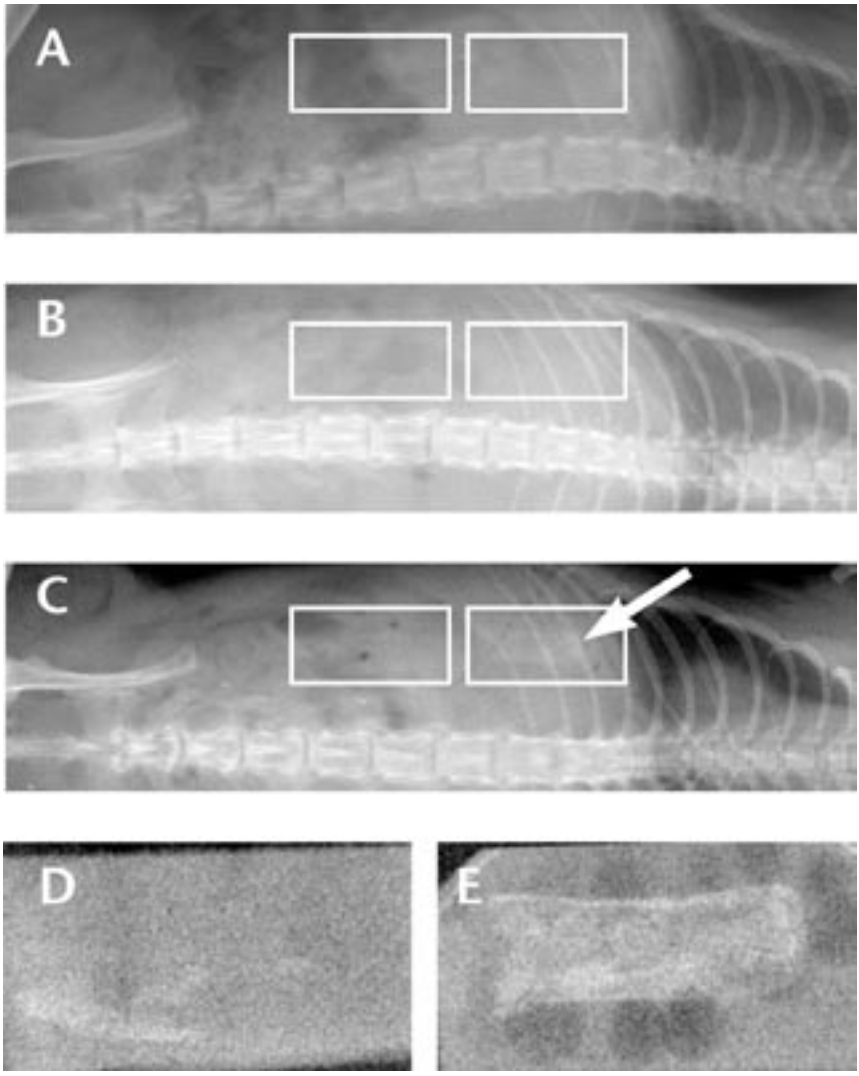
After X-raying, the MMA embedded samples were prepared for histological analysis. Therefore, thin sections (thickness 10 mm) were made using a modified sawing microtome technique[6]. Transverse sections were made on 5 levels in each implant. These levels corresponded with the transverse slices of the MRI evaluation. The sections were stained with methylene blue and basic fuchsin. To evaluate the tissue response to the implants, both histological and histomorphometrical evaluation was performed. The histological evaluation consisted of a complete description of the observed thin sections.
Image analysis techniques were performed for the histomorphometrical analysis. We used a computer based image analysis system (Qwin Pro, Leica). The following parameters were assessed: (1) bone surface area and (2) DBM / iDBM area. All histomorphometrical measurements were performed in 5 different transverse sections per implant. Presented results are based on the average of these measurements.

Results

X-ray images

At week three none of the implants was visible on whole body soft X-ray images (Figure 3). At week 5 and 7 implant DBM_prox could clearly be seen. On the other hand, no evidence of bone formation was observed in the other DBM implant (DBM_dist) or in the two iDBM implants (iDBM_prox and iDBM_dist). In the post-explantation PMMA X-ray images implant DBM_prox was clearly detectable as well as the distal part of implant DBM_dist.

Figure 3. Soft X-ray images of DBM implants DBM_dist (left) and DBM_prox (right) at four different times after implantation. (a, b, c) In-vivo soft X-ray images at weeks 1, 3, and 5, respectively. The estimated location of both implants is indicated with a white box. (d) Post-explantation soft X-ray images at week 7. The first ossification-related contrast appears in block DBM_prox at week 5 (arrow). The post-explantation X-ray image of implant DBM_prox shows complete, uniform, bone formation. In implant DBM_dist bone formation is not found in the in-vivo soft X-ray images, not even at week 5. However, partial ossification is observed in the post-explantation X-ray images at week 7.



In the longitudinal MR imaging study (Figure 4), low-intensity (dark) areas became gradually apparent at week 3 (implant DBM_prox) or week 5 (implant DBM_dist). These areas were located at the surface of the original DBM implants and were supposed to represent bone. At week 7 (Figure 5) the complete surface of block DBM_prox seemed to be uniformly calcified, whereas block DBM_dist showed only partial calcification. This was most noticeable at the distal side. Similar appearing low-intensity areas could no be found in iDBM implants iDBM_prox (Figure 5) and iDBM_dist (not shown).

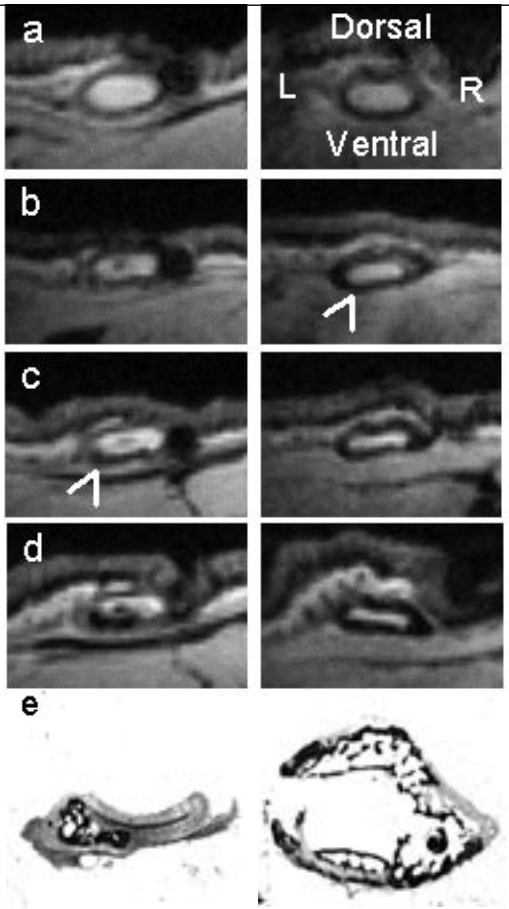


Figure 4.

Figure 4. Transverse images (perpendicular to the X-ray images in Figure 3) of the distal side of the implants DBM_dist (left) and DBM_prox (right). (a-d) MRI at weeks 1, 3, 5, and 7, respectively, after implantation. (e) Histological images post-explantation at week 7. The first signs of bone formation appear in the MRI at week 3 (implant DBM_prox) or week 5 (implant DBM_dist) as low-intensity areas located at the surface of the original implants (indicated by arrows). Both MRI and histology show that ossification in the distal section of implant DBM_prox (right column) appears evenly distributed, whereas the distal section of implant DBM_dist (left column) is ossified at the left side mainly. The dark circular spot in the MRI at the right side of Block DBM_dist is attributed to a hemorrhage.

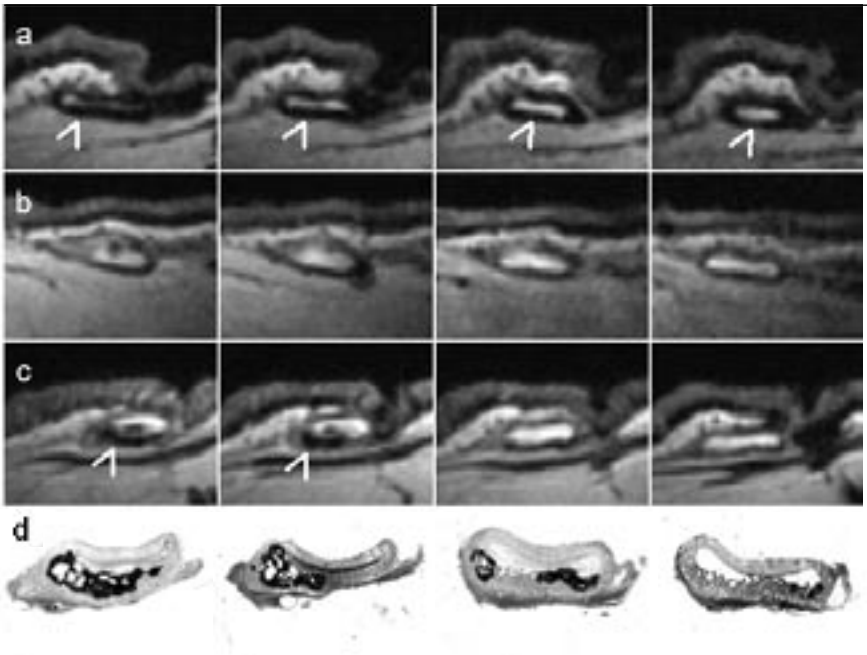


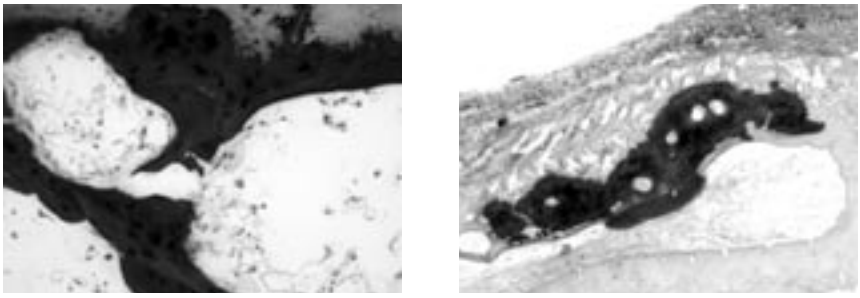
Figure 5. Bone formation at 7 weeks post implantation in three implants, DBM_prox (a), iDBM_prox (b) and DBM_dist (c), as studied by MRI. For each implant four equidistant transverse slices (distance 2.0 mm) are shown from the distal side (left) to the proximal side of the implant (right). Low-intensity areas associated with bone formation are indicated by arrows.

Corresponding histological slices of the partially ossified implant DBM_dist are shown for comparison (d). Despite the difference in slice thickness and the significant deformation of the implants during the post-explantation treatment for histology, MRI and histology show strong similarities in the geometry of bone formation of implant DBM_dist.

Light microscopy

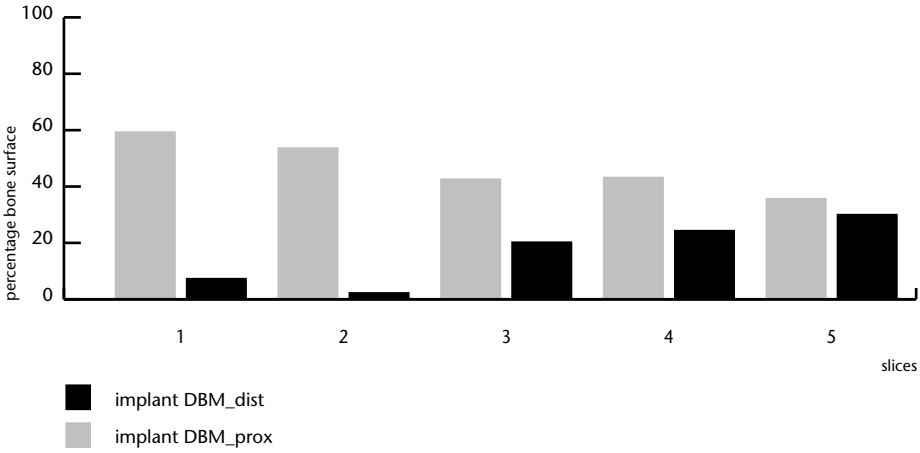
In DBM implants DBM_prox and DBM_dist bone formation was seen (Figure 4). The deposited bone had a trabecular appearance and could be associated with the presence of bone marrow-like tissue within the porosity of the bone trabeculae. The bone was also characterized by the presence of osteocytes that were embedded in the mineralized matrix. At the outside, the bone was surrounded by a periosteum-like tissue layer. In the part of the implant with incomplete bone formation (implant DBM_dist), the collagenous structure of the DBM was still visible together with fibrous tissue ingrowth. Hardly any inflammatory cells were present. In iDBM implants iDBM_prox and iDBM_dist (not shown) no bone formation was seen. Only remnants of the iDBM implant were observed together with fibrous tissue ingrowth. The enlargements in Figure 6 show an even distribution of bone formation throughout implant DBM_prox. Implant DBM_dist, however, showed only partial bone formation, most notably at the distal side.

Figure 6. Histology of adequate ectopic bone formation in block DBM_prox (40x) and only partial ectopic bone formation in block DBM_dist (10x) confirms findings on MRI.



Results of histomorphometric analysis of the sections of implants DBM_prox and DBM_dist are shown in Figure 7. Implant DBM_prox had an average bone formation of 51%, implant DBM_dist of 14%. In implant DBM_prox distribution of bone formation was rather uniform, in implant DBM_dist bone formation was most pronounced at the distal part. Histomorphometric analysis of the sections of implants iDBM_prox and iDBM_dist did not reveal any significant bone formation (less than 1%).

Figure 7. Histomorphometrical analysis of implants DBM_prox and DBM_dist at week 7. Implant DBM_prox shows an average bone surface area of 51%, which is evenly distributed over the DBM. Implant DBM_dist shows an average bone surface area of 14%, predominantly located at the distal side (slices 3-5) of the DBM.



Discussion

Demineralized bone matrix has been shown to induce ectopic endochondral bone formation, when subcutaneously implanted in rats[7]. The temporal sequence of recruitment/chemotaxis and proliferation of undifferentiated mesenchymal cells, chondrocyte differentiation, calcified cartilage formation, neovascularization, osteoblast differentiation, bone formation, osteoclastic bone remodelling, and hematopoietic marrow

development is complete by 21 days. After 4M guanidine-HCl extraction of the native bone morphogenetic proteins (BMP's), iDBM ceases to induce any bone formation. In table 1 we summarize the evidence of ectopic bone formation from the four different techniques on all four implants. Both block DBM_prox and DBM_dist proved to form ectopic bone, where block iDBM_prox and iDBM_dist did form almost no bone at all, quite as expected. Residual bone formation in block iDBM_dist (less than 1%) may be attributed to incomplete decalcification or incomplete extraction of BMPs. Decalcification and Guanidine deactivation is usually performed on DBM particles[8-10], whereas in our study we chose to deactivate an intact femoral diaphysis, as shape was an important feature in our study.

	Block A	Block B	Block C	Block D
Soft X-ray images	yes	no	yes[1]	no
MRI	yes	no	yes[2]	no
Light microscopy	yes	no	yes	no
Histomorphometry	yes[3]	no	yes[4]	no

[1)] Only partial ectopic bone formation is observed

[2)] Ectopic bone formation is observed at the distal side only

[3)] 50%

[4)] 14%

Table 1. Evidence of ectopic bone formation from four different techniques on all four blocks of implanted DBM. As expected, only block DBM_prox and DBM_dist showed bone formation. Block DBM_prox had an even distribution of bone formation, in block DBM_dist bone was formed at the distal side only

In this study different methods to assess bone formation (X-ray images, MRI and light microscopy) are compared. Implant DBM_prox exhibited bone formation, which was detected by X-ray examination at week 5. Implant DBM_dist showed partial bone formation at the distal side of the implant. This could be detected by X-rays in the post-explantation phase only. In our experiment we could not demonstrate ectopic bone formation radiographically before week 5. In a study by Viljanen et al. ectopic bone

formation was found as early as in week 3, using radiomorphometric quantification[11]. Moreover, in this study almost complete ossification was reported, whereas in our experiment we find 51% and 14% ossification only. The variable osteogenic potential of DBM is well recognized[12-14]. In contrast to our study, Viljanen et al. implanted DBM in a muscle flap, which is known to have greater potential for ectopic bone formation than subcutaneous tissue[15]. Furthermore, the age of the acceptor rats that were used was 5 to 6 weeks. In our experiment we used a 10 week old rat. Aged animals show a decreased ability to produce bone in response to DBM[16]. Because induction is related to the surface area of exposed matrix, the use of DBM powder instead of femoral shafts would have given more bone formation in our experiment[17].

MRI was able to detect bone formation in an earlier phase both for implant DBM_prox (3 weeks) and DBM_dist (5 weeks). Besides detection of bone formation, MRI was able to assess the localization of bone formation at the distal side of the implant. Bone formation was confirmed by light microscopy for both implant DBM_prox and DBM_dist. Histomorphometry confirmed that the bone formation of implant DBM_dist was localized at the distal side only. Recently Traoré described the use of MRI for the monitoring of biological changes of implanted porous biomaterials[18], but to our knowledge MRI has not been used for the monitoring of ectopic bone formation in the study of bone tissue engineering[19].
In the present study all MRI scans were carried out at 7.0 T, whereas most clinical MRI scanners presently use field strengths between 0.5 and 1.5 T. This relatively high magnetic field strength was used to obtain sufficient signal-to-noise ratio and spatial resolution necessary for the small size of the rat DBM implants. However, we may expect that the presented proton-density weighted MRI method can be used at lower field strengths for human studies as well[3].

Conclusion

This explorative animal study showed that MRI appears to be a valuable tool in bone tissue engineering studies. Using MRI bone formation can be detected earlier than by soft X-rays. Besides, the complete three-dimensional shape of the newly formed bone can be monitored noninvasively. An additional advantage is that in comparison with histology, this technique requires less animals for longitudinal studies and that interanimal variation can be prevented, on the other hand a comparative study will be needed in which all diagnostic studies are performed at each follow-up, and this should be performed blindly.

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chapter three

DBM induced ectopic bone formation in rats: in vivo study with follow-up by MRI, MRA and DEXA

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Tissue Engineering 2004; 10 (5/6): 747-754

Introduction

In reconstructive bone surgery donor site morbidity is a consequence of the use of autogenous grafts[1]. Tissue engineering is a promising new technique which can help to minimize this donor site morbidity by creating a Bone Graft Substitute (BGS). As part of our efforts to produce BGS's, we explored in a previous study the feasibility of using Magnetic Resonance Imaging (MRI) as a method to assess bone formation in tissue engineered bone constructs[2]. In this pilot study, demineralized bone matrix (DBM) implants were inserted subcutaneously in just one single rat. Subsequently, at 1, 3, 5 and 7 weeks post implantation, MRI was done to monitor bone formation in the implants. The results showed that: (1) in vivo MRI is suitable to check bone formation non-invasively, and (2) MRI results match well with histology results obtained at 7 weeks. However, in this preliminary study, no attempt was made to quantify the MRI results and to compare the quantitative MRI data with standard histomorphometry. Therefore, the first aim of the current study is to continue and expand our in vivo MRI studies for bone engineering applications.

Besides the use of MRI to produce pictures of body tissue, MRI technology can also be utilized to detect blood vessels. This so-called MR Angiography (MRA) provides detailed information about the functionality of blood vessels without using any contrast material. At this point, it appears appropriate to emphasize that the final goal in our bone engineering studies is to create a prefabricated and preshaped bone graft consisting of an osteoinductive scaffold combined with a vascularised muscle flap, which can be transplanted microsurgically to the receptor area. Maintenance of blood flow is of major importance for the final success of such free flap constructs. Consequently, the second aim of the present study was to determine whether MRA is indeed a useful method to determine the functionality of the supplying blood vessel in such pedicled bone graft substitutes.

A third aim of the current study was to evaluate also another technique for the assessment of bone formation, i.e. Dual Energy X-ray Absorptiometry (DEXA). This is a fast, cheap and reproducible method to examine bone

mineral content and density in clinical practice[3, 4]. In addition, the efficacy of this technique for the measurement of bone formation in porous implants has been reported[5, 6].
Considering these three aims, we created pedicled muscle flaps in rats, which were wrapped around DBM implants. Two groups of rats were formed in our study: a so-called early (six weeks) and late (twelve weeks) group. In vivo MRI and post-mortem histology were used to evaluate bone formation in the early group. Also, MRA was performed to watch the functionality of the blood vessel in the pedicled DBM implant. For the late group, in vivo DEXA and post-mortem histology were used for bone formation analysis.

Materials and methods

Implants

The femora of seven male Wistar donor rats (10 weeks old) were used to prepare 14 DBM-implants. From each femur the bone marrow was removed and one cm sections of the diaphyses were made. These were immediately chilled to 0°C and then decalcified in hydrochloric acid (HCl) (1 g bone per 100 ml 0.6 N HCl) under constant stirring during 48 hours at a temperature of 20ºC. The HCl was refreshed after 24 hours. After dilution of the acid with 0.15 N NaCl, the implants were lyophilized and stored at minus 20°C. The average weight of the DBM implants was 19 ± 4 mg.

Implantation procedure

Fourteen male Wistar rats (W.U., 10 weeks of age, average weight 280 g) were used in the study. Before surgery, the animals were anaesthetized using a mixture of 50% oxygen with 50% nitrous oxide and 1.2% Isoflurane (1-chloro-2,2,2-trifluoroethyl-difluoromethyl-ether). For the insertion of the implants, the animals were immobilized and placed in a supine position. During the implantation procedure the core temperature was registered with a rectal probe and maintained with a variable-temperature circulating-water bed. The skin of the animals was shaved, washed and disinfected with povidone-iodine. After incising the skin on the medial side of the hind

limb, the saphenous vessels were distally ligated, just above the point where the vessels split in two or three branches. The anterior and posterior gracilis muscles were elevated from distal to proximal, preserving the branches of the saphenous vessels to these muscles. Proximally from the muscles, the saphenous vessels originate from the femoral vessels. All other branches from the femoral vessels were ligated and cut to provide an optimal blood flow to the muscle flap. Each animal received one DBM implant, which was wrapped in the adductor thigh muscle flap[7]. The flap was closed with non-absorbable monofilament 7x0 sutures (Nylon). Around this muscle flap, a 1.5 x 1.0 cm silicon sheet with a thickness of 0.5 mm was wrapped to prevent vascular ingrowth from the surrounding tissues. Consequently, the flap was vascularised solely from its pedicle. At the end of the operation, the skin was closed with stainless steel staples.

After surgery, the animals were randomly assigned to two groups. The early group (n=7) was maintained for six weeks and the late group (n=7) was maintained for twelve weeks. At six weeks after implantation, MRI/MRA was obtained from all rats of the early group. Thereafter, the animals were killed by an overdose of ether anesthesia and implants together with surrounding tissues were retrieved for histological evaluation. Twelve weeks after implantation, DEXA scans were obtained for all rats belonging to the late group. Subsequently, also these animals were killed and implants plus surrounding tissue retrieved for histological analysis.
The used animal protocol was approved by the Animal Care Ethics committee of the University of Nijmegen. The animal experiments were performed according guidelines for animal experiments for scientific research of the Dutch government.

Evaluation techniques

Magnetic Resonance Imaging (MRI) / Magnetic Resonance Angiography (MRA)
All MRI and MRA experiments were carried out on an S.M.I.S. 7 Tesla MRI scanner with a 200 mm bore size dedicated for small test animals. Animals were placed into the magnet, face down, on top of a 22 mm radiofrequency (RF) surface coil covering one DBM-implant at a time. During the MRI/MRA investigation, the rat was anesthetized as indicated in the implantation

procedures, the respiration rate was continuously monitored, and the core temperature was maintained as indicated in the implantation procedures. For the MRI experiment we used a gradient-echo sequence (TE = 6 ms; TR = 1000 ms; flip angle = 60°; field of view = 55 mm; matrix size = 512 x 512 data points). The images show mainly proton-density weighting. As shown in a previous paper, areas with newly formed bone will appear dark ("calcification") in proton-density weighted images in contrast to non responding DBM, which appear white ("no calcification")[2]. After a trial scan to establish the orientation of the DBM implants, 24 parallel slices (thickness 0.7 mm; separation 1.0 mm) were acquired perpendicular to the long axis through the DBM. As the length of the DBM implants was 10 mm, each DBM was visible in 10 contiguous slices. The percentage of "calcification" in each slice was calculated. Thereafter, the data for the separate slices were averaged, which resulted in a volume percentage calcification of the total implant volume, i.e. 45% calcification means 45% of the total implant volume. Finally, the same slices were imaged with an MRA sequence (TE = 6 ms; TR = 200 ms; flip angle = 90°) as well. In these images, regions with large flow (functional blood vessels) appear white, and other areas appear dark. Thus the MRA images can be used to demonstrate the integrity of the vascular pedicle.

DEXA

DEXA scans were performed on a Hologic QDR 1000 scanner (Waltham, MA). The rats were anesthetized using halothane/nitrous oxide/oxygen inhalation anesthesia and placed prone on the scanner. The hind limbs were fixed to prevent projection of the femur over the implant. After acquiring a scan of the hind limb area, the femora and the DBM implants were identified. It was attempted to quantify the bone mineral content of the DBM by positioning regions of interest over the location of the implant and over the surrounding tissues.

Histological analysis

The specimens were retrieved for histological analysis: they were fixed and stored in phosphate-buffered 4% formalin solution (pH = 7.4). Then, the tissue blocks were dehydrated in a graded series of ethanol and embedded in methyl-methacrylate (MMA). Subsequently, 10 µm sections of the

MMA embedded samples were made using a modified sawing microtome technique[8]. At least three transversal sections were made on five different levels in each implant. These levels corresponded with the transverse slices of the MRI evaluation. The sections were stained with methylene blue and basic fuchsin. To evaluate the tissue response to the implants, both histological and histomorphometrical analysis was performed. The histological evaluation consisted of a complete description of the observed thin sections.

A computer based image analysis system (Qwin Pro, version 2.3, Leica, Wetzlar, Germany) was used for histomorphometrical analysis. Two parameters were assessed: (1) the surface area of the newly formed bone, and (2) the surface area of the DBM implant. In this way the relative bone formation could be measured. All histomorphometrical measurements were performed in the five different transverse sections of each implant, which were averaged to obtain the final result.

Statistical procedure

Results were expressed as mean + one standard deviation (SD). To test the correlation between MRI and histomorphometry a linear correlation analysis was done. Data of the histomorphometrical analysis were compared with an unpaired t test. The test was performed at 95% confidence level. The t test assumes that the data are sampled from populations that follow Gaussian distributions. This assumption was tested using the method of Kolmogorov and Smirnov. All calculations were performed in GraphPad Instat version 3.05 for Windows 95/NT (Graphpad Software, San Diego, California, USA).

Results

Clinical observations

One of the rats of the early group died after surgery. Therefore, this group finally consisted of 6 rats. All rats showed an undisturbed wound healing without any clinical signs of inflammation at the surgical sites during the various implantation times.

Early group

MRI/MRA

Six implants were evaluated by MRI and MRA. The quantitative data for these six implants are presented in Fig. 1. In four implants clear bone formation was observed on MRI, all of them having an intact vascular pedicle on MRA (Fig. 2). Two implants showed only minimal bone formation, while the patency of the pedicle could not be proven by MRA. The mean relative bone formation for all implants was 42% ± 35%.

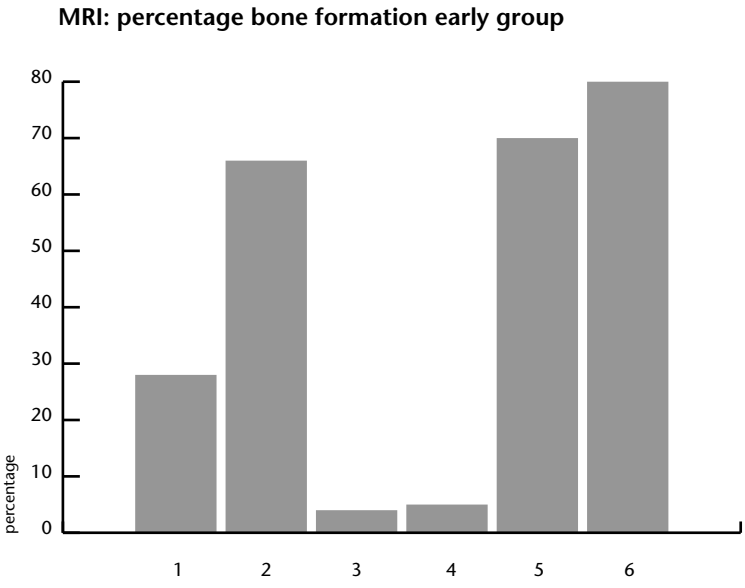

Figure 1. Quantitative MRI data for implants in the early group: four implants (1, 2, 5 and 6) show marked bone formation, two implants show almost no bone formation.

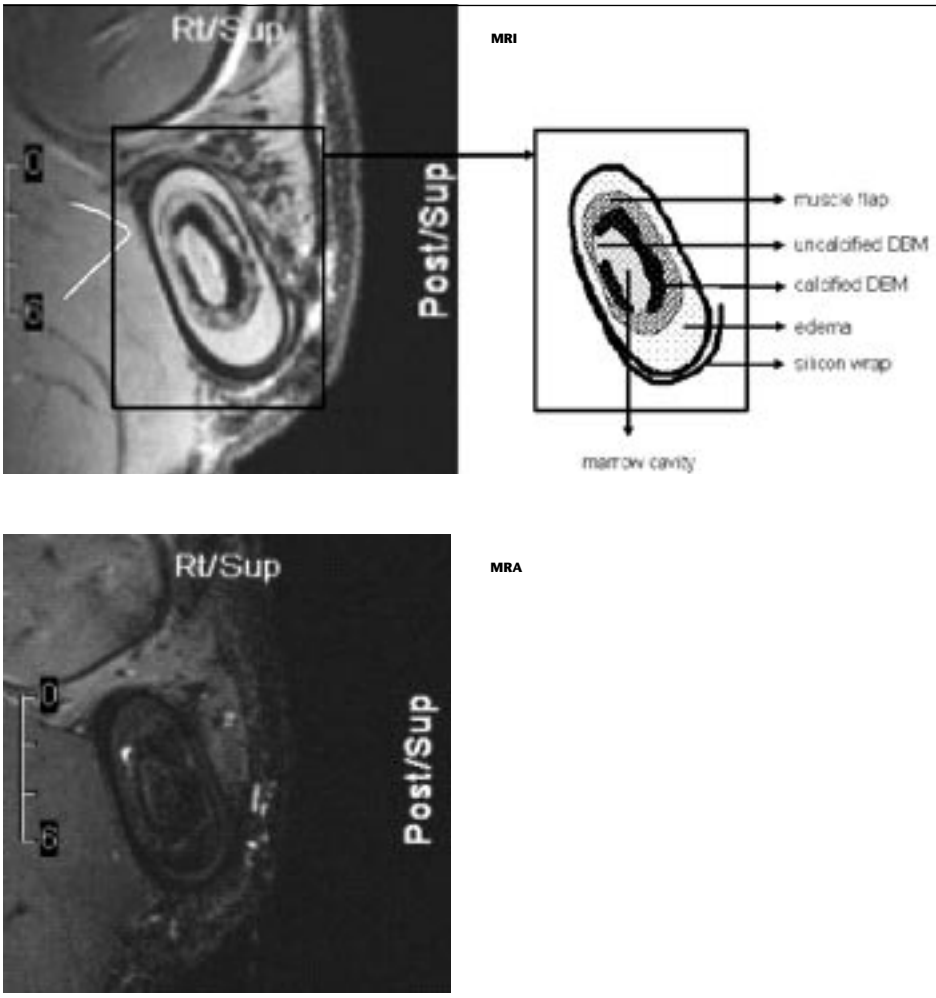

Figure 2. MRI and MRA of DBM implant. Upper figure (MRI): bone formation is visible as a dark ring. A silicon sheet is clearly visible (caret). Explanation of the tissue structures as visible in the MRI image. Lower figure (MRA): the white spot represents flow in the vascular pedicle of the flap.

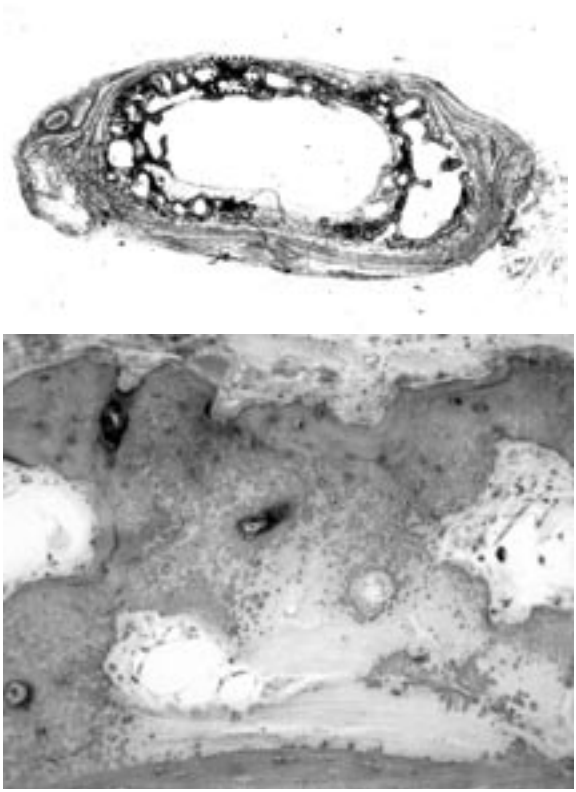

Figure 3. Histology of an early group specimen: ectopic bone formation at 6 weeks (original magnification: 1.6x; enlargement 20x).

Descriptive light microscopy

Light microscopy confirmed the MRI results, i.e. bone formation was seen in only four of the six specimens. Bone formation was characterized by the occurrence of mineral deposits inside the DBM material. Bone was deposited in islands and was not present as a continuous phase in the DBM. Bone formation could be associated with the presence of osteoblasts, osteoid and osteocytes (Fig. 3). Inside the bony islands, cavities were present that were filled with bone marrow-like tissue. Bone formation did not expand outside the original geometry of the DBM specimens. At the outer surface, the specimens were surrounded by a fibrous tissue capsule. No inflammatory response inside the capsule or at the capsule-DBM interface was observed.

Histomorphometry

Unfortunately, one specimen could not be used for histomorphometrical evaluation, since the available sections were of insufficient quality to allow a reliable quantification of the relative bone formation. The results of the other five measurements are depicted in Fig. 4. Three implants showed bone formation of 25% or more of their total surface area. The other two implants had bone formation of less than 10%. The mean relative value of bone formation for all implants was 25 ± 19%.

Bone formation of early group assessed by histomorphometry

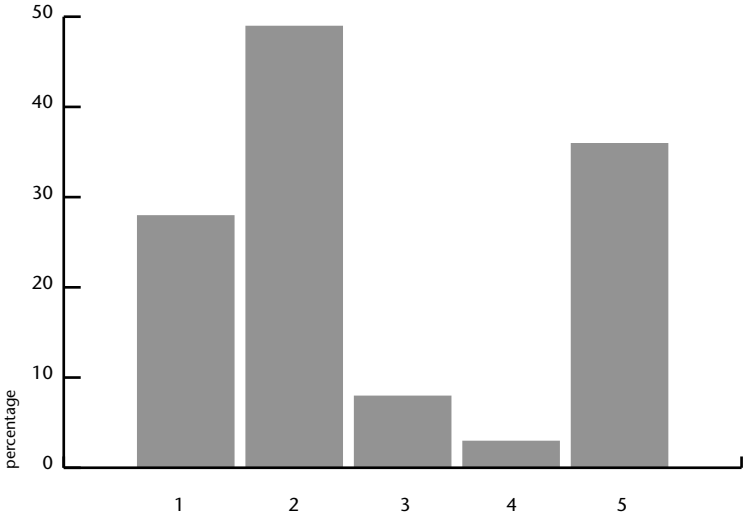

Figure 4. Histomorphometrical analysis of bone formation in early group: three implants (1, 2 and 5) show marked bone formation, two implants show almost no bone formation.

Comparison of MRI and histomorphometry data

To determine the existence of a relationship between MRI and histomorphometrical evaluation, a linear correlation analysis was done. The calculated correlation coefficient r was 0.9395 and the coefficient of determination (r^2) was 0.8827. This indicates the existence of a strong correlation.

Late group

DEXA

The autologous rat femurs were well-visualized. However, the density of the implants was hardly discernable from the soft tissues. At best, the implants were only faintly visualized, indicating a very low bone mineral content. Consequently, region-of-interest quantification did not yield any reproducible results, since it was impossible to reliably discriminate the implant from the surrounding soft tissues.

Descriptive light microscopy

Histological analysis seemed contradictory to the DEXA observations. In five of the DBM specimens, extensive bone formation was observed. Bone formation was again characterized by the presence of osteoblasts, osteoid and osteocytes in a mineralized matrix. Also, inside the deposited bone, bone marrow-like tissue was observed. Occasionally, bone formation extended throughout the DBM and filled at some sites the DBM almost completely. The newly deposited bone never expanded in the original medullar cavity of the DBM or outside the DBM material. In the other two specimens, also bone formation could be observed. However, the amount of bone was clearly less. All DBM specimens maintained their original shape. No sign of DBM degradation was seen. All specimens were further surrounded by a fibrous capsule without the presence of inflammatory cells.

Histomorphometry

The bone formation data of the seven DBM implants of the late group are depicted in Fig. 5. Five implants showed bone formation of 45% or more of their total surface area. The other two implants revealed a much lower bone formation percentage (less than 25%). The mean percentage bone formation for all implants was 48 ± 15%.

Bone information of late group assessed by histomorphometry

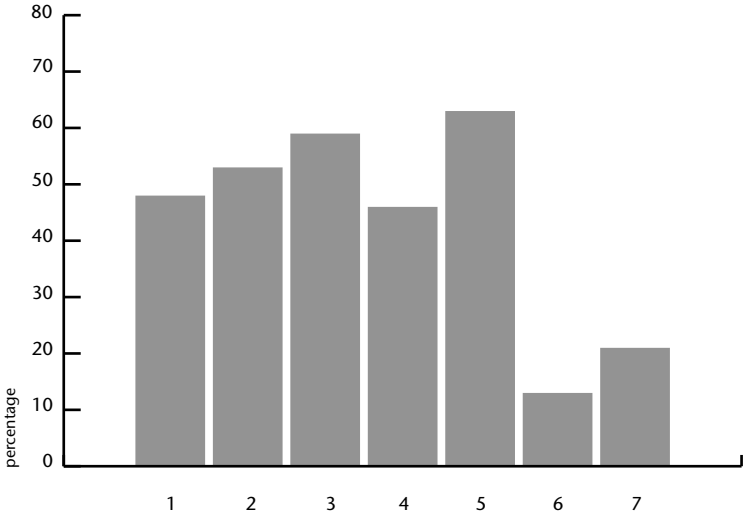

Figure 5. Histomorphometrical analysis of bone formation in late group: five implants (1, 2, 3, 4 and 5) show marked bone formation, two implants show no marked bone formation (less than 20%).

Comparison of early and late group data

Comparison of the histomorphometrical data for bone formation between the early and late groups reveals that significantly more bone is formed in the late DBM group ($P = 0.0463$). Additional statistical testing confirmed that the two SD's of the early and late group did not differ significantly from each other ($P = 0.3011$). Also, the data were shown to be sampled from Gaussian distributions ($P > 0.10$).

Discussion

The current study was an expanded follow-up analysis of an earlier performed pilot experiment and aimed at the use of MRI and DEXA to follow the progress of the bone development process in pedicled bone graft substitutes[2]. Additionally, MRA was used to study the blood circulation in the created muscle flaps. Bone formation was evaluated in DBM implants, wrapped in pedicled muscle flaps and inserted in rats. MRI and DEXA results were always compared with histomorphometrical measurement. Unfortunately, MRI/MRA and DEXA could not be performed on the same animals due to organizational and regulatory affairs. The used MRI equipment was available at the animal facility. DEXA equipment was available in the hospital and is used for patient diagnosis. As a consequence, DEXA measurements had to be done outside regular working hours. In addition, MRI for experimental animals is still a costly and time elaborative technique. Therefore, we decided to create two separate groups and to correlate the obtained data to histomorphometrical measurements. Considering the use of MRI for the analysis of bone development, the major advantage of MRI, compared to the golden standard histology, is the ability to perform in vivo longitudinal studies. The interanimal variation, which cannot be avoided in histological studies, can be prevented in this way. On the other hand, disadvantages of MRI are the availability of the equipment, costs as well as the necessity of anesthesia with the inherent risk of animal death. Comparison of the available MRI with histological data reveals the existence of a strong relationship between both methods. This confirms that MRI is of sufficient accuracy for comparative and sequential bone formation studies. Consequently, MRI can be a competitive

technique for the long term follow-up evaluation of bone graft substitutes, especially when larger and less elaborative equipment becomes available in the future. Despite this favorable outcome, we have to notice that MRI and histomorphometry do not provide exactly the same amounts of bone formation. An explanation can be found in the number of slices and the used bone quantification technique. For MRI, 10 slices were evaluated representing the total implant. However, the imaging of these relatively thick slices is still a 2-D representation of a 3-D subject, which can result in over- or underestimation on the amount of bone formation. On the other hand, for the histomorphometrical analysis only a very limited number of sections out of a complete specimen can be analyzed, which can also result in over- or underestimation.

Besides MRI, longitudinal studies can also be performed using DEXA scans. Similar to MRI, the animals have to be anesthetized for each investigation. However, the investigation itself is generally very fast, cheap and widely available. We chose to use DEXA for the late group and to compare it with the golden standard of histology and histomorphometry. However, the bone mineral content was so low in comparison to surrounding soft tissue density that the graft could hardly be identified. Consequently, DEXA is unsuitable to reliably assess small variations in mineralization, which makes this technique not suitable for evaluation of bone tissue regeneration in the current experimental design in small laboratory animals. However, given the procedure's highly reproducible evaluation of bone density and bone mineral content in a clinical setting, DEXA may be more suitable when larger animal models and larger bone constructs are used.
Bone inductive activity of demineralized bone preparations is determined by the amount and activity of BMPs present in the material. Increased bone formation is observed at higher concentrations of BMP[9-12]. These growth factors are present in cortical bone, but only in minute amounts, approximately 1-2 µg BMP/kg of cortical bone[13]. The amount of BMPs may vary widely depending on source[14], preparative technique[15] and sterilization method used[16]. The quantity of bone formation is also related to the surface area of exposed matrix[17, 18]. Therefore, in most studies DBM is used as particulate and not as a massive implant[19, 20]. Such DBM particles were reported to generate 58% (± 25%) of bone in a rat craniotomy defect after

three weeks of implantation[10]. In the present study, we used DBM as bulk one centimeter sections of a femur. Such DBM implants have a smaller surface area than powder DBM. Nevertheless, bone formation after six and twelve weeks proved to be respectively, 25% (±19%) and 48% (± 15%). Concerning the maintenance of the deposited bone during time, we found that bone formation did not stop at six weeks, but instead continued so that after twelve weeks the amount of developed bone is significantly higher than at six weeks. No signs of osteolysis could be detected. This corroborates with the findings of Viljanen et al, who also described a time-related increase in new bone in a rat latissimus dorsi flap model with DBM, using radiomorphometry to quantify bone formation[21]. They reported a relative increase of the radiographic reference from 55% to 80% of rat normal bone average intensity at 10, 21 and 35 days.

Additionally, we observed that two of the six week samples showed almost no bone formation (less than 10%). MRA suggests that this is evidently due to a lack of blood supply. This can be due to either kinking of the vascular pedicle with subsequent lack of circulation preventing formation of bone, or compression of the flap by the silicone envelope. Post operative edema of the muscle flap can result in a relative tightness of this silicone envelop with flap failure as a result. Viljanen et al. already warned for this complication, although in their series of twelve DBM implants in a rat latissimus dorsi muscle flap model, almost complete ossification was found in all implants[21].
Although, MRA was found to be a suitable technique to evaluate the maintenance of circulation in the supplying blood vessel in pedicled BGS's, we cannot exclude that the lack of bone formation could also have been caused by the known and marked variation in the bone inducing capacity of DBM[21-25]. Evidently, the processing technique is important for the effectiveness of DBM and should therefore be standardized[26]. Despite of our efforts to be as accurate as possible, still some variation in the processing of the samples can have occurred. In this respect, we have to emphasize that DBM material has to be bioassayed prior to use in order to prevent the variation in osteoinductive effect[27].

Conclusion

On basis of our observations, we conclude that in vivo MRI proved to be a reliable method for monitoring ectopic bone formation in a rat model, while in vivo DEXA was unable to detect the implants. However, since MRI is time consuming and expensive, it is currently still not suited to replace histology and histomorphometry. Furthermore, MRA proved to be a very useful technique for studying the circulation of muscle flaps in this animal model.

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chapter four

DBM induced ectopic bone formation in the rat: the importance of surface area

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In press: Journal of Materials Science, 2004

Introduction

In reconstructive bone surgery donor site morbidity is a consequence of the use of autologous tissue[1]. Tissue engineering is a promising tool, which could help to minimise this donor site morbidity. One of the materials that has been studied extensively in bone regeneration procedures is demineralized bone matrix (DBM). As an osteoinductive agent, DBM induces osteogenesis via endochondral ossification[2-4]. The pioneering studies on bone induction were performed by Urist[5, 6]. He reported the isolation of a bone morphogenic protein (BMP) derived from bovine bone matrix gelatin by dissociative extraction in guanidine-HCl. This purified BMP, similar to DBM, was found to induce differentiation of mesenchymal cells into cartilage and bone when implanted into thigh-muscle pouches in mice. BMP has subsequently been isolated from rat, rabbit, guinea pig, porcine and human bone[4].

Glowacki and co-authors[7, 8] have recommended the following choices of DBM implant forms:

- powdered (75-250µm) for small irregular defects
- small chips (5mm) for septic lesions
- corticocancellous blocks or strips for segmental defects

They caution that very large pieces of DBM should be avoided, since smaller ones have increased surface area, which ensures a greater accessibility to BMP[9]. This encourages the proliferation and differentiation of osteogenic cells and makes DBM granulate more osteoinductive[7, 8].
In earlier studies we have used blocks of DBM obtained from the femora of donor rats. These blocks were inserted subcutaneously[2] as well as intramuscularly[3] in rats. Although significant bone formation occurred in both acceptor sites, we found a strong variation in bone forming capacity. This in contrast to Viljanen et al., who found almost complete ossification in their series of 12 DBM implants in a rat latissimus dorsi muscle flap model[10]. This difference in results can be due to the variation in bone forming capacity of the DBM itself, which has been described before[11]. It is possible that this effect was even more enhanced in our study, because we inserted the DBM as a block and not as a granulate.

On basis of the above mentioned, we decided to further explore the osteoinductive properties of DBM implants. We hypothesized that morsellized DBM (MDBM) has greater osteoinductive properties than non-morsellized DBM. Therefore, morsellized and non-morsellized DBM was inserted in a rat muscle flap. Bone formation was investigated by histology and histomorphometry.

Materials and methods

Implants

Demineralized bone matrix (DBM)

Ten 10-week male Wistar (W.U.) donor rats were sacrificed by an overdose CO_2. The femora of these donor rats were used to prepare 20 DBM-implants. Donor femora were cleaned of adherent periosteum, muscle and connective tissue. From the diaphysis of the resected femora, pieces with a length of 1.0 cm were cut and placed on ice, after flushing with saline to remove the bone marrow. The fragments were decalcified in 0.6 N HCl (1g bone per 100 ml 0.6 N HCl) under constant stirring during 72 hours at a temperature of 4°C. After elution of the acid in 0.15 N NaCl, the fragments were lyophilized and stored at -20˚C. The average weight of the DBM was 3.1 (± 0.4) mg.

Morsellized Demineralized bone matrix (MDBM)

Ten pieces of the previously demineralized bone matrix were morsellized by mortar and pestle and bone chips were remodelled in a cylindrical mould by impaction. The average weight of the morsellized DBM implants was 2.8 (± 0.5) mg.

Experimental design and surgical procedure

Surgery

For implantation ten 10-week old male Wistar acceptor rats (W.U., with an average weight of 280 grams) were used. Surgery was performed under general inhalation anesthesia using a mixture of fifty percent oxygen with

fifty percent nitric oxide and 1.2% Isoflurane [Forene® (1-chloro-2, 2,2-trifluorethyl-difluoromethyl-ether)]. For the insertion of the implants, the animals were immobilized and placed in a supine position. Longitudinal skin incisions were made on the medial surface of both hind legs of the rat, from the ankle to the groin above the inguinal ligament. After the incision was made, an intramuscular pocket in the adductor thigh muscle was created by blunt dissection. The DBM implants were placed in the pocket, and the muscle and skin were closed using Agraven suture material. Each animal received one DBM implant as well as one morsellized DBM implant on the contralateral side. To assure statistical randomization, DBM implants were alternately placed on the right or left side. In total 20 implants were placed. Six weeks after surgery the animals were sacrificed using an overdose of CO_2 anesthesia.
The used animal protocol was approved by the Animal Care Ethics committee of the University of Nijmegen. The animal experiments were performed according guidelines for animal experiments for scientific research of the Dutch government.

Light microscopic evaluation

After sacrificing the animals, the implants with their surrounding tissue were retrieved and prepared for histological and histomorphometrical evaluation. For one implant histological and histomorphometrical evaluation was not possible as it was completely resorbed during the 6 weeks of implantation in the intramuscular pocket. All other implants were fixed in 4% buffered formaldehyde solution. Subsequently, the tissue blocks were dehydrated in ethanol and embedded in methylmethacrylate.

After polymerization, thin sections (thickness 10 µm) were made using a modified sawing microtome technique[12]. Transversal sections were made on 3 levels in each implant, with 3 slices per level. To be able to differentiate cartilage, bone, marrow and muscle the sections were stained with methylene blue (15 sec.) and basic fuchsin (30 sec.) and examined with a light microscope. To evaluate the tissue response to the implants, both histological and histomorphometrical evaluation was performed. The histological evaluation consisted of a complete description of the observed thin sections. For the histomorphometrical measurements image analysis

techniques were performed. Therefore a computer based image analysis system (Leica Qwin) was used. The following parameters were assessed:

- DBM respectively MDBM surface area
- Surface area bone formation
- All histomorphometrical measurements were performed in 9 different sections per implant. Presented results are based on the average of these measurements.

Results

Histological analysis

In DBM implants and morsellized DBM implants bone formation was seen (Figure 1). However, a wide variation in the occurrence as well as amount of new bone formation was observed. In four out of nine DBM and one out of nine MDBM implants even no bone formation at all occurred. The deposited bone in the DBM implant had a trabecular appearance and could be associated with the presence of bone marrow-like tissue within the porosity of the bone trabeculae. The newly formed bone in the MDBM implants showed a more compact appearance than seen in the DBM implants. The bone was always characterized by the presence of osteocytes that were embedded in the mineralized matrix. At the outside, a periosteum-like tissue layer surrounded the bone. As shown in Figure 1 the shape and size of the MDBM implants are different from the DBM implants.

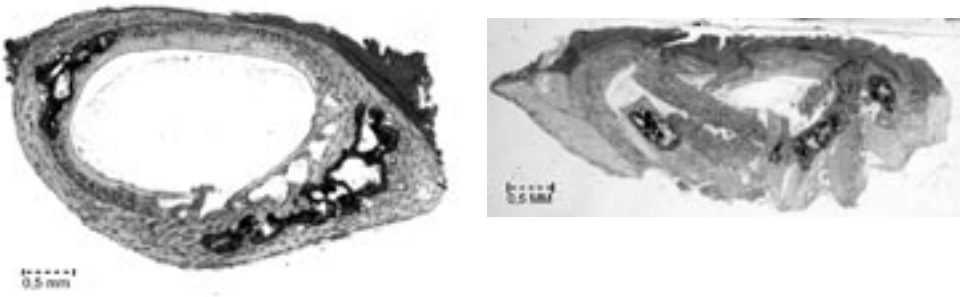


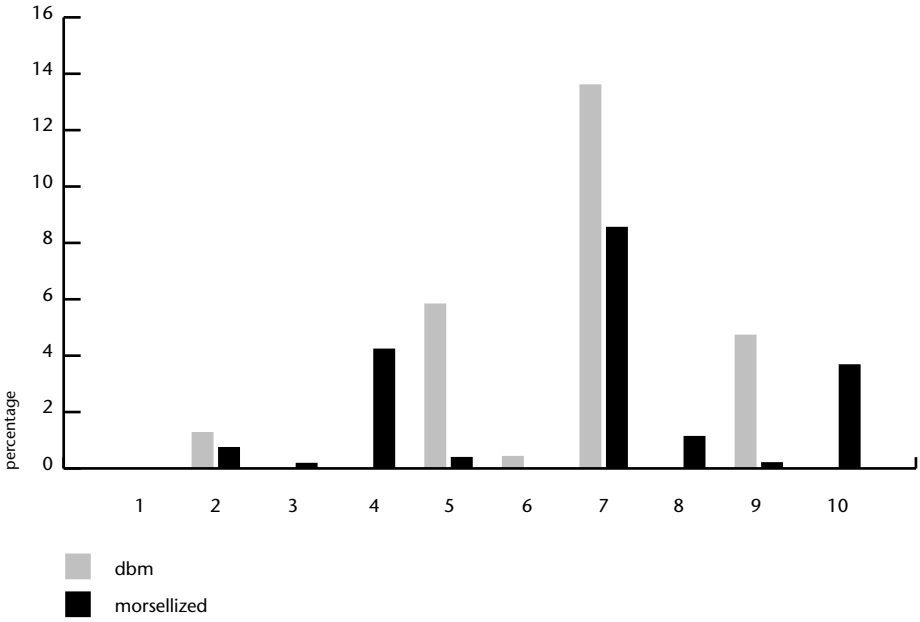
Figure 1a and b. Bone formation at 6 weeks implantation in two implants, DBM (a) and MDBM (b) as studied by histology shows bone formation in both groups.

The natural oval-shape and collagenous structure of the DBM is hardly recognizable, as the MDBM implants have a more elongated form. Also, the location of the bone formation differed, i.e. in non-morsellized DBM distribution of bone formation was most pronounced at the distal part, while in morsellized DBM bone formation was most pronounced centrally. Almost no inflammatory cells were present close to the DBM or in between the MDBM particles, except for one DBM implant, where an inflammation was seen, which was associated with a lack of new bone formation.

Histomorphometrical evaluation

Results of histomorphometric analysis of the sections of both implants are shown in Figure 2. In average 2.6% of bone formation was measured in DBM implants with a range from 0% to 14%. In MDBM implants, in average 1.9% bone formation was measured with a range from 0% to 9%.

Figure 2. Histomorphometrical analysis of non-morsellized DBM and morsellized DBM showed limited bone formation for both groups. Non-morsellized DBM had an average bone formation of 2.6 %, morsellized DBM 1.9%. There was no significant difference between these two groups.



Discussion

Demineralized bone matrix has been shown to induce ectopic endochondral bone formation, when intramuscularly implanted in rats. This study was designed to investigate whether increasing the surface area of DBM by morsellizing makes it more osteoinductive. This was done by comparing the osteoinductive properties of morsellized versus non-morsellized DBM by histology and histomorphometry. As described in previous reports, the induction of bone is related to the surface area of the exposed matrix. By morsellizing the DBM implant, the surface area increases and the structure of the implant becomes less compact and therefore more osteoinductive[7, 8]. Nevertheless, in this study we did not observe a difference between the amount of bone formation of DBM versus MDBM. Besides, we found that the new bone formation was very limited and showed a wide variance for both material preparations.

Earlier performed studies[5, 8, 11, 13]. showed general guidelines and test methods for assessment of the effectiveness of materials intended to induce bone formation when implanted in vivo. Following these guidelines we used in our experiment 10 week old rats of the male gender. Though gender is not a factor, the animals should be adolescent or young adults as older animals do not respond as effectively to osteoinductive materials[14]. Furthermore, we placed the implants in an intramuscular pocket in the adductor thigh muscle, as this site has proven to have greater potential for ectopic bone formation than subcutaneous tissue[15]. Also, we included on basis of previous studies, a sufficient number of samples per group in order to confirm the activity of DBM and to detect differences. Considering the fact that we met all these study design criteria, the following explanations can be given and remarks can be made dealing with our observations. Firstly, the limited amount of new bone formation confirms again that demineralized bone is variable in its osteoinductive potential[10, 11, 16]. Of course, failure to detect reproducible bone induction using demineralized freeze-dried rat bone powder may be due to processing problems. For example, we know that the complete removal of all potentially inflammatory or antigenic substances is very important, since this can inhibit the osteoinductive properties of the DBM. Therefore, we

followed a detailed procedure for the preparation of demineralized bone powder, which had been proven successful before[8]. In view of this, we have to notice that the inherent variability between donors and also unknown surgical as well as donor conditions can interfere with bone inductivity. In order to learn more about the importance and relevance of such difficult to control variables, it appears to be a prerequisite that in future studies controls with proven osteoinductive characteristics are included in the study design.

Secondly, comparison with our earlier subcutaneous DBM suggests that implant location is not a very relevant parameter for bone inductivity. This corroborates with one of our other studies in which we inserted titanium fiber mesh implants provided with bone marrow cells in the thigh muscle and subcutaneous tissue of rats[17]. Bone formation, as induced by the marrow cells, was observed in all titanium meshes, but the location of the construct did not have an effect at all on the initiation as well as amount of bone formation.

Finally, we have to notice that we cannot exclude that more bone formation occurred in our specimens than determined in the histomorphometrical analysis. Histology is a destructive and time consuming technique. For example, only nine sections (at three different locations) could be analyzed in the current study. Bone formation in between the sections and selected areas will be missed and is impossible to avoid. Therefore, besides histology, other non-destructive methods, like micro-computer tomography (micro-CT), have to be recommended for the evaluation of bone formation in osteoinductivity studies.

Conclusion

Although in the literature it has been stated that the surface area of DBM is an important factor in the bone forming capacity of DBM, our results do not support this theory: enlargement of the surface area by morsellizing DBM implants did not lead to more bone formation. Significant amounts of newly formed bone were present in some DBM as well as MDBM implants while in others no or very little new bone was found. Still, the amount of bone formation was limited compared with previous studies. Therefore, in order to learn more and exclude unknown experimental variables, the inclusion of positive bone inductive controls as well the use of non-destructive analysis methods has to be recommended for future osteoinductivity studies.

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chapter five

Ectopic bone formation in rats: the importance of vascularity of the acceptor site

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Biomaterials, 2004; 25(27): 5831-7

Introduction

Bone Graft Substitutes (BGS's) can be fabricated by the interaction of three key components: 1) competent bone-forming cells, 2) a suitable framework or scaffold, and 3) the presence of biological stimulants[1]. Based on these three components, a classification system for BGS has been developed by Laurencin and Khan[2]. According their description, cell-based, factor-based, ceramic-based, polymer-based and metal-based BGS can be discerned. Worldwide a lot of work has already been done to develop the ideal BGS that can be applied in patients for the repair and regeneration of large skeletal defects[2, 3]. Although some promising results have been reported, till now the outcome with such so-called tissue engineered bone grafts are not so consistent as hoped for. This is especially true for the cell-based approaches[4-6]. Consequently, the ideal BGS is not available yet. In view of this, the overall aim of the research as performed in our laboratory is to obtain more knowledge and understanding of the osteoinductive capacity of cell-based bone graft systems and how such constructs can be used to direct the bone healing process.

Considering the inconsistent results with cell-based BGS, we know that the cellularity and vascularity of the recipient bed are very important parameters in the bone formation process[7, 8]. The importance of vascularity has also been confirmed in animal studies where factor-based BGS were used. For example, Khouri found skeletal muscle the optimal responding tissue for BMP-3 induced bone formation in the rat and hypothesized that muscle tissue with its blood vessels and their associated pericytes is a rich source of stem cells capable of responding to bone morphogenetic proteins[9]. Yoshida used recombinant human bone morphogenetic protein-2 and atelopeptide type-I collagen as a carrier in intramuscular and subcutaneous sites in the rat and found also a significant difference in favor of the intramuscular implants at 7 and 21 days[10]. Okubo observed a similar preference for intramuscular bone formation and claimed that the variations of osteoinductive activity of rhBMP-2 in various sites might be due to differences in the blood supply[11]. This importance of the blood supply was also confirmed by Kusumoto, who used rhBMP-2 and atelopeptide type-I collagen in a rat latissimus dorsi flap[8, 12]. The

osteoinductive activity of rhBMP-2 in tissue with low blood flow has been proven to be less than in normal tissue[13]. Besides the presence of pericytes as a source for stem cells, vascularity has also an effect on oxygen and nutrient supply, Hereby, a low oxygen concentration favors chondrogenesis, while a higher oxygen supply supports bone formation[14].
To this end, it can be hypothesized that vascularity of the recipient site is also important for the osteoinductive capacity of a cell-based BGS. When this is true, then a muscle recipient site has to be preferred above a subcutaneous recipient site due to the higher vascularity of muscle tissue[15, 16]. To prove this hypothesis, titanium (Ti) fiber mesh implants were seeded with rat bone marrow cells (RBM) and implanted either subcutaneously of intramuscularly in the adductor thigh muscle of the rat. The amount of bone formation after one, three and six weeks was evaluated by histology and histomorphometry as well as by determining the calcium content inside the constructs.

Materials and methods

Implants:

Forty-eight sintered porous Ti-fiber mesh implants with a volumetric porosity of 86%, density of 600 g/m^2 and a fiber diameter of 50 mm were used as scaffold material. The titanium fiber mesh is fabricated by interengaging and intertwining a multiplicity of thin titanium fibers. The fibers are bond at their points of contact using a sinter process. This results in a very open interconnected porosity. The average pore size of the mesh was about 250 µm. The diameter of the implants was 6 mm, the thickness 0.8 mm and the weight about 15 mg. All specimens were sterilized by autoclavation at 121°C for 15 min.

Cell culture technique:

The rat bone marrow cell culture technique was used as described by Maniatopoulos[17]. RBM cells were obtained from femora of 16 syngeneic male Fisher 344 rats (150-170 g, 6 weeks old). Femora were washed twice in αMEM (Minimal Essential Medium; MEM Gibco BRL, Life Technologies B.V.) with 0.5 mg/ml gentamycin and 3 µg/ml fungizone. Epiphyses were

cut off, and both diaphyses were flushed out, using a-MEM supplemented with 10% fetal calf serum (FCS; Gibco), 50 mg/ml freshly prepared ascorbic acid, 10 mM Na-β-glycerophosphate, 10^{-8}M dexamethasone and 50 µg/ml gentamycin (Sigma Chemicals, St. Louis, MO). Per two femora 15 ml of this medium was used to generate a cell suspension. This cell suspension was distributed over three tissue culture flasks per two femurs. Finally, cultures were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37ºC.

After six days of primary culture (day 0), cells were detached using trypsin/EDTA (0.25% w/v trypsin/0.02% EDTA) (Sigma) and concentrated by centrifugation at 1500 rpm for five minutes and resuspended in medium. A Coulter counter was used to count the cells. The cell suspension was diluted to 5 * 10^6 cells/ml in complete media. Subsequently, cells were seeded into the carrier materials. The specimens were loaded by placing the carrier materials in a cell suspension of 5 * 10^6 cells/ml (four ml cell suspension per 24 Ti-mesh discs). After a brief exposure to a vacuum with a 50 ml syringe, the tubes with the cell suspension and meshes were placed in an incubator (95% air, 5% CO_2, at 37ºC). Every 30 minutes the tubes were shaken manually. After four hours the meshes were removed from the suspension and placed in 24 well-plates. Subsequently, one ml of medium was added to each well. All constructs were cultured for one day prior to implantation[18]. Subsequently, all constructs were transported to the animal facility.

Implantation procedure

For implantation 24 four-week-old syngeneic Fisher 344 male rats were used. Surgery was performed under general inhalation anesthesia with nitrous oxide and oxygen. The RBM loaded meshes were subcutaneously implanted into the back of the animals and intramuscularly in the adductor thigh muscle of the leg. For the insertion of the subcutaneous implants, the animals were immobilized and placed in a ventral position. The back of the animals was shaved, washed and disinfected with povidone-iodine. A longitudinal incision was made on one side of the vertebral column. Lateral of the incision a sub-cutaneous pocket was created using blunt dissection. After placement of the implants the skin was closed, using Agraven suture material.
For the insertion of the intramuscular implants in the adductor thigh

muscle, the animal was placed in a supine position. The groin and leg area were shaved, washed and disinfected with povidone-iodine. A longitudinal incision was made in the groin and leg, the incision was through the fascia and a pocket was made into the adductor thigh muscle. The implants were placed into this pocket. Each animal received one subcutaneous implant and one intramuscular implant. A total of 48 implants were placed:
24 subcutaneous Ti implants suspension-loaded with RBM cells (Ti/R-BM/s) and 24 intramuscular Ti implants suspension-loaded with RBM cells (Ti/R-BM/im).
All subcutaneous implants were placed on the left or right side of the animal, alternately and the intramuscular implants were subsequently placed in the contralateral leg.
Implantation periods were one, three and six weeks. At the end of each implantation period eight rats were sacrificed (n=8 for each material, acceptor site and time period).
Further, four rats out of the six week group received sequential triple fluorochrome labeling. Fluorochrome labels (i.e. tetracycline, alizarin-complexon and calcein (25 mg/kg body weight) were administered at one, three and five weeks respectively.
The animal experiments were performed according guidelines for animal experiments for scientific research of the Dutch government. The used animal protocol was approved by the Animal Care Ethics committee of the University of Nijmegen.

Evaluation techniques

Histology

Four specimens of every type and time period were retrieved for histological analysis: they were fixed and stored in phosphate-buffered 4% formalin solution (pH = 7.4). Then, the tissue blocks were dehydrated in a graded series of ethanol and embedded in methyl-methacrylate (MMA). Subsequently, 10 µm sections of the MMA embedded samples were made using a modified sawing microtome technique[19]. At least three transverse sections were made of each implant. The sections were stained with methylene blue and basic fuchsin. Additionally, in the six week group, two slightly thicker sections (30 mm) were made per implant for analysis with

a fluorescence microscope. To evaluate the tissue response to the implants, both histological and histomorphometrical analysis was performed. The histological evaluation consisted of a complete description of the observed thin sections.
A computer based image analysis system (Qwin Pro, version 2.3, Leica, Wetzlar, Germany) was used for histomorphometrical analysis. Two parameters were assessed: (1) the surface area of the newly formed bone and (2) the surface area of the titanium implant. In this way the relative bone formation could be measured. All histomorphometrical measurements were performed in the three different transverse sections of each implant, which were averaged to obtain the final result.

Calcium content

The other four implants of every type and time period were used for assessment of calcium content. These implants were lyophilized and subsequently weighed. After that, the implants were cut in half and then weighed again. All measurements were corrected for weight. The specimens were kept at -80ºC.
After thawing, one ml of acetic acid was added to every sample and stored overnight under constant shaking. The calcium content was determined using the orthocresolphthalein OCPC method with an Elisa reader at 575 nm[20].

Statistical analysis

Obtained data were compared among and within the groups using an ANOVA with a Tukey multiple comparison post-test. Differences were considered to be significant at P values less than 0.05. Before testing, all data were log transformed because Bartlett's test suggested that the differences among the standard deviations of the various experimental groups were significant. All statistical analyses were performed with GraphPad® Instat 3.05 software (GraphPad Software Inc., San Diego, Ca, USA).

Results

Clinical observations

All rats showed an undisturbed wound healing without any clinical signs of inflammation at the surgical sites during the various implantation times. At retrieval, both subcutaneous and intramuscular implants were surrounded by a thin reaction-free fibrous capsule.

Descriptive light microscopy

One week implants

One week after implantation, all intramuscular and subcutaneous implants were surrounded by a thin fibrous capsule, which was five to eight cell layers thick. No inflammatory reaction was found outside the capsule. Already fibrous tissue had penetrated in between the Ti fibers. This fibrous tissue still lacked a clear organization and contained only a very low number of inflammatory cells. In both the subcutaneous and intramuscular implants, there was an abundant ingrowth of capillaries. In three subcutaneous and three intramuscular implants, bone formation was observed inside the mesh porosity. This bone had a granular appearance and was deposited in small islands. Occasionally, cells were observed to be included in the deposited bone matrix. The bone islands were always surrounded by hypertrophic cartilage-like cells (Fig. 1).

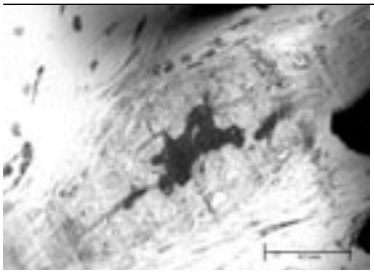


Figure 1. One week post-implantation, deposited bone mineral in an intramuscular implant is surrounded with hypertrophic cells. Original magnification 40x.

Three week implants

All three week implants were still surrounded by a thin fibrous capsule without the presence of inflammatory cells. Inside the mesh porosity of the subcutaneous and intramuscular implants, the fibrous tissue had become more organized, as characterized by the occurrence of collagen bundles. Almost no inflammatory cells were seen in the mesh porosity. Bone formation in the mesh porosity was observed in two subcutaneous and three intramuscular implants. The amount of deposited bone was definitely increased compared with the one week implants. Osteocytes could clearly be recognized within the mineralized bone matrix (Fig. 2). At the periphery of the deposited bone, osteoid and osteoblasts could be seen. Bone formation appeared to grow from the centre to the periphery of the implant (Fig. 3).

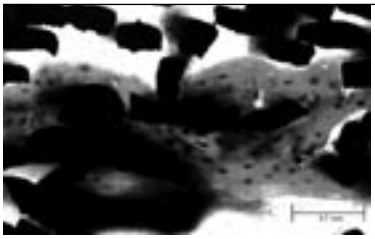


Figure 2. Three weeks post-implantation, osteocytes can be recognized in the mineralized bone matrix. Original magnification 20x.

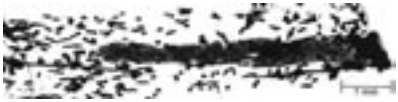


Figure 3. Three weeks post-implantation: bone appears to grow from the center to the periphery of the subcutaneous implant. No bone formation outside the area of the implant is observed. Bone formation appears to grow from the centre to the periphery of the implant. Original magnification 1.6x.

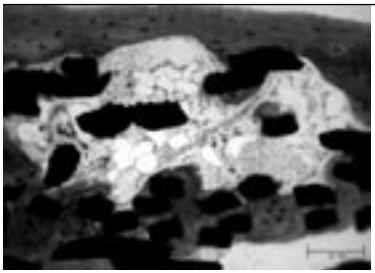


Figure 4. Six weeks post-implantation: cavities filled with bone marrow-like tissue were seen within the deposited bone. Original magnification 20x.

Six week implants

In the six week group, the appearance of the fibrous capsule around the intramuscular as well subcutaneous implants had not changed compared with the three week implants. The same was true for the fibrous tissue inside the mesh porosity. Bone formation was now observed in three of the subcutaneous and two of the intramuscular implants. The deposited bone had further matured compared with the three week specimens. In addition, cavities filled with bone marrow-like tissue were seen within the deposited bone (Fig. 4). The appearance of the bone in the subcutaneous as well as intramuscular implants was the same. Subjectively, the amount of bone appeared to be not further increased in the subcutaneous or intramuscular implants compared with the three week specimens.

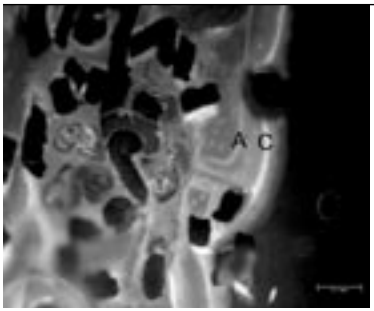


Figure 5. Six weeks post-implantation: a fluorescent light micrograph shows the three weeks alizarin layer (A, red) and the five weeks calcein layer (C, green). Bone formation starts in the center of the implant and grows to the periphery of the implant. Original magnification 20x.

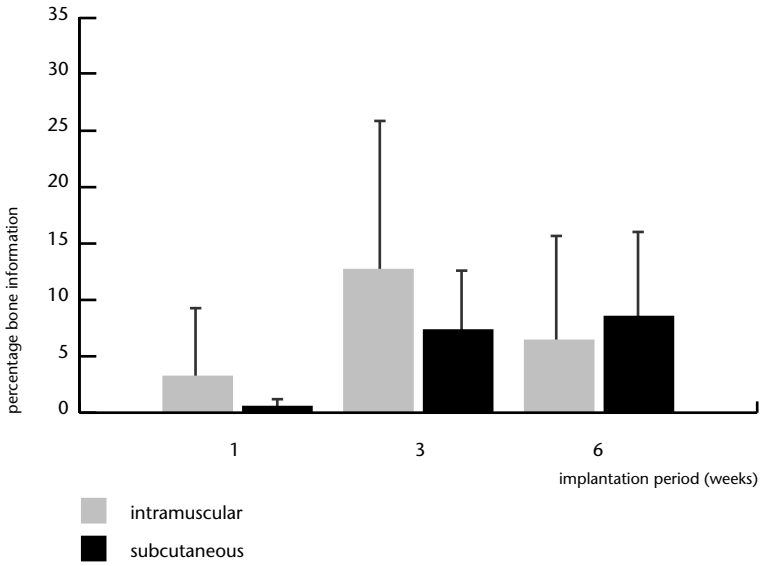
Fluorescence microscopy

Fluorescence microscopy confirmed the formation of mineralized tissue after six weeks. The fluorescent labels that stained the mineralization front, demonstrated that bone formation started from the center of the implant and proceeded to the periphery (Fig. 5). The mineralization speed could be measured for week three till week five by measuring the distance between the two differently stained mineralization lines: this amounted to seven µm in two weeks.

Histomorphometry

The results of the measurements are depicted in figure 6. After one week of implantation, bone formation for both the intramuscular and subcutaneous group is very limited. At three weeks, the amount of bone formation is increased till about 12 percent and stays at this level till six weeks of implantation. No significant difference between the two groups could be observed. Although, the data suggest a decrease in bone formation for the intramuscular implants after 6 weeks of implantation, this difference is not significant.

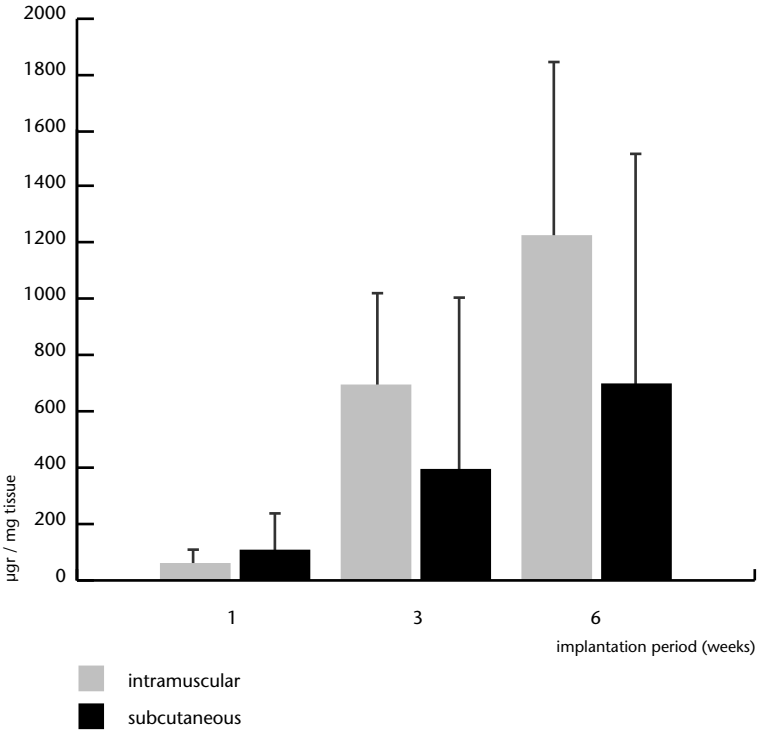
Figure 6. Percentage bone formation for each implant type and implantation period as determined by histomorphometry. No significant difference could be observed between the two groups.



Calcium content

The results of the measurements are depicted in figure 7. The data appear to indicate a continuous increase in calcium content during the implantation period of the constructs in both the subcutaneous as well intramuscular recipient sites. Statistical testing revealed that this increase was indeed significant for the intramuscular implants. Also, the data suggest that calcium content at three and six weeks is higher for intramuscular than subcutaneous specimens. However, statistical testing revealed that these differences were not significant.

Figure 7. Ca content as a marker for each implant type and implantation period. No significant difference between the two groups could be observed.



Discussion

In this experiment we hypothesized that the vascularity of the recipient site will have an effect on bone formation in cell-based BGS. Therefore, we implanted 48 titanium fiber mesh scaffolds, seeded with RBM cells, in a subcutaneous and muscle recipient site of rats. Bone formation was assessed by histomorphometry and calcium content at one, three and six weeks after implantation.

First, we have to notice that the results of the two analysis methods do not completely corroborate. In the calcium content measurements, a continuous increase in calcium content was seen during implantation time, while in the histomorphometry an increase in bone formation was only observed between one and three weeks, with even a tendency to decrease at six weeks. Probably, this difference is due to the fact that for the histomorphometrical analysis only three sections out of each specimen were used in contrast to the calcium assay where the whole specimen is used. We know already from other studies, that a wide variation in histological observations can occur within the same sample[21]. Therefore, we measure for our histomorphometrical analyses always at least three sections. Nevertheless, this can still result in underestimation of the amount of bone formation due to lacking data points. On the other hand, overestimation can occur in the calcium content analysis, since all calcium as present in the sample is measured, which includes the calcium as there is in all cells in the scaffold. Therefore, it is essential that the analysis of bone formation is always performed by using different methods.

Further, bone formation in our titanium mesh was very limited and hardly exceeded 10% of the total construct content. Also, no significant differences existed between the two recipient sites. Unfortunately this does not completely agree with various other reports, since literature suggests that muscle is a good donor site in BMP induced ectopic bone formation. Apparently, the advantage of blood vessels for oxygen and nutrient delivery as well as their associated pericytes as a rich source of stem cells are less important when RBM cells are already present in abundance through cell seeding.

Another factor, which is important in cell-based constructs is the optimal time of prolonged culturing after cell seeding in the porous scaffold material. In our previous experiment with titanium implants, seeded with RBM cells and inserted in a subcutaneous rat model without prolonged culturing of the constructs, only a very limited amount of ectopic bone formation was found[22]. In follow-up studies, the optimal time of culturing after cell seeding was found to be one day[18, 23]. Our results confirm these results: the consequent rise in calcium content from week one till week six for both groups indicates an ongoing process of osteogenic differentiation of the marrow stromal cells. Also, the values for the subcutaneous implants at six weeks (1231 (± 606) µg/ml) are comparable to the four week results of den Dolder (550 (± 200) µg/ml Ca)[18]. The results of the fluorescence microscopy further indicate that bone growth started at the center of the titanium mesh and proceeded subsequently during time to the periphery. This confirms that the suspension technique is capable of seeding cells not only to the surface of the implant, but also deep in the implant.

Finally, the ultimate goal of our bone engineering experiments is to reconstruct large bone defects in human patients. These defects can be caused by trauma, tumor or infection. An example of such a defect is a resected mandible after tumor surgery. An average mandible of an adult male weighs about 80 grams and has a volume of about 60 cm^3 [24].
The engineering of such a large bone part will give rise to much more vascularity problems during the bone formation, than in the rat model as used in this experiment. Besides that, we know that after ablative surgery for malignancies, the soft tissues are scarred by surgery and often radiation therapy[25, 26]. This leaves the osteoinductive potential of these tissues doubtful. In view of this, bone tissue engineered constructs should be well vascularised and the amount of bone should be much more than we can currently form. These new reconstructions should be at least as good as conventional reconstruction techniques, like the vascularised fibula bone graft[27]. With this conventional technique an adequate volume of bone can be made, which is in addition very well vascularised. Comparison with the current results, where bone formation is at the most 12%, learns that our final goal is still very far away. It can even be doubted whether cell-based constructs are the right way to go. Evidently, more efficient ways of

culturing, scaffold materials with bone inductive capacity and probably simultaneous use of growth factors, are required to solve the problems.

Conclusion

In summary, our results did not confirm that the vascularity of the recipient site plays a significant role in the osteoinductive capacity of cell-based titanium fiber mesh BGS.

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chapter six

Ectopic bone formation in rats: the importance of the carrier

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In press: Biomaterials, 2004

Introduction

Much research has been done on Bone Graft Substitutes (BGS). These BGS can be fabricated by the interaction of three key components: 1) competent bone-forming cells, 2) a suitable framework or scaffold, and 3) the presence of biological stimulants[1]. Treatment of bone defects with growth factors alone provides a direct osteoinductive stimulus, but this strategy is still dependent upon the presence of osteoprogenitor cells able to respond to this signal. Thus, implantation of these exogenous bioactive factors may have limited efficacy in clinical scenarios with reduced osteogenic potential, such as those involving significant bone loss, poor vascularity and inadequate soft tissue coverage[2]. An alternative to the use of growth factors is a cell-based approach. An advantage of this approach is that in principle immediately sufficient cells with bone forming capacity are delivered at the defect site. On the other hand, we know that this cell-based approach also faces a lot of problems and challenges[3, 4]. For example, the success of this method depends on multiple factors like the source and amount of cells used[5], the technique used for culturing[6], the environment and the scaffold material that is used[7]. In our laboratory, we try to get a better understanding of the effect of all these parameters in cell-based bone engineering strategies. For example, in earlier research we investigated the osteodifferentiation of marrow stromal cell/scaffold constructs in vitro[6, 8, 9]. Further, we have shown in an ectopic rat model that a titanium fiber mesh scaffold seeded with rat bone marrow cells produced a limited amount of bone. This was independent of the implantation site[10], seeding technique[11] and culture method[12]. Up till now we have mainly used titanium fiber mesh scaffolds in our studies because of the excellent mechanical properties and the bone-compatibility of this material[13]. On the other hand, we know that from a bone point of view calcium phosphate (CaP) ceramic has to be preferred above titanium. CaP ceramics are known for their excellent osteoconductivity and there are even suggestions that this material is osteoinductive[14]. As a consequence, the aim of the current study is to investigate the influence of the carrier in a cell-based bone regeneration approach, whereby we hypothesize that CaP ceramic implants will induce more bone formation than titanium fiber implants, in the same animal model as our previous experiment. To prove this hypothesis, titanium

(Ti) fiber mesh implants and ceramic implants were seeded with rat bone marrow cells (RBM) and implanted subcutaneously. The amount of bone formation after one, three and six weeks was evaluated by histology and histomorphometry.

Materials and methods

Implants:

Twenty-four sintered porous Ti-fiber mesh implants (Fig. 1a) with a volumetric porosity of 86%, density of 600 g/m^2 and a fiber diameter of 50 mm were used as scaffold material (Bekaert NV, Zwevegem, Belgium). The Ti-fiber mesh is fabricated by interengaging and intertwining a multiplicity of thin titanium fibers. The fibers are bond at their points of contact using a sinter process. This results in a very open interconnected porosity. The average pore size of the mesh was about 250 µm. The diameter of the implants was 6 mm, the thickness 0.8 mm and the weight about 15 mg. Twenty-four control implants consist of a "Biphasic Material" of porous Hydroxyapatite (HA) and tricalcium phosphate (TCP) in a relationship of 60%/40% (CAM implants BV, Leiden, the Netherlands) (Fig. 1b). The pore size is 300-500 µm with a volumetric porosity of 75%, The implants have a diameter of 6 mm and the height is 3 mm. All specimens were sterilized by autoclavation at 121ºC for 15 min. before use.

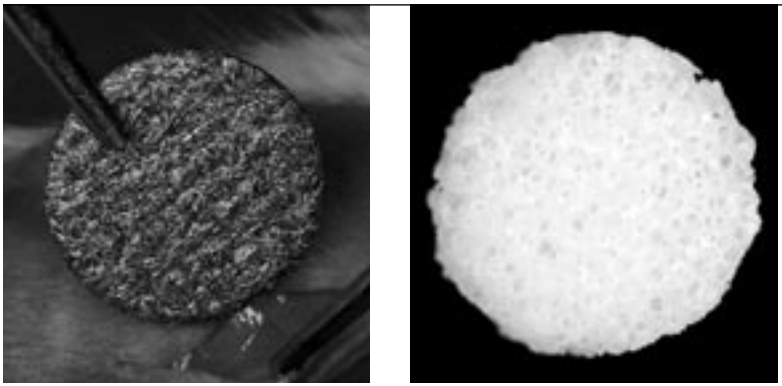


Figure 1a and b. Titanium fiber mesh implant with an average pore size of 250 um. The diameter of the implants is 6 mm, the thickness 0.8 mm. Figure 1b. "Biphasic Material" of porous Hydroxyapatite (HA) and tricalcium phosphate (TCP) in a relationship of 60%/40%. The pore size of the material is 300-500 µm^2 and the porosity is 75%, the implants have a diameter of 6 mm and the height is 3 mm (CAM implants BV).

Cell culture technique:

The rat bone marrow cell culture technique was used as described by Maniatopoulos[15]. RBM cells were obtained from femora of 16 syngeneic male Fisher 344 rats (150-170 g, 6 weeks old). Epiphyses were cut off, and both diaphyses were flushed out, using α-Minimal Essential Medium (Gibco BRL) supplemented with 10% fetal calf serum (FCS), 50 mg/ml freshly prepared ascorbic acid, 10 mM Na-β-glycerophosphate, 10^{-8}M dexamethasone and antibiotics. Per two femurs 15 ml of this medium was used to generate a cell suspension. This cell suspension was distributed over three tissue culture flasks per two femurs. Finally, cultures were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37ºC.
After 7 days of primary culture (day 0), cells were detached using trypsin/EDTA and concentrated by centrifugation at 1500 rpm. for five minutes and resuspended in a known amount of medium. A Coulter counter was used to count the cells. The cell suspension was diluted to 5 * 10^6 cells / ml in complete media. Subsequently, cells were seeded onto the carrier materials. The specimens were loaded by placing the carrier materials in a cell suspension of 5 * 10^6 cells/ml. After a brief exposure to a vacuum with a 50 ml syringe, the tubes with the cell suspension and scaffolds were placed in an incubator (95% air, 5% CO_2, at 37ºC). Every 30 minutes the tubes were shaken manually. After 4 hours the scaffolds were removed from the suspension and placed in 24 well-plates. Subsequently, 1 ml of medium was added to each well. All constructs were cultured for 1 day prior to implantation[12]. Then the medium was replaced by serum-free medium. Subsequently, all constructs were transported to the animal facility.

Implantation procedure

For implantation 24 four-week-old syngeneic Fisher 344 male rats were used. Surgery was performed under general inhalation anesthesia with nitrous oxide and oxygen. The RBM loaded scaffolds were implanted subcutaneously into the back of the animals. For the insertion of the subcutaneous implants, the animals were immobilized and placed in a ventral position. The back of the animals was shaved, washed and disinfected with povidone-iodine. Two longitudinal incisions were made on both sides of the vertebral column. Lateral of the incisions a subcutaneous

pocket was created using blunt dissection. After placement of the implants the skin was closed, using Agraven suture material. All titanium implants were placed at the left or the right side alternately and the ceramic implant was subsequently placed at the contralateral side.. A total of 48 implants were placed: 24 Ti implants suspension-loaded with RBM cells (Ti/R-BM) and 24 CaP implants suspension-loaded with RBM cells (CaP/R-BM). Implantation periods were one, three and six weeks. At the end of each implantation period eight rats were sacrificed (n = 8 for each material and time period).
The animal experiments were performed according guidelines for animal experiments for scientific research of the Dutch government. The used animal protocol was approved by the Animal Care Ethics committee of the University of Nijmegen.

Evaluation techniques

Histological analysis

Eight specimens of every type and time period were retrieved for histological analysis: they were fixed and stored in phosphate-buffered 4% formalin solution (pH = 7.4). Then, the tissue blocks were dehydrated in a graded series of ethanol and embedded in methyl-methacrylate (MMA). Subsequently, 10 µm sections of the MMA embedded samples were made using a modified sawing microtome technique[16]. At least three transversal sections of sufficient quality for evaluation were made of each implant. The sections were stained with methylene blue and basic fuchsin. To evaluate the tissue response to the implants, both histological and histomorphometrical analysis was performed. The histological evaluation consisted of a complete description of the observed thin sections.

A computer based image analysis system (Qwin Pro, version 2.3, Leica, Wetzlar, Germany) was used for histomorphometrical analysis. Two parameters were assessed: (1) the surface area of the newly formed bone, and (2) the surface area of the titanium respectively CaP ceramic implant. In this way the relative bone formation could be measured. All histomorphometrical measurements were performed in three different transverse sections of each implant, which were averaged to obtain the final result.

Statistical procedure

The histomorphometrical measurements of CaP ceramic and Ti scaffolds were statistically evaluated with GraphPad® Instat 3.05 software (GraphPad Software Inc, San Diego, CA, USA), using an unpaired T-test with Welch correction. To test whether the data were sampled from populations that followed Gaussian distributions, the test method of Kolmogorov and Smirnov was used.

Results

Clinical observations

All rats showed an undisturbed wound healing without any clinical signs of inflammation at the surgical sites during the various implantation times. At retrieval, the implants were surrounded by a thin reaction-free fibrous capsule.

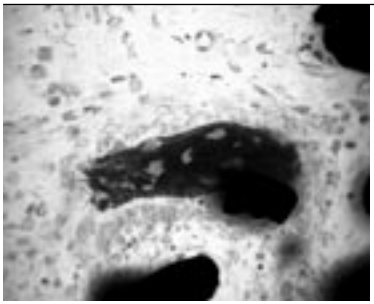
Descriptive light microscopy

One week implants

One week after implantation, all titanium and ceramic implants were surrounded by a thin fibrous capsule, which was five to eight cell layers thick. No inflammatory reaction was found outside the capsule. Already fibrous tissue had penetrated in between the Ti fibers and inside the CaP porosity. This fibrous tissue still lacked a clear organization and contained only a very low number of inflammatory cells. In both the titanium and ceramic implants, there was an abundant ingrowth of capillaries. In three titanium and seven ceramic implants, bone-like tissue formation was observed inside the scaffold porosity. This bone-like tissue had a granular appearance and was deposited in small islands. Occasionally, cells were observed to be included in the deposited bony matrix. The bone-like islands were always surrounded by hypertrophic cartilage-like cells (Fig. 2).

Three week implants

All three week implants were still surrounded by a thin fibrous capsule without the presence of inflammatory cells. Inside the mesh porosity of



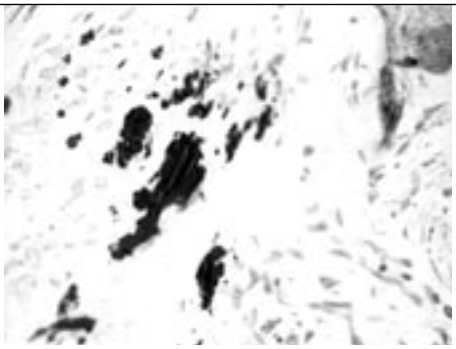
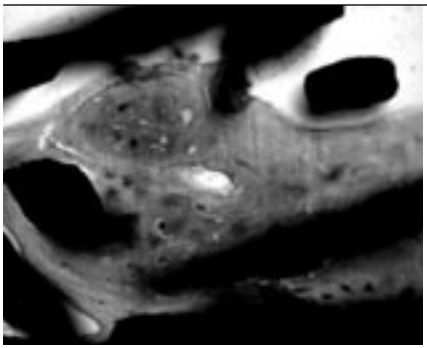


Figure 2a and b. After one week in both the titanium group (2a) as well as in the ceramic group (2b), the bone islands were always surrounded by hypertrophic cartilage-like cells (both figures: original magnification 40x).



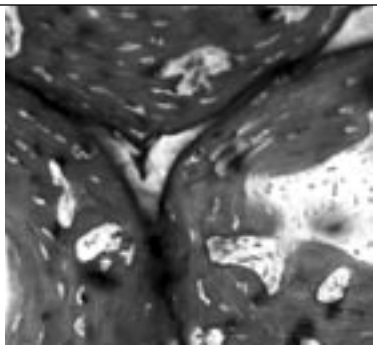


Figure 3a and b. After three weeks in both the titanium group (fig. 3a) as well as in the ceramic group (fig. 3b), osteocytes could be clearly recognized within the mineralized bone matrix. At the periphery of the deposited bone, osteoid and osteoblasts could be seen (both figures: original magnification 40x).

the titanium and ceramic implants, the fibrous tissue had become more organized, as characterized by the clear occurrence of collagen bundles. Almost no inflammatory cells were seen in the scaffold porosity. Bone formation in the scaffold porosity was observed in seven titanium and eight ceramic implants. The amount of deposited bone was definitely increased compared with the one week implants, especially for the ceramic group. At three weeks, osteocytes could be clearly recognized within the mineralized bone matrix both in the titanium group (Fig. 3a) as well in the ceramic group (Fig. 3b). At the periphery of the deposited bone, osteoid and osteoblasts could be seen.

Six week implants

In the six week group, the appearance of the fibrous capsule around the titanium and ceramic implants had not changed compared with the three week implants. The same was true for the fibrous tissue inside the mesh porosity. Bone formation was now observed in seven titanium and eight ceramic implants. The appearance of the bone formation differed between the titanium and ceramic implants. Bone formation in the titanium mesh was observed as a dense entity localized centrally in the implants. The bone appeared to grow from the centre to the periphery of the implant in the titanium group (Fig. 4a). In contrast, bone formation in the ceramic implants occurred through the whole implant. Bone formation was characterized by apposition as a shell-like layer on the pore surface with subsequent centripetal fill of the pore (Fig. 4b).The deposited bone had always further matured compared with the three week specimens. In addition, cavities filled with bone marrow-like tissue were seen within the deposited bone in both groups (Fig.5a and b respectively).

Histomorphometry

The results of the measurements are depicted in figure 6. After 1 week of implantation, bone formation for both the titanium and ceramic group is very limited. At three weeks, the amount of bone formation is increased till about 10 percent for the titanium group and 18 percent for the ceramic group. No significant difference between the two groups could be observed ($P>0.05$). In the six week group, the bone formation is 6 (Ti) and 23 (CaP) percent, respectively. Statistical testing showed that this difference is extremely significant ($P<0.0001$).

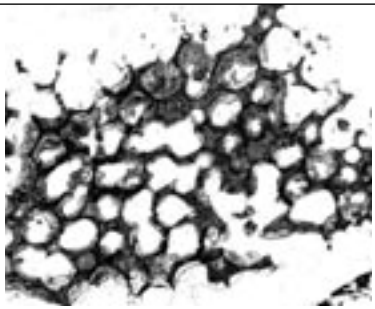
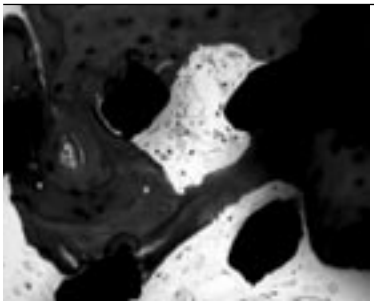


Figure 4a and b. After six weeks, in the titanium group bone formation appeared to grow from the centre to the periphery of the implant, while no bone formation outside the area of the implant was observed. Figure 4b. In the ceramic group bone formation was by apposition of bone to the inner surface of the implant (both figures: original magnification 2.5x).



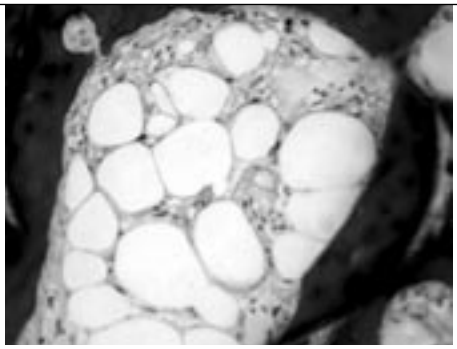
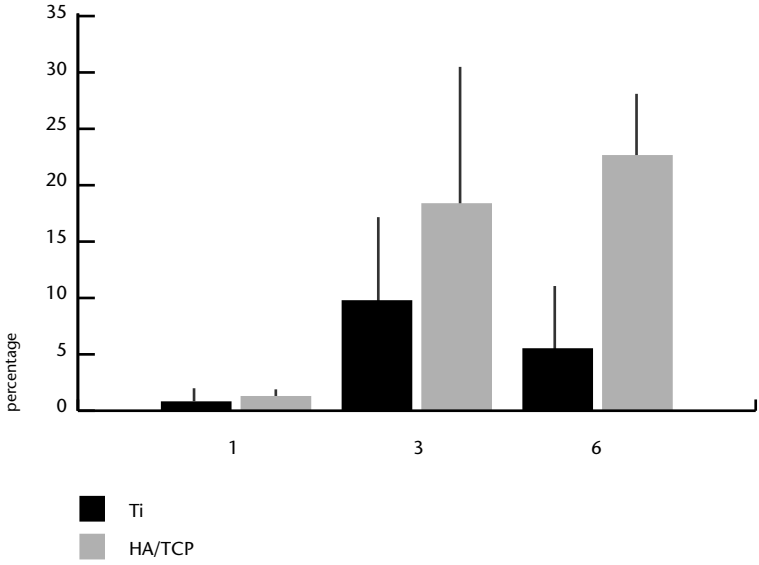


Figure 5a and b. In the titanium group, cavities filled with bone marrow like tissue were seen within the deposited bone. Figure 5b. Cavities filled with bone marrow tissue could be observed in the ceramic group as well (both figures: original magnification 40x).

Figure 6. Percentage bone formation for each implant type and implementation period as determined by histomorphometry shows a significant difference for the six week group: the titanium group shows 6 percent and the ceramic group 23% bone formation.



Discussion

We hypothesized that the use of a ceramic scaffold in a cell-based BGS technique would lead to more bone formation than a titanium fiber implant. Therefore, we implanted 24 titanium fiber mesh implants and 24 biphasic ceramic implants, seeded with rat bone marrow cells, in a subcutaneous recipient site. The amount of bone formation after one, three and six weeks was evaluated by histology and histomorphometry. Bone formation started earlier in the CaP ceramic scaffolds than in the Ti scaffolds (7 vs. 3 positive specimens at one week of implantation). Further, after six weeks a significant difference in amount of bone formation was observed between the titanium and the CaP-ceramic group. The low amount of bone formation in the titanium implants corresponds well with our earlier studies, in which we found that bone formation hardly exceeded ten percent.

As mentioned above, the amount of bone formation in the titanium implants was limited, while in the CaP-ceramic group this was much higher. In addition, the deposition pattern of the bone in the CaP scaffolds was different. Bone formation in the ceramic implants occurred through the whole implant and was characterized by apposition as a shell-like layer on the pore surface with subsequent centripetal fill of the pore. Consequently, the bone in the CaP scaffolds resembles much more trabecular bone. In contrast, in the titanium scaffolds the bone appeared to grow from the centre to the periphery of the implant. At itself, this is remarkable, because due to the diffusion dependent bone formation in porous implants growth from the periphery to the centre would be expected. Still, such a growth pattern has been described before and is probably caused by the fast invasion of our titanium scaffolds with new blood vessels[11]. The observed differences in bone deposition between titanium and CaP-ceramic can be explained by the known excellent bone biocompatibility of CaP-ceramic. In view of this, biphasic CaP ceramics, composed of a mixture of hydroxyapatite and tricalcium phosphate, are considered to be even more bioactive and more efficient than hydroxyapatite alone[17]. Combined with a BMP (osteogenin), biphasic CaP ceramic, has already shown to provide good results in the healing of a segmental defect in a long bone in

the rat[18]. Also, biphasic calcium phosphate ceramics have been shown to possess osteoconductive and osteogenic activity in rabbits[19]. Probably, this favorable behavior of CaP-ceramics is the result of the preferential binding of growth factors and/or bone marrow cells to CaP-ceramic surfaces[20]. We also know that cell adhesion molecules play an important role in the binding of bone forming cells to an implant surface[20]. Recently, Ripamonti et al. even described that sintered hydroxyapatite can spontaneously induce bone formation in adult primates without exogenously applied BMPs, whereby the implants are thought to adsorb endogenously produced osteogenic molecules[21]. Despite the clear biological advantage of CaP-ceramic, the weak mechanical properties and the poor resorbability of e.g. hydroxyapatite remain a problem for clinical application[22]. Currently, we can only hypothesize that due to the extensive bone formation inside the porosity, the biomechanical properties of our CaP scaffolds will be completely different from the as received material. In future experiments, this hypothesis has to be proven.

Besides the difference in chemical composition, we have to notice that our scaffold materials also differed from each other in pore size and pore geometry. It has been shown before that these geometrical parameters can affect the amount as well as the pattern of bone growth into the scaffold[23-26], since they control the diffusion of oxygen, growth factors and nutrients to the cells. Although such an effect cannot be excluded completely for our Ti-mesh and CaP material, we still do not suppose that these are the driving parameters for the observed pattern of bone formation. In a previous study, we investigated ectopic bone formation in non-coated and CaP-coated Ti-mesh in the same animal model[27]. Histology showed that after 2, 4 and 8 weeks always more bone was formed in the coated compared to the non-coated titanium mesh and we concluded already that CaP ceramic can have a beneficial effect on the bone-generating properties of a scaffold material.

Another material that is used as bone graft substitute is demineralized bone matrix (DBM). Its bone inductive activity is determined by the amount and activity of bone morphogenic proteins (BMP). Bone formation in a DBM implant after six weeks has been established before as 25%[28], which is about the same value as we have found for the ceramic implants. Nevertheless,

a disadvantage of DBM compared to cell-based CaP constructs is the variability of the osteogenic potential[29].

Finally, we have to mention that to improve the bone inductive properties of titanium, a surface pretreatment method can be applied[30]. Such a method makes the titanium surface more bioactive and can promote cell differentiation. Through chemical and heat treatment, the surface of titanium implants is changed to get an appropriate macrostructure and microstructure. In this way an apatite layer can form (in vitro as well in vivo) onto which bone matrix integrates more easily than just blank titanium-oxide.

Conclusion

Our hypothesis that ceramic implants, seeded with rat bone marrow cells produce more bone formation than titanium fiber implants in the same rat model was confirmed. We conclude that the biphasic ceramic implants are promising material for bone tissue engineering. Our findings also support the need for further research to assess the efficacy of these materials in larger animal models and in orthotopic locations.

Acknowledgements

We would like to thank Jan-Paul van der Waerden for his help with the image analysis, Anja de Ruijter for helping out with the cell culturing and CAM Implants BV, Leiden, The Netherlands for the provision of the CaP scaffolds.

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chapter seven

Ectopic bone formation in rats: comparison of biphasic ceramic implants seeded with cultured rat bone marrow cells in a pedicled and a revascularised muscle flap

Ed H.M. Hartman, P. Quinten Ruhé, Paul H.M. Spauwen, John A. Jansen

In press: European Journal of Plastic Surgery, 2004

Introduction

The aim in reconstructive surgery is restoration of function and form. Reconstructions in head and neck surgery are performed by tissue transfer. Local flaps have been used extensively in the past, but they are limited in their application. In contrast, microvascular free flaps give the opportunity to transfer tissue, which can be tailor-made to the defect to be reconstructed. For example, one of the major challenges in head and neck reconstructive surgery is the reconstruction of the mandible. In this case, the application of microsurgically revascularised bone flaps with robust vascularity produces higher success rates than the use of non-vascularised bone flaps, especially in irradiated patients[1]. The most often used microsurgical flaps for bone reconstruction are: fibula, iliac crest, scapula and radial forearm flap. Unfortunately, such flaps leave the patient with inherent donor site morbidity[2]. Another problem with the use of autogenic bone can be a lack of sufficient bone tissue. Therefore, the search for other treatment methods with less associated problems has to continue. In view of this, recent progress in tissue engineering offers perhaps a solution for the current problems with donor site morbidity associated with microsurgical flaps, since tissue engineering allows the creation of a preshaped, vascularised bone graft at a convenient donor site. For this reason, we are carrying out a series of studies with the ultimate goal of developing a method for microsurgical reconstruction of bone defects with help of a cell-based tissue engineering approach. In our strategy, bone marrow is the source for the bone forming cells, which are subsequently added to an appropriate carrier or scaffold material. Potential carriers, as tested by us, were porous sintered titanium fiber mesh and porous biphasic calcium phosphate (CaP) ceramic. The titanium mesh has excellent mechanical properties in terms of stiffness and elasticity[3-6]. However, an inherent disadvantage of titanium is that it is non-degradable and does not further improve bone formation as induced by the bone marrow cells. Recently, we found that the porous biphasic CaP ceramic appeared to be more suitable[7]. Bone formation was not only increased in this material compared with titanium mesh, but also the distribution of the newly formed bone throughout the scaffold as well as the morphological appearance was significantly better. The next step in our development is to

determine the appropriate acceptor site for the final microsurgical flap[8] and to determine how microsurgery affects the bone forming capacity of the final construct.

Considering the above mentioned, the present experiment is focused on the microsurgical aspect of tissue engineered bone constructs. We implanted sixteen porous ceramic implants containing cultured bone marrow cells in the adductor thigh muscle flap of the rat. To be sure that the flap relied on the vascular pedicle only, just like in a clinical situation, the flap was enveloped by a silicone sheet. After four weeks the pedicles of eight flaps were cut and directly microsurgically anastomosed. The other eight flaps were given a sham operation, in which all branches of the pedicle were cut and ligated, but the main pedicle was left intact. Four weeks after the second surgery, all implants were harvested and analyzed by histology and histomorphometry to study the effect a microvascular operation might have on the bone forming process. We hypothesized that the amount of bone formation in the microsurgical group would be similar to the conventionally pedicled group.

Materials and methods

Implants:

Sixteen implants were used consisting of a "Biphasic Material" of porous Hydroxyapatite (HA) and tricalcium phosphate (TCP) in a relationship of 60%/40% (CAM implants BV, Leiden, the Netherlands) (Figure 1). The pore size was 300-500 µm with a volumetric porosity of 75%. The implants had a diameter of 6 mm and the height was 3 mm. All specimens were sterilized by autoclavation at 121ºC for 15 min. before use.

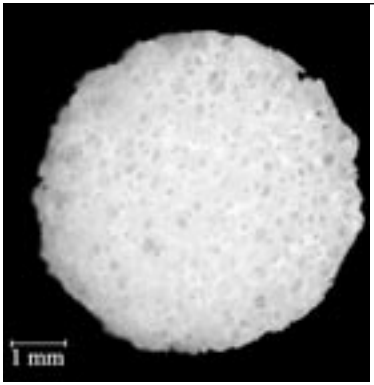

Figure 1. "Biphasic Material" of porous Hydroxyapatite (HA) and tricalcium phosphate (TCP) in a relationship of 60%/40%. The pore size of the material is 300-500 µm, the porosity is 75%, the implants have a diameter of 6 mm and the height is 3 mm (CAM implants BV).

Cell culture technique:

The rat bone marrow cell culture technique was used as described by Maniatopoulos[9]. RBM cells were obtained from femora of 12 syngeneic male Fisher 344 rats (150-170 g, six weeks old). Epiphyses were cut off, and both diaphyses were flushed out, using α-Minimal Essential Medium supplemented with 10% fetal calf serum (FCS), 50 mg/ml freshly prepared ascorbic acid, 10 mM Na-β-glycerophosphate, 10^{-8}M dexamethasone and antibiotics. Per two femurs 15 ml of this medium was used to generate a cell suspension. This cell suspension was distributed over three tissue culture flasks per two femurs. Finally, cultures were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

After seven days of primary culture (day 0), cells were detached using trypsin/EDTA and concentrated by centrifugation at 1500 rpm. for five minutes and resuspended in a known amount of medium. A Coulter counter was used to count the cells. The cell suspension was diluted to 5 * 10^6 cells/ml in complete media. Subsequently, cells were seeded onto the carrier materials. The specimens were loaded by placing the carrier materials in a cell suspension of 5 * 10^6 cells/ml (8 ml cell suspension per 16 implants). After a brief exposure to a vacuum with a 50 ml syringe, the tubes with the cell suspension and implants were placed in an incubator (95% air, 5% CO_2, at 37°C). Every 30 minutes the tubes were shaken manually. After 4 hours the implants were removed from the suspension and placed in 24 well-plates. Subsequently, 1 ml of medium was added to each well. All constructs were cultured for 1 day prior to implantation[10]. Then the medium was replaced by serum-free medium. Subsequently, all constructs were transported to the animal facility.

Surgical procedure

For implantation eight four-week-old syngeneic Fisher 344 male rats were used. Surgery was performed under general inhalation anesthesia with nitrous oxide and oxygen. A total of sixteen implants were placed in the adductor thigh muscle flap[11], two implants were used per rat (Figure 2). For the insertion of the implants, the animals were immobilized and placed in a supine position. During the implantation procedure the core temperature was registered with a rectal probe and maintained with a

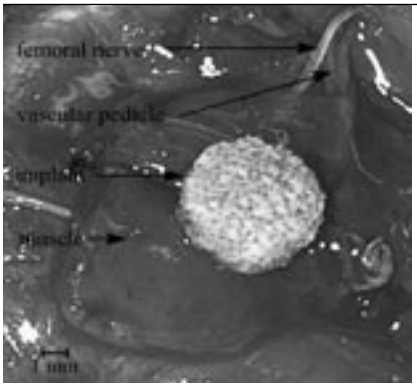

Figure 2. The implant is placed on the adductor thigh muscle flap. The pedicle of the flap can be seen at the right upper side of the figure. The femoral nerve has been spared and is seen lying under the muscle flap. The muscle flap will be folded over the implant and a silicone sheet will prevent vascular ingrowth from the surrounding tissues.

variable-temperature circulating-water bed. The skin of the animals was shaved, washed and disinfected with povidone-iodine. After incising the skin on the medial side of the hind limb, the saphenous vessels were ligated distally, just above the point where the vessels split in two or three branches. The anterior and posterior gracilis muscles were elevated from distal to proximal, preserving the branches of the saphenous vessels to these muscles. The flap was closed with non-absorbable monofilament 7x0 sutures (Nylon). Around this muscle flap, a 1.5 x 1.0 cm silicon sheet with a thickness of 1.0 mm was wrapped to prevent vascular ingrowth from the surrounding tissues. Consequently, the flap was vascularised solely from its pedicle. At the end of the operation, the skin was closed with stainless steel staples. After four weeks all rats were re-operated: at one side the vascular pedicle was cut and microsurgically anastomosed using an operation microscope (Zeiss OPMI 6 - DF). After clamping the vessels with Ackland microvessel clamps and cutting the artery and the vein of the pedicle, the vessels were irrigated with heparin solution and anastomosed using Ethilon® 10x0 sutures. At the other side a sham operation was done: dissection of the pedicle without cutting the vessels, but with ligation of all side branches. Four weeks after the second surgery, all rats were sacrificed and implants with surrounding tissue retrieved for histological evaluation.

Also, all rats received sequential triple fluorochrome labeling. Fluorochrome labels (i.e. tetracycline, alizarin-complexon and calcein (25 mg/kg body weight) were administered at week 1, 2 and 3 for the first four animals and at week 4, 5 and 6 for the second four animals.
The animal experiments were performed according guidelines for animal experiments for scientific research of the Dutch government. The used animal protocol was approved by the Animal Care Ethics committee of the University of Nijmegen.

Evaluation techniques

Histological analysis

Four specimens of every type and time period were retrieved for histological analysis: they were fixed and stored in phosphate-buffered 4% formalin solution (pH = 7.4). Then, the tissue blocks were dehydrated in a graded series of ethanol and embedded in methyl-methacrylate (MMA). Subsequently, 10 µm sections of the MMA embedded samples were made using a modified sawing microtome technique[12]. Three transversal sections were made of each implant. The sections were stained with methylene blue and basic fuchsin to determine bone formation. Additionally, two slightly thicker sections (30 mm) were made per implant for analysis with a fluorescence microscope. To evaluate the tissue response to the implants, both histological and histomorphometrical analysis was performed. The histological evaluation consisted of a complete description of the observed thin sections. The evaluators were blinded to the treatment groups.
A computer based image analysis system (Qwin Pro, version 2.3, Leica, Wetzlar, Germany) was used for histomorphometrical analysis. Two parameters were assessed: (1) the surface area of the newly formed bone, and (2) the surface area of the ceramic implant. In this way the relative bone formation could be measured. All histomorphometrical measurements were performed in the three different transverse sections of each implant, which were averaged to obtain the final result.

Statistical procedure

The histomorphometrical measurements were statistically evaluated with GraphPad® Instat 3.05 software (GraphPad Software Inc, San Diego, CA,

USA), using an unpaired T-test with Welch correction. To test whether the data were sampled from populations that followed Gaussian distributions, the test method of Kolmogorov and Smirnov was used.

Results

Clinical observations

After the first operation, which consisted of implantation of the ceramic implants, all rats showed an undisturbed wound healing without any clinical signs of inflammation at the surgical sites, except for one side in one rat. Here a hematoma developed postoperatively that showed spontaneous resorption and did not interfere with the second operation. During the second operation at four weeks, in both groups almost no muscle tissue over the implants was observed (Figure 3). A striking postoperative observation was that all rats, except one, showed a hematoma at the microsurgical side but not at the sham operation side. At retrieval, all implants in both groups were surrounded by a thin reaction-free fibrous capsule without any sign of remaining muscle tissue. Two of the eight microsurgical implants were surrounded by a hematoma and one microsurgical implant was surrounded by a seroma. No hematomas were observed in the pedicled group. All vascular pedicles were patent, as tested by milking the vessels. Some minor ingrowth of fibrous tissue along the folds of the silicone envelope to the implants was observed.

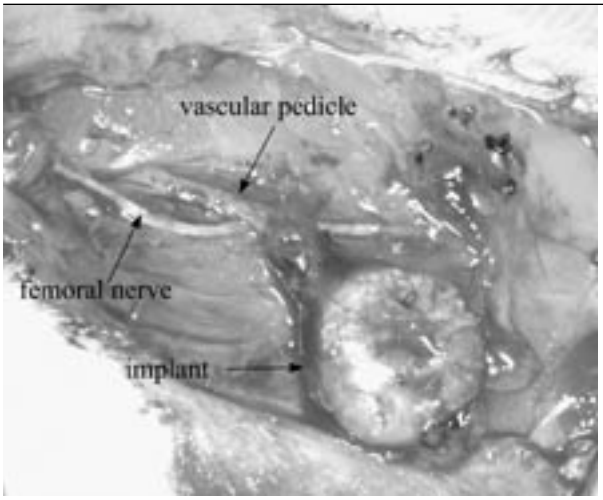

Figure 3. At explantation, no muscle could be observed surrounding the implants. Instead a thin fibrous capsule was found. All vascular pedicles were patent, as tested by milking the vessels.

Descriptive light microscopy

All implants were surrounded by a slightly inflamed capsule. No remaining muscle tissue from the adductor thigh flap was observed. Also, no vascular ingrowth from the surroundings was visible. Bone formation was observed in seven out of eight implants in the pedicled group and in all eight implants in the microsurgical group. Bone formation in the ceramic implants occurred through the whole implant and was characterized by apposition as a shell-like layer on the pore surface with subsequent centripetal fill of the pore (Figure 4a and 4b). In addition, cavities filled with bone marrow-like tissue were seen within the deposited bone. The appearance of the bone was the same in both groups. In one implant of the pedicled group, some calcifications outside the original ceramic implant and inside the surrounding fibrous capsule were observed (Figure 5).

Fluorescence microscopy

Fluorescence microscopy revealed that formation of bone was characterized by apposition as a shell-like layer on the pore surface with subsequent centripetal fill of the pore (Figure 6). This observation agrees with previously described bone deposition in porous ceramics[8,13] and emphasizes the osteocompatibility of CaP ceramics.

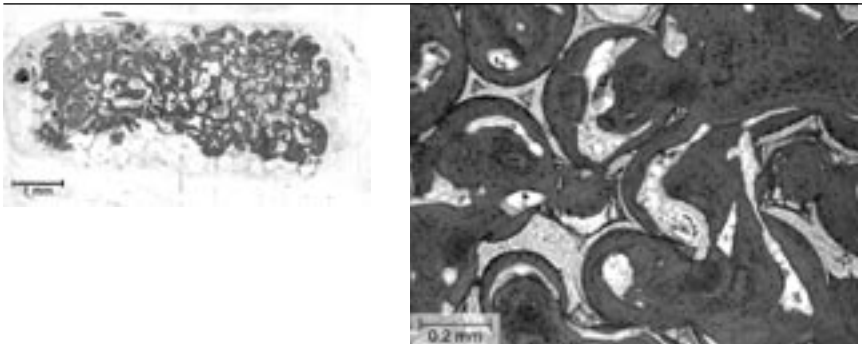

Figure 4a and b. Bone formation in the ceramic implants occurred through the whole implant. The absence of muscle tissue, which was found at explantation, was confirmed histologically. Instead a fibrous capsule was observed. Magnification in 4b.

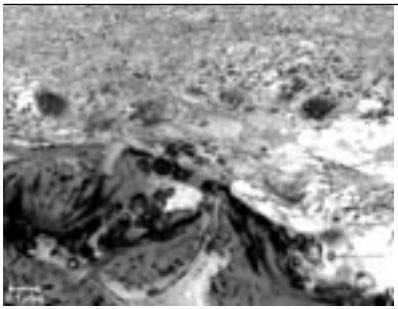

Figure 5. Calcifications are visible in the surrounding fibrous capsule, outside of the original implant. The absence of muscle tissue, which was found at explantation, was confirmed histologically. Instead a fibrous capsule was observed.

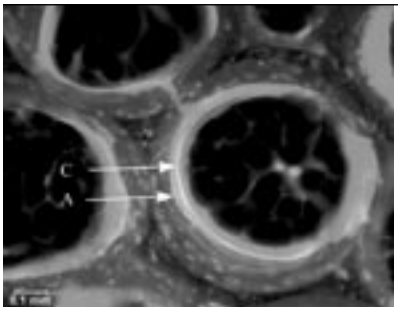

Figure 6. Fluorochrome markers show bone formation from the surface of the implant to the center. Alizarine (A) staining at two weeks (red) and calcein (C) staining at three weeks (green).

Histomorphometry

One implant from the pedicled group did not show any evidence of bone formation, all other implants revealed bone formation. Bone formation as determined by histomorphometry in the pedicled and the microsurgical group is 41 and 38 percent respectively (Figure 7). The standard deviation is 18 and 14 percent respectively. There is no significant difference between both groups (P>0.05).

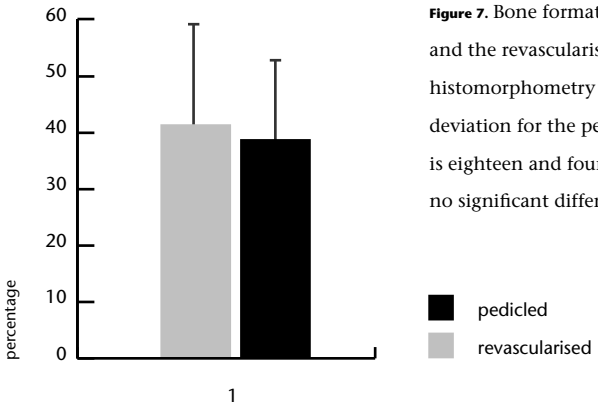

Figure 7. Bone formation in both the pedicled and the revascularised group as determined by histomorphometry is about forty percent. The standard deviation for the pedicled and the revascularised group is eighteen and fourteen percent respectively. There is no significant difference between both groups.

Discussion

Currently, we are performing a series of studies with the ultimate goal of developing a cell-based tissue engineering technique for the microsurgical reconstruction of bone defects. In previous studies, we evaluated already the efficacy of scaffold material as well as donor site with respect to the bone forming capacity of the construct. In this experiment we have explored the influence of a microvascular transplantation on the construct. Histological and histomorphometrical analysis confirmed that cutting and anastomosing the vascular pedicles did not influence the amount of bone formation of cell seeded CaP ceramic implants in the adductor thigh muscle flap of rats. This corroborates with the findings of Khouri, who demonstrated in a rat model viable bone tissue, eight months after prefabrication and microsurgical transplantation of muscle transformed into bone by using BMPs[14]. Similar observations have been reported in a minipig model, where viable bone was demonstrated three months after microsurgical transplantation of an in this way prefabricated muscle flap[15]. In both studies the vascular pedicle was functional, which is a prerequisite for bone formation. Insufficient blood supply reduces bone formation[16].

Our study differed from the aforementioned studies because we induced bone marrow cells to induce bone formation instead of BMPs. Also, we made a microvascular anastomosis, but did not transplant the prefabricated flap to an acceptor site. We wanted to prove first the feasibility of our cell-based technique before entering the next stage of transferring the construct.

Comparison of the results of our present study with an earlier study in which we induced bone formation with similar ceramic implants and cultured cells in a subcutaneous pocket, learns that the amount of bone formation correlates very well. In the previous study we found 23 percent bone formation after six weeks[7]. In our present study the amount of bone formation was 41 and 38 percent after eight weeks. Also the appearance of the bone formation between these two studies did not show differences either.

Nevertheless, we have to notice that some problems occurred in the current study. One of these is the finding that in almost all cases a hematoma was observed after the microsurgical anastomosis, while no hematoma was seen in the control group with the sham operation. In most cases this hematoma resorbed spontaneously, but in two rats this hematoma still existed at the time of explantation. The exact origin of this hematoma remains unclear. Most probably the anastomoses have leaked, especially since no hematoma were observed in the sham operated sides. Still, at the time of operation the anastomoses were patent and non-leaking, so there must have been a secondary leaking after the operation. However, the hematoma did not necessarily have a negative influence on the amount of bone in the flap because there was no difference in bone formation between the two groups. Evidently, the patency of the pedicle was not hampered due to the hematoma because all anastomoses were patent[17].

Further, in one of our specimens some small calcifications without evidence of osteoblasts were seen outside the implant. Occasionally, so-called external ectopic bone tissue is observed when superphysiological concentrations of exogenous BMP are used[18]. Evidently, this is not the case in our study. We suppose that due to the brittleness of our porous ceramic material some particles of the implant had broken off, which caused calcification in the capsule.

Another comment we have to make is that the muscle flap around all implants had completely disappeared, macroscopically as well as microscopically. To our knowledge this has not been described before. For example, no atrophy of a pedicled adductor muscle was seen three weeks after implantation of a coral scaffold combined with type I collagen and BMP-2 in the gracilis muscle of rats[19]. Also, a pedicled latissimus dorsi muscle flap was still maintained three weeks after bone formation by wrapping this muscle around just a collagen carrier with BMP-2[20]. In cases when only a bony reconstruction is needed, this loss of muscle tissue is no disadvantage when no bulky soft tissue is desired. Besides, we have to notice that a microsurgical reconstruction is even now possible because the vascular pedicle, which has been supplied by the muscle is still functioning. In other cases where a composite reconstruction is needed, other tissue like skin can be used, by including it in the prefabrication process.

Finally, we noticed some ingrowth of fibrous tissue along folds of the

silicone rubber envelope. The occurrence of such an ingrowth was not described before in another study where also the muscle was isolated form the surrounding tissue[21]. We suppose that this ingrowth did not influence the bone formation inside our specimens, because we could not detect vascular ingrowth from the surroundings into the ceramic scaffold in the histological slices.

Conclusion

Our results show that in rats viable microsurgical bone flaps can be obtained by using a cell-based tissue engineering approach. The amount of bone formation in a pedicled flap and in a microsurgical flap did not differ significantly in porous CaP ceramic scaffolds provided with bone marrow cells. These results warrant the further pursuit of this method in order to solve the problems associated with conventional microsurgical free flaps.

Acknowledgement

We would like to thank Jan-Paul van der Waerden for his help with the image analysis, Anja de Ruijter for helping out with the cell culturing and CAM implants BV, Leiden, The Netherlands for the provision of the CaP scaffolds.

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chapter eight

Summary, address to the aims, closing remarks and future perspectives

Summary and address to the aims

Microvascular osteocutaneous free flaps have given reconstructive surgeons a powerful tool in the treatment of large defects involving hard and soft tissues in head and neck surgery. For example, radial forearm, scapula, iliac crest and fibula flaps have been used extensively in mandibular reconstruction. The inevitable donor site morbidity of these osteocutaneous flaps has received less attention than the reconstructive advantages. In chapter one, a review of the literature is presented for each type of flap to determine the kind, incidence and consequences of flap associated morbidity. In the future tissue-engineered prefabricated free flaps might play an important role to solve this morbidity problem. In view of this, the objective of this thesis is to develop a method for the microsurgical reconstruction of bone defects based on a tissue engineering approach. Using this technique, cultured bone marrow cells can be seeded in an appropriate carrier or scaffold in a suitable site. This so called prefabricated flap can then be transplanted with microsurgical techniques to a defect, e.g. a segmental mandibular gap. In the subsequent chapters of this thesis, the aims as described in chapter one of this thesis will be addressed. Each subsequent chapter comprises a separate investigation.

Aims

1. Can MRI be used in a longitudinal study to noninvasively detect bone formation in a rat model and can it give quantitative as well as morphological information about the construct?

In animal studies on tissue engineering of bone, histology remains the standard for assessing bone formation. As longitudinal studies with this method are feasible only at the cost of large numbers of animals, we looked for an alternative. Therefore, demineralized bone matrix (DBM) and inactivated demineralized bone matrix (iDBM) implants were subcutaneously implanted in a rat. At 1, 3, 5, and 7 weeks postimplantation soft X-ray and magnetic resonance imaging (MRI) were done to monitor bone formation in the implants. At 7 weeks, the animal was killed and the

implants were retrieved for histology. The results showed that in vivo MRI is well suited to assess bone formation larger than 0.5 mm in diameter and to monitor the complete three-dimensional shape of the newly formed bone in a noninvasive manner in this longitudinal study. The MRI results matched well with the histology results as obtained at 7 weeks. In contrast, X-ray imaging appeared inappropriate to monitor the bone formation process in DBM.

2. What is the best method for noninvasive monitoring of bone formation, MRI or DEXA? And can MRA be used to demonstrate the vascularity of the pedicle of a prefabricated flap?

The aim of the study was to further explore the utilization of Magnetic Resonance Imaging (MRI), Magnetic Resonance Angiography (MRA) and Dual-Energy X-ray Absorptiometry (DEXA) to assess bone formation and blood circulation in a pedicled bone graft substitute. In 14 Wistar rats, initially ten weeks old, heterogeneous demineralized femur bone matrix implants were wrapped in pedicled adductor thigh muscle flaps. One rat died after surgery. Subsequently, bone formation and maintenance of blood flow was evaluated in six rats after six weeks of implantation by means of in vivo MRI/MRA and post-mortem histomorphometry. The other seven rats were left for 12 weeks, after which bone formation was evaluated by in vivo DEXA and post-mortem histomorphometry.

The results demonstrated that after six weeks bone formation was present in four out of six animals, quantified as 42% (± 35%) and 25% (± 19%) by means of MRI and histomorphometry respectively. MRA was able to show patency of the pedicles of these four rats only, which suggests that the lack of blood supply in the other two rats was the cause of failure to form bone. In the 12 week group, histology showed increased bone formation without signs of osteolysis, which was quantified histomorphometrically to be as high as 48% (± 15%). DEXA failed to show bone formation.

It is concluded that in vivo MRI proved to be a reliable method for monitoring ectopic bone formation in a rat model, while in vivo DEXA was unable to detect the implants. Furthermore, in vivo MRA proved to be

a very useful technique for studying the circulation of muscle flaps in this animal model.

3. What is the influence of the surface area of the carrier for the bone forming capacity?

Demineralized bone matrix (DBM) has been shown to induce ectopic endochondral bone formation, when intramuscularly implanted in rats. In chapter two and three we have found a variation in bone formation capacity of this DBM. This might be due to the properties of the DBM itself, but the use of DBM blocks could be of influence as well. Therefore, this study was designed to investigate whether increasing the surface area of the DBM by morsellizing, influences the bone formation capacity. In view of this, DBM implants and morsellized DBM (MDBM) implants were placed intramuscularly in a rat model. At six weeks the implants were retrieved and evaluated by histology and histomorphometry. The results demonstrated that significant amounts of newly formed bone were present in some DBM as well as some MDBM implants while in others no, or very little new bone was found. Histomorphometric analysis showed an average bone formation of 2.6% in DBM implants and an average of 1.9% in MDBM implants. Still, the amount of bone formation was limited compared with previous studies. It is concluded that enlargement of the surface area by morsellizing DBM implants is not an important factor in bone forming capacity.

4. What is the most useful carrier for a cell-based construct, titanium fibre mesh or a ceramic implant?

Much research has been done to develop the ideal bone graft substitute (BGS). One approach to develop this ideal BGS is the use of growth factors, but for this approach osteoprogenitor cells are needed at the site of reconstruction. An alternative is a cell-based approach, where enough cells are provided to form bone in a carrier material. In previous studies of our group, titanium (Ti) carriers have been used, because of the excellent mechanical properties and the bone-compatibility of this material. On the other hand, calcium phosphate (CaP) ceramics are known for their excellent osteoconductivity. The aim of this study is to investigate the

influence of the carrier in a cell-based bone regeneration approach, whereby we hypothesize that CaP ceramic implants will induce more bone formation than Ti-fiber implants, in the same animal model as our previous experiment. Ti-fiber mesh implants and ceramic implants were seeded with rat bone marrow cells (RBM) and implanted subcutaneously. Histological analysis after one, three and six weeks showed differences in the way of bone formation in the two groups: bone appeared to grow from the centre to the periphery of the implant in the titanium group, while bone formation in the ceramic group occurred through the whole implant. Histomorphometrical analysis after one week showed very limited bone formation for both the titanium and ceramic group. At three weeks, the amount of bone formation was increased till about 10 percent for the titanium group and 18 percent for the ceramic group. No significant difference between the two groups could be observed. In the six week group, the bone formation was 6 (Ti) and 23 (CaP) percent, respectively ($P<0.0001$). Further, bone formation started earlier in the CaP ceramic scaffolds than in the Ti scaffolds. Our hypothesis could be confirmed: ceramic implants induce more bone formation than titanium implants.

5. What is the most useful acceptor site for a prefabricated flap, muscle or subcutaneous tissue?

Bone Graft Substitutes (BGS) can be fabricated by the combination of three key ingredients: 1) competent bone-forming cells, 2) a suitable framework or scaffold, and 3) the presence of biological stimulants. Although much research has been done to develop the ideal BGS, the results are still not very consistent. In view of this, the cellularity and vascularity of the recipient site are supposed to be important for the osteoinductive capacity of BGS. Therefore, we hypothesized that a muscle recipient site could favor bone formation in a cell-based BGS compared to a subcutaneous recipient site due to the higher vascularity of muscle tissue. To prove this hypothesis, 48 titanium (Ti) fiber mesh implants were seeded with rat bone marrow stromal cells (RBM) and implanted subcutaneously and intramuscularly in the adductor thigh muscle of rats. The amount of bone formation after one, three and six weeks was evaluated by histology and histomorphometry as well as by calcium content. Analysis revealed that bone formation

increased during implantation. However, bone formation did not exceed above 12 percent of the implant surface, both for the intramuscular and subcutaneous recipient site. Also, no significant differences in bone amount between these two sites existed. Consequently, our hypothesis could not be confirmed.

6. What is the influence of a microsurgical anastomosis of the vascular pedicle of a prefabricated muscle flap on the tissue engineered bone in this flap?

We have conducted a series of studies with the ultimate goal of developing a method for microsurgical reconstruction of bone defects with the help of tissue engineering techniques. In this study we determined how microsurgery affects the bone forming capacity of the final construct. Therefore we implanted 16 ceramic implants with cultured cells in the adductor thigh muscle flap of the rat, which was enveloped by a silicone sheet. After four weeks the pedicles of eight flaps were cut and directly microsurgically anastomosed. The other eight flaps were given a sham operation, in which all branches of the pedicle were cut and ligated, but the main pedicle was left intact. Four weeks after the second surgery, all implants were harvested and analyzed by histology and histomorphometry. The histological appearance of the bone was found to be similar in both groups. Histomorphometry did not reveal statistical significant differences in bone formation. We conclude that in a rat model viable microsurgical bone flaps can be obtained by using a cell-based tissue engineering approach.

Closing remarks and future perspectives

In this thesis, we have described the development of a method for microsurgical reconstruction of bone defects with the help of tissue engineering techniques. We have created a model of a prefabricated bone in a pedicled muscle flap, which was revascularised. The ultimate goal of reconstructing a mandible in a human being has yet to be achieved. First of all, the next step should be the use of a larger animal model like a goat, to study mandibular reconstruction[1, 2]. In larger animals, new challenges will arise. Nevertheless, the effectiveness of the combination of cell-based scaffolds and prefabrication has always to be proven in higher animals, before its application in humans. In this context, the combination of growth factors and cells with a carrier might be advantageous compared to the use of growth factors or cells alone. For example, it can allow a decrease in the concentration of these growth factors that have to be used to induce bone formation. Further, it can also solve the problems with the final yield of osteogenic cells that can be derived out of bone marrow. Considering growth factors and cells, perhaps an even more promising method is the implantation of genetically modified cells. This combines cell transplantation and delivery of osteogenic factors at the same time and has already been shown to increase the effectiveness of both approaches[3].

The current investigation revealed that further research is required to achieve the final goal, i.e. the manufacturing of preshaped tissue engineered bone flaps. This will undoubtedly imply major investments both financially and in terms of man power. However, we also experienced that the actual clinical applicability is still in its infancy[4]. Although the community could put questions to the enormous costs versus the small benefits of tissue engineering, this thesis showed that tissue engineering is in potency an important development in health care. In the future it will certainly be possible to reconstruct osseous defects in various parts of the human body with these techniques.

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hoofdstuk acht

Samenvatting, evaluatie van de doelstellingen, afsluitende opmerkingen en toekomstperspectief

Samenvatting en evaluatie van de doelstellingen

Door de ontwikkeling van microvasculaire osteocutane lappen hebben reconstructief chirurgen een belangrijk wapen in handen gekregen bij het herstel van complexe hoofd-halsdefecten. Zowel de radialis onderarmslap, als de scapula-lap, crista iliaca-lap en fibula-lap worden frequent gebruikt voor het herstel van de mandibula. De reconstructieve voordelen van deze osteocutane lappen hebben in de literatuur meer aandacht gekregen dan de onvermijdelijke donorplaatsmorbiditeit. In hoofdstuk één wordt voor bovengenoemde lappen een overzicht van de beschikbare literatuur gegeven, waarbij aard, incidentie en consequenties van deze lap-gerelateerde morbiditeit wordt behandeld. In de toekomst kunnen zogenaamde 'tissue engineered' geprefabriceerde lappen een belangrijke rol spelen bij het oplossen van dit morbiditeitsprobleem. Het doel van de studie ten grondslag liggend aan dit proefschrift is dan ook om een methode te ontwikkelen voor de microchirurgische reconstructie van botdefecten, gebaseerd op tissue engineering technieken. Gebruik makend van deze technieken, kunnen gekweekte beenmergcellen gezaaid worden in een geschikt dragermateriaal. Vervolgens kan dit als implantaat in een geschikte plaats in het lichaam ingebracht worden. Nadat er voldoende botvorming is opgetreden in dit implantaat, kan deze zogeheten geprefabriceerde lap met behulp van microchirurgische technieken verplaatst worden naar een botdefect, zoals dat bijvoorbeeld is ontstaan na een segmentale mandibularesectie. In de opeenvolgende hoofdstukken van dit proefschrift, zullen de doelen, die in hoofdstuk één van dit proefschrift zijn gesteld, behandeld worden. Elk hoofdstuk is gebaseerd op een separaat onderzoek.

Vraagstellingen

1. kan MRI gebruikt worden om op een non-invasieve wijze botvorming in een ratmodel in tijd te volgen en kan op deze wijze quantitatieve en morfologische informatie verkregen worden over het gevormde bot?

Histologisch onderzoek is de gouden standaard voor evaluatie van botvorming in proefdierexperimenten. Dit maakt dat longitudinale

studies alleen uitgevoerd kunnen worden door gebruik te maken van grote aantallen dieren. Daarom is gezocht naar een alternatieve methode om botvorming te bepalen. Hiertoe werden implantaten van gedemineraliseerde botmatrix (DBM) en geïnactiveerde gedemineraliseerde botmatrix (iDMB) subcutaan bij een rat ingebracht. Om de botvorming te volgen werden op één, drie, vijf en zeven weken na implantatie röntgenfoto's gemaakt en werden MRI (magnetische resonantie imaging) opnames gemaakt. Na zeven weken werd de rat gedood en werden de implantaten uitgenomen voor histologisch onderzoek. Onze resultaten laten zien dat in-vivo MRI uitstekend botvorming met een minimale afmeting van 0,5 mm kan aantonen. Met behulp van deze techniek kan ook de driedimensionale vorm van het nieuw gevormde bot gereconstrueerd worden. Tevens kan de botvorming in de tijd vervolgd worden. De resultaten van de MRI komen ook goed overeen met de histologische resultaten die na zeven weken werden verkregen. In tegenstelling hiermee, bleken de röntgenologische afbeeldingen niet geschikt om het proces van botvorming in DBM te volgen.

2. wat is de beste niet-invasieve methode om botvorming te volgen, MRI of Dual-Energy X-ray Absorptiometry (DEXA)? Kan magnetische resonantie angiografie (MRA) gebruikt worden om de doorbloeding van de vaatsteel van een geprefabriceerde lap aan te tonen?

Het doel van deze studie was om verder onderzoek te doen naar het gebruik van MRI, MRA en DEXA om de botvorming en de bloedvoorziening van een gesteeld bottransplantaatsubstituut te beoordelen. In 14 Wistar ratten werden heterologe gedemineraliseerde botimplantaten in een gesteelde adductordijspierlap gewikkeld. Eén rat stierf direct na de operatie. Van zes ratten werd botvorming en functie van de bloedvaten na zes weken geëvalueerd middels in-vivo MRI/MRA en post-mortem histologisch en histomorfometrisch onderzoek. Van de overige zeven ratten werd botvorming na 12 weken bepaald middels in-vivo DEXA en post-mortem histologisch en histomorfometrisch onderzoek.

Na zes weken was er botvorming opgetreden bij vier van de zes dieren. De gemiddelde botvorming gemeten met MRI en histomorfometrie was

respectievelijk 42% (±35%) en 25% (±19%). MRA-onderzoek toonde alleen bij deze vier dieren een intacte vaatsteel aan. Dit suggereert dat het verlies van de bloedvoorziening bij de andere twee dieren de oorzaak is van het niet optreden van botvorming. Histologisch onderzoek na 12 weken liet zien dat er meer botvorming was opgetreden, zonder tekenen van osteolyse. De histomorfometrisch bepaalde gemiddelde botvorming was 48% (±15%). Met behulp van DEXA kon deze botvorming niet aangetoond worden. De conclusie luidt dat in-vivo MRI een betrouwbare methode is om ectopische botvorming aan te tonen in een ratmodel. DEXA daarentegen kan niet gebruikt worden om de implantaten aan te tonen. Verder blijkt in-vivo MRA een zeer bruikbare techniek om de bloedvoorziening van spierlappen in dit diermodel te bestuderen.

3. wat is de invloed van de grootte van het oppervlak van het dragermateriaal voor de botvormende capaciteit?

Als gedemineraliseerde botmatrix (DBM) intramusculair in ratten wordt geïmplanteerd, wordt ectopische botvorming geïnduceerd. In hoofdstuk twee en drie is beschreven dat de botinducerende capaciteit van verschillende DBM-implantaten sterk verschilt. Dit wordt mogelijk veroorzaakt door de eigenschappen van het DBM zelf. De vorm waarin het DBM gebruikt wordt, als één implantaat of in kleinere fragmenten, zou hierbij een rol kunnen spelen. Daarom werd in deze studie onderzocht of de vorm waarin DBM wordt geïmplanteerd inderdaad de botvormende capaciteit van DBM beïnvloedt. Zowel DBM implantaten uit één stuk, als DBM implantaten die uit kleinere fragmenten bestonden (MDBM), werden intramusculair in een ratmodel geïmplanteerd. Na zes weken werden de implantaten uitgenomen en werd de botvorming geëvalueerd middels histologisch en histomorfometrisch onderzoek. In enkele DBM- en enkele MDBM-implantaten werden significante hoeveelheden nieuw gevormd bot aangetoond, terwijl in andere implantaten geen of zeer weinig bot werd aangetroffen. Histomorfometrische analyse liet een gemiddelde botvorming van 2,6% zien voor de DBM-implantaten en een gemiddelde van 1,9% voor de MDBM-implantaten. Deze hoeveelheid botvorming was minder dan in voorgaande studies. Geconcludeerd wordt dat vergroting van het oppervlak door het fragmenteren van DBM-implantaten geen belangrijke factor is in de botinducerende capaciteit.

4. wat is de meest geschikte drager voor een construct, dat gebaseerd is op het gebruik van cellen?

Er is veel onderzoek gedaan om het ideale bottransplantaat(BGS) te ontwikkelen. Een benadering om dit ideale bottransplantaatsubstituut te ontwikkelen is door middel van het gebruik van groeifactoren. Hiervoor is het nodig dat er osteoprogenitorcellen aanwezig zijn op de plaats van reconstructie. Een alternatief voor deze methode is het gebruik van cellen. De cellen worden in een dragermateriaal geladen en vervolgens wordt het geheel in lichaamsweefsel geïmplanteerd. In voorgaande studies van onze groep is voor deze celtechniek gekozen, waarbij titanium (Ti) werd gebruikt als dragermateriaal. Titanium werd gekozen in verband met de uitstekende mechanische eigenschappen van dit materiaal en de compatibiliteit van titanium met bot. In plaats van titanium zou ook gebruik gemaakt kunnen worden van een keramiek als calciumfosfaat (CaP). Dit materiaal staat met name bekend om zijn uitstekende osteoconductieve eigenschappen. Het doel van deze studie was om de invloed van het dragermateriaal te bestuderen voor een op cellen gebaseerde benadering van botregeneratie. De hypothese was dat poreuze calciumfosfaat dragers meer botvorming induceren dan titaniumvezelgaas implantaten. Titanium vezelgaas en CaP implantaten, gezaaid met gekweekte beenmergcellen (RBM), werden subcutaan geïmplanteerd in ratten. Histologische analyse na een implantatieperiode van één, drie en zes weken laat zien dat de manier waarop de botvorming plaatsvond in deze twee materialen verschilt: in de titaniumgroep bleek botvorming op te treden vanuit het centrum naar de periferie van het implantaat, terwijl de botvorming in de keramiekgroep uniform door het hele implantaat plaatsvond. Histomorfometrische analyse liet verder zien dat na één week zeer weinig botvorming optrad in zowel de titaniumgroep als de CaP groep. Na drie weken was de hoeveelheid botvorming toegenomen tot 10 procent voor de titanium groep en tot 18 procent voor de CaP groep. Na zes weken was er significant ($P<0,0001$) meer bot gevormd in de CaP groep (23%), dan in de titanium groep (6%). De hypothese dat poreuze CaP dragers meer botvorming induceren dan titanium vezelgaas werd dan ook bevestigd.

5. wat is de optimale acceptor site voor een geprefabriceerde lap?

Bottransplantaatsubstituten kunnen verkregen worden door de combinatie van drie bestanddelen: 1) botvormende cellen, 2) een geschikte drager, en 3) de aanwezigheid van biologische groeifactoren. Hoewel veel onderzoek is verricht om het ideale bottransplantaatsubstituut te ontwikkelen, zijn de resultaten nog niet erg consistent. De aanwezigheid van cellen en de doorbloeding van de receptorplaats worden als belangrijke factoren gezien voor de botinductieve eigenschappen van een bottransplantaatsubstituut. De hypothese is dat in een op cellen gebaseerd bottransplantaatsubstituut meer botvorming optreedt in spierweefsel, dan in subcutaan weefsel, omdat spierweefsel een betere doorbloeding heeft dan subcutaan weefsel. Om deze hypothese te bewijzen werden beenmergcellen (RBM) gezaaid op 48 titaniumvezelgaasimplantaten, waarna deze implantaten zowel subcutaan als intramusculair in de adductordijbeenspier van ratten werden geplaatst. De hoeveelheid botvorming na één, drie en zes weken werd geëvalueerd middels histologisch en histomorfometrisch onderzoek, alsook door het calciumgehalte in de dragermaterialen te bepalen. De resultaten lieten zien dat de botvorming toenam gedurende de implantatietijd. Echter, zowel in de intramusculaire groep als in de subcutane groep bleef de botvorming beperkt tot 12 procent. Tussen de twee locaties kon geen significant verschil in botvorming aangetoond worden. Derhalve kon onze hypothese niet bevestigd worden.

6. wat is de invloed van een microchirurgische anastomose op bot, dat met behulp van tissue engineering technieken in een geprefabriceerde spierlap is verkregen?

Het uiteindelijke doel van deze studies is om een methode te ontwikkelen voor microchirurgische reconstructie van botdefecten met behulp van tissue engineering technieken. In de onderhavige studie is gekeken naar de invloed van het microchirurgisch anastomoseren van de vaatsteel van een geprefabriceerde spierlap op de botvormende capaciteit van het uiteindelijke bottransplantaatsubstituut. Daartoe werden 16 poreuze CaP keramische implantaten, geladen met beenmergcellen, aangebracht in de adductordijbeenspierlap van ratten. De spierlappen werden omwikkeld met

een siliconen membraan om vaatingroei uit de omgeving te voorkomen. Na vier weken werd de vaatsteel van acht lappen doorgenomen en direct hierna microchirurgisch hersteld. De andere acht lappen kregen een controle operatie, waarbij alle zijtakken van de vaatsteel werden geligeerd, maar waarbij de vaatsteel zelf niet werd doorgenomen. Vier weken na de tweede operatie werden alle implantaten uitgenomen en geanalyseerd met behulp van histologisch en histomorfometrisch onderzoek. In alle implantaten werd vitaal botweefsel waargenomen. Histomorfometrisch onderzoek liet geen statisch significante verschillen in hoeveelheid botvorming zien. Daarom luidt de conclusie dat in een ratmodel levensvatbare microchirurgische botlappen verkregen kunnen worden door gebruik te maken van een op cellen gebaseerde tissue engineering benadering.

Afsluitende opmerkingen en toekomstperspectief

In dit proefschrift is de ontwikkeling beschreven van een methode voor de microchirurgische reconstructie van botdefecten met behulp van tissue engineering technieken. Er is een model gecreëerd van geprefabriceerd bot in een gesteelde en gerevasculariseerde spierlap in ratten. Het uiteindelijke doel om een mandibula te reconstrueren in de mens, moet nog worden bereikt. Hiertoe zal nog meer onderzoek moeten worden verricht. Een eerste stap zal het gebruik van een groter proefdier, zoals bijvoorbeeld de geit[1, 2] zijn. In grotere dieren zullen andere omstandigheden voorkomen, die nieuwe vraagstellingen zullen opleveren. Echter alvorens de methode toepassing te laten vinden in mensen, zal de effectiviteit van de combinatie van op cellen gebaseerde dragers en prefabricage altijd eerst bewezen moeten worden in hogere dieren,. In dit kader dient opgemerkt te worden dat de combinatie van groeifactoren en cellen met een drager voordelen kan hebben ten opzichte van het gebruik van uitsluitend groeifactoren of cellen. Op deze wijze zou bijvoorbeeld de concentratie van groeifactoren, die gebruikt dient te worden om botvorming te induceren, verminderd kunnen worden. Verder zou wellicht ook het probleem van de hoeveelheid cellen die uit beenmerg geoogst moet worden om botvorming te bewerkstelligen, opgelost kunnen worden. Dit zal een tweede stap zijn in het komend onderzoek. Ten aanzien van groeifactoren en cellen, zou implantatie van

genetisch gemodificeerde cellen nog veelbelovender kunnen zijn. Hiermee worden op hetzelfde moment cellen getransplanteerd en osteogene factoren aangeleverd. Er is al aangetoond dat deze methode de effectiviteit van beide benaderingen verhoogt[3]. Dit zal een derde stap zijn in het komend onderzoek.

Het gedane onderzoek heeft geleerd dat er een grote investering in tijd en geld nodig is om het uiteindelijke doel, te weten het vervaardigen van door tissue engineering verkregen voorgevormde bottransplantaten, te bereiken. Wij hebben ook ervaren dat de huidige klinische toepasbaarheid van tissue engineering nog in zijn kinderschoenen staat[4]. Hoewel de gemeenschap vragen kan stellen bij de enorme kosten afgezet tegen de op het moment nog geringe voordelen van tissue engineering, laat dit proefschrift zien dat tissue engineering in potentie een belangrijke ontwikkeling in de gezondheidszorg is. In de toekomst zal het zeker mogelijk zijn om met deze technieken botdefecten op diverse plaatsen in het menselijk lichaam te reconstrueren.

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Dankwoord

Het is al zo vaak gezegd, dat het een cliché lijkt: promoveren doe je niet alleen. Dat het geen cliché is, dat heb ik inmiddels aan den lijve mogen ervaren. Veel mensen hebben direct of indirect meegewerkt aan de totstandkoming van dit proefschrift. Ik wil iedereen hiervoor hartelijk danken.

Prof. dr. J.A. Jansen. Beste John, onder jouw inspirerende leiding is dit onderzoek tot een goed eind gekomen. Ik heb veel respect voor jou als persoon en hoe je jouw afdeling in korte tijd op zo´n hoog internationaal niveau hebt gebracht. Ik ben er van overtuigd dat de samenwerking tussen onze beide afdelingen nog veel onderzoek en vele promoties op gaat leveren. Daarnaast zullen wij ook onze minder formele contacten ongetwijfeld blijven onderhouden.

Prof. dr. P.H.M. Spauwen. Beste Paul, nadat je een belangrijk deel van mijn opleiding voor je rekening hebt genomen, heb je mij de kans gegeven om mijzelf als staflid te profileren. Jij had er vertrouwen in dat ik zou promoveren, en dat blijkt niet onterecht. Dank voor je vertrouwen, je aanmoedigingen en geduld: het heeft resultaat gehad! Ik hoop dat we ons gezamenlijk nog vele jaren kunnen inzetten voor de afdeling.

Dr. J.M.H.M. Borghouts. Beste Hans, als opleider heb jij mij destijds de kans gegeven om plastisch chirurg te worden, hartelijk dank daarvoor. Om Julien van Rappard aan te halen: "zonder opleiding wordt het nooit wat …". Het doet me deugd dat ik je verzuchting `Ed, die promoveert nooit´ nu kan weerspreken.

Dr. J.W.M. Vehof. Beste Johan, samen zijn we het avontuur begonnen. Bedankt voor je hulp en ondersteuning bij dit proefschrift. Ik ben er trots op dat je een fraai proefschrift hebt geschreven en dat je nu je opleiding tot plastisch chirurg in het UMCN aan het afronden bent. Ik denk dat we beiden een goede keuze gemaakt hebben, destijds … Ik ben benieuwd hoe je toekomst er uit gaat zien: kies je voor een academische loopbaan of ligt je toekomst in de periferie?

Drs. P.Q. Ruhé. Beste Quinten, als 'opvolger van Johan' heb je het uitstekend gedaan. Ook jij bedankt voor je hulp en ondersteuning bij dit proefschrift.

Ons tissue engineering congres in Orlando en de paar uur in Miami zal ik niet snel vergeten. Ik hoop je ook snel op onze afdeling terug te zien als plastisch chirurg in opleiding, en wie weet …

Dr. J.A. Pikkemaat. Beste Jeroen, ik herinner me de uren die we samen hebben doorgebracht in de krochten van het Centraal Dierenlaboratorium (waar de MRI-apparatuur staat opgesteld) en de vele correcties van de eerste twee hoofdstukken, die we elkaar via de e-mail heen en weer stuurden. Hartelijk dank voor deze vruchtbare samenwerking. Ongetwijfeld komen we elkaar weer tegen in de Vereeniging tijdens een fraai concert.

Drs C.C. Schouten. Beste Corinne, de eerste stap heb je al gezet: je eerste artikel is geaccepteerd voor publicatie en staat in dit proefschrift. Ik zal je carrière in de plastische chirurgie met belangstelling en vertrouwen volgen.

Ik wil ook bedanken: Jacques van Asten en prof. dr. Arend Heerschap voor de hulp bij het MRI-onderzoek, prof. dr. Wim Oyen voor de ondersteuning bij het onderzoek met de DEXA-scan, drs. Yu Lan Khouw voor een deel van de operaties voor hoofdstuk 3; Elise van Haalen voor ondersteuning bij de operaties op het dierenlab, drs. (hoe lang nog?) Henriëtte Deutman dr. Juliëtte den Dolder en drs. Esther Bodde voor hun aandeel in de inspirerende promotiebesprekingen.

De medewerkers van het laboratorium biomaterialen wil ik met nadruk noemen: Anja de Ruijter, dank voor je hulp bij de celkweken en je verdere steun en medeleven: het was altijd prettig om met je van gedachten te wisselen; Jan-Paul van der Waerden dank voor het vele werk achter de computer voor de histomorfometrie en de fraaie prints; ik denk met genoegen terug aan onze discussies over automatisering en netwerken; Jacky den Bakker, dank voor het vele zaagwerk van de coupes. Dr. Joop Wolke, bedankt voor je hulp met de implantaten en de siliconen sheets, het was fijn om naast het wetenschappelijke werk ook even over andere onderwerpen van gedachten te kunnen wisselen.

De medewerkers op het Centraal Dierenlaboratorium mogen uiteraard ook niet ongenoemd blijven: Debbie Smits, Geert Poelen, Kai Poelen, Theo van den Ing en Alex Hanssen. Jullie gaven me altijd het gevoel welkom te zijn en jullie hulp was altijd professioneel.

Eddy Kopp en Philip van Damme wil ik hierbij alvast bedanken voor het feit dat zij mij als paranimf op mijn promotiedag terzijde zullen staan. Eddy, na vele jaren van vriendschap hoop ik dat je deze keer op tijd weet te komen ... Philip, nog hartelijk dank voor je ondersteuning in moeilijke promotietijden … En als je ooit nog eens zonder auto komt te staan …

Ik dank staf, assistenten en secretariaat van de afdeling plastische chirurgie van het Universitair Medisch Centrum Nijmegen (UMCN) evenals OK-assistenten en verplegend personeel op de poli en de verpleegafdelingen N20 en B21/22 voor de genoeglijke samenwerking. Graag wil ik alle anderen in het UMCN bedanken voor de vaak jarenlange samenwerking: de chirurgische specialismen, de anaesthesiologie, maar ook het management waar ik veel mee heb samengewerkt. Peter van der Linden verdient een extra Pluim, voor de langjarige samenwerking die uiteindelijk in een ziekenhuisbrede implementatie van een fraai operatieplanning- en registratiesysteem (Okapi) heeft geresulteerd.

Een speciale band heb ik met alle teamleden van onze humanitaire missies voor Interplast Holland en de Nederlandse Nomastichting: het 'hug team' is in de annalen bijgeschreven. We gaan zeker weer terug naar Afrika!

De lagere school is een belangrijke stap op weg naar een wetenschappelijke loopbaan, maar dit wordt nogal eens onderschat. Ik wil met name de 'meesters' Vermolen en Kremers bedanken: zij hebben destijds de kiem gelegd en deden dat op hun eigen bijzondere wijze.

Mijn ouders en zusjes wil ik bedanken voor de liefdevolle interesse, die zij gedurende al die jaren hebben getoond. Emil Gunsing, bedankt voor je vriendschap, al meer dan 45 jaar lang! Eddie Hagen is zeer belangrijk geweest voor mijn vorming, maar helaas veel te vroeg uit mijn leven verdwenen: 'uit de tijd gevallen'.

Tot slot, maar niet in de laatste plaats, wil ik Marlies bedanken: liefste Marlies, zonder jouw steun had ik het niet gered, en dat geldt niet alleen voor dit proefschrift. Ik ben er trots op dat je mijn levensgezellin bent en ik hoop dat je nog vele malen 'een jaartje bijtekent'.

Curriculum vitae

Ed Hartman – geboren op zes februari 1959 in Amsterdam – behaalt in Amstelveen het gymnasium-diploma op het Keizer Karel College, na het volgen van de lagere school in Mijdrecht. In 1977 start hij met de studie Geneeskunde aan de Vrije Universiteit te Amsterdam. Tijdens deze studie woont hij een microchirurgische reconstructie bij, die door Frits Groeneveld en Reinier Schoorl in Beverwijk wordt uitgevoerd. Dit is een openbaring: "microchirurgie, dat wil ik!" Na het artsexamen in 1985, volgt een jaar postdoctorale Medische Informatica met als onderwerp 'expert systemen'. Deze studie wordt omgezet in de vooropleiding Algemene Heelkunde in het Sint Joannes de Deo Ziekenhuis te Haarlem bij dr. B.W. van Neerijnen, waarin de vaatchirurgie onder leiding van Johan Coosemans bijzondere aandacht krijgt. In 1990 wordt de vervolgopleiding Plastische Chirurgie in het Academisch Ziekenhuis Nijmegen gestart bij dr. J.M.H.M. Borghouts.

Na afronding van de opleiding wordt de relatie met Nijmegen niet verbroken: bewust wordt gekozen voor werken in een academisch ziekenhuis. Speerpunten zijn de microchirurgische reconstructies en de schisis. Voor het optimaliseren van microchirurgische facialisreconstructies wordt prof. dr. Ronald Zuker in Toronto bezocht, terwijl voor de perforator flaps prof. dr. Fu Chen Wei in Taiwan wordt bezocht.

Daarnaast worden managementtaken ontwikkeld: reeds jaren is hij waarnemend chef de clinique en sinds 2003 is hij tevens waarnemend hoofd van de afdeling.

Uiteraard betekent een academische carrière ook participatie in het studentenonderwijs en de opleiding van plastisch chirurgen: sinds 2003 is hij officieel plaatsvervangend opleider in de Plastische Chirurgie.

In 2004 wordt het redacteurschap van het European Journal of Plastic and Reconstructive Surgery verworven.

Hij is lid van de Nederlandse Vereniging voor Plastische Chirurgie, Nederlandse Vereniging voor Handchirurgie, Nederlandse Vereniging voor Aesthetische Plastische Chirurgie, Nederlandse Vereniging voor Schisis en Craniofaciale Afwijkingen en de World Society for Reconstructive Microsurgery. In 1999 wordt hij gevraagd om toe te treden tot de European Plastic Surgery Workshop.

In het kader van dit proefschrift zijn internationale voordrachten gehouden in Helsinki (2001, European Conference of Scientists and Plastic Surgeons) en Orlando (2003, Tissue Engineering Society International).

Onder de vlag van Interplast Holland en de Nederlandse Noma Stichting voert hij regelmatig humanitaire missies uit in Afrika en het Midden Oosten.

Marlies van Melick is zijn levensgezellin, samen wonen zij in de stad Nijmegen.

Curriculum vitae

Ed Hartman – born on February 6th in Amsterdam – followed elementary school in Mijdrecht and he graduates from high school and college (gymnasium) in Amstelveen at the Keizer Karel College. In 1977 he starts medical school at the Free University in Amsterdam. During this study, a microvascular reconstruction performed by Frits Groeneveld and Reinier Schoorl in Beverwijk is a revelation: "microsurgery, that's what I want!" After finishing his medical studies in 1985, he starts one year of Medical Information Science with as main subject 'expert systems'. This study is stopped in order to start his general surgery residency with dr. B.W. van Neerijnen in the Joannes de Deo Hospital in Haarlem. After this residency, during which vascular surgery with Johan Coosemans is his main interest, he starts his plastic surgery residency in 1990 at the University Hospital Nijmegen with dr. J.H.M.H. Borghouts.

After his registration as a plastic surgeon, he stays in Nijmegen: he chooses to work in a University Hospital. His main interests are microsurgical reconstructions and cleft lip and palate surgery. He visits prof. dr. Ronald Zuker in Toronto for his microsurgical facial reanimations, and prof. dr. Fu Chen Wei in Taiwan for his perforator flaps.

Management becomes important: he becomes interim chef de clinique and deputy chief of the department since 2003

Of course working in a university hospital means training of medical students and residents: in 2003 he becomes Assistant Professor.

Since 2004 he is appointed editor of the European Journal of Plastic and Reconstructive Surgery.

He is a member of the Netherlands Society for Plastic Surgery, the Netherlands Society for Hand Surgery, the Netherlands Society for Aesthetic Plastic Surgery, the Netherlands Society for Cleft Lip and Palate and Craniofacial Malformations and the World Society for Reconstructive Microsurgery. In 1999 he is invited member for the European Plastic Surgery Workshop.

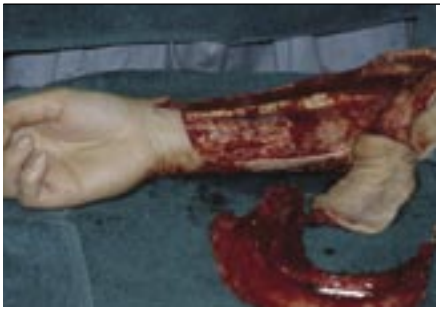
For this thesis he presents at international congresses in Helsinki (2001, European Conference of Scientists and Plastic Surgeons) and Orlando (2003, Tissue Engineering Society International).

He regularly engages in humanitarian missions for Interplast Holland and the Dutch Noma Foundation. These missions lead him to Africa and the Middle East.

He shares his life with Marlies van Melick in the city of Nijmegen, the Netherlands.

appendix

Color images



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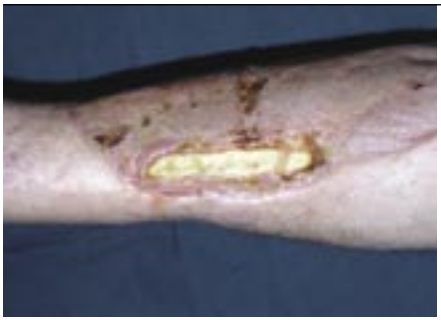

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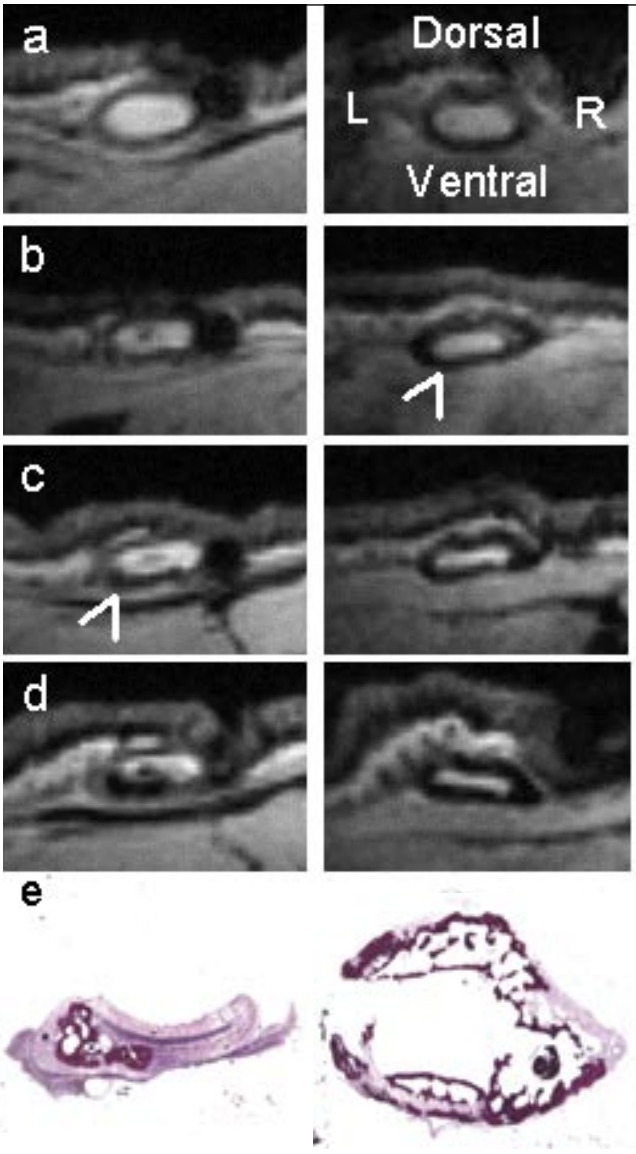
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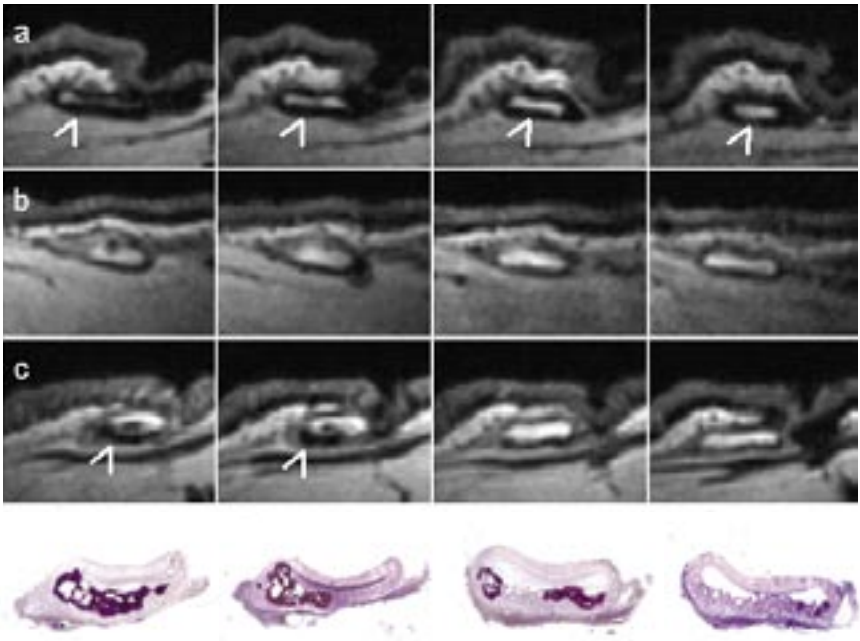
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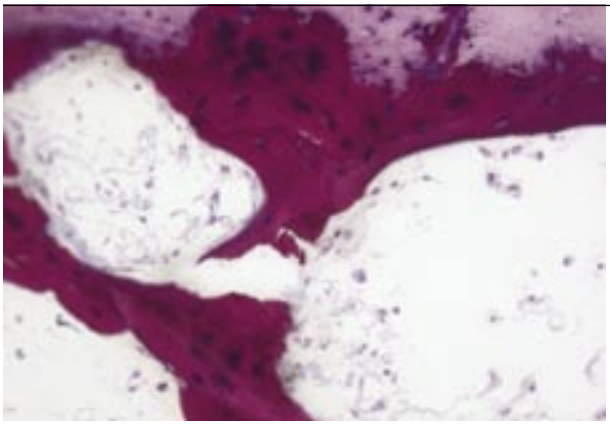

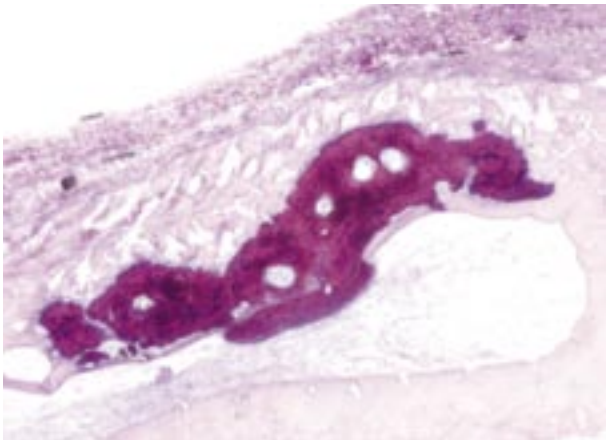

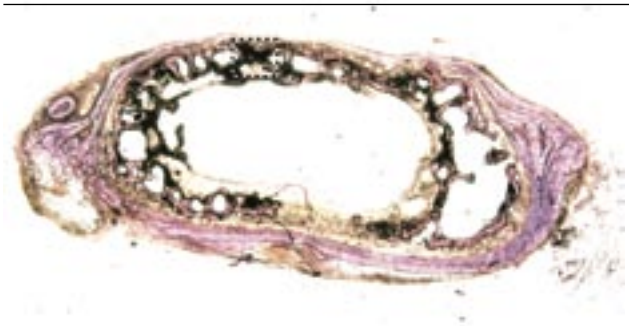

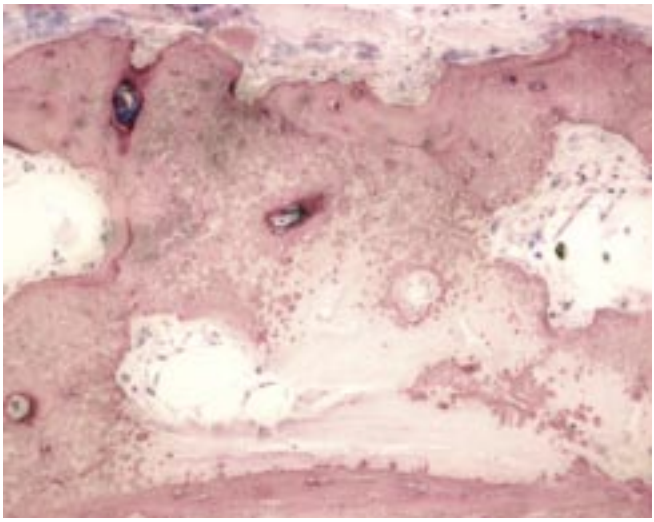

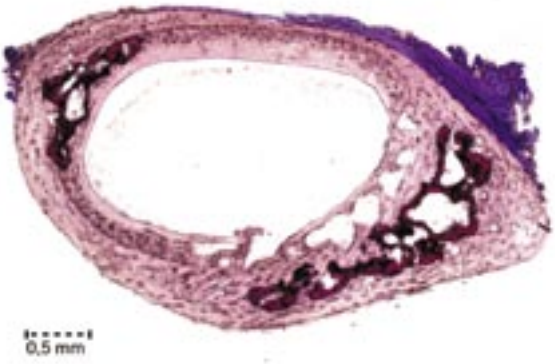
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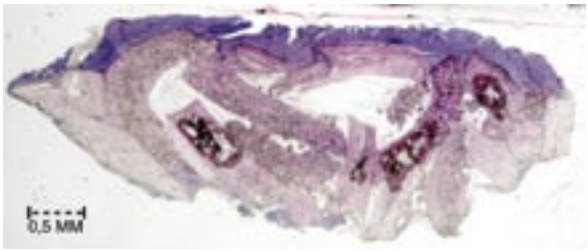
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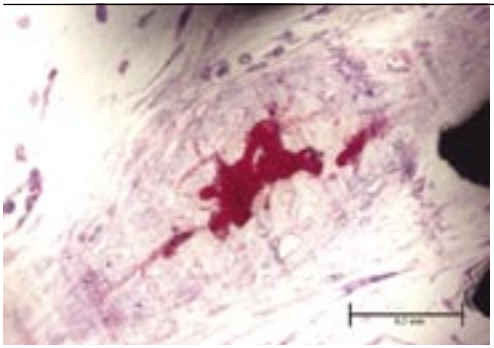

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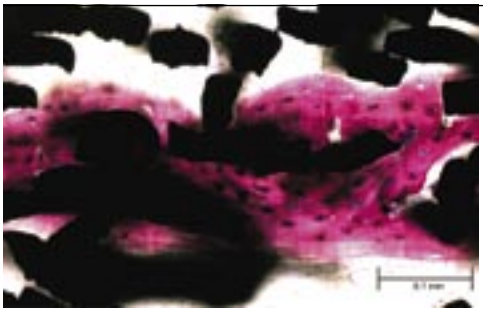

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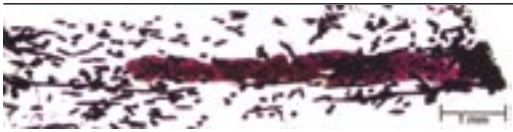

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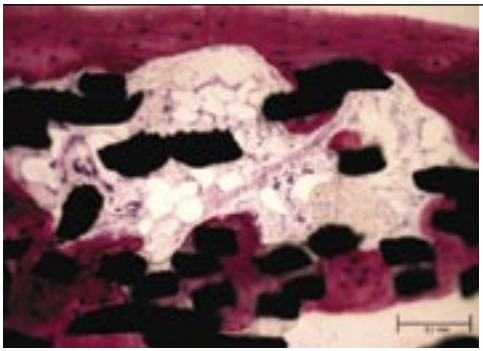

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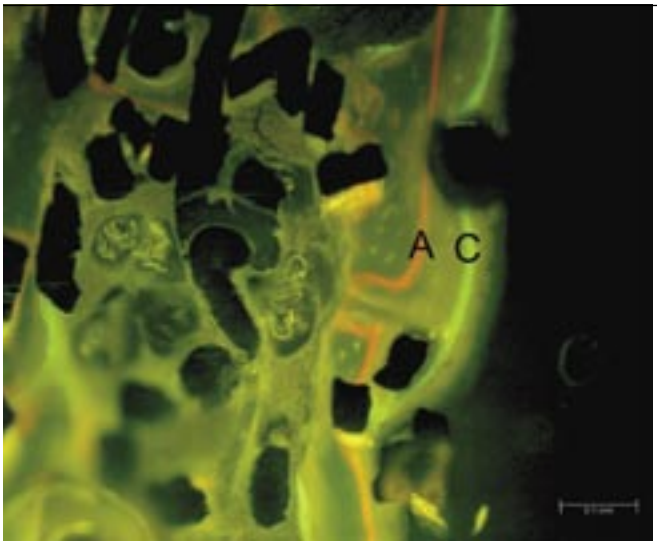
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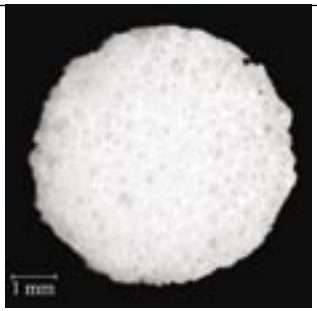
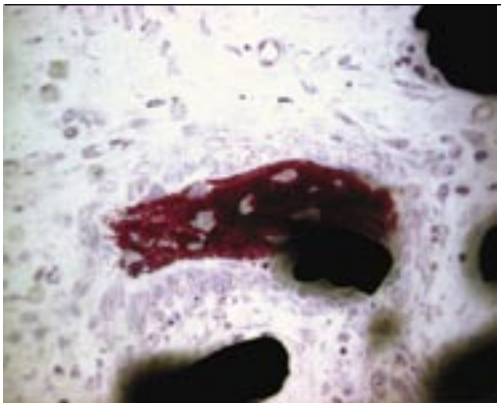
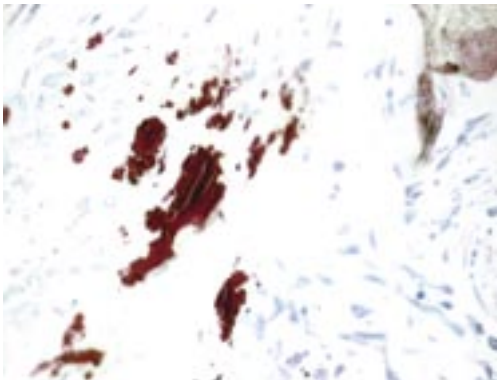


chapter 6 figure 1a and b





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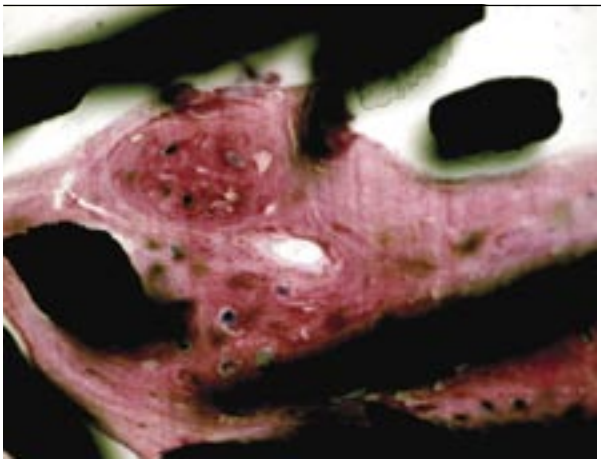

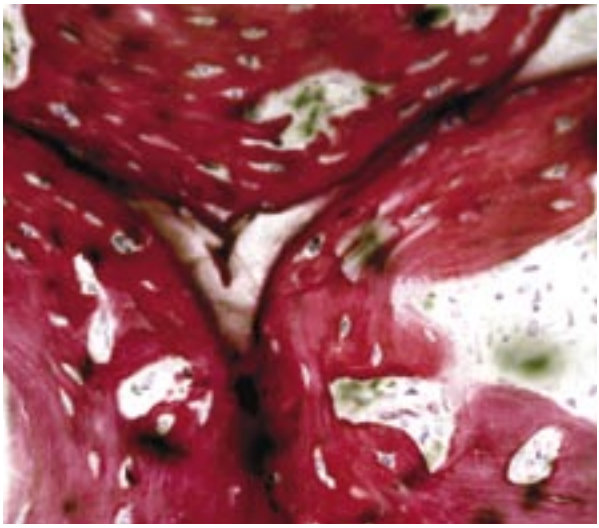

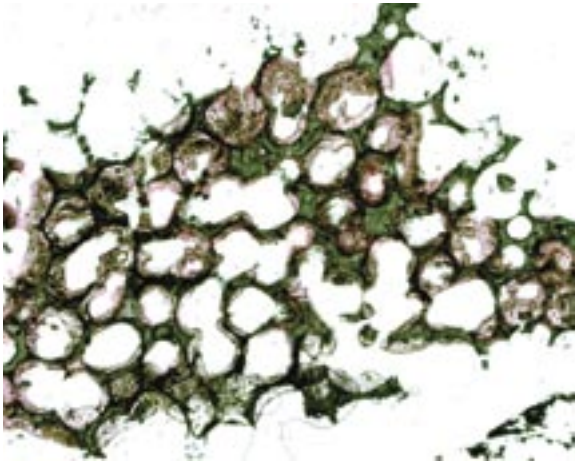

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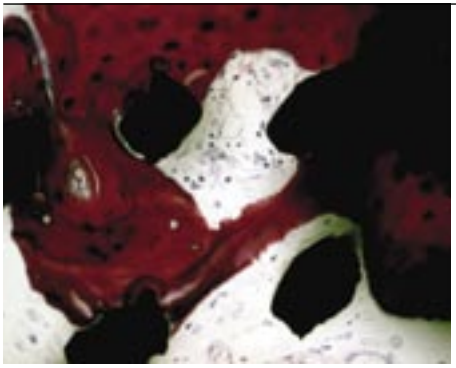

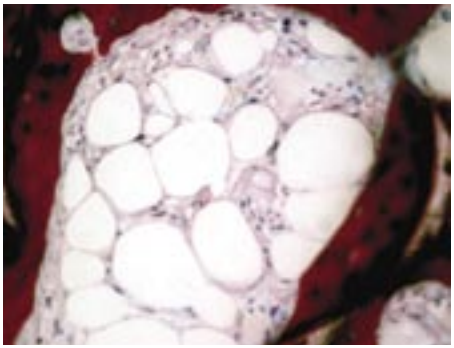

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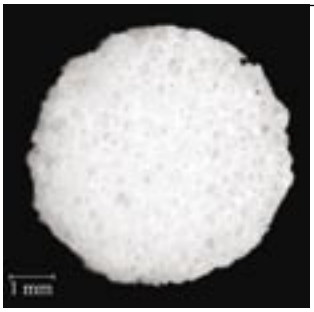
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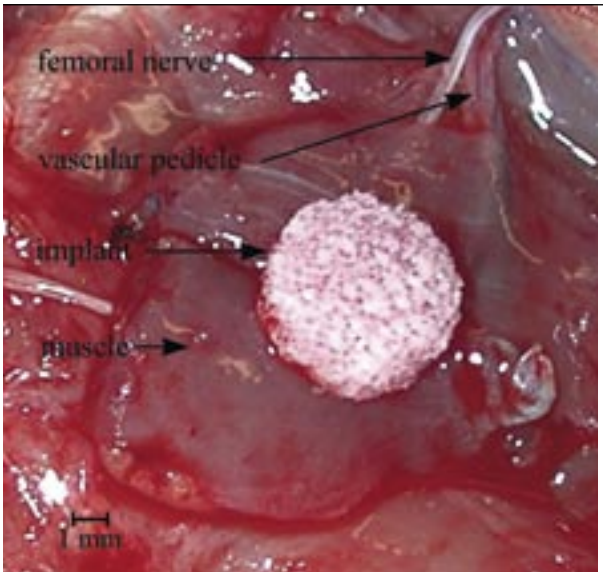
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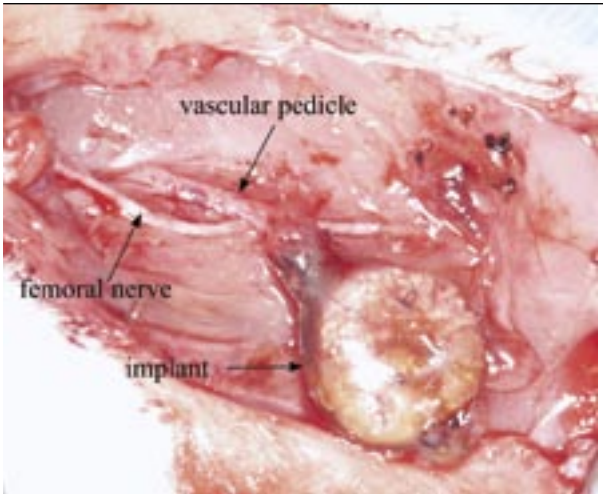
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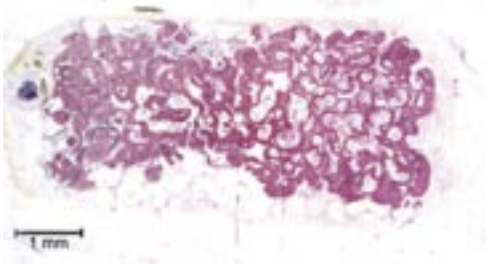
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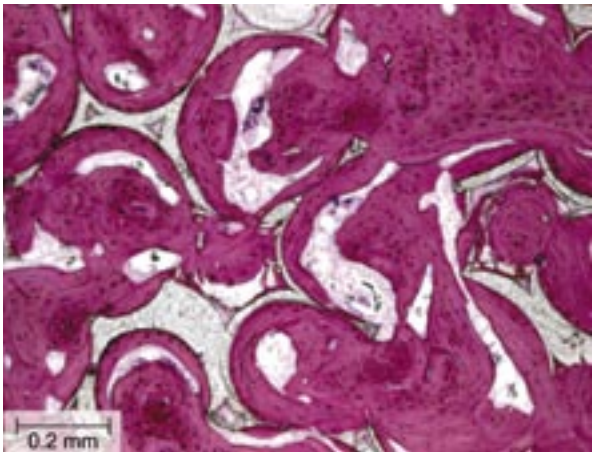
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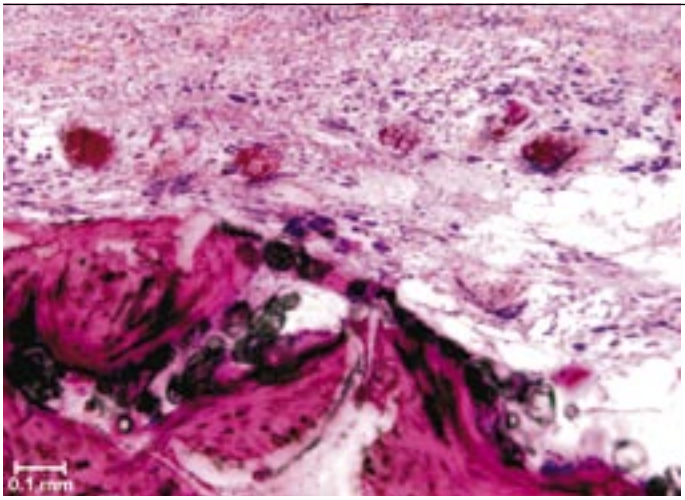
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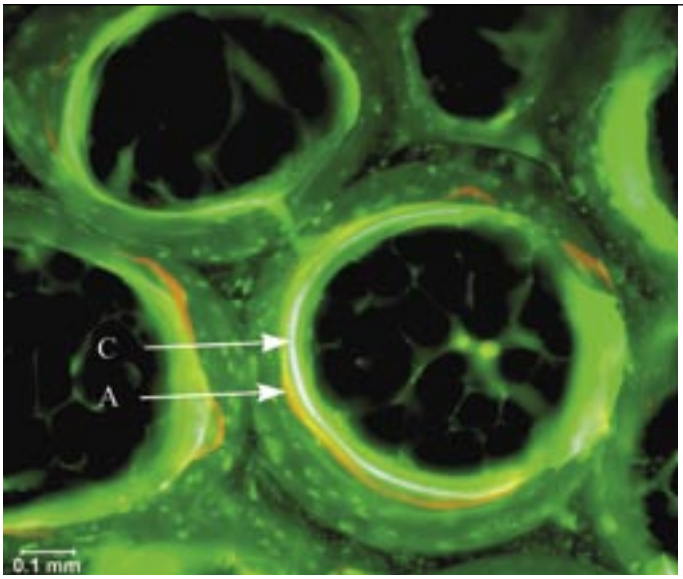
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