In vivo bone regeneration in sheep model of sterile polymeric scaffolds processed by supercritical CO_2 technology

V. Santos-Rosales[a], L. Diaz-Gomez[a], B. Magariños[b], C. Alvarez-Lorenzo[a] and C.A. García-González[a,*]

[a]*Departamento de Farmacología, Farmacia y Tecnología Farmacéutica, I+D Farma (GI-1645), Faculty of Pharmacy, iMATUS and Health Research Institute of Santiago de Compostela (IDIS), Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain.*

[b]*Departamento de Microbiología y Parasitología, Facultad de Biología, CIBUS, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain.*

carlos.garcia@usc.es

1. Introduction

The limited availability of bone tissue for transplantation has prompted the development of innovative synthetic grafts, known as scaffolds, that promote the self-healing capacity of the tissue patients. Scaffolds are 3D-porous structures that temporary replace the damaged tissue due to their similar morphological and mechanical properties [1]. To date, there is a broad portfolio of manufacturing techniques for scaffold fabrication, but supercritical (sc) CO_2 technology offers outstanding advantages. Indeed, $scCO_2$ foaming allows the production of drug-loaded polymeric scaffolds for bone regeneration purposes under mild temperature and pressure conditions [2]. A combination of polyesters can be prepared and foamed to match specific degradation kinetics and mechanical performance. Among others, the use of poly(ε-caprolactone) (PCL) and poly(lactic-co-glycolic acid) (PLGA) blends are of special interest for bone scaffolding.

On the other hand, sterility is a critical quality attribute of any implantable medical device, including scaffolds. $ScCO_2$ is a recognized sterilizing agent achieving high sterilization levels (6-log reductions) against bacterial endospores of high resistance (*B. stearothermophilus, B. atrophaeus, B. pumilus*) [3]. The exploitation of $scCO_2$ as a foaming and sterilizing agent in a combined protocol is still a major technological challenge in the supercritical fluid field. Recent results from our research group provided outstanding *in vitro* and *in ovo* results [2,4], encouraging the further translation to *in vivo* models.

In this work, admixtures of PCL, PLGA and pregelatinized starch (St) were subjected to a proprietary $scCO_2$ sterilization+foaming integrated process and evaluated *in vivo* in a sheep model. Scaffolds containing platelet-rich-plasma (PRP) to enhance the bone regeneration were also prepared by the same methodology. The obtained scaffolds were tested *in vivo* in critical size bone defects of femur and tibia from a Manchega sheep model and compared to negative (unfilled defects) and positive (autograft) controls.

2. Materials and Methods

1200 ppm of hydrogen peroxide were added in the liquid form in a high-pressure autoclave (100 mL) containing scaffolds components (PCL, PLGA, St, PRP) in cylindrical moulds (D=8,5 mm, L: 15 mm). The system was heated to 39 ºC and pressurized at 140 bar, maintaining the setup in the batch mode for 2 h, followed by a $scCO_2$ flow (5 g/min) during 1 h. Finally, the system was depressurized at a constant venting rate of 3 bar/min until atmospheric pressure. The physicochemical and morphological properties of the obtained scaffolds were evaluated by scanning electron microscopy (SEM), mercury intrusion porosimetry (MIP), and helium pycnometry.

In vivo evaluation was approved by the Hospital Universitario de A Coruña (CHUAC) Bioethics Committee for Animal Studies and conducted in accordance with the European regulation on care and use of animals in experimental procedures and the ARRIVE guidelines. Cylindrical defects (9.5 mm diameter) were performed in the distal femoral epiphyses and proximal tibial epiphyses. After 16 weeks, animals were sacrificed, the treated bone defects were fixed and stored in 70% ethanol for further processing. Bone regeneration was evaluated by microcomputed tomography (μ-CT) and histological/immunohistochemical analysis. Non-treated defects were used as controls.

3. Results and Discussion

Sterile PCL/PLGA-St scaffolds loaded with PRP with suitable morphological criteria to be used as bone grafts were manufactured by means of $scCO_2$ technology. Preliminary *in vitro* tests revealed the retained bioactivity of PRP after $scCO_2$ treatment. No segregation of components was observed along the scaffolds and the PRP incorporation yields were close to 100%.

After implantation, animals did not show any sign of inflammation response, thus ensuring the compatibility of the designed scaffolds. After 16 weeks of implantation, the significant formation of new bone in the defect area was observed by µ-CT (Figure 1) for animals treated with scaffolds, compared to controls. The regeneration of the injured region in scaffold-treated defects was also confirmed by the quantification of the new bone tissue from the µ-CT reconstructions. Histological and immunohistochemical analysis confirmed the formation of mineralized bone tissue and vascularization promoted by the implanted scaffolds.

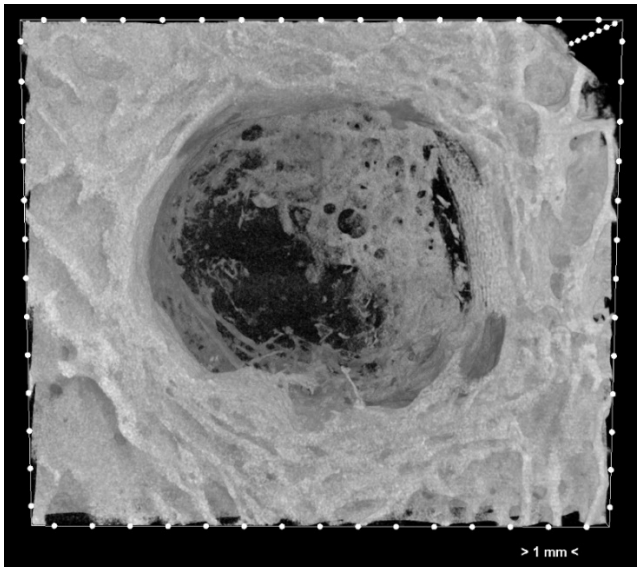

Figure 1. Representative µ-CT reconstruction of a filled defect with a scaffold on sheep femur after 16 weeks. The formation of new bone is observed as new radio dense material in the damaged area.

4. Conclusions

Sterile PCL/PLGA-St scaffolds loaded with PRP were manufactured with suitable morphological criteria to be used as bone grafts in a sterile manner by means of $scCO_2$ technology. Scaffold performance after 16 weeks of implantation proved their effectiveness in regenerating bone tissue in critical size femoral and tibial defects. Overall, this work highlights the advantages of $scCO_2$ technology for the manufacturing of sterile and drug-loaded polymeric scaffolds in a single-step, with promising performance in a relevant *in vivo* model. The technological transfer and valorization of these scaffolds within the clinical arena is currently ongoing.

5. Acknowledgments

This research was funded by MICINN [PID2020-120010RB-I00], Xunta de Galicia [ED431C 2020/17; ED481D-2021-014], Agencia Estatal de Investigación [AEI] and FEDER funds. Work supported by Ignicia Programme from Axencia Galega de Innovación (Xunta de Galicia, IN855A 2021/13).

References

[1] V. Santos-Rosales, A. Iglesias-Mejuto, C. García-González. Polymers. 12 (**2020**) 533.
[2] V. Santos-Rosales, B. Magariños, C. Alvarez-Lorenzo, C.A. García-González, International Journal of Pharmaceutics. 612 (**2022**) 121362.
[3] N. Ribeiro, G.C. Soares, V. Santos-Rosales, A. Concheiro, C. Alvarez-Lorenzo, C.A. García-González, A.L. Oliveira. Journal of Biomedical Materials Research Part B. Applied Biomaterials. 108 (**2020**) 399–428.
[4] V. Santos-Rosales, B. Magariños, R. Starbid, J. Suárez-González, J.B. fariña C. Alvarez-Lorenzo, C.A. García-González, International Journal of Pharmaceutics. 605 (**2021**) 120801.