Appendix

Lyophilized Human Bone Chips, Platelet Gel, and Bone Marrow Stromal Cells Preparation

The bone for freeze-drying was retrieved from the cortical-spongiosa bone of the femoral epiphyses of living or deceased donors, or from the femoral condyles, tibial plateau, humeral epiphyses, vertebrae, or pelvis of cadavers. The bone was cut and shaped, and cells, blood, and adipose tissue were removed by soaking in distilled water, chloroform, methanol, and oxygenated water. After a final wash, the pieces were frozen at –80°C overnight. Freeze-drying was carried out with the Heto FD 6-55 apparatus (A. de Mori, Milan, Italy), in two phases. Finally, the pieces were quickly vacuum-packed, in a triple polyethylene bag, labeled with the production lot number, general information of the donor, particle size (mean diameter, 3 mm) and expiration date, and sterilized by gamma radiation (Gammarad Italia, Bologna, Italy).

Platelet-rich plasma (PRP) was obtained from the patient's venous blood (450 mL) the day before surgery, and CPD (citrate-phosphate-dextrose anticoagulant) was used as an anticoagulant (1 mL CPD/7 mL blood). Blood was centrifuged at 20°C and 1000 g for fifteen minutes to remove red blood cells, then at 3000 g for ten minutes to obtain PRP. By this separation technique, platelet counts of about 1×10^6/μL were routinely obtained.

Preoperatively, the patient platelet concentrations in plasma, expressed as the mean number (and standard deviation) per microliter, were 240,000 ± 58,000 (minimum-maximum range, 147,000 to 354,000), without any significant differences identified between groups. The plasma concentration procedure induced a concentration of 5.8 times (6.16 times in Group A and 5.5 times in Group B, without a significant difference) and the final PRP platelet concentration was 1,399,000 ± 372,000 (minimum-maximum range, 919,000-2,033,000).

Platelet-rich plasma (16 mL) was mixed with the lyophilized bone (1 to 3 mL, depending on the size of the opening wedge) and autologous cryoprecipitate (8 mL), which was prepared from autologous fresh-frozen plasma by freeze-thaw precipitation of proteins and subsequent resuspension in plasma. Autologous thrombin was generated on the addition of 330 μL calcium gluconate (100 mg/mL) to 10 mL of plasma. By mixing 4 mL of thrombin to 16 mL of PRP, the platelet gel (PG) was obtained[21].

Autologous bone marrow stromal cells (BMSCs) were obtained from the bone marrow retrieved from the iliac crest during surgery, and heparin (500 U in 10 mL of saline solution) was employed as anticoagulant. The sample was centrifuged at 3800 g for ten minutes, and 50 mL of buffy coat was harvested and newly washed by adding 300 mL of saline solution and centrifuging at 4000 g for five minutes in order to reduce the heparin inhibition on thrombin.

An aliquot of the bone marrow-derived cells of each patient in Group B was cultured in vitro for the osteogenic potential analysis. All cultures expressed the osteoblastic phenotype: cells were positive for alkaline phosphatase (ALP) activity and calcium deposition, and expressed mRNAs specific for Osterix, ALP, and osteocalcin[25].

The red blood cells were not reinfused, but they were stored for the autotransfusions.

Histology and Histomorphometry

Bone tissue biopsy specimens fixed in 10% formalin solution buffered at pH 7.2 (Bio-Optica, Milan, Italy) were partly decalcified (50 mL of formic acid, 40 mL of hydrochloric acid, 1 L of distilled water) and were embedded in paraffin. They were partly dehydrated and embedded in methylmethacrylate. Five to 10-μm thick serial sections of the decalcified fragments were obtained with use of a rotator microtome (RM2155; Leica, Nussloch, Germany) and were stained with hematoxylin-eosin (Bio-Optica, Milan, Italy); 10 to 20-μm-thick slices were obtained from the resin-embedded specimens by means of a diamond saw microtome (model 1600; Leitz, Wetzlar, Germany), and stained with Paragon (Merck, Darmstadt, Germany), as well as with Goldner trichromic stain (Bio-Optica, Milan, Italy), in order to identify red-orange osteoid or connective tissue and green mineralized bone.

Solochrome cyanine stain (Sigma-Aldrich, Milan, Italy) was employed, too, in order to visualize the cells better and to identify immature and/or mature bone. Two evaluators, according to a system published in a previous paper[23], performed the histopathological evaluation blindly, using a light and polarizing microscope (Eclipse E800M; Nikon, Tokyo, Japan), and evaluated the biopsy specimens at ten times and sixty times original microscope magnifications.

The bone tissue morphology at the level of the junction between the bone graft and host bone was analyzed. A scoring system was used to assess the type and degree of bone remodeling: osteogenesis, angiogenesis, and inflammation were estimated. For each patient, the mean values of all histomorphometric parameters were calculated from five sections per each sample.

Five micro-areas at sixty times magnification, corresponding to 1.8 mm^2 (window area) were analyzed: osteoblasts (OBs), osteoclasts (OCs), vascular buds, and inflammatory cells were counted and the average from the five micro-areas was calculated. Polymorphonuclear cells (PMNs) were considered cells positive for infection, whereas chronic infection could be suspected by the presence of lymphocytes and plasma cells or lymphoid follicles (hematoxylin-eosin staining).

Angiogenesis and/or neovascularization was evaluated immunohistochemically by employing monoclonal antibody to CD31 or the platelet endothelial adhesion molecule (PECAM-1), a type-I integral membrane glycoprotein and a member of the immunoglobulin super family of cell surface receptors expressed strongly by all endothelial cells (Chemicon International, Temecula, California).

Fibrous tissue as a percentage filling in the gaps of bone chips, bone-forming areas, and seam areas were measured by light microscopy at low magnification (×10), equipped with a digital still camera-based system (DXM1200; Nikon) and color image analysis software (LUCIA G v4.21; Laboratory Imaging, Prague, Czech Republic). The fraction of trabecular surface covered by mature bone and the bone-forming surfaces, i.e., the number of osteoid seams covering the trabecular surface, was estimated on the undecalcified bone sections after Goldner trichromic staining (Fig. E-1) and solochrome cyanine staining (Fig. E-2).

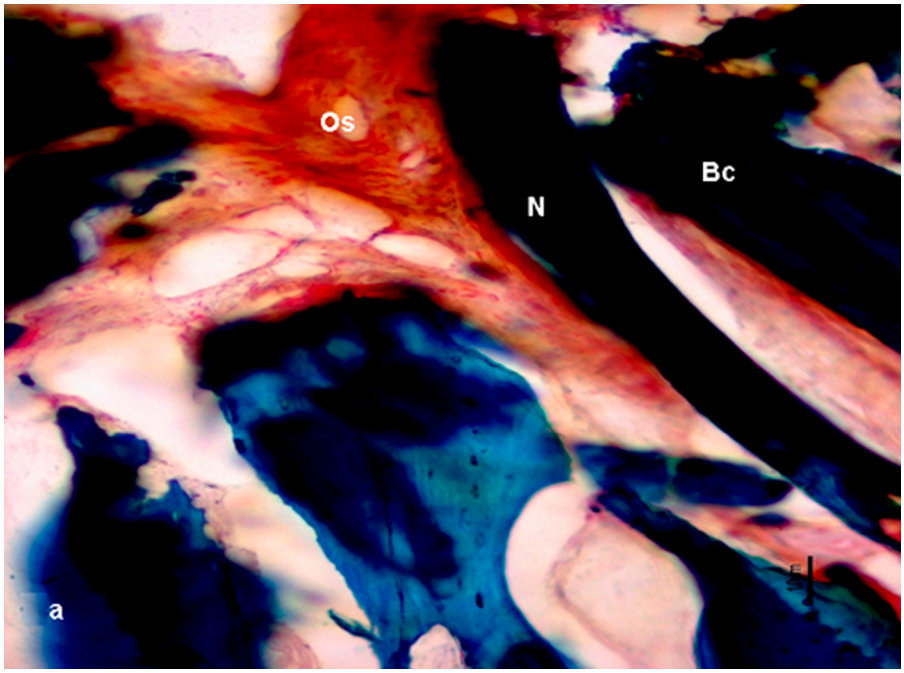

Fig. E-1

A bone biopsy specimen from a patient in Group C with lyophilized bone chips showing new bone (N) and osteoid seams (Os) around bone chips (Bc) (Goldner trichromic stain; original magnification, ×10).

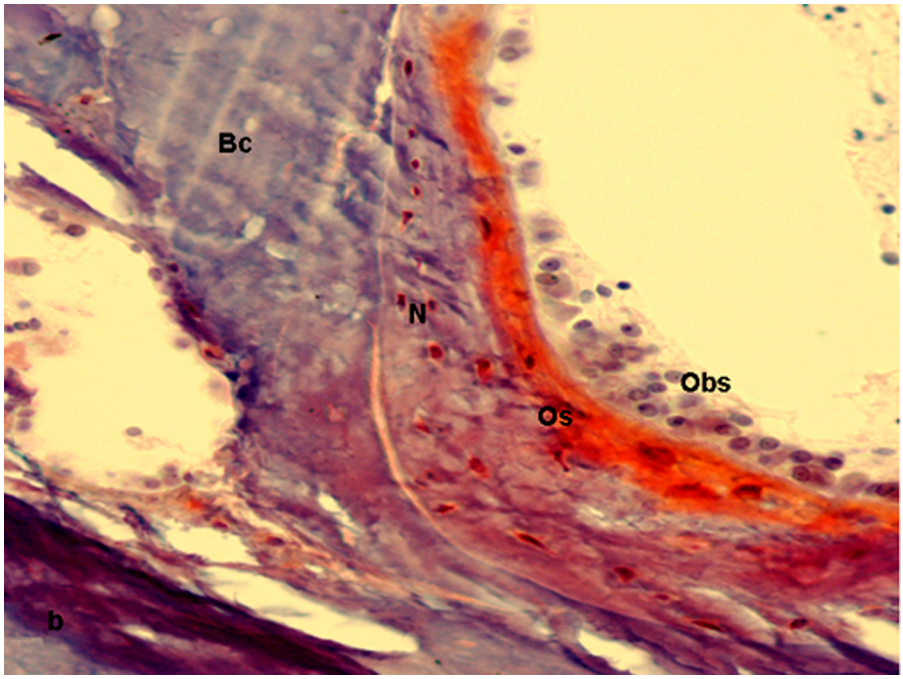

Fig. E-2

A bone biopsy specimen from a patient in Group C with lyophilized bone chips showing new linings of osteoblasts (Obs), osteoid seams (Os) and new bone (N) around bone chips (Bc) (solochrome cyanine stain; original magnification, ×20).