Incorporation of morsellized bone graft under controlled loading conditions. A new animal model in the goat

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Abstract

The aim of this study was to develop a new animal model in which we could assess the in vivo effects of mechanical stimuli in the incorporation process of impacted morsellized bone grafts. The subcutaneous pressure implant SPI was developed for use in the goat. This device can generate controlled loading conditions onto a fixed amount of bone graft in the distal femur. Twenty goats were divided into three groups: non-loaded, 2 or 4 MPa loads (1 Hz, 1 h/day). The goats were sacrificed after 3, 6 or 12 weeks. The results were documented by clinical observations, quantitative bone density from QCT-scanning and histomorphometry. Nine post-mortem knee specimens were prepared in a similar manner to the experimental knees to determine the reproducibility and mechanical stability of the grafting method. Three goats were lost due to complications, the others functioned clinically well. Histology showed invasion of the bone graft by a front of vascular fibrous tissue after which osteoclasts resorbed the dead bone graft, followed by woven bone apposition on the graft remnants. At 12 weeks the loaded grafts had transformed into a vital trabecular structure. QCT bone density measurements revealed persistently high densities in the 12-weeks 4 MPa specimens, but reduced densities in the 2 MPa and non-loaded specimens. Morphometrically, the mineralising surface was larger in the 4 MPa group ($P = 0.02$) and the incorporation and remodelling processes had advanced more rapidly in the 2 MPa specimens ($P = 0.04$). Although the numbers investigated in this study in each group were low, statistical differences were found in the amount of graft left after incorporation and in the apposition rate of the new bone. In the future this model will be used to study the incorporation potential of different types of bone graft and bone graft substitutes.

Keywords: Animal model; Bone graft; Bone incorporation; Goat

1. Introduction

Bone stock deficiency around failed arthroplasties can be reconstructed with bone grafts. In the late 1970s, a revision technique was developed for hip replacements using impacted morsellized bone graft in the acetabulum [1]. Later this technique was adopted for revisions on the femoral side [2]. The short-term results in the femur are very promising [3]. The long-term result in the acetabulum after a mean follow up of 11.8 yr is 90% [4,5].

The histological evaluation of biopsies taken from acetabulae, previously reconstructed with the morsellized chip grafting technique, demonstrated graft incorporation with the formation of a new trabecular structure after 8 months [6]. However, in an animal experiment to test this impaction revision technique, it was found that at mid shaft levels around a stiff stem, resorption of the allograft predominated [7]. Various factors, such as loading of the graft, the type of graft and the size of graft chips may all influence the incorporation process.

To study these variables, an animal model was developed. The subcutaneous pressure implant (SPI) allows us to study the effect of loading-conditions on the incorporation of different types of bone graft. In this paper this implant is described and the first results are presented of a loading experiment in the goat using impacted morsellized bone allograft in the SPI.

2. Materials and methods

2.1. Implant

The subcutaneous pressure implant (SPI) is composed of 3 elements (Fig. 1). Part A is made out of titanium and
consists of a hollow screw (8 × 20 mm), opening into a cylindrical pressure chamber (50 × 20 mm). A stainless-steel piston (B) with a 5 mm diameter and a driving plateau (diameter 25 mm) fits into part A. A stainless-steel screw-cap (C) (diameter 38 mm) closes the implant. The screw cap is connected to a subcutaneous air pressure cannula. Compressed air is conveyed through this cannula to the implant. The cannula is made of high-pressure-resistant polyurethane and has a length of about 1.3 m. A titanium cuff (2 cm length, moulded fibres, diameter 50 μm, porosity 80%, NV Beheart S.A. Zwevegem, Belgium) is glued to the distal end of this cannula. An apparatus that generates the selected air pressure, which is connected to the distal end of the cannula during the daily loading period, regulates the loading regime. Frequency (Hz) and air pressure can be adjusted with this equipment. In this way, the piston is force-controlled and creates its own displacement dependent on the visco-elastic behaviour of the graft.

2.2. Graft preparation

The allografts were harvested manually as small chips from the sternal bone of non-relative donor goats. The size of the individual chips was ca. 5 × 5 mm with a content of ca. 0.125 cm³. This is slightly smaller than prescribed for use in impaction grafting in the acetabulum where chip sizes of ca. 0.5–1 cm are used [1,4,8]. The graft material was cultured, packed in sterile bags and stored at −70°C until use. Before implantation the grafts were thawed in saline.

2.3. Surgical procedure

The goats (Capra Hircus Sana) were positioned on their left or right side and the area of interest on the hind-leg was prepared according to a standard sterile surgical procedure. The side to receive the SPI was chosen by random and every animal only received one implant. The goats were anaesthetized with pentobarbitonal (Narcovet® 60 mg/ml, Apharmo) at a dose of 0.5 ml/kg body weight, incubated and maintained using halothane and oxygen in a semiclosed ventilation system. The knee was approached laterally, visualising the collateral ligament. A hole, 30 mm deep, 6.7 mm diameter, was drilled at the femoral insertion of the lateral ligament, using a water-cooled diamond-tipped drill (Surgical Diamond Instruments®, Scientific Developments GMBH, Munich). The defect was filled with impacted morsellized bone graft over a distance of 10 millimetres. Then the implant was screwed into the pre-tapped hole. The amount of implanted morsellized graft was weighed. The air-pressure cannula was passed subcutaneously through a skin opening in the mid line at the level of the first thoracic spine and cannulated towards the implant on the lateral side of the knee. The cannula was fixed to the nipple on the cap of the implant, while the distal titanium-cuffed end of the cannula permanently extruded through the skin in the midline. This titanium cuff was placed subcutaneously near the skin opening to obtain fibrous tissue ingrowth and provide a stable canula–skin interface. The facia and skin were closed separately with resorbable sutures. After the implantation procedure, the animals received subcutaneous ampicillin (Albupen LA® 100 mg/ml, Mycofarm) 7.5 ml per day for 5 days. During the remaining follow-up, no medication was administered to the animals. From the second week onwards, the implanted grafts were subjected to a daily loading regime.

2.4. Experimental design for ex vivo study

To determine the reproducibility of the degree of graft impaction and the influence of the loading regime on the initial stability of the graft, nine post-mortem goat knees were prepared in a similar way as in the experimental animals. In six post-mortem specimens, the density of the graft was determined using QCT. The coefficient of variation for graft density due to repeated placement in the scanner was 4%. Three post mortem specimens were
subjected to loading, one knee with 2 MPa and two with 4 MPa. Radiographs were taken after 36 000, 90 000, 144 000 and 198 000 loading cycles of 1 Hz, corresponding with 3, 6, 9 and 12 weeks of follow-up, respectively.

2.5. Experimental design for in vivo study

Eighteen goats were scheduled to receive an SPI. Six goats served as controls with non-loaded pistons. Twelve goats were subjected to loading. The loading regimes were cyclic, 2 \( (n = 6) \) or 4 MPa \( (n = 6) \), with a ramp waveform pressure profile (Fig. 2) and a frequency of 1 Hz, for 1 h/day. The stress values chosen were within the range of physiological stresses around hip prosthesis components as calculated from finite element analyses [9–11]. The goats were grouped according to 3, 6 or 12 weeks of follow-up (Table 1). Three failures occurred during the experiment. One goat in the non-loaded group developed an infection; there was air leakage in one goat in the 2 MPa group; one other goat in the non-loaded group developed aseptic loosening of the screw after 12 weeks. The first two goats were replaced by new ones to compensate for the failures, so that each group comprised 6 goats, except for the 12-week-follow-up group, which comprised 5 goats. To allow fluorescence microscopy all the goats received two doses of subcutaneous Calceine Green (20 mg/kg/day) seven days and one day before sacrifice.

2.6. Clinical evaluation, QCT and X-ray analysis

Clinical performance was graded according to Ypma et al. [12] ((grade 0) leg not used at all; (grade 1) supported incidentally; (grade 2) loaded in standing position and incidentally while walking; (grade 3) loaded in standing position and while walking but with a limp; (grade 4) normal walking and standing pattern). The skin reactions around the cannula were graded according to Hoggers et al. [13] ((grade 0) no irritation; (grade 1) slight irritation; (grade 2) red and slightly moist tissue, no granuloma; (grade 3) red and moist tissue and granuloma tissue; (grade 4) infection). Plain radiographs were taken in the A–P direction post-operatively and post-mortem. After excision of the total distal femur the bone graft mineral density was measured using QCT (Stratec XCT-960A).

2.7. Histological and histomorphometrical analysis

Specimens (total distal femur with bone graft in situ) were immediately fixed after sacrifice in 4% phosphate-buffered formaldehyde. After densitometry measurements, the cylinder of graft with at least 2 mm of surrounding host bone was excised from the distal femur, dehydrated in ethanol and embedded in PMMA. Undecalcified sections were sectioned with a Leica microtome (RM 2155) parallel to the long axis of the bone graft specimens (7 μ) and used for fluorescence microscopy or stained with Haematoxylin–Eosine or Goldner–Masson. Histomorphometry was performed using the PC-Image system (Foster Findlay UK), an automated computerised system or a semi-automated system with a microscope connected to a computerised video digitising tablet system (Videoplan, Kontron Bildanalyse GMBH, Germany). For every variable in the analysis, two sections at 300 μm distance were used from the centre of the defect. The reproducibility of the morphometrical variables was expressed as the coefficient of variance (SD/x) for repeated measurements. The Graft Volume expressed as a percentage (GV = bone graft area/total tissue area of the defect; 25x, reproducibility 2.5%) was measured with the PC-Image system. The semi-automated interactive system was used to measure the Graft-Re-Vascularization percentage (GRV = area of revascularized graft/total graft area; 25x, reproducibility 5%), the amount of Graft Remnant expressed as a percentage (GRM = amount of dead graft in incorporated bone; 25x, reproducibility 12%), the mineralizing surface (MS = double label surface + \( \frac{1}{2} \) single label surface/total bone perimeter; 100x, reproducibility 0.2%) and the mineral apposition rate in \( \mu \)m/day (MAR = amount of bone apposition between a double label/6 days; 250x, reproducibility 4.4%).

### Table 1

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<th>Follow-up</th>
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<th>4 MPa (#)</th>
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<td>2 (1b)</td>
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<td>2</td>
<td>2</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>12 Weeks</td>
<td>1 (1c)</td>
<td>2</td>
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<td>5 (1)</td>
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<tr>
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<td>5 (2)</td>
<td>6 (1)</td>
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<td>17 (3)</td>
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*The numbers in brackets are failures; excluded from the analysis: (a) infection; (b) pneumatic complication; (c) aseptic loosening of the screw.

Fig. 2. The ramp waveform pressure profile of the loading impulse. The lag-time until maximal loading was 0.4 s.
2.8. Statistical analysis

The data were analysed statistically using 2-way ANOVA for the factors for the follow up and loading regime (2, 4 Mpa and non-loaded).

3. Results

3.1. Clinical results

Peroperatively, 0.8 ± 0.1 g of impacted bone graft was implanted in the defects. The goats showed good function with no restraint at all (Ypma grade 4). All the implants and cannulae were encapsulated in fibrous tissue. The cannula skin passage showed only slight redness and some epithelial debris (Holgers grade 1). When tested after removal, all the implants were functioning smoothly and no body fluid had entered the inner chamber.

3.2. QCT and radiographic analysis

The average density of the trabecular bone surrounding the defect was 487.8 ± 36 mg/cm³, while for the femoral cortical bone it was 1400 ± 84 mg/cm³. In the ex vivo specimens, the average density of the impacted graft was 664.7 ± 22.5 mg/cm³. Irrespective to the loading regime, the QCT values of the 3-week follow-up specimens did not differ from the immediate postoperative values (Table 2). After 6 weeks of loading with 2 or 4 MPa, the density had decreased, whereas a persistently high density was observed in the non-loaded grafts (Table 2). At the graft–piston interface, the QCT values of the 3-week follow-up specimens (Table 2). However, these differences were not statistically significant (P = 0.55). The 12-week follow-up group was too small to conduct a separate statistical analysis.

The plain radiographs of the knees in the ex vivo specimens were identical to the postoperative films. The prostheses were uneventfully integrated into the subchondral bone and the proximal femoral cortex. The prosthesis had been well integrated into the bone in all the specimens. The bone apposition rate (MAR) between a double label/6 days.

3.3. Histological and morphometric analysis

Histologically, only marginal graft revascularization had occurred at 3 weeks and there was no difference in histological appearance between the loaded and unloaded grafts (Fig. 3A and B). A front of vascular mesenchymal tissue had advanced into the graft and osteoclasts had resorbed the dead bone graft. In the direct vicinity of the resorption activity, osteoblasts had apposited woven bone on the graft remnants, irrespective of the loading regime (Fig. 3A and B). At the graft–piston interface, a thin fibrous tissue layer was observed in three of the loaded specimens. In all the specimens loaded for 6 weeks remodelling was seen in the host bone and adjacent graft, but incorporation and remodelling were more advanced in the 2 MPa loaded grafts, with large areas of woven bone apposited on dead trabeculae (Fig. 3C and D). At 12 weeks, the loaded grafts had transformed into a vital trabecular structure, which demonstrated load-dependent trabecular orientation and density, especially in the area on top of the piston (Fig. 3G). Non-loading, in contrast, had resulted in the disappearance of the graft and trabeculae in this specimen (Fig. 3F). Morphometrically, bone density followed the same pattern as in the QCT measurements and demonstrated lower densities in the non-loaded and 2 MPa loaded specimens after 12 weeks, whereas the density remained high in the 4 MPa specimens (Table 2). However, these differences were not statistically significant (P = 0.2). The bone grafts had revascularized most rapidly in the non-loaded group and the 2 MPa loaded group, whereas revascularization in the 4 MPa group was slower until 6 weeks (P = 0.09). There were significantly fewer dead graft remnants in the incorporated bone in the 2 MPa group than in the other specimens (P = 0.04). The surface onto which woven bone had been apposited (MS) was significantly larger in the 4 MPa loaded specimens (P = 0.02), but no statistically significant difference was observed in the bone mineral apposition rate (MAR) (P = 0.9).

<table>
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<tr>
<th>FU (wk)</th>
<th>F (MPa)</th>
<th>QCT (mg/cm³)</th>
<th>GV (%)</th>
<th>GRV (%)</th>
<th>GRM (%)</th>
<th>MS (g)</th>
<th>MAR (μm/day)</th>
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<td>100</td>
<td>4.7</td>
<td>0.50</td>
<td>2.1</td>
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*GV* bone graft area/total tissue area of the defect; *GRV* area of revascularized graft/tot graft area; *GRM* amount of deadgraft in incorporated bone; *MS* double label surface + single label surface/tot bone perimeter; *MAR* amount of mineral apposition between a double label/6 days.
Fig. 3. (A) Revascularization, bone resorption by osteoclasts (large arrow) and bone apposition on dead graft (G) remnants (small arrows) in a non-loaded impacted morsellized graft after 6 weeks, ×130. (B) Magnification of osteoclastic resorption (arrow) of the graft (G), ×300. (C) Incorporated bone in 2 MPa loaded specimen after 6 weeks. A new immature trabular structure has been formed which is a mixture of remnants of the graft (G) and new bone (NB), ×130. (D and E) Details of incorporated graft 12 weeks after surgery. G, graft remnants, NB new bone, ×100, and ×300, respectively. (F and G) Overview of incorporated bone 12 weeks after implantation in a non-loaded (F) and 4 MPa loaded (G) bone graft. Notice difference in bone density. The large arrow indicates the location of the piston and the direction of load transfer, ×12.

4. Discussion

The subcutaneous pressure implant will enable us to investigate the influence of controlled loading on the bone graft incorporation process in vivo in the absence of any functional restriction or impairment of the well-being of the animals. Mechanically induced bone repair has been described for cortical fracture models [14–16] and
also more recently for trabecular repair and adaptation [17,18]. Although the latter systems might also be used to study load-dependent bone graft incorporation, it was decided to design an uncomplicated implant that operates on air pressure, is easy to apply, is fairly invulnerable to technical complications and involves less burden to the animals. The disadvantage of the experimental set-up was that only one type of graft was investigated per animal. Besides the study of morsellized bone grafts, the model can also be used to study the influence of loading on the incorporation of processed grafts or any other biomaterials. When starting this project, a percutaneous implant similar to the subcutaneous one was designed. This percutaneous implant, which permanently pierced the skin in the lateral knee region of the goat, was designed to prevent and control potential pneumatic complications, such as air leakage and body fluid penetration into the implant. Several percutaneous implants were applied successfully [19], but a stable implant–skin interface could not be created [20], due to tissue movement at the knee, which resulted in infection and loosening of the implant. The specially designed implant as described in this paper, did not display these clinical problems. Plain radiographs and QCT of the cadaver specimens demonstrated a reproducible experimental graft-impacting technique. The fact that the piston remained stationary after extensive in vitro loading demonstrated initial mechanical stability of the graft. Radiographic evidence of migration of the piston after 6 weeks of loading, combined with histological evidence of graft incorporation and remodelling, can be explained by a temporary reduction in mechanical rigidity due to the simultaneous occurrence of graft resorption and bone apposition. This local remodelling process, together with the postoperative observation of permanent deformation of the impacted bone chips, can to a certain extent explain the subsidence of the femoral component in clinical studies [21,22].

The incorporation behaviour of the morsellized bone graft used in this experiment is in accordance with histological findings in other animal experiments [7,23,24]. The same pattern of revascularization was described, with an invasion front of loose connective tissue, osteoclastic bone destruction whereafter woven bone apposition on the remnants of the graft takes place. In this model, however, this process advanced more rapidly. In the studies by Schreurs et al. [7] and Schimmel et al. [23], the graft layer was almost fully revascularized after 12 weeks, whereas in this experiment, this had already occurred after 6 weeks. Three explanations can be given for this phenomenon. First, in the acetabulum and femur the vessels can only penetrate into the graft layer from one side, whereas in this model, this could occur from all sides. Secondly, the amount of vascular damage in reamed host bone is more extensive than in the water-cooled diamond-drilled defect. Thirdly, the size of the defects was larger in the animal studies.

The present experiment showed that in the first 6 weeks of follow-up, the bone graft repair reaction was relatively independent of the loading regime. No differences were observed in bone density and the bone mineral apposition rate between the three groups. It seems that at the very beginning of repair after surgical trauma, there is an all-or-none reaction probably caused by various cytokines and pluripotent mesenchymal cells [25,26]. At longer-term follow-up, the size of the bone-mineralizing surface was directly correlated with the amount of load applied. This suggests that subsequent loading can influence the later stages of graft incorporation and remodelling. The final trabecular orientation in the loaded specimens suggests that loading plays an important role in transforming the incorporated bone allograft into a new vital bone structure that is able to withstand load and that it adapted to the local mechanical environment. To determine the actual receptors and effectors in the bone graft remodelling process and to distinguish more clearly between initial trauma and loading effect, longer-term experiments are needed. The sequence of events that occurred in this experiment was similar to that in the incorporation process of impacted morsellized bone graft in the human acetabulum and femur [27,28]. The rate of incorporation, however, is much slower in the human than in the goat. Revascularization of the graft in patients takes at least four months, while the complete conversion of the dead graft into a vital trabecular structure with various amounts of remnant takes at least eight months [6].

To our knowledge no studies have been performed in which the incorporation patterns of morsellized fresh frozen bone allograft have been investigated under controlled mechanical conditions. The influence of mechanical loading on the fracture healing process has been investigated using segmental defect models in sheep [14,23]. The cytokines present in the cortical callus tissue will play the same role in this case as in the healing of bone grafts. In fractures stabilised with external fixation, that generates controlled mechanical loading, there were no obvious histological differences in healing until 10–12 weeks of loading. Underloading resulted in immature callus tissue [15], whereas subsequent increased physiological loading and cyclic loading resulted in enhanced bone formation [14,15]. From these and the present experiment it appears that in the early response to trauma, a consistent cellular repair reaction occurs that is unrelated to loading, whereas the final healing and remodelling of bone tissue is dependent on loading.

In conclusion, this new animal model, the subcutaneous pressure implant, is suitable to study bone graft incorporation under controlled experimental loading conditions. This experiment demonstrated an effect of loading on the percentage of dead bone graft left after
incorporation and on the surface on which woven bone had been appositioned.

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References


