



Biological parameters for quality evaluation of allografts from the Brazilian National Institute of Traumatology and Orthopedics tissue bank

Rafael A. D. Prinz · Leonardo Rosa da Rocha · Thiago Penna Eirado ·
Jonathan da Silva Pinto · João Antônio Matheus Guimarães ·
Fabricio Fogagnolo · Rhayra Braga Dias 

Received: 19 December 2022 / Accepted: 10 January 2024 / Published online: 17 February 2024
© The Author(s), under exclusive licence to Springer Nature B.V. 2024

Abstract Bone allografts are clinically used in a variety of surgical procedures, and tissue banks are responsible for harvesting, processing, quality testing, storing, and delivering these materials for transplantation. In tissue banks, the bone is processed for the removal of all organic content, remaining only the tissue structure (scaffold). However, several studies have shown that even after using different processing methods, viable cells, functional proteins, and DNA may still persist in the tissue, which constitute the main causes of graft rejection. Therefore, the objective of this study was to establish techniques and biological parameters for quality validation of allografts. To this end, we propose the use of 3 combined

methods such as microscopy, histology, and molecular biology techniques to evaluate the quality of allografts harvested and processed by the Brazilian National Institute of Traumatology and Orthopedics (INTO) tissue bank according to the donation criteria of the Brazilian National Health Surveillance Agency and the Brazilian National Transplant System. Bone fragments from different processing stages showed no viable cells on histology, an intact extracellular matrix on scanning electron microscopy, and gradual reduction in DNA amount. Different techniques were used to demonstrate the quality of allografts produced by the INTO tissue bank and to establish biological parameters for ensuring the safety and quality of

R. A. D. Prinz
Transplant Area and Tissue Bank, Instituto Nacional de Traumatologia e Ortopedia Jamil Haddad (INTO), Av. Brasil, 500, Rio de Janeiro, RJ 0940-070, Brazil
e-mail: rafprinz@gmail.com

R. A. D. Prinz
Director of the Institute of Medical Education, Campus Vista Carioca University of Estácio de Sá, Rio de Janeiro, RJ, Brazil

L. R. da Rocha · T. P. Eirado · J. da Silva Pinto ·
J. A. M. Guimarães · R. B. Dias (✉)
Teaching and Research Division, Instituto Nacional de Traumatologia e Ortopedia Jamil Haddad (INTO), Av. Brasil, 500, Rio de Janeiro, RJ 20940-070, Brazil
e-mail: rhayrabraga@outlook.com

L. R. da Rocha
e-mail: leonardorocha@me.com

T. P. Eirado
e-mail: thiagoeirado@hotmail.com

J. da Silva Pinto
e-mail: jonjonathan.99@gmail.com

J. A. M. Guimarães
e-mail: jmatheusguimaraes@gmail.com

F. Fogagnolo
Department of Orthopaedics and Anaesthesiology, Ribeirão Preto Medical School, Universidade de São Paulo (USP), Av. Bandeirantes, 3900, São Paulo, SP 14049900, Brazil
e-mail: ffogagnolo@gmail.com

R. B. Dias
Institute of Biomedical Sciences, Federal University of Rio de Janeiro, Av. Carlos Chagas Filho, 373/J-10, Rio de Janeiro, RJ 21941590, Brazil

these products. Future studies need to be undertaken to assess and validate the efficacy of the decellularization process in larger bone grafts with diverse architectural configurations.

Keywords Allograft · Decellularization · Tissue bank · Bone regeneration

Introduction

Fracture incidence is expected to increase with a proportional increase in the prevalence of complex clinical situations, such as pseudarthrosis, bone defects caused by high-energy trauma, infection, and tumor resection (Curtis et al. 2016). In these situations, tissue integrity is not restored spontaneously, requiring surgical treatment with the use of grafts for mechanical stabilization and filling of the bone defect (Wiese and Pape 2010). Although autologous bone graft is considered the gold standard for stimulating bone healing, there is limited supply and high morbidity associated with autograft harvest (Hernigou et al. 2005; Sohn and Oh 2019). Allografts are an alternative to autologous bone grafts, as they are taken from human cadavers, which can include a greater variety of sizes and shapes and cause no pain to the patient. Allografts are obtained from tissue banks that harvest, process/decellularize, and store these tissues (Damien and Parsons 1991).

Decellularization methods may be physical or mechanical (using freeze-drying, cryopreservation or freezing), chemical, (using oxidizing agents that hydrogen peroxide), or enzymatic (using proteins such as trypsin and dispase) (Crapo et al. 2011; Gardin et al. 2015; Chen and Lv 2017). All decellularization protocols aim to remove the cellular content, reduce tissue antigenicity, and produce a scaffold with a preserved extracellular matrix (ECM) that supports tissue regeneration (Varettas and Taylor 2011; Urciuolo and De Coppi 2018).

However, several studies have shown that viable cells, functional proteins, and DNA may persist in the tissue even after the employment of different decellularization methods (Civril et al. 2013; Coutinho et al. 2017; Fretwurst et al. 2014). There have also been reports of inflammatory reactions and anti-HLA sensitization due to the presence of allograft-associated DNA (Lacerda et al. 2016).

These studies raise questions about allograft quality and show the lack of consensus and parameters to assess allograft quality and degree of decellularization. Therefore, the objective of this study was to establish techniques and biological parameters for validation of allografts from the Brazilian National Institute of Traumatology and Orthopedics (Instituto Nacional de Traumatologia e Ortopedia-INTO) tissue bank.

Materials and methods

Study design, ethical aspects, and eligibility criteria

This was an *in vitro*, prospective study with human bone samples from the INTO tissue bank. The research project was approved by the Institutional Research Ethics Committee (CAAE protocol no. 50696321.7.0000.5273). Study samples were harvested by the INTO tissue bank for clinical use and are in accordance with the criteria for allograft donation of the Brazilian National Health Surveillance Agency (RDC 220/2006 ANVISA) and the Brazilian National Transplant System.

Allograft harvesting and preparation

All processing operations were conducted in an ISO 5 clean room at the INTO tissue bank. After harvesting, the femoral heads were frozen in a mechanical freezer at -80°C for at least 6 months. Then, the femoral head was thawed, removed from its sterile triple bag, placed in a surgical lathe, and finally sectioned into 2 parts by a longitudinal cut along its greatest diameter.

A Jamshid biopsy needle was used to extract 30 cylindrical fragments of approximately 30 mg from different tissue sites. The fragments were placed in a sterile flask with 30 mL of 10% hydrogen peroxide and subjected to 2 washing cycles at 40 Hz for 5 min in an ultrasonic cleaner (L100, Schuster, Rio Grande do Sul, Brazil). The hydrogen peroxide solution was discarded, the bone fragments were collected with a stainless steel sieve and relocated to a new sterile flask with absolute ethanol for an additional cycle of 40 Hz for 5 min in the ultrasonic cleaner. The fragments were then placed in a glass basin for washing with 0.9% saline and antibiotic solution. After processing, the bone fragments were stored at -80°C .

or assigned to allograft quality assessment assays (Fig. 1).

Histological processing

After processing, the bone fragments were fixed in 4% paraformaldehyde in phosphate-buffered saline solution for 24 h and decalcified for approximately 3 days in 10% nitric acid solution (Vetec-Sigma-Aldrich). The samples were then embedded in paraffin to obtain 5- μ m thick histological sections and stained with hematoxylin and eosin (H&E). Images were obtained using an EVOS optical microscope (Thermo Fisher).

Immunofluorescence

The allografts were decalcified in 10% EDTA (Sigma-Aldrich) for 4 weeks and processed for paraffin embedding and sectioning as previously described. To confirm the presence of cells in the tissue, the histological sections were incubated in citrate buffer (pH

6.0) at 96 °C for 40 min and subsequently with 10% bovine serum albumin solution (Sigma-Aldrich) for 1 h. The slides were washed 3 times with tris-buffered saline, and the nuclei were stained with 1 μ g/mL of 4',6-diamidino-2-phenylindole solution (sc-3598, Santa Cruz Biotechnology, Texas, USA). Images were obtained using an EVOS microscope (Thermo Fisher).

Scanning electron microscopy

The allograft was fixed with 2% glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 M of sodium cacodylate buffer for 2 h and post-fixed with 1% osmium tetroxide solution for 30 min. The allograft was subsequently dehydrated through a series of ethyl alcohol solutions (Sigma-Aldrich, Missouri, United States) at increasing concentrations from 30 to 100%. At the end of the dehydration process, the samples were immersed in an ethanol-hexamethyldisilazane (HMDS) (Sigma-Aldrich) solution (1:1, v/v) for 30 min and then incubated with HMDS for 12 h.



Fig. 1 Allograft processing and quality assessment. Allograft processing: the femoral head was thawed and cut longitudinally, and a section of the bone was subsequently supported in a press. A biopsy needle was used to obtain cylindrical fragments of bone tissue from different parts. Finally, the frag-

ments underwent washing cycles in an ultrasonic bath with hydrogen peroxide, alcohol, saline, and antibiotic solution. Quality assessment methods: after processing, the bone fragments underwent histological evaluation, immunofluorescence, scanning electron microscopy, and DNA quantification

For visualization under scanning electron microscopy (SEM) (Quanta 400, Thermo Fisher), the samples were mounted on carbon tape and coated with a layer of gold for 3 min. Image acquisition and analysis were performed under low vacuum at 15 kV.

DNA quantification

The allograft fragments were incubated with the lysis solution from the Wizard® Genomic DNA Purification Kit (Promega, Wisconsin, USA) in a water bath at 55 °C for 12 h. Magnetic beads were subsequently placed in the tubes, and the tissue was fragmented through 4 rotation cycles at 3,733 rpm for 40 s with a disruptor tissue (L-BEADER 24, Loccus, São Paulo, Brazil). DNA extraction was conducted according to the protocol recommended by the kit's manufacturer

(Wizard® Genomic DNA Purification Kit). The DNA was quantified with a spectrophotometer (Nanodrop, Thermo Fisher, Massachusetts, USA) (Fig. 4a).

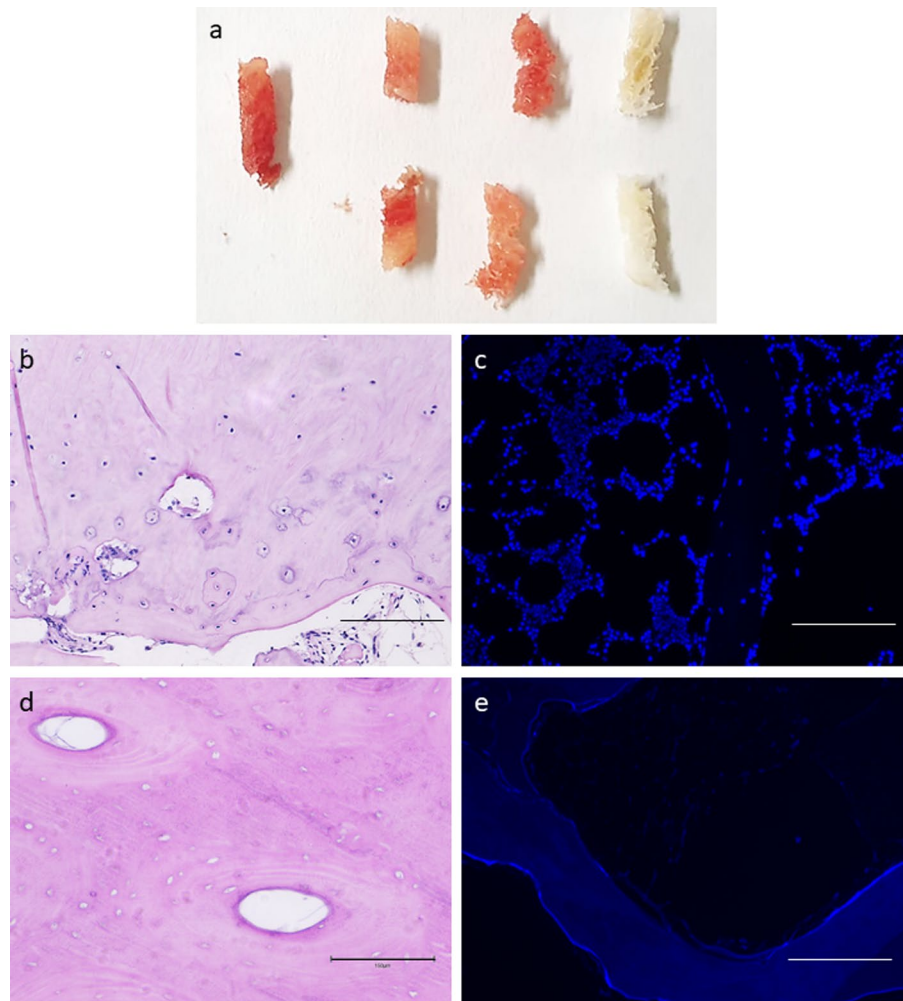
Statistical analysis

The results were expressed as mean $\pm$ SD and compared using ANOVA. The nonparametric Kruskal–Wallis test was used to compare 3 samples, and a p value < 0.05 was considered significant.

Results

Two femoral heads from male donors aged between 43 and 52 years, were sourced from the INTO Tissue Bank. The samples were collected between

Fig. 2 (a) Bone fragments from different processing stages. The recently harvested tissue is very reddish, and during the process, the color gradually fades until it becomes whitish. (b, d) Histological section of the allograft pre- and post-processing with H&E staining, respectively. DAPI staining showing resident cells before processing (c) and elimination of resident cells after processing (e). Scale bar = 100 μ m



December 2018 and January 2019, respectively. The allografts were harvested and frozen for at least 6 months before being subjected to the decellularization process, with successive washings, as described in Fig. 1.

A macroscopic evaluation of the bone fragments at different processing stages revealed gradual loss of color until the tissue was whitish in the last stage, suggesting the absence of cellular content (Fig. 2A). To confirm the elimination of resident cells, a comparative microscopic analysis of the tissue was performed before (Fig. 2B, D) and after (Fig. 2C, E) the application of the decellularization protocol. The tissue initially contained numerous osteocytes in the matrix (Fig. 2B), as well as cell nuclei stained with DAPI (Fig. 2C). After the decellularization process, these cells were eliminated (Fig. 2D and E).

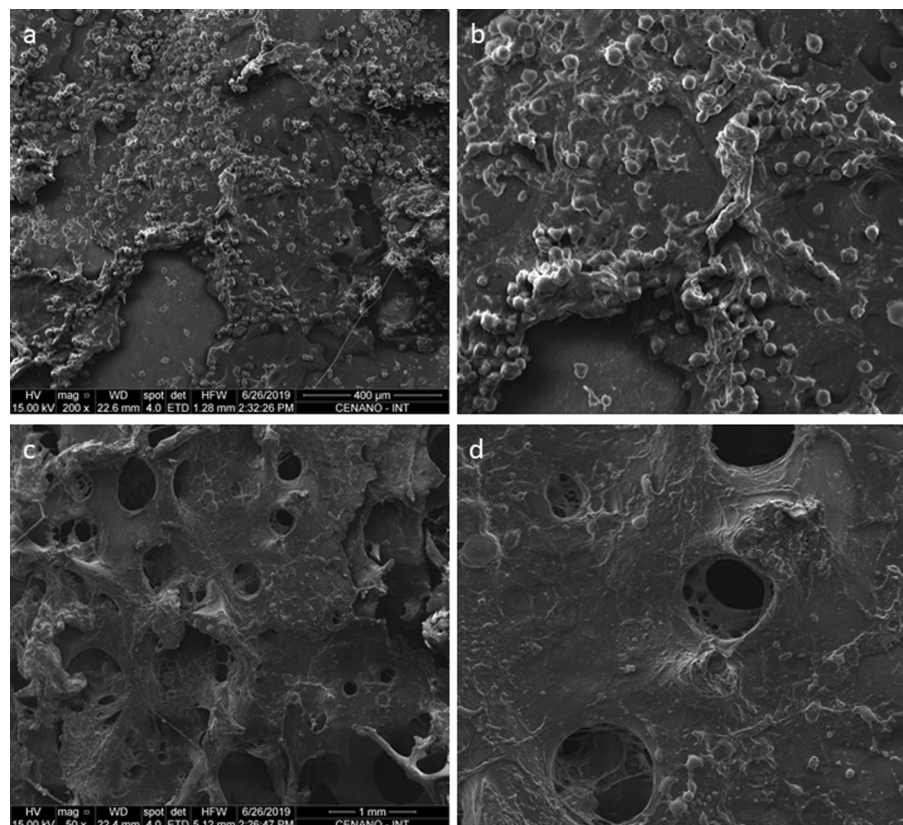
A complementary analysis with SEM showed loss of the cells adhered to the bone matrix and that the three-dimensional structure, structural architecture, and ECM of the allograft remained intact after the decellularization protocol was employed (Fig. 3).

Tissue fragments from 3 different processing stages (*in natura*, post-frozen, final product) underwent a DNA extraction protocol (Fig. 4A). DNA content decreased from 85.66 ± 9.01 ng DNA/mg tissue in the *in natura* sample to 22.44 ± 2.17 ng DNA/mg tissue in the post-frozen sample and to 17.76 ± 1.18 ng DNA/mg tissue in the final product sample, accounting for a 79.26% reduction from the *in natura* stage to the final product stage ($p=0.005$) (Fig. 4B).

Discussion

The increased incidence of bone defects, especially of those caused by high-energy trauma or nonunion, requires increasing amounts of bone grafts, and tissue repair with bone allografts is a relevant alternative to autologous transplantation, which has major supply limitations despite being the gold standard (Sohn and Oh 2019). Bone allografts provide a unique natural microstructure that not only has similar components

Fig. 3 Structural aspects of the allograft on scanning electron microscopy. (a, b) Allograft before processing showing numerous cells adhered to the bone matrix. (c, d) Allograft after processing showing absence of cells and intact extracellular matrix



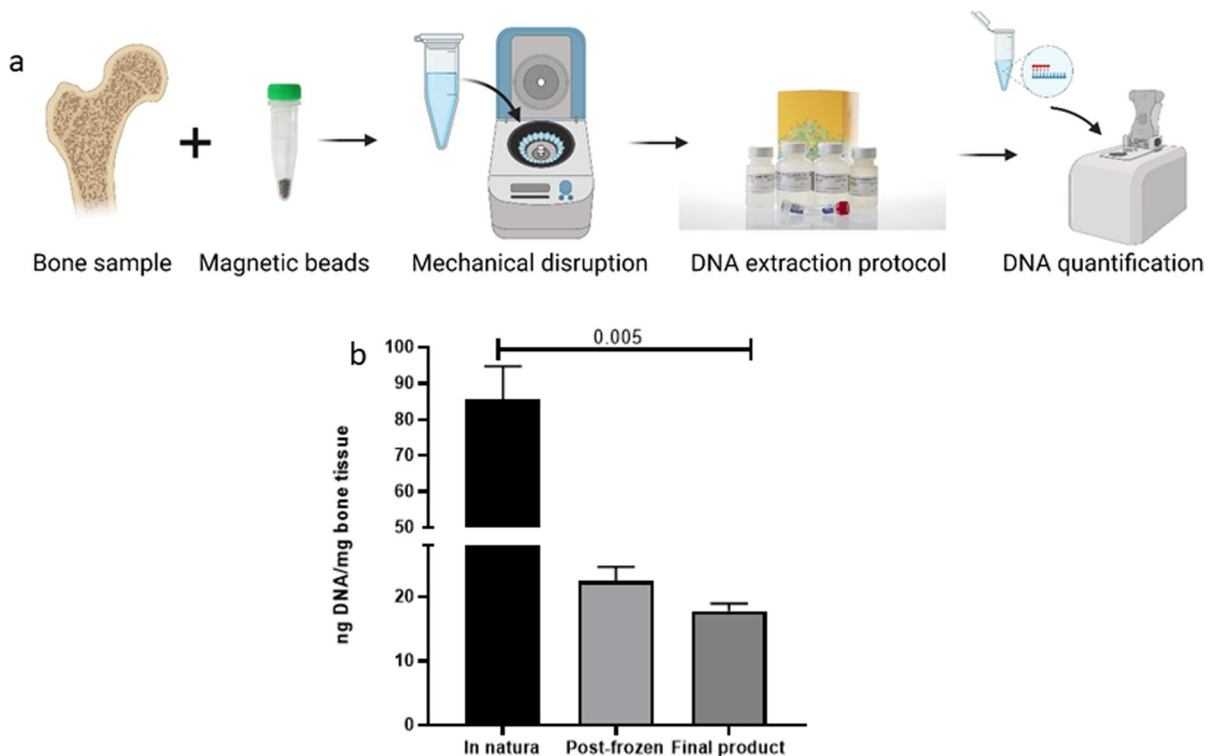


Fig. 4 DNA quantification in allografts. **(a)** Protocol steps used for DNA extraction. **(b)** DNA amount in the *in natura*, post-frozen, and final product stages of 10 fragments from allo-

grafts obtained from 2 femoral heads. The graph represents the mean and standard deviation

to the bone, but also has similar porosity and pore size, which can promote osteoinduction and osteogenesis, leading to bone mineralization.

Despite the clear importance of allografts, consensus about the best decellularization method and about which biological parameters should be used to evaluate allograft suitability for transplantation is lacking. An optimal decellularization method should remove all cellular components without changing the mechanical and biological properties of the native bone, allowing cellular function, cellular interaction, and cellular differentiation (Kim et al. 2019; Keane et al. 2015). Due to the great national relevance of the INTO tissue bank, the INTO should play a key role in the establishment of acceptable biological parameters for allograft validation.

The femoral head was chosen because it is one of the bone segments most commonly requested for orthopedic surgery due to the good quality and quantity of trabecular tissue. Therefore, we included samples from 2 patients for analysis by our study group.

The gradual loss of color and the macroscopic structure observed throughout the decellularization process suggest that the samples were decellularized. This evaluation parameter is commonly used in the devitalization of organs such as the liver (Crapo et al. 2011), heart (Wainwright et al. 2010), and skeletal muscle (Miranda et al. 2021).

On slides stained with H&E, osteocytes within the bone defect were eliminated after material processing. This is the most commonly used parameter to indicate tissue devitalization (Lee et al. 2016; Xu et al. 2017). Likewise, SEM and nuclear labeling with DAPI were consistent with the results obtained with H&E and support the hypothesis that cell components were successfully eliminated, as observed in decellularization assessment tests (Gardin et al. 2015).

Quantification of genetic material revealed residual DNA still present in the sample. At the end of the process, DNA content was consistent with other studies that produced decellularized bone (Hashimoto et al. 2011; Rasch et al. 2019) and was < 50 ng DNA/

mg tissue, which is the value proposed by Crapo in 2011 as a criterion for bone tissue decellularization (Crapo et al. 2011). According to the literature, the presence of DNA in small amounts is acceptable and found even in commercially available allografts. This is due to technical limitations that make it impossible to completely remove biological material (Badylak and Gilbert 2008). Finally, by SEM analysis, we demonstrated preserved ECM properties.

Thinking about the use of this allograft as a scaffold capable of stimulating bone regeneration, we associated it with bone marrow mesenchymal stem cells and, through a murine model, we demonstrated that allografts were able to promote bone neoformation (da Rocha et al 2023). Now, further studies need to be conducted first to demonstrate the efficacy of the decellularization process in larger bone grafts and second, demonstrate that this bones with distinct architecture also have the ability to stimulate bone formation and ability to support/form a medullary microenvironment.

Conclusion

With the set of techniques used in this study, it was possible to perform macroscopic and microscopic analyses that allowed us to ensure the quality of the material offered and the safety of bone transplantation with the use of allografts. In addition, because the techniques are reproducible, we believe that the establishment of these techniques may help other tissue banks validate their processing operations and improve quality and safety standards.

Funding The study was partially supported by the AO Trauma Latin America Research Office.

Data availability All relevant data are within the paper.

Declarations

Conflict of interest The authors have no conflicts of interest to disclose.

Human and animal participants The research project was approved by the Institutional Research Ethics Committee (CAAE protocol no. 21778619.9.0000.5273). Study samples were harvested by the INTO tissue bank and are in accordance with the criteria for allograft donation of the Brazilian National Health Surveillance Agency (RDC 220/2006 ANVISA) and the Brazilian National Transplant System.

References

- Badylak SF, Gilbert TW. (2008) Immune response to biologic scaffold materials. *Semin Immunol.* 20(2):109–16. <https://doi.org/10.1016/j.smim.2007.11.003>
- Chen G, Lv Y (2017) Decellularized bone matrix scaffold for bone regeneration. In: Turksen K (ed) Decellularized scaffolds and organogenesis. *Methods in molecular biology.* Springer, New York, pp 239–254
- Civril F, Deimling T, de Oliveira Mann CC, Ablasser A, Moldt M, Witte G, Hornung V, Hopfner KP (2013) Structural mechanism of cytosolic DNA sensing by cGAS. *Nature* 498:332–337. <https://doi.org/10.1038/nature12305>
- Coutinho LF, Amaral JBD, Santos EBD, Martinez EF, Montalli VAM, Junqueira JLC, Araujo VC, Napimoga MH (2017) Presence of cells in fresh-frozen allogeneic bone grafts from different tissue banks. *Braz Dent J* 28:152–157. <https://doi.org/10.1590/0103-6440201701206>
- Crapo PM, Gilbert TW, Badylak SF (2011) An overview of tissue and whole organ decellularization processes. *Biomaterials* 32:3233–3243. <https://doi.org/10.1016/j.biomaterials.2011.01.057>
- Curtis EM, van der Velde R, Moon RJ, van den Bergh JP, Geusens P, de Vries F, van Staa TP, Cooper C, Harvey NC (2016) Epidemiology of fractures in the United Kingdom 1988–2012: Variation with age, sex, geography, ethnicity and socioeconomic status. *Bone* 87:19–26. <https://doi.org/10.1016/j.bone.2016.03.006>
- da Rocha LR, Dias RB, Fernandes MBC, Prinz R, Eirado TP, Costa IS, Monteiro MJ, da Silva CER, Dos Santos CT, Fogagnolo F (2023) A new option for bone regeneration: a rapid methodology for cellularization of allograft with human bone marrow stromal cells with in vivo bone-forming potential. *Injury* 54(Suppl 6):110777. <https://doi.org/10.1016/j.injury.2023.05.008>
- Damien CJ, Parsons JR (1991) Bone graft and bone graft substitutes: a review of current technology and applications. *J Appl Biomater* 2:187–208. <https://doi.org/10.1002/jab.770020307>
- De Lacerda PE, Pelegrine AA, Teixeira ML, Montalli VA, Rodrigues H, Napimoga MH (2016) Homologous transplantation with fresh frozen bone for dental implant placement can induce HLA sensitization: a preliminary study. *Cell Tissue Bank* 17:465–472. <https://doi.org/10.1007/s10561-016-9562-9>
- Fretwurst T, Spanou A, Nelson K, Wein M, Steinberg T, Stricker A (2014) Comparison of four different allogeneic bone grafts for alveolar ridge reconstruction: a preliminary histologic and biochemical analysis. *Oral Surg Oral Med Oral Pathol Oral Radiol* 118:424–431. <https://doi.org/10.1016/j.oooo.2014.05.020>
- Gardin C, Ricci S, Ferroni L, Guazzo R, Sbricoli L, De Benedictis G, Finotti L, Isola M, Bressan E, Zavan B (2015) Decellularization and delipidation protocols of bovine bone and pericardium for bone grafting and guided bone regeneration procedures. *PLoS ONE* 10:e0132344. <https://doi.org/10.1371/journal.pone.0132344>
- Hashimoto Y, Funamoto S, Kimura T, Nam K, Fujisato T, Kishida A (2011) The effect of decellularized bone/bone marrow produced by high-hydrostatic pressurization on

- the osteogenic differentiation of mesenchymal stem cells. *Biomaterials* 32:7060–7067. <https://doi.org/10.1016/j.biomaterials.2011.06.008>
- Hernigou P, Poignard A, Beaujean F, Rouard H (2005) Percutaneous autologous bone-marrow grafting for nonunions. Influence of the number and concentration of progenitor cells. *J Bone Joint Surg Am* 87:1430–1437. <https://doi.org/10.2106/JBJS.D.02215>
- Keane TJ, Swinehart IT, Badylak SF (2015) Methods of tissue decellularization used for preparation of biologic scaffolds and in vivo relevance. *Methods* 84:25–34. <https://doi.org/10.1016/j.ymeth.2015.03.005>
- Kim YS, Majid M, Melchiorri AJ, Mikos AG (2019) Applications of decellularized extracellular matrix in bone and cartilage tissue engineering. *Bioeng Transl Med* 4:83–95. <https://doi.org/10.1002/btm2.10110>
- Lee DJ, Diachina S, Lee YT, Zhao L, Zou R, Tang N, Han H, Chen X, Ko CC (2016) Decellularized bone matrix grafts for calvaria regeneration. *J Tissue Eng* 7:2041731416680306. <https://doi.org/10.1177/2041731416680306>
- Miranda C, Leonel L, Canada RR, Maria DA, Miglino MA, Del Sol M, Lobo SE (2021) Effects of chemical and physical methods on decellularization of murine skeletal muscles. *An Acad Bras Cienc* 93:e20190942. <https://doi.org/10.1590/0001-3765202120190942>
- Rasch A, Naujokat H, Wang F, Seekamp A, Fuchs S, Kluter T (2019) Evaluation of bone allograft processing methods: impact on decellularization efficacy, biocompatibility and mesenchymal stem cell functionality. *PLoS ONE* 14:e0218404. <https://doi.org/10.1371/journal.pone.0218404>
- Sohn HS, Oh JK (2019) Review of bone graft and bone substitutes with an emphasis on fracture surgeries. *Biomater Res* 23:9. <https://doi.org/10.1186/s40824-019-0157-y>
- Urciuolo A, De Coppi P (2018) Decellularized tissue for muscle regeneration. *Int J Mol Sci* 19:2392. <https://doi.org/10.3390/ijms19082392>
- Varettas K, Taylor P (2011) Bioburden assessment of banked bone used for allografts. *Cell Tissue Bank* 12:37–43. <https://doi.org/10.1007/s10561-009-9154-z>
- Wainwright JM, Czajka CA, Patel UB, Freytes DO, Tobita K, Gilbert TW, Badylak SF (2010) Preparation of cardiac extracellular matrix from an intact porcine heart. *Tissue Eng Part C Methods* 16:525–532. <https://doi.org/10.1089/ten.TEC.2009.0392>
- Wiese A, Pape HC (2010) Bone defects caused by high-energy injuries, bone loss, infected nonunions, and nonunions. *Orthop Clin North Am* 41:1–4. <https://doi.org/10.1016/j.ocl.2009.07.003>
- Xu K, Kuntz LA, Foehr P, Kuempel K, Wagner A, Tuebel J, Deimling CV, Burgkart RH (2017) Efficient decellularization for tissue engineering of the tendon-bone interface with preservation of biomechanics. *PLoS ONE* 12:e0171577. <https://doi.org/10.1371/journal.pone.0171577>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.