Materials and methods-IANDOLO

**2. Materials and Methods**

**2.1 Study design and site allocation**

Three bone defects (5 mm diameter and 10 mm length) were created bilaterally in the medial part of each femoral condyle and one defect (5 mm diameter and 10 mm length) was generated in the lateral-proximal major tubercle of the humerus of two sheep (figure 1). The sheep were 2.9 and 3.6 years old and they were therefore skeletally mature. A total of 8 defects were created to test nacre powder and 8 used as control samples (randomly attributed as specified in table 1 and table 2).

Defects were either filled with the nacre powder (Test) (figure 2) or they were left empty (Control) according to the site allocation described in table 1.

Sheep were terminated 8 weeks after surgery.

**2.2 Model choice and regulatory aspects.**

The study was carried out in accordance with the EU Directive 2010/63/EU for animal experiments. The study was also reviewed in accordance with the OECD Good Laboratory Practice regulations, ENV/MC/CHEM (98) 17, with the European Good Laboratory Practice regulations, 2004/10/EC Directive, and with the United States Food and Drug Administration Good Laboratory Practice regulations, 21 CFR 58. The study was run by the Medical Research Organization NAMSA (Chasse-sur-Rhône, France).

The sheep is an animal model identified for evaluating materials and is recommended in the ISO-10993 standard (part 6, 2007, Biological evaluation of medical devices — Part 6: Tests for local effects after implantation) for intraosseous implantations. In addition, a large animal allows for testing relevant size implant material 1,2. Moreover, this model is well characterized and it has historically been used in femoral implant studies. In accordance with the ISO-10993 standard, both test material and control were performed in the same animal.

The time period was chosen to evaluate the local tissue effects and the bone healing performance after mid-term implantation (8 weeks), taking into account the kinetics of nacre biodegradation 3. Control sites were evaluated to determine the innate healing after 8 weeks in similar defects.

**2.3 Surgical procedure**

**2.3.1 Nacre-based paste preparation.** Nacre powder (mean particle size: 42.7 ± 5.1 µm), provided by Stansea (Saint-Etienne, France) and produced from the nacreous part of the shells of the pearl oyster *Pinctada maxima*, was sterilized at 121°C for 20 min in an autoclave 4,5. Nacre powder was reconstituted as follows: 0.25 mL of autologous blood was sampled and mixed with 1 g of powder just before implantation. The blood was added progressively (drop by drop) and mixed to the powder using a spatula until obtaining a paste to be implanted in the bone defects. The blood/powder ratio was determined during a preliminary feasibility test (data not published).

**2.3.2 Pre-operative procedure**. The sheep were fasted approximately 24 hours for food and 12 hours for water before implantation. At the time of implantation, the sheep were weighed and then anesthetized.

**2.3.3 Anesthesia, premedication and preparation of the surgical sites.** Pre-medication was performed by intravenous (IV) injection of diazepam (Valium®, Roche) and butorphanol (Torbugesic®, Zoetis). Anesthesia was induced by intravenous injection of propofol (Propovet®, Abbot Laboratories) and maintained by inhalation of an O2 – isoflurane mixture (IsoFlo®, Axience, 1-5%). Each sheep was infused with Ringer lactate, and received intramuscularly a nonsteroidal anti-inflammatory drug (flunixine, Meflosyl®, Pfizer) and an antibiotic (amoxicillin). The surgical areas were clipped free of wool, scrubbed with povidone iodine (Vetoquinol), wiped with 70% isopropyl alcohol (LPG), painted with providone iodine solution (Vetoquinol) and draped. The vital parameters of the sheep were monitored throughout surgery, which was performed by an experienced veterinary surgeon using standard aseptic techniques.

**2.3.4 Implantation procedure.** The sheep was placed on its back. During surgery, a rectal temperature probe and a rumen tube were placed. Electrocardiogram (ECG) and oxygen saturation were monitored. The sheep was infused with electrolyte solution (Ringer Lactate, Baxter) to maintain iso-volumetric conditions.

**2.3.5 Surgical approach to the femoral condyle.** A cutaneous incision was made on the medial side of each femoral condyle. The vastus medialis muscle was retracted to access the femur. The periosteum was carefully removed from the femoral epiphysis to expose the implant sites.

**2.3.6 Surgical approach to the humeral major tubercle.** A skin incision over the shoulder joint was made from the acromion to the middle of the proximal third of the humerus on each lateral humeral major tubercle. Subcutaneous tissues and deep fascia were dissected and muscles were split longitudinally from the deltoid muscle space, and then the muscle fibers were retracted with blunt dissection. The interspinalis muscle was then retracted caudally by blunt dissection. The periosteum was carefully removed to expose the implant sites. The drill was placed centered in the groove of the humeral major tubercle.

**2.3.7 Creation of the defects and implantation of the article.** Defects with a diameter of 5 mm and a depth of approximately 10 mm were drilled. Drilling was accompanied and followed by extensive rinsing with saline solution to control any temperature increase at the implantation site and to remove bone debris. In the femoral condyle, the sites were spaced by at least 3.0 mm. The defects were cleaned with sterile saline before implantation to avoid any blood clot at the bottom of the defect.The created bone defects were filled with the graft material or left empty (control) (figure 1 c, d).

**2.3.8 Closure of the implanted sites.** The incision was closed by suturing both capsule and muscles with absorbable thread (PDS™ II 1, Ethicon). The subcutaneous layer was closed with absorbable thread (Vicryl™ 2.0, Ethicon). The skin layer was closed using surgical staples. The wounds were disinfected using an iodine solution (Vetedine® solution, Vetoquinol) and then sprayed with oxytetracycline (Oxytetrin® spray, Intervet). The operated legs were not restrained in any manner.

**2.4 Post-operative procedures**.

The sheep were left to recover from the anesthesia in the operating room and returned to their individual cages and kept under close observation. An intramuscular injection of buprenorphine was administered at the end of the surgery day, then daily for 2 days post-surgery. An anti-inflammatory drug (flunixine) was administered daily for 5 days post-surgery and an antibiotic (amoxicillin, Duphamox LA®, Zoetis, long action) was given every other day for 8 days following surgery. The surgical staples were removed after complete healing (2 weeks following surgery). The wounds were disinfected with oxytetracycline (Oxytetrin® spray, Intervet) every other day until 2 days after the removal of the surgical staples. After this period of recovery, the sheep returned to a farm setting.

**2.5 Termination.** At 8 weeks, the sheep were weighed and then euthanized by a lethal intravenous injection of a pentobarbital solution. One additional defect (4 mm diameter) was created in the tibial plateau of one sheep as described before and filled with the nacre paste, to be used as reference sample (T0) for all characterizations. The implanted sites of each sheep were harvested and fixed in 10% neutral buffered formalin.

**2.6 Histopathological analysis**

After complete fixation, all samples (n = 6 for test sites, n = 5 for control sites and n=1 for T0) were dehydrated in alcohol solutions at increasing concentration, cleared in xylene and embedded in polymethylmetacrylate (PMMA). One central longitudinal section was obtained by a microcutting and microgrinding system (EXAKT System - thickness of each section ranging between 30 to 40 µm) and it was stained with Paragon. Slices of PMMA-embedded samples were stained with both Safranin/Fast Green to detect cartilage and the modified Goldner's trichrome method to account for the mineralized tissue and osteoid. Images were acquired using a DMRB Microscope (Leica).

**2.7 Qualitative and semi-quantitative analyses.**

Qualitative and semi-quantitative histopathologic evaluation of the local tissue effects and the performance was conducted for each selected site by anatomopathologists from NAMSA. The analysis was conducted according to the ISO 10993-6 Standard. The following parameters were graded from 0 to 4: cellular inflammatory parameters (polymorphonuclear cells, lymphocytes, plasma cells, macrophages and giant cells/osteoclastic cells); necrosis fibrosis (ultimate inflammatory stage, characterized in histology by an organized deposit of mature collagen); neovascularization; fatty infiltrate/bone marrow; fibrin; osteolysis and tissue degeneration. The irritation score of the test and control groups was calculated as described in ISO 10993, Part 6, Annex E. It corresponded to the sum of the tissue damage and cellular inflammatory parameters scores (e.g. lymphocytes, macrophages, Giant cells/osteoclastic cells) weighted with a factor 2, plus the inflammation scores of the repair phase (e.g. fibrosis, neovascularization and fatty infiltrate and bone marrow).

The Irritant Ranking Score (IRS) reflecting the inflammatory reaction and the local tissue effects was determined by subtracting the irritation score of the control from the score of the test article. A negative difference was recorded as zero. The IRS was graded as non-irritant (0.0 to 2.9), slightly irritant (3.0 to 8.9), moderately irritant (9.0 to 15.0) or severely irritant (> 15.0). The following parameters were graded from 0 to 4: cellular inflammatory parameters (polymorphonuclear cells, lymphocytes, plasma cells, macrophages and giant cells/osteoclastic cells); necrosis fibrosis (ultimate inflammatory stage, characterized in histology by an organized deposit of mature collagen); neovascularization; fatty infiltrate/bone marrow; fibrin; osteolysis and tissue degeneration and any other relevant parameters.

The T0 site served as a reference for structural characterization of the test article.

**2.8** **Micro-Computed tomography.**

Scans of the PMMA-embedded femurs and humeri were acquired using a Viva CT40 microtomograph (μCT, Scanco Medical, Bassersdorf, Switzerland). The scanning parameters were set at 70 kV, 114 µA, 250 ms and the voxel size at 10.5 µm. Three-dimensional reconstructions were generated using the following parameters: σ =1; support = 2; threshold = 225.

**2.9 Statistical analysis**

Median as well as average and standard deviation were calculated for most of the analyzed parameters using GraphPad Prism version 5.03 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Samples were compared with the non-parametric Mann Whitney U test. Significance level was set at 0.05.

**References**

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