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Human tissue allograft processing: impact on in vitro and in vivo biocompatibility

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Abstract This work investigates the impact of chemical and physical treatments on biocompatibility for human bone/tendon tissues. Nontreated and treated tissues were compared. In vitro testing assessed indirect and direct cytotoxicity. Tissues were subcutaneously implanted in rats to assess the immunological, recolonization, and revascularization processes at 2–4 weeks postimplantation. No significant cytotoxicity was found for freeze-dried treated bones and tendons in comparison to control. The cellular adhesion was significantly reduced for cells seeded on these treated tissues after 24 h of direct contact. A significant cytotoxicity was found for frozen treated bones in comparison to freeze-dried treated bones. Tissue remodeling with graft stability, no harmful inflammation, and neo-vascularization was observed for freeze-dried chemically treated bones and tendons. Frozen-treated bones were characterized by a lack of matrix recolonization at 4 weeks postimplantation. In conclusion, chemical processing with freeze-drying of human tissues maintains in vitro biocompatibility and in vivo tissue remodeling for clinical application.

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1 Introduction

The transplantation of human tissue (bone, tendon, cartilage, etc.) is an important component of the treatment of bone and soft tissue defects [1]. Tissues are extensively used in orthopedic (trauma, revision of total hip and knee arthroplasty), neurosurgery (dura mater substitutes), abdominal (wall defects repair), otorhinolaryngological, and periodontal surgery [1–8]. Human tissue allograft has many advantages over autograft, for example, lack of donor morbidity (postoperative pain, decrease of surgical time), and it is relatively quick and easy to use. However, allograft tissues remain associated with a potential for infection and rejection, as compared with autograft tissues.

One complication of both organ and tissue allotransplantation is the transmission of infectious diseases from the donor to the recipient. Among the potential transmissible diseases, viruses and prions are the most difficult to track from the donor. The transmission of hepatitis C virus (HCV), bacterial contamination, and HIV through human grafts has been well documented [9–13]. Lelie et al. [14] have estimated that the risk of virus transmission is 10-fold higher in cases of organ tissue transplantation than in those of blood transfusion. Although the selection of donor is very severe for tissue grafting, the most important preoccupation among administrators of tissue banks, since the last decade, remains the reduction of the risk of conventional and nonconventional agent transmission.

In general, allograft tissue is a composite graft made up of a variety of cells (osteogenic, chondroblastic, fibroblastic) contained in an organic matrix (collagen and proteoglycans). A collection of glycoproteins are present on the cell surface membrane (human leukocyte antigens) and remain strongly immunogenic. In contrast, antigens present in matrix and collagen are weakly antigenic

[15, 16]. Clinical studies have shown that allograft tissue reconstruction tends to incorporate and heal more slowly than autograft tissue. It has been suggested that this delay is due to the immunoresponse against histocompatibility antigens. Minami et al. [17, 18] found that the cells of a tendon tissue were antigenic, whereas the collagen was not.

To avoid previously encountered complications, chemical and physical treatments were developed in our tissue bank to reduce the immunogenicity of the graft material as well as the risk of agent transmission [7, 19, 20]. However, human processed tissues as well as synthetic biomaterials must remain biocompatible to promote an adapted cellular recolonization by an optimal interaction with their microenvironment. The aim of the study was to demonstrate the impact of chemical and physical procedures on the in vitro and in vivo biocompatibility of tissue properties. The objective of the in vitro study was to evaluate the cytotoxicity of human bone/tendon tissues. The cell culture response against human allografts was estimated using indirect and direct methods. To provide information on cellular responses, metabolic activity (succinate dehydrogenase activity) and cell membrane integrity (lactate dehydrogenase activity in culture medium) were investigated after contact between cells and extracts of grafts. Initial cell attachment and the prolonged cellular adhesion after 3 h and 24 h were used to study the direct contact between cells and grafts, respectively. The in vivo biocompatibility was assessed by the transplantation of tissue on the subcutaneous space of immunocompetent Wistar rats. The immunologic response (lymphocyte-CD3 and macrophage-CD68 infiltration), recolonization, and revascularization (Masson's trichrome) processes were studied at 2 and 4 weeks after implantation.

2 Materials and methods

2.1 Experimental groups and human tissue processing

Bone and tendon from selected donors in our hospital were procured according to the common standards of the European Association of Muskulo Skeletal Transplantation (EAMST, Vienna, 1997) (mean ± SD: 57 ± 34 years old for five human tissue donors, one male/four female; range: 19–90 years old). In our study, donors were selected considering a review of medical history, including risk factors for subacute spongiform encephalopathies. Serological testing, which included detection of HIV-1 and -2, HTLV 1, hepatitis B and C, and syphilis, was performed. Human tissues were treated as previously described [20]. Briefly, procurement was accomplished in an operating room or adequate mortuary facility. Tissues were washed in sterile physiological saline solution and mechanically stripped of external loose connective tissue, including adipose tissue, vessels, and nerves. At the tissue bank, the remaining tissue was chemically treated by a succession of multiple steps. First, the pieces were extensively defatted in baths of absolute acetone. Next, prion inactivation was obtained with 1 N NaOH at room temperature. Reduction of immunogenicity for both tissues was obtained by protein coagulation, nuclear acid precipitation, and cell membrane degradation following sodium chloride and oxygenated water treatment. After each procedure, tissues were intensively washed with a continuous distilled water flow. Allografts were conserved at −80°C or at room temperature by freeze-drying.

Finally, treated grafts were sterilized by gamma irradiation at 25,000 Gy (IBA Mediris, Fleurus, Belgium). This treatment led to the inactivation of viruses such as HIV and hepatitis B and C and diminished the bacterial activity [21].

Different experimental groups were assessed as follows: (1) fresh-frozen untreated bone (FFB), (2) freeze-dried treated irradiated bone (FDTB), (3) frozen treated irradiated bone (FTB), (4) fresh-frozen tendon (FFT), and (5) freeze-dried treated irradiated tendon (FDTT).

No frozen treated tendon was performed since no clinical indications can be associated to this type of tissue.

2.2 In vitro biocompatibility testing

For each bone and tendon tissue, Saos2 (osteosarcoma cell line, ATCC: HB-85™) and MRC-5 (human lung cells, ATCC: CCL-171™) were used for cytotoxicity testing, respectively.

2.2.1 Indirect cytotoxicity assays

2.2.1.1 Extraction method The extraction method was carried out according to the International Standard Organization (ISO 10993-5: 1999 ISO/EN 10993-5, Biological Evaluation of Medical Devices, Part 5, Tests for Cytotoxicity, in vitro methods 8.2, Tests on Extracts); the ratio between the tendon area (cm^2) and the medium volume (ml) was 6 [20, 22]. For technical reasons, the ratio was determined with bone sample weight at 0.2 g/ml. Under sterile conditions, fragments of tendon and bone were immersed in the culture medium for 72 h at 37°C without agitation. The complete liquid culture medium was used as negative or nontoxic control (Ctrl−). Triton X-100 (0.1%) was used as positive control (Ctrl+) to induce cell death.

2.2.1.2 Cell and culture conditions MRC-5 and Saos2 were grown in media (Dulbecco's modified Eagle's medium [DMEM] or DMEM with Ham's F12 [50% v/v]) supplemented with 10% heat inactivated fetal bovine serum (FBS), 50 U/ml penicillin, 50 µg/ml streptomycin,

2 mM L-glutamine, and 2.5 μg/ml fungizone. Cells were maintained at 37°C (95% air and 5% CO_2) until about 85–90% confluence. The medium was changed every 2 days.

To suspend cells for cytotoxicity assay, cells were detached from the culture flask with a 0.25% trypsin-EDTA mixture for 10 min at 37°C and were resuspended in the culture medium. Cells were seeded in 96-well microplates for MTS (bromure de 3-[4,5diméthylthiazol-2yl]-5-[3-carboxyméthoxyphényl]-2-[4-sulfophényl]-2H-tetrazolium) and lactate dehydrogenase (LDH) assays at a density of 1 $\times$ 10^4 cells/well. They were grown near confluency at 96 h at 37°C prior exposition to the extract tissue medium at 24 h.

2.2.1.3 MTS assay After 24 h of extract-cell contact, 20 μl of "Cell titer 96® AQ_{ueous} One Solution Cell Proliferation Assay" (Promega, Madison, WI) was added directly to each well containing 100 μl of extract medium. Cells were incubated for 3 h at 37°C. The absorbance was measured at 492 nm using a microtiter plate spectrophotometer (Multiskan Ex, Labsystems, Brussels, Belgium). The reference wavelength was 690 nm. The optical density difference OD = 492–690 nm was estimated.

2.2.1.4 LDH assay After 24 h of extract-cell contact, 100 μl of Cyto Tox–ONE Reagent (Cyto Tox–ONE Homogeneous Membrane Integrity Assay, Promega) was added directly to each well containing 100 μl of extract medium and incubated at 22°C for 10 min. The stop solution was supplemented (100 μl) and the fluorescent signal was measured with a fluorescence spectrophotometer (Fluoroscan ASCENT, Labsystems) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

2.2.2 Direct cytotoxicity assays

2.2.2.1 Initial cell attachment and cell adhesion The initial attachment and cell adhesion were studied at 3 h and 24 h postincubation, respectively. They were measured by fluorescent Hoechst 33342 incorporation into cell DNA according to the procedure of Papadimitriou and Lelkes [23].

Bone and tendon samples, recovering a well surface of a 24-well plate, were incubated with cells. The control (Ctrl) for cellular adhesion and attachment assays was a plastic well used for cells culture. Cells were seeded at a density of 2 $\times$ 10^4 and 1 $\times$ 10^4 cells/well for cell attachment and cell adhesion, respectively. A higher number of cells were seeded, for cells attachment, in view to specifically investigate the early contact between cells and human tissues. In contrast, cellular adhesion with spreading and replication were investigated with a lower number of cells. In view to avoid a non-specific cellular adhesion on the plastic well (situated beside the tissue), implants were removed and placed into a new 24-well plate. We controlled that no cells were seeded on the plastic well. Free-floating cells were removed by washing with phosphate-buffered saline solution (PBS), and adherent cells were fixed for 10 min with absolute methanol at −20°C. Then cells were washed twice with PBS to ensure complete removal of methanol. Prior to each assay, the dye stock solution Hoechst 33342 (Sigma, Bornem, Belgium), solublized in distilled water at 1 mg/ml, was diluted at 1/50 in Dulbecco's medium without red phenol. Then 500 μl of this solution was added to each well. After 30 min, the dye solution was removed, and cells were washed twice (for 2 min) with Dulbecco's medium to eliminate any unbound dye. The DNA-bound dye was extracted by adding to each well 1 ml of denatured ethanol. After 5 min of incubation, 1 ml of the mixture was transferred to another 24-well plate that contained 1 ml of denatured ethanol. Fluorescence intensity was measured with a fluorescence spectrophotometer (Fluoroscan Ascent, Labsystems) with excitation and emission wavelengths set at 355 nm and 460 nm, respectively. Denatured ethanol was used as a blank standard.

2.3 In vivo biocompatibility testing: implantation procedure

Each experimental group of human tissues (bone and tendon) was implanted in immunocompetent Wistar rats (6- to 8-week-old males, ± 150 g, $n = 15$). A sterile surgical procedure was used to implant human tissues alternately in the right and left side of subcutaneous paravertebral spaces. Each rat ($n = 2$ per group) received two implants per tissue (see above). At 2 and 4 weeks postimplantation, animals were sacrificed by TI61 (Intervet, Mechelen, Belgium) under general anesthesia (20% xylazine [Rompun®, Bayer, Brussels, Belgium] and 50 mg/ml ketamine [Pfizer, Brussels, Belgium]), and the transplanted tissue was explanted and processed for histology (see below). All procedures were approved by the Animal Care and the Research and Development Ethics committees of our medical faculty.

2.4 Histological evaluation

After graft removal, tissues were immediately fixed overnight in 4% Formalin, decalcified with formic acid, dehydrated in graded alcohols, and embedded in paraffin, sectioned to a thickness of 5 μm, mounted on glass slides with demineralized water, dried for 12 h at 37°C, and stained with hemalaun-eosin (H&E) for examination by light microscopy. Cells in treated and untreated tissues

(bone and tendon) were counted by the slide section stained with H&E; an original magnification at ×12.5 with a grid 200 × 200 μm was used to quantify the cells at each point (Jenamed 2, Carl Zeiss, Germany).

To assess the cellular infiltration, sections were processed by immunohistochemistry for CD3 (lymphocyte) (1:250; NeoMarkers, CA,USA) and CD68 (macrophage) (1:100; Clone ED1, Acris Antibodies, Hiddenhausen, Germany) [24]. Revascularization was investigated by Masson's trichrome staining. Masson's trichrome staining identifies muscle fibers as red, collagen as blue or green, cytoplasm as light red or pink, and cell nuclei as dark brown to black.

2.5 Histomorphological analysis

Immunostaining for CD3 and CD68 cells was studied by histomorphology analysis with a view to quantify the total implant cellular infiltration and the distribution of immunological response in different tissues at 2 and 4 weeks postimplantation. Five nonoverlapping areas (0.04 mm^2/area) per slide and an original magnification at ×50 with a grid 200 × 200 μm were used to quantify cellular infiltration in the matrix (Jenamed 2, Carl Zeiss).

Under ×25 magnification with a grid of 400 × 400 μm, blood vessel distributions in all explanted tissues (bone and tendon) were evaluated under fluorescence microscopy (Jenamed 2, Carl Zeiss), and the number of blood vessels was determined in four nonoverlapping areas (three zones/area, 0.16 mm^2/area).

2.6 Statistical analysis

The one-sample Kolmogornov–Smirnov test was used to compare the shape and the location of replicates in each experiment. The values are reported as means ± SD. The statistical significance of differences between different experimental groups was tested by a one-way analysis of variance (ANOVA) with a Bonferroni post hoc test. The statistical tests were carried out using SPSS version 15.0. Differences were considered to be significant at $P < 0.05$.

3 Results

Non-treated tissues (FFB and FFT) were characterized by the presence of native donor cells inside the matrix (a mean of 31 and 27 cells/mm^2 were counted for FFB and FFT, respectively) and were considered as 100% reference in view to promote decellularization. Total decellularization of human tissues was obtained by chemical treatment followed by freeze-drying (FDTB and FDTT for bone and tendon, respectively) (Table 1). In contrast, a mean of 23% of native bone cells remained, after chemical and physical treatments, in the FTB ($P < 0.05$: FTB vs FDTB) (Fig. 1f, Table 1).

Table 1 Effect of chemical and physical treatment on the decellularization of human tissues

	n	Median of cell (min–max)
Bone		
FFB	3	31 (28–35)
FTB	3	7 (7–11)$^{\$}$
FDTB	3	0*
Tendon		
FFT	3	27 (22–31)
FDTT	3	0$^{\#}$

Statistical significance was determined by Kruskal–Wallis Test

* $P < 0.05$: FDTB versus FFB/FTB; $^{\$}$ $P < 0.05$: FTB versus FFB; $^{\#}$ $P < 0.05$: FDTT versus FFT

3.1 In vitro biocompatibility testing: indirect cytotoxicity assays

In both tissues, toxic control (Ctrl+) induced a significant reduction of mitochondrial activity associated with an increase of lactate dehydrogenase activity (Fig. 2).

For bone, no modification of mitochondrial activity was found for Saos2 incubated with FFB extract (Fig. 2a). In contrast, the lactate dehydrogenase activity was significantly increased in comparison to Ctrl-/FDTB/FTB groups (Fig. 2b, $P < 0.005$). No cytotoxic effect was observed for FDTB extract in contact with Saos2 cells (Figs. 2a, b). Although cellular metabolism was not affected following incubation with FTB extracts (Figs. 2a, b), a significant lower activity of MTS metabolization was found for cells exposed to FTB in comparison to FDTB extracts (Fig. 2a, $P = 0.002$).

For tendon, no significant difference of FFT and FDTT extracts was observed in the mitochondrial activity of MRC-5 cells (Fig. 2c). In addition, the LDH activity was significantly lower for cells incubated with FFT/FDTT than control groups (Fig. 2d, $P < 0.005$).

3.2 Direct cytotoxicity assays

After 3 h of direct contact between Saos2 and bone tissues, a significant decrease of adhesion was demonstrated in the case of FTB in comparison to FFB, FDTB, and Ctrl (Fig. 3a, $P < 0.005$). At 24 h of contact, no significant modification was detected with FFB as compared to Ctrl- (Fig. 3b). In contrast, cellular adhesion was significantly decreased for cells incubated on FDTB and FTB tissues in comparison to FFB and Ctrl- (Fig. 3b, $P < 0.005$). Moreover, a significantly lower adhesion was observed for Saos2 cells on FTB than FDTB (Fig. 3b, $P < 0.005$).

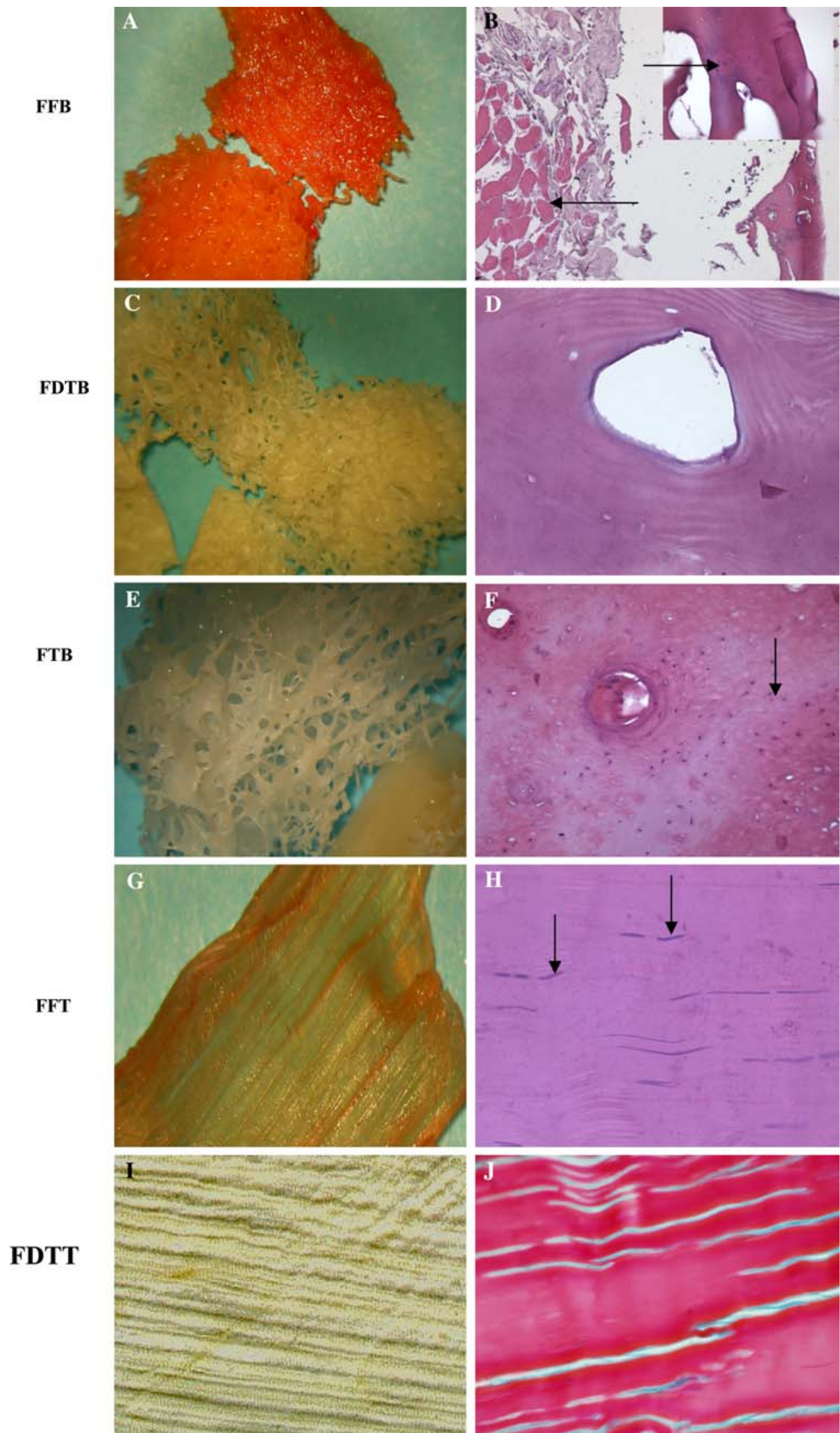

Fig. 1 Photomacrographs (*left*) and micrographs (*right*) (**h** and **e** staining, original magnification: ×20) of bone and tendon tissues prior to (**a/b** and **g/h**, respectively) and after (**c**–**f** and **i/j**, respectively) treatment. Note delipidation and decellularization for treated tissues (FDTB and FDTT). Arrow shows cells prior (FFB, FFT) and after treatment for FTB

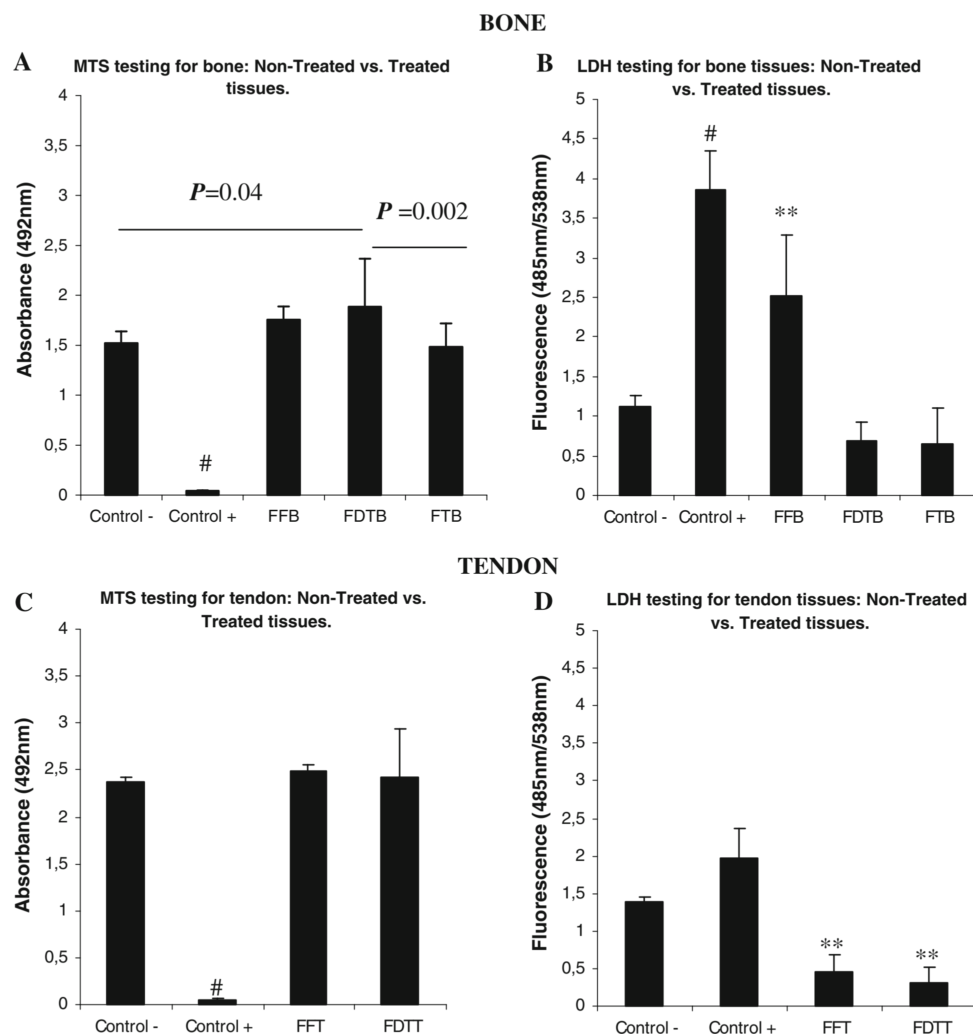

Fig. 2 Indirect cytotoxicity of cells incubated with bone/tendon tissue extracts at 24 h **a**, **c** MTS formazan formation by Saos2 and MRC-5 cells after incubation with fresh and chemically treated bone/tendon tissue extracts, respectively. **b**, **d** Extracellular level of LDH in Saos2 and MRC-5 cells after incubation with fresh and chemically treated bone/tendon extracts, respectively. Values are mean ± SD ($n = 16$ per each sample, except Ctrl+ and Ctrl− at $n = 8$) ($^{\#}P < 0.005$: Ctrl+ versus all other groups; $^{**}P < 0.005$: FFB versus Ctrl−/FDTB/FTB and FFT/FDTT versus Ctrl+/Ctrl− for Figs. 2b, d, respectively). Note that the LDH level in FFB can be associated to remaining donor native cells

After 3 h of direct contact between MRC-5 and tendon tissues, adhesion of cells on FFT and FDTT was significantly decreased compared to cells incubated on treated plastic wells (Fig. 3c, $P < 0.005$). A significant lower adhesion was also found at 3 h for FDTT in comparison to FFT (Fig. 3c, $P < 0.005$). Similar results were confirmed at 24 h with a significantly lower adhesion rate for FDTT (Fig. 3d, $P < 0.005$) than Ctrl- and FFT.

3.3 In vivo studies

3.3.1 Immunological reaction

Explanted tissues were quantitatively analyzed to determine the degree of lymphocyte (CD3) and macrophage (CD68) infiltration at 2 and 4 weeks postimplantation (Figs. 4 and 5).

For bone, no significant difference in both lymphocyte and macrophage infiltrations was observed between experimental groups at 2 and 4 weeks after implantation (Figs. 4 and 5a, b).

For tendon, a significantly lower CD3 infiltration was found for FDTT in comparison to FFT at 2 weeks postimplantation (Fig. 5c, $P = 0.004$). The CD3 infiltration was significantly higher for FDTT as compared to FFT after 4 weeks postimplantation (Figs. 4 and 5c, $P < 0.003$). The lymphocyte infiltration was significantly decreased for FFT at 4 weeks as compared to 2 weeks (Fig. 5c, $P < 0.001$). In contrast, no significant modification of lymphocyte infiltration was demonstrated for FDTT at 2 and 4 weeks after implantation. No significant difference of macrophage infiltration was observed in both tissues (FFT/FDTT) at 2 weeks. A significantly lower amount of macrophage cells was found at 4 weeks for FDTT than FFT group (Figs. 4 and 5d, $P < 0.001$). Moreover, a significant decrease of CD68 cells was found in FDTT after 4 weeks versus 2 weeks postimplantation (Fig. 5d, $P < 0.001$).

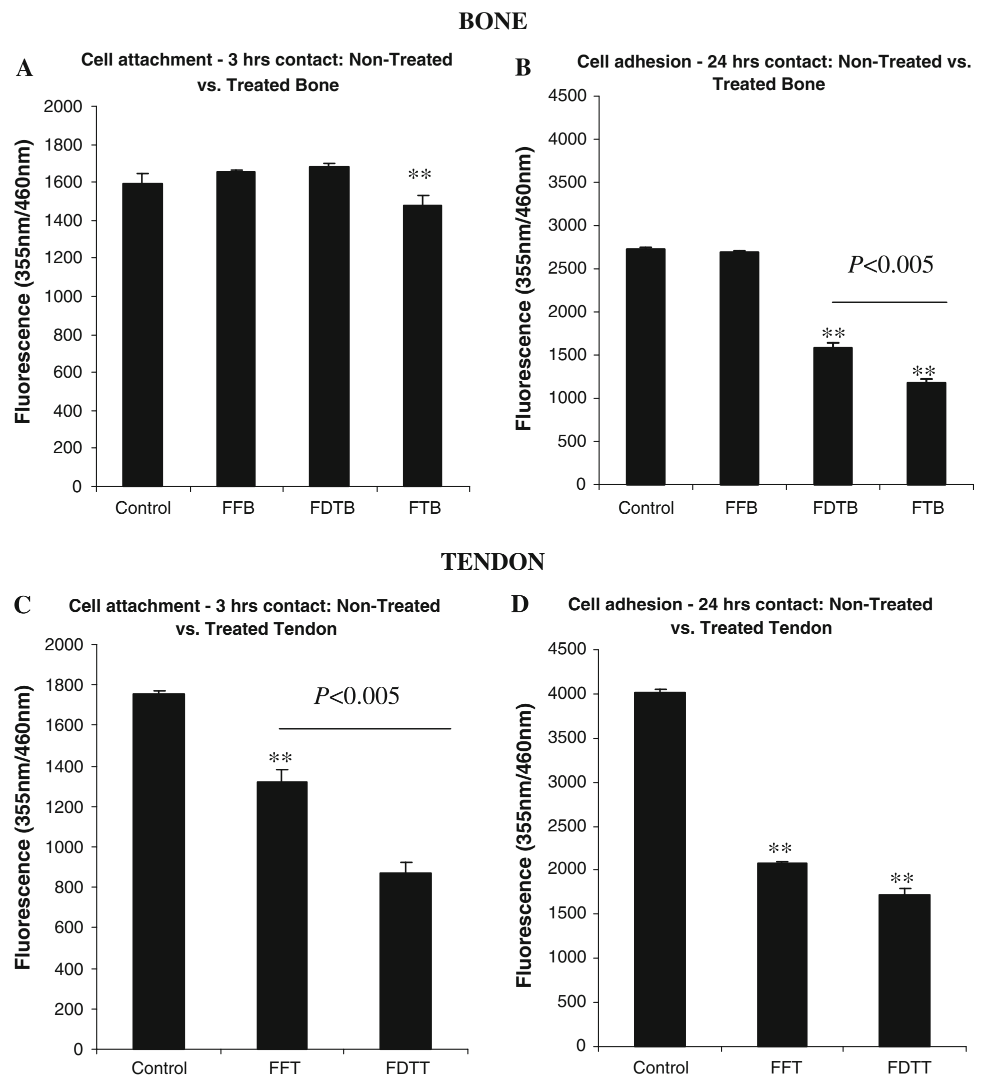

Fig. 3 Direct cytotoxicity assays (cell attachment at 3 h and cell adhesion at 24 h on tendon and bone tissue). **a**, **b** Saos2 cells were cultured directly on fresh and chemically treated bone tissues for 3 and 24 h. **c**, **d** MRC-5 cells were cultured directly on fresh and chemically treated tendon tissues for 3 and 24 h. Each datum represents the mean ± SD ($n = 4$, $^{**}P < 0.005$)

3.3.2 Revascularization processes

The number of blood vessels was determined in histological sections stained with Masson's trichrome staining.

For bone, a significant increase in blood vessel numbers was shown for FFB and FDTB experimental groups between 2 and 4 weeks postimplantation (Figs. 4 and 6a, $P < 0.05$). In contrast, the number of vessels was significantly decreased in the case of FTB at 4 weeks postimplantation (Fig. 6a, $P < 0.001$). After 2 weeks of transplantation, a higher concentration of vessels was found in FTB in comparison to FFB and FDTB (Fig. 6a, $P < 0.001$). The number of vessels was significantly decreased for the FTB group as compared to the FDTB group (Figs. 4 and 6a, $P < 0.05$) after 4 weeks.

For tendon, a significant decrease in blood vessel numbers was detected for both FFT and FDTT tissues between 2 and 4 weeks (Fig. 6b, $P < 0.05$). A significantly lower level of vessels was found in FDTT in comparison to FFT for both explantation times (Figs. 4 and 6b, $P < 0.005$).

4 Discussion

An optimal human tissue allograft should satisfy the following criteria: (1) to be free from any potential risk of known and unknown infections, (2) to be storable and readily available when needed, (3) to be biologically active for tissue recolonization, and (4) to induce no harmful foreign body reaction. Although our University Tissue Bank developed a procedure for securization of human bones and tendons, processing must respect biological properties required for clinical indications.

Human tissue allograft may elicit immune responses in the host that interfere with cellular recolonization of the implant. After composite tissue allotransplantation as the so-called hand transplantation, transplant immunologists

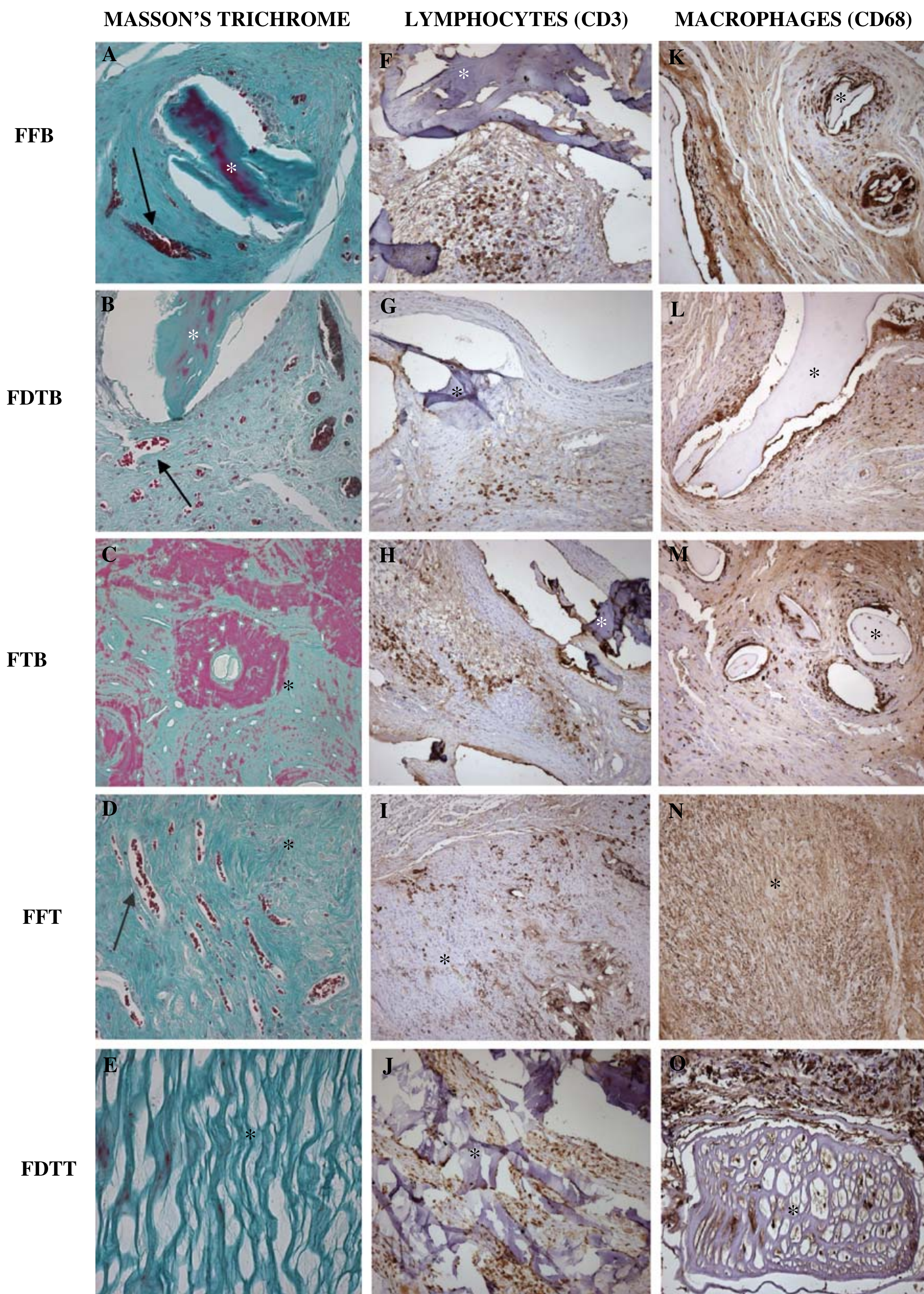

Fig. 4 **a–e** Masson's trichrome staining of explanted tissues (bone/tendon) from a Wistar rat after 4 weeks of subcutaneous implantation, original magnification: ×20. **f–j** Lymphocyte and (**k–o**) macrophage immunostaining for explanted tissues (bone/tendon) at 4 weeks postimplantation, original magnification: ×10. *Bone and tendon tissues; arrow blood vessel. Note a gap between the bone implant and the host tissue which can be due to tissue fixation and slide cutting

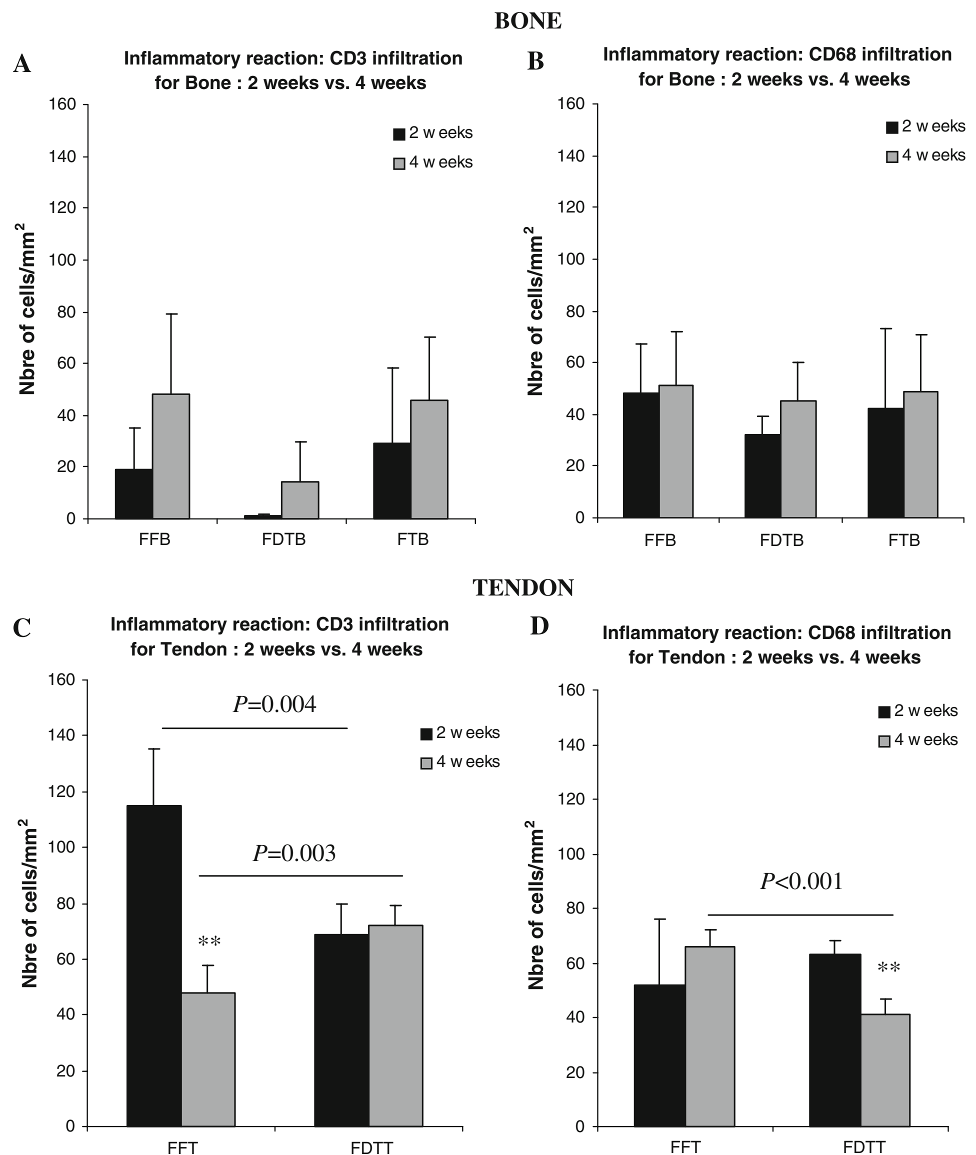

Fig. 5 Histomorphological analysis to quantify the inflammatory reaction (CD3, CD68) at 2 and 4 weeks after implantation for **a**, **b** bone and **c**, **d** tendon tissues. $^{**}P < 0.005$

anticipated that the global immunogenicity of a composite allograft would be the sum of the immunogenicity of muscle, skin, subcutaneous tissue, and bone allografts [25, 26]. Although human bone/tendon tissue allotransplantation is not a vascularized graft, the immunological response against human massive tissues remains underestimated. Most human tissues (bone/tendon) are currently preserved at −80°C with a view to maintain mechanical properties until defrosting and implantation in a recipient body. This work confirms that nontreated frozen grafts are composed of a massive donor cellular component of muscular/fatty/osteogenic and fibroblastic cells from bone and tendon, respectively (as revealed by specific immunohistochemistry by dystrophin, oil red, osteocalcin stainings, respectively) (data not shown) (Fig. 1), which remain a potential immunological barrier between donor and recipient [27]. Stevenson [28] already demonstrated that cells of the musculoskeletal tissue display Class I MHC antigens and, frequently, a subset of cells displaying class II MHC antigens [28, 29]. Then our tissue bank performed a serial step of chemical and physical treatments, applied on human bone and tendon, to induce decellularization [20]. The total decellularization of tissue is reported for human treated fascia lata used as dura mater and abdominal wall substitutes [7, 19, 20]. This is confirmed by this study (Table 1). For human bone tissues, a total decellularization is only found for chemically treated bones with freeze-drying and gamma irradiation for preservation and sterilization, respectively. In the case of a frozen process (−80°C) for treated bone preservation, 23% of native donor osteogenic cells remain in the bone matrix. Since the chemical procedure is the same for both

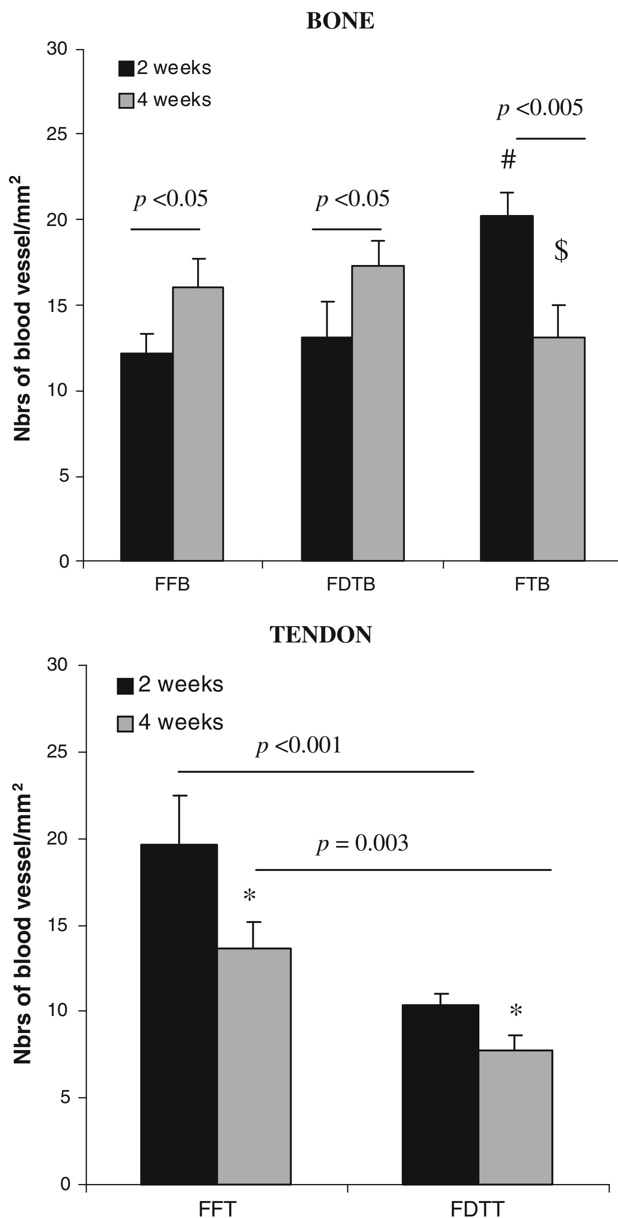

Fig. 6 Histomorphological analysis to quantify the blood vessel at 2 and 4 weeks after implantation for bone and tendon tissues. Each datum represents the mean ± SD ($n = 4$, $^{\#}P < 0.005$ for FTB versus FFB/FDTB at 2 weeks, $^{\$}P < 0.05$ for FTB versus FDTB at 4 weeks). $^{*}P < 0.05$ for FDTT and FFT at 4 weeks versus 2 weeks

treatments, only the storage method can explain the difference in decellularization level. In contrast to freezing, the freeze-drying procedure can induce physical damage to bone cells insufficiently removed by chemical treatments. Indeed, additional experiments demonstrated that a mean of 32% of donor cells are still found, in the bone tissue, after chemical treatments prior to storage (freeze-drying or frozen) (data not shown). With a view to obtain a sterile graft for clinical application, gamma irradiation is applied at the end of the tissue treatment. However, we previously demonstrated that gamma irradiation of a nondecellularized human tissue (for fascia lata) induces an in vitro cytotoxicity and a lack of cell adhesion required for tissue recolonization [20]. Prior to introducing a novel processing for a tissue graft, it is important to demonstrate that the treatment does not unacceptably affect the in vitro biocompatibility and in vivo immunological response of human bone/tendon allografts.

Measurement of in vitro cytotoxicity is essential to ensure that the applied treatments have not rendered the tissue liable to induce cytotoxic response in vivo, either by direct alterations in the composition of the tissue or by retention of traces of cytotoxic processing reagents. Cytotoxicity was assessed, in this study, using the methodologies recommended in ISO 10993-5 to assess both extract and contact cytotoxicity, using osteogenic and fibroblastic cell lines for bone and tendon tissues, respectively. To evaluate the cytotoxicity of the substances that leach out of the developed human tissues, viability (MTS test) and suffering (LDH test) assays were performed. For tendon and bone tissues, the treatment did not render the tissue cytotoxic, as assessed by extract assays. In contrast, treatment applied to bone tissues significantly affects the cytotoxicity of extracts. As already reported for the antigen depletion by decellularization process, the storage procedure directly influences the cytoxicity of bone extracts. A lower cellular viability was observed for freeze-dried treated bones in comparison to frozen tissues. It has already been described that the sterilization of fresh bone and tendon grafts (with fatty tissues and osteogenic/fibroblastic cells) by gamma irradiation generates toxic substances for the microenvironment and cellular recolonization of the tissue [20, 30]. The absence of indirect cytotoxicity does not confer any information about the total biocompatibility of treated graft. To verify whether the developed tissues support the functions shared by host cells (for wound filling and tissue adaptation), such as membrane integrity, adhesion to surfaces, and replication, adhesion studies with cell lines were performed.

In tissue regeneration and repair, the matrix provides an initial physical support and extracellular signaling. Adhesion and signaling proteins binding sites in the matrix are known to affect cell repopulation and matrix remodeling [31, 32]. A nontreated tissue was considered as our reference. In this latest, bone tissue promotes comparable cell attachment (3 h contact) and adhesion (24 h contact) to a culture plastic well. In contrast, a lower degree of cell adhesion is observed for a nontreated tendon in comparison to a cell culture well. This difference may be related to a more hydrophilic surface in tendon than bone tissues [33]. This impairs the adsorption of serum proteins and, consequently, cell adhesion. Another explanation for the difference between untreated bone and tendon tissues could

be the difference in topography since a higher level roughness in bone appears to enhance cell adhesion and subsequent proliferation [34]. Although the collagen, in most animals, is the most abundant protein (90%) in the extra-cellular matrix, the difference of non-collagenous proteins (as proteoglycans, g-carboxylated-proteins, glycoproteins and others) between bone and tendon may also influence the cellular adhesion [33]. After chemical and physical treatments of both bone and tendon tissues, cellular adhesion is significantly affected with a lower degree of cellular replication after seeding on the support in comparison to nontreated implants. In correlation to indirect cytotoxicity assays, the frozen, chemically treated bone followed by gamma irradiation revealed the lowest capacity of cell adhesion, confirming that this treatment does not create an appropriate microenvironment for cell recolonization. After 24 h of direct contact between cell and human matrix, a lower degree of cell adhesion is found for osteoblastic and fibroblastic cell lines on freeze-dried chemically treated grafts (bone and tendon, respectively) in comparison to native tissues. Physical modifications of the matrix, by freeze-drying and gamma irradiation, can damage the collagen ultrastructure (intra/intermolecular cross-linking, fibril reorganization, protein denaturation, molecular fragmentation, and/or secondary structure disorganization) and then reduce adhesion sites for cells [35–39]. Although these in vitro data remains very relevant, it is important to correlate if tissue treatments directly influence the graft incorporation and functional capacity after in vivo implantation.

The goal of using tissue allograft is to initiate a healing response from the host bed that will produce new tissue at the host-graft interface and within the porous body of the graft material. The biological response to implant materials can be characterized by the inflammation/foreign body reaction, the formation of collagenous tissue, and neovascularization. Assessment of the capacity of the tissue to induce an inflammatory response is necessary to determine whether or not the graft is likely to provoke an inflammatory response in vivo, which could result in an increased rate of graft resorbtion. Then nontreated and treated human bone and tendons were subcutaneously implanted in immunocompetent rats. Surprisingly, there was no significant difference in host reaction to the various human bone tissues. Indeed, the cellular content of nontreated/treated bone tissues did not influence the immunological response. This reaction was comparable to the implantation of synthetic materials (clinically applicable as polypropylene and polyester meshes) [7] The reduction of antigenicity by decellularization did not reduce lymphocyte and macrophage infiltrations of treated human bones after implantation. Although Friedlaender et al. [40] established a direct relationship between the immunologic activity both in allografting events and bone remodeling phenomena, tissue remodeling (with collagen deposition and neo-vascularization) was similar between cellular (Fresh-Frozen untreated bone) and acellular (chemically treated freeze-dried bone) bone tissues. In relation to in vitro cytotoxicity, frozen, chemically treated bones elicited a lower remodeling with encapsulation of implant, lack of angiogenesis at 1 month posttransplantation. In contrast, for tendon allografting, the tissue remodeling is delayed in case of chemically treated tissue. The lymphocyte infiltration is delayed at 4 weeks postimplantation with a lack of macrophage activation in comparison to untreated tissue. Then a lower level of neo-vascularization in treated tissue is found at 4 weeks postimplantation [41]. Our results are in contradiction to those reported by Gouk et al. [42] with a higher in vivo degradation rate of collagen matrix irradiated at 25 kGy. However, the degradation rate should not persistently outpace extracellular matrix deposition to avoid remodeling failure. In the clinical indication of a human treated fascia lata as a dura mater substitute and the repair of abdominal wall defects, it is important to achieve a balance between human matrix resorbtion and newly formed collagen matrix with a view to reduce the implant failure due to a high degree of degradation. We have previously demonstrated that the use a human acellular collagen matrix (derived from chemically treated fascia lata) in abdominal wall repair elicits adaptate remodeling (angiogenesis, collagen deposition, tissue resorbtion, and neo-peritonealization) to mechanical constraint at 8 weeks postimplantation in the same model of transplantation in Wistar rats [7].

5 Conclusions

This work demonstrated that human tissue processing must be severely controlled prior to clinical application since intrinsic properties of grafts can be affected and modify their biological faculty (recolonization/remodeling).

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References

1. Delloye C, Cornu O, Druez V, Barbier O. Bone allografts: what they can offer and what they cannot. J Bone Joint Surg Br. 2007;89:574–9.
2. Docquier PL, Paul L, Mousny M, Cornu O, Delloye C. The use of allografts in paediatric orthopaedic surgery. Acta Orthop Belg. 2007;73:551–7.

3. Engh GA, Ammeen DJ. Use of structural allograft in revision total knee arthroplasty in knees with severe tibial bone loss. J Bone Joint Surg Am. 2007;89:2640–7.
4. Nevins M, Hanratty J, Lynch SE. Clinical results using recombinant human platelet-derived growth factor and mineralized freeze-dried bone allograft in periodontal defects. Int J Periodontics Restorative Dent. 2007;27:421–7.
5. Gok A, Erkutlu I, Alptekin M, Kanlikama M. Three-layer reconstruction with fascia lata and vascularized pericranium for anterior skull base defects. Acta Neurochir (Wien). 2004;146:53–6.
6. Errani C, Schuster S, Biagini R, et al. Reconstruction with fascia lata allograft of the posterior vertebra elements after resection for aneurysmal bone cyst in a child. Eur Spine J. 2007;16:1531–5.
7. Dufrane D, Mourad M, van Steenberghe M, Goebbels RM, Gianello P. Regeneration of abdominal wall musculofascial defects by a human acellular collagen matrix. Biomaterials. 2008;29:2237–48.
8. Jang YJ, Wang JH, Sinha V, Song HM, Lee BJ. Tutoplast-processed fascia lata for dorsal augmentation in rhinoplasty. Otolaryngol Head Neck Surg. 2007;137:88–92.
9. Delloye C. Tissue allografts and health risks. Acta Orthop Belg. 1994;60(Suppl 1):62–7.
10. Carlson ER, Marx RE, Buck BE. The potential for HIV transmission through allogeneic bone. A review of risks and safety. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1995;80:17–23.
11. Conrad EU, Gretch DR, Obermeyer KR, et al. Transmission of the hepatitis-C virus by tissue transplantation. J Bone Joint Surg Am. 1995;77:214–24.
12. Tomford WW. Transmission of disease through transplantation of musculoskeletal allografts. J Bone Joint Surg Am. 1995;77:1742–54.
13. Eastlund T. Bacterial infection transmitted by human tissue allograft transplantation. Cell Tissue Bank. 2006;7:147–66.
14. Lelie PN, Zaaijer HL, Cuypers HT. Risk of virus transmission by tissue, blood, and plasma products. Transplant Proc. 1996;28:2939.
15. Pinkowski JL, Rodrigo JJ, Sharkey NA, Vasseur PB. Immune response to nonspecific and altered tissue antigens in soft tissue allografts. Clin Orthop Relat Res. 1996;326:80–5.
16. Pinkowski JL, Reiman PR, Chen SL. Human lymphocyte reaction to freeze-dried allograft and xenograft ligamentous tissue. Am J Sports Med. 1989;17:595–600.
17. Minami A, Usui M, Ishii S, Kobayashi H. The in vivo effects of various immunoreactive treatments on allogeneic tendon grafts. J Hand Surg (Am). 1983;8:888–93.
18. Minami A, Ishii S, Ogino T, Oikawa T, Kobayashi H. Effect of the immunological antigenicity of the allogeneic tendons on tendon grafting. Hand. 1982;14:111–9.
19. Dufrane D, Marchal C, Cornu O, Raftopoulos C, Delloye C. Clinical application of a physically and chemically processed human substitute for dura mater. J Neurosurg. 2003;98:1198–202.
20. Dufrane D, Cornu O, Delloye C, Schneider YJ. Physical and chemical processing for a human dura mater substitute. Biomaterials. 2002;23:2979–88.
21. Anastasescou M, Cornu O, Banse X, Konig J, Hassoun A, Delloye C. Ethanol treatment of tendon allografts: a potential HIV inactivating procedure. Int Orthop. 1998;22:252–4.
22. Dufrane D, Delloye C, McKay IJ, et al. Indirect cytotoxicity evaluation of pseudowollastonite. J Mater Sci: Mater Med. 2003;14:33–8.
23. Papadimitriou E, Lelkes PI. Measurement of cell numbers in microtiter culture plates using the fluorescent dye Hoechst 33258. J Immunol Methods. 1993;162:41–5.
24. Dufrane D, Steenberghe M, Goebbels RM, Saliez A, Guiot Y, Gianello P. The influence of implantation site on the biocompatibility and survival of alginate encapsulated pig islets in rats. Biomaterials. 2006;27:3201–8.
25. Thaunat O, Badet L, El Jaafari A, Kanitakis J, Dubernard JM, Morelon E. Composite tissue allograft extends a helping hand to transplant immunologists. Am J Transplant. 2006;6:2238–42.
26. Lee WP, Yaremchuk MJ, Pan YC, Randolph MA, Tan CM, Weiland AJ. Relative antigenicity of components of a vascularized limb allograft. Plast Reconstr Surg. 1991;87:401–11.
27. Elves MW, Salama R. A study of the development of cytotoxic antibodies produced in recipients of xenografts (heterografts) of iliac bone. J Bone Joint Surg Br. 1974;56:331–9.
28. Stevenson S. Biology of bone grafts. Orthop Clin North Am. 1999;30:543–52.
29. Skjodt H, Moller T, Freiesleben SF. Human osteoblast-like cells expressing MHC class II determinants stimulate allogeneic and autologous peripheral blood mononuclear cells and function as antigen-presenting cells. Immunology. 1989;68:416–20.
30. Moreau MF, Gallois Y, Basle MF, Chappard D. Gamma irradiation of human bone allografts alters medullary lipids and releases toxic compounds for osteoblast-like cells. Biomaterials. 2000;21:369–76.
31. Wozniak MA, Modzelewska K, Kwong L, Keely PJ. Focal adhesion regulation of cell behavior. Biochim Biophys Acta. 2004;1692:103–19.
32. Rosso F, Giordano A, Barbarisi M, Barbarisi A. From cell-ECM interactions to tissue engineering. J Cell Physiol. 2004;199:174–80.
33. Martins AM, Santos MI, Azevedo HS, Malafaya PB, Reis RL. Natural origin scaffolds with in situ pore forming capability for bone tissue engineering applications. Acta Biomater. 2008;4:1637–45.
34. Montanaro L, Arciola CR, Campoccia D, Cervellati M. In vitro effects on MG63 osteoblast-like cells following contact with two roughness-differing fluorohydroxyapatite-coated titanium alloys. Biomaterials. 2002;23:3651–9.
35. Cheung DT, Perelman N, Tong D, Nimni ME. The effect of gamma-irradiation on collagen molecules, isolated alpha-chains, and crosslinked native fibers. J Biomed Mater Res. 1990;24:581–9.
36. Bowes JH, Moss JA. The effect of gamma radiation on collagen. Radiat Res. 1962;16:211–23.
37. Bailey AJ, Rhodes DN, Cater CW. Irradiation-induced crosslinking of collagen. Radiat Res. 1964;22:606–21.
38. Davidson RJ, Cooper DR. The effect of gamma-irradiation on soluble collagen. Biochem J. 1968;107:29–34.
39. Leontiou I, Matthopoulos DP, Tzaphlidou M, Glaros D. The effect of gamma irradiation on collagen fibril structure. Micron. 1993;14:13–6.
40. Friedlaender GE, Strong DM, Tomford WW, Mankin HJ. Long-term follow-up of patients with osteochondral allografts. A correlation between immunologic responses and clinical outcome. Orthop Clin North Am. 1999;30:583–8.
41. Horowitz MC, Friedlaender GE. Induction of specific T-cell responsiveness to allogeneic bone. J Bone Joint Surg Am. 1991;73:1157–68.
42. Gouk SS, Lim TM, Teoh SH, Sun WQ. Alterations of human acellular tissue matrix by gamma irradiation: histology, biomechanical property, stability, in vitro cell repopulation, and remodeling. J Biomed Mater Res B Appl Biomater. 2008;84:205–17.