Evaluation of biocompatibility, osteointegration and biomechanical properties of the new Calcemex® cement: An in vivo study

Tommaso Maluta,1 Umberto Lavagnolo,1,2 Lydia Segalla,2 Nicholas Elena,1 Paolo Bernardi,2 Daniele Degl’Innocenti,2 Andrea Sbarbati,2 Bruno Magnan1

1Department of Surgery, Orthopaedic and Traumatology Clinic, University of Verona
2Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona, Italy

The mixture of polymethylmethacrylate (PMMA) and β-tricalciumphosphate (β-TCP) is the most widely used bone graft. Common features of bone cement are the biocompatibility, bioactivity, mechanical stability and ability to fuse with the host's bone tissue. However, there are still few studies that have evaluated these characteristics in vivo. Our study aims to acquire these parameters, using an animal model with functional characteristics similar to those of humans. The analyzed cement is Calcemex®, evaluated both in compact and fluid formulation. The chosen animal models were 5 pigs, treated with femoral and tibial implants of Calcemex® samples. After one year, the pigs were sacrificed and the specimens explanted for morphological, histological, ultrastructural and mechanical evaluations. For both formulations, the investigation highlighted the absence of foreign body reactions in the host, the histological integration with the surrounding tissues and the preservation of mechanical compression resistance.

Key words: Polymethylmethacrylate; calcium phosphate cement; osteointegration; osteoconduction; biocompatibility; compression; environmental scanning electron microscopy.

Correspondence: Umberto Lavagnolo, Department of Surgery, Orthopaedic and Traumatology Clinic, University of Verona, Ospedale Borgo Trento, Piazzale A. Stefani 1, 37126 Verona, Italy. Tel. +39.045.8121489 - Fax: +39.045.8022811. E-mail: umberto.lavagnolo@gmail.com

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Availability of data and material: All data (both raw and processed) are available for public consultation by contacting the corresponding author. All materials and instruments can be viewed in the laboratories of the University of Verona.
Introduction

In the orthopedic practice, polymethylmethacrylate (PMMA)-based cement was first used for prostheses fixation and as a bone substitute.\(^1\)\(^2\) Excellent mechanical properties, coupled with strength and radiopacity are the best characteristics for medical uses.\(^3\)\(^4\) Nowadays, the role of this material has been extended to fill bone defects in orthopedic surgery, neurosurgery and dentistry.\(^5\)\(^6\) However, all bone cements generate a highly exothermic polymerization reaction, carry inherent monomer toxicity, are unable to bind to microscopic bone architecture, and can induce a foreign body reaction with granulomas and fibrous tissue at the interface.\(^10\) Therefore, new bone cements with bioactive characteristics were developed and investigated.\(^11\)\(^12\)

Autologous bone remains the gold standard for stimulating bone repair and regeneration.\(^13\) Bone graft substitutes can either replace or expand an existing amount of autologous bone graft.\(^14\) Calcium phosphate based cement (CPC) has been tested and clinically used as bony filler in traumatic bone loss for its excellent self-setting ability, biocompatibility, partial osteoconductivity and biosorbability.\(^15\) Its downside are the poor mechanical characteristics (e.g. compression strength).\(^16\)\(^17\) In addition, its handling problems and limited radiopacity prevents extended clinical uses.\(^18\)

Dall’Oca et al.\(^19\) studied a new porous and acrylic partially resorbable bone cement, composed of β-tricalcium phosphate (β-TCP) and polymethylmethacrylate (PMMA). The first in vivo studies of this cement were conducted on rabbits to evaluate its bio-compatibility and osteointegration, through histological evaluations. The good clinical outcomes were confirmed by a further methodological approach based on the histological embedding in LR white resin and performing environmental scanning electron microscopy (ESEM) analyses.\(^20\) These assessments revealed the excellent histological properties of the cement and provided an operational methodology, thus requiring the execution of further studies on animals of larger size and weight to understand the behaviour of the samples under weight-bearing conditions.

The purpose of this experimental study is to improve the methodological approach in evaluating the in vivo biomechanical characteristics of Calcemex\(^6\) (Tecres S.p.A., Sommacampagna, VR, Italy). It consists of a mixture based on β-TCP and PMMA with a resorption rate similar to that of new bone formation. It possesses a characteristic macroscopic structure with macropores filled with β-TCP linked by microcanaliculi, all supported by a spongy matrix of PMMA.\(^21\) The reabsorption of β-TCP and the resulting lacunae give the cement a marked tendency to integrate with the native trabecular structure, allowing for better cellular colonization and faster bone formation.\(^22\)

The animals used for this study were 5 common pigs (Sus scrofa domesticus L.), who underwent implant to cement the thimble epiphysis of the femur and in the proximal tibia of the right limb. To evaluate the biomechanical effects due to the physical form of the cement, two different physical forms were used: one in fluid phase and one in solid phase.

Materials and Methods

Animals

Five 4-month-old pigs with an initial weight of 60 kg and which grew up to 250 kg after 12 months were used for the experiments. The experiments were approved by Ethics Committee of Verona University and performed in accordance with the Guideline for Animal Experimentation of the Italian Ministry of Health. The study was carried out at the “Bruno Ubertini” Experimental Zooprophylactic Institute of Lombardy and Emilia Romagna where the European Directives (CE Directive 86/609), UNI EN ISO 10993-2 (2006 - Animal welfare requirement) and Decree n. 116/02 was respected for the protection of animals bred for experimental purposes.

The pigs were housed in 6 m\(^2\) single stalls on a concrete floor. They were fed with a mix of barley, corn, alfalfa, oat bran, soy, flax cake, multivitamin and mineral supplement in the amount of 10 g/kg prepared directly on site, and free water. The five animals were identified by numbered tags applied to the ear, the identification numbers were: 241, 242, 243, 255, 256. The animals were followed for the duration of the experimentation by veterinary and trained personnel.

Cement samples

A composite bone cement was used, consisting of an organic part and an inorganic part. The organic component is insoluble in biological liquids, is not reabsorbed and consists of (PMMA); the inorganic component is soluble in biological fluids, resorbable and consists of a calcium salt, β-tricalcium phosphate (β-TCP). In the formulation there is also a portion of saline solution which, once the polymerization reaction has taken place, acts as an interface between the β-TCP (strongly hydrophilic) and the lipophilic PMMA. After reviewing the results of previous in vivo studies,\(^16\)\(^20\) it was decided to use the PMMA + β-TCP formulation in powder and granules (Table 1).

Femoral implants

Prepolymerised Calcemex\(^6\) samples were used for the femoral implants. Cylindrical specimens measuring 10 x 20 mm were obtained from a solid mold of Calcemex\(^6\) with a coaxial tip cutter. Given the large size of the specimens, implantation in the femoral condyles was envisaged. The central cavity of the implant allowed the recovery of the same even after many months: the insertion of the drill tip into the canal allowed to create a guide on which to make the coaxial tip cutter act exactly at the periphery of the specimen. 10 samples were used, two for each femur, and one femur (right limb) was treated for each pig. Samples implanted at the femoral level were intended for biomechanical testing.

Tibial implants

Two types of specimens were used for the tibial implants. Both prepolymerized Calcemex\(^6\) in the form of cylinders measuring 4 x 8 or 4 x 15 mm, and fluid Calcemex\(^6\) (Table 1) were implanted. In particular, the fluid samples were injected into bone hollows created by using an electric 4 mm tip perforator. The implant was performed in the region immediately distal to the tibial plateau. 10 solid Calcemex\(^6\) samples, 2 for each tibia and 10 fluid Calcemex\(^6\) samples, 2 for each tibia were used. For each pig, only the right tibia was treated. Specimens implanted at the tibial level were intended for histological study.

Surgery

All animal specimens underwent the following anesthetic and operative protocols. Only the right hind limb was treated, to ensure the possibility of autonomous movement immediately after sur-

| Table 1. Composition of the analyzed cement. |
|--------------------------------------------|
| Powder       | Fluid       |
| PMMA         | 48.6 %      | PMMA       | 99.5%       |
| TCP          | 50.1 %      | N,N dimethyl-p-toluidine | 0.5%    |
| Benzoyl peroxide | 1.3 %     | Hydroquinone | 75 ppm    |
After washing, disinfection and preparation of sterile operative field, the skin was incised at the level of the right knee and the tissues dissected until the underlying bone was exposed. The chosen implantation site was the epiphyseal region of the femur, in direct contact with cancellous bone. Housing for cannulated bone cement sample was achieved using a surgical drill bit with a diameter of 10 mm. After drilling, bone debris were removed by rinsing and suction. Then, the cement samples were inserted into the created housing and finally the soft tissues were sutured, and the wound was medicated. For each femur, two specimens were implanted in the condyle-epiphysial region approximately 2 cm apart, one median and the second one more laterized.

Simultaneously with samples insertion at the femoral level, samples used for histological tests were inserted in the corresponding tibiae, one with solid Calcemex® in 4 mm specimens and the other in the form of extruded paste for the verification of chemical-toxicity on tissues. Surgical tissue suturing was performed with not reabsorbable spin Prolene (Ethicon, Bridgewater, NJ, USA) n. 3-0. After a final disinfection, an adhesive elastic dressing was applied to the surgical wound.

**Post-operative clinical follow-up and animal sacrifice**

The pigs were followed up daily, the dressings were periodically renewed after disinfection with iodized antisepic. Cutaneous stitches were removed on post-operative day 10.

All the animals immediately used quadrupedal walking, despite a slight limp in the operated limb which disappeared within 48-72 h. Post-operative antibiotic therapy was administered for 4 days, using enrofloxacin 5% 2.5 mg/kg IM and/or amoxicillin 1.5 mg/kg IM. In some clinically suggestive cases, pain relief therapy with ketoprofen 3 mg/kg IM was administered. During the follow-up period, the pigs underwent an X-ray examination of the knees.

Throughout the entire follow-up year, the animals were always in excellent health, with routine physiological (feeding, urine and stool) assessments and check-ups for complications related to the intervention. The growth of the 5 pigs was regular and their weight went from 60 kg to over 250 kg at the age of 16 months. Twelve months after the operation, the pigs were sacrificed by stunning with a penetrating captive bolt and subsequent cerebral hemorrhage. When the pigs were slaughtered, their average weight was 220 kg. Since the load on the fore and hind limbs is equivalent, the weight-bearing on the treated extremity could be estimated to be about 55 kg.

**Collection of samples**

After the suppression, disarticulation of the operated limb was performed at the level of the coxo-femoral joint. X-rays, using a Philips Practix 360 mobile radiography system, were taken to evaluate the actual position of the specimens and their degree of integration with the surrounding tissue (Figure 1). Mechanical tests were performed on the femoral samples, once adequately refrigerated and transported to Tecres S.p.A. As well the refrigerated tibias, were transported to the Anatomy Section of the University of Verona.

Subsequently, dissection of the femoral condyle was carried out to extract the bone cement specimen. The sampling represented the most critical step of the whole process, being the integration with the surrounding bone highly represented and lacking a clear margin for the identification of the specimen itself. Therefore, the extraction was possible thanks to the central canal of the specimen which acted as a guide for the insertion of the central tip of a cup bur. The tip, entering the canal of the concrete cylinder, served to perfectly center it. Therefore, since the blade of the cup bur has a diameter exactly equal to the original cylindrical specimen (10 mm), a clean and constant cut for the extraction was obtained.

**Histological sample preparation**

Upon arrival of the tibia specimens, the locations of the implants were identified based on radiographic images and visual appearance. Subsequently, on the basis of the radiographic reports, the necessary cuts were planned for the isolation of the implant areas. For each subject, 3 samples were isolated, identified and processed for microscopic analysis.

The obtained tibia sections were stored for three days in a 10% formaldehyde solution for fixation; followed by an embedding method in resin according to the following protocol: progressive dehydration in ethyl alcohol with increasing gradation (30%, 50%, 70%, 95% and absolute), leaving the sample for at least three days in each alcoholic solution and performing three passages in absolute alcohol; infiltration of LR white resin, three steps for a total of fifteen days; UV curing of the resin. Following this preparation, a bone microtome was used to cut the samples into approximately 60 µm thick sections. These were stained according to the following protocol: sections were stained by immersion in a solution of 1% toluidine blue for 5 min, rinsed in running water, immersed in a solution of 2% acid fuchsin for 3-5 min, rinsed in running water, immersed in a 0.02% solution of acetic acid for 1 min, stained with fast green (Diaphath) for 5 min, and finally rinsed.

The stained sections were mounted on a slide with an aqueous medium and observed with an Olympus BX51 optical microscope.

**Ultrastructural analysis**

For scanning electron microscopy (SEM), 2-3 mm-thick sections were fixed in 2% glutaraldehyde in Sorensen buffer for 4 h,
postfixed in 1% osmium tetroxide in Sorensen buffer for 1 h, and dehydrated in graded acetones (Fluka, Buchs, Switzerland). The specimens were then treated by a critical point dryer (CPD 030; BAL-TEC AG, Balzers, Liechtenstein), mounted on aluminum stubs with sticky carbon and coated by gold (MED 010; BAL-TEC AG). A XL 30 ESEM (FEI-Philips) was used for the ultrastructural examination.

**Compressive resistance**

The mechanical property considered in this test was compressive resistance, defined as the maximum stress level reached during the test at a constant speed (20 mm/min) before specimen fracture (normal force per unit area of the original cross-section of the specimen, expressed in Mega Pascal, MPa).

The protocol used for the experiment was the ISO5833 (E attachment): measurement of the average specimens’ diameters; specimen placement in the test machine; machine activation to produce a curve of displacement against load, using constant cross-head speed; machine lock when the cylinder fractures. This evaluation was repeated for each cylinder.

The method is the one specified in ISO5833. However, the sample size was different from what is reported to be the standard cylinder used in literature (20 mm x 10 mm for cylinders with hole vs 12 mm x 6 mm for standard cylinders). Due to this mismatch, our results were not comparable with the data in literature reporting compressive strength of bone cement. The following equipment were used for testing: testing machine Galdabini SUN 1000 with 10 kN cell load; digital micrometer. Each measuring instrument was recalibrated before the experiments began.

According to the described protocol, the following samples were examined: Calcemex®: pre-polymerized pure hollow cylinder; cancellous bone tissue obtained from the tibial plateau; fluid cement sample taken from the tibia; hollow cylindrical cement sample explanted from the femoral condyles after one year.

**Results**

**Post-operative clinical follow-up**

All the animals subjected to surgery survived. The surgical procedure was well tolerated and the animals were able to feed and move from post-operative day 1. We reported neither surgical site alterations such as swelling or inflammation, nor systemic signs of cement-mediated toxicity or adverse reactions appeared.

**Macroscopical and histological evidence**

In both femoral and tibial specimens, the cortical bone showed no discontinuity. There was no bone rarefraction around the specimens which appeared to be well integrated with the native bone matrix. Macroscopically, it is noted that the specimen has assumed the same color as the surrounding cancellous bone, as a consequence of its integration. At the same time, the cortical region also adapted to the surface remodeling itself to the presence of the specimen, without showing any solution of continuity or irregularity. Inside the concrete cylinder, a fibrous cylindrical structure had developed. No sign of bone distress was reported.

The optical microscope investigation evidenced the two types of preparation: the cement sample injected in vivo (Figure 2A) and the prepolymerized cement cylinder (Figure 2B). A first observation was the absence of inflammatory and necrotic aspects both in the tissues surrounding the injected cement sample and in those surrounding the cylinders. The margins of the specimens appear regular and the trophism of the surrounding bone is preserved. At the interface, the contact between the sample and the bone is very close, with no visible spaces. At this level no infiltrates of hematic origin, interpretable as inflammatory cells, were found. The two types of preparation therefore did not induce a foreign body reaction in the host. This demonstrates a high biocompatibility even over time.

A second important point concerns the biomechanical properties of Calcemex®, as the samples remained compact despite the load and growth the limbs underwent throughout the follow-up year.

Regarding the osseointegration of the cement, two main aspects were highlighted. Where the sample interfaced with the compact cortical bone, the cement matrix appeared partially replaced by structurally normal bone tissue, more evident in the injected preparations (Figure 3 A,B). Where the specimen interfaced with the medullary cavity or with cancellous bone, the formation of a bony capsule was observed which almost completely envelops the specimen. The thickness of this capsule is about 200 µm, comparable to that of normal bone trabeculae. This aspect is evident both for the injected cement and for the prepolymerized preparations. Two faces of the capsule can be described: the outer one appears smooth; meanwhile the internal one, in contact with the cement, appears more anarthrous. The bone-cement contact surface is therefore very large, indicating excellent osseointegration. Again, the bone is structurally normal. The external face of the capsule is in continuity with the trabeculae of the cancellous bone. The sample is therefore perfectly integrated with the cancellous bone and the spatial trend of the bone trabeculae suggests the hypothesis that they are directed along force lines.

![Figure 2. Histological sample of fluid cement injected into the native bone matrix (A) and of polymerized cement inserted into the native bone matrix (B). Toluidine blue / fast green stain. Both cements (c) are regularly surrounded by bone tissue (b) with no signs of inflammation or necrosis, demonstrating complete biocompatibility one year after implantation. Polymerized cement sample (B) has a more regular contact surface (arrowhead). Scale bars: 1 mm.](image-url)
Ultrastructural evidence

At SEM examination, the interface between the cement and the cortical bone was crossed by serial microcavities parallel to the interface itself. These cavities have an irregular internal surface and are present at the edges of the bone cement. In the contiguous areas, these cavities are not present and the interface between cement and cortical bone does not present continuity solutions (Figure 4). The central area of the cement is dense and, at higher magnifications, consists of both a compact matrix and a granular matrix. Some areas have a higher level of irregularity whose genesis can reasonably be traced back to the moment of insertion of the cement. In the central area of the cement there are very elongated micro cavities with an irregular internal surface, very similar to those found at the interface between cement and cortical bone (Figure 5).

Compression test

The study showed that Calcemex® is a material with minimal reduction in mechanical performance after 12 months of permanence in vivo and under weight-bearing conditions. We obtained the following results (mean ± SD): 30.69±1.97 MPa for pure hollow cylinder of pre-polymerized Calcemex®; 5.45±2.54 MPa for cancellous bone tissue obtained from the tibial plateau; 27.48±0.09 MPa for Calcemex® (no TCP) fluid cement sample taken from the tibia; 29.24±2.53 MPa for Calcemex® hollow cylindrical cement sample explanted from the femoral condyles after one year (Figure 6). Evaluating the compressive strength tests, the extreme strength of the cement compared to the naïve bone is evident, with a minimum loss of strength over time. This strength is 6 times higher than natural cancellous bone, although lower than pure Calcemex® (Figures 6 and 7).

It is important to underline that Calcemex® tested according to ISO5833 (method and specimen dimension) reaches a compressive resistance of 53±5 MPa, higher than any value detected in our study. In particular, probably the specimen dimension affects the result.
Discussion

This study evaluated the biocompatibility, osseointegration, osteoconductive properties and mechanical response of Calcemex® in the bone tissue of an animal model after one year. The mechanical stresses on the limbs and on the implanted devices are comparable to those in humans, therefore with an environmental context similar to the clinical one.

All animals survived the surgery and the follow-up period. No signs of systemic or local toxicity were evident. Radiographic evaluations showed that the cement did not interfere with the growth of the limb in which it was previously implanted and with its contralateral, as evidenced by the absence of limping during the observation period. Microscopically, there were no signs of inflammation or necrosis, no fibrosis or reaction from a foreign body. These considerations are valid for both types of preparation: prepolymerized Calcemex® and fluid Calcemex® have demonstrated perfect biocompatibility, confirming the observations made by Dall'Oca et al.\textsuperscript{20} In this study, Calcemex® was evaluated in 8 rabbits by injecting 1 mL of fluid cement in femoral bone marrow channel, with good outcomes in term of osteointegration.

Upon radiological examination, the fluid and polymerized Calcemex® preparations clearly appear radiopaque. This radiopacity is less intense in post-mortem radiograms after limb disarticulation than in previous controls. In particular for the fluid preparations, this radiopacity assumes a grainy aspect, less evident for the prepolymerized Calcemex®, which appears more compact. The grainy appearance would be related to the radiopaque PMMA scaffold and to the partial reabsorption of β-TCP.

PMMA cement is widely used for surgical fixation, due to its mechanical properties, without high osteogenic potential as described by Goto et al.\textsuperscript{21} In particular, the absence of porosity and the fibrous peripheral layer prevent the osteointegration. On the contrary, cements enriched with TCP favor bone osteoconduction both by the release of the TCP itself and by the porosity of the scaffold (pores of 200-300 μm), aiding the autologous bone rehabilitation of the substitute.

Upon histological investigation, both preparations appear perfectly integrated with the surrounding bone tissue and these considerations are valid for cortical and cancellous bone as well. Calcemex® therefore demonstrated excellent osseointegration potential.

Fluid Calcemex® intertwines with newly formed bone tissue in some area, even in deeper layers, unlike prepolymerized Calcemex® in which the presence of ossified matrix is limited only to the periphery. The osteoconductive properties of the cement are more evident in the fluid preparation, instead more limited in the prepolymerized preparation. Perfect integration with the surrounding tissue is fundamental for the biomechanical properties of the bone-cement complex. Both fluid and prepolymerized Calcemex® were incorporated in the surrounding compact and cancellous bone. In particular, the appearance of the bone trabeculae that branch off from the capsule suggests the full integration of the cement as a consequence of mechanical response to the stresses to which the implantation bone segment is subjected.

The results received from biomechanical tests on prepolymerized Calcemex® preparations (10x20 mm cylinders) reveal that the cement is much more resistant to compression than the cancellous bone taken from the same models. As expected, explanted Calcemex® proved slightly less resistant than the native prepolymerized cement. This is related to the modifications occurred in vivo, which highlight the osteoconductive properties of this material.\textsuperscript{18} Although a direct comparison with other studies is not possible since we used different methods, we can assume that Calcemex® has sufficient biomechanical capabilities to be used as a bone substitute in humans. As reported in literature, when compared with cancellous bone or other fillers [e.g., bioglasses or Norian® (DePuy Synthes, Warsaw, IN, USA)],\textsuperscript{12,24} we suggest that Calcemex® can provide potential osteoinduction, osteosynthesis and osteoconduction.

Calcemex® is registered for clinical use, currently it can only be used as a filler and the results are promising. However, further studies are needed before Calcemex® can be registered for other indications, such as the augmentation and kyphoplasty.

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