

Assessment of Sterility and Residual Solvent Agents in Antler-derived and Bovine Xenograft Bone Substitutes: An In-vitro Study

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Objectives This study aimed to assess and compare the results of sterility and residual solvent testing in a newly developed antler-derived xenograft versus a bovine-derived xenograft.

Methods First, test and control samples were prepared using thermal and chemical procedures, involving immersion in deionized water for 24 hours, drying, boiling in sterile water, chemical treatment with chloroform and methanol, and heating at 650°C in a furnace. Next, they were sterilized via gamma radiation at 25 kGy. The sterility test was then performed based on the ISO 11737-2:2019 standard, using the direct inoculation method. Finally, residual solvent testing was carried out via gas chromatography-mass spectrometry.

Results The sterility test showed no evidence of bacterial or fungal growth in any of the samples during 14 days of incubation. Also, residual solvent testing indicated no sign of residual solvents in the samples.

Conclusion Antler-derived xenograft was safe to use in terms of the sterility and removal of residual solvents. Further studies should be carried out regarding other important laboratory tests as well as the animal and clinical studies.

Keywords Xenograft; Bone substitute; Deer antler; Sterility testing; Residual solvent testing

Introduction

Considering the recent advances in bone regeneration, alveolar bone defects can be managed with the aim of reconstructing the natural anatomy, improving aesthetic outcomes, and placing dental implants. Bone grafting substitutes are widely used for bone reconstruction. Currently, several bone replacement materials with variable properties are available.¹ Autogenous bone is considered the gold standard for bone defect reconstruction due to its osteoinductive, osteoconductive, and osteogenic properties with predictable outcomes. However, owing to the drawbacks of autografts, such as possible harvesting morbidity, unpredictable resorption, and limited available bone volume for intraoral donor sites, other sources of grafting material have been also introduced to the market.² Allografts obtained from the cadaveric tissue have several advantages over autogenous bone, while the possibility of disease transmission and immunological reaction are their disadvantages.^{1, 3, 4}

Due to problems associated with allografts and autografts, there is a tendency toward using xenogeneic grafts. Biocompatibility, porous structure, reasonable cost, and mechanical strength are the advantages of xenografts.⁵ Although xenografts have been widely accepted, they have some disadvantages. Two of the most important disadvantages of available xenografts are the transmission of common diseases of cattle and humans, and ethical

issues associated with animal sacrifice. Consequently, introduction of a safe, effective, and ethically acceptable xenograft is warranted.^{5, 6}

The deer antler is the only organ in mammals which grows quickly and can regenerate on its own. The remarkable growth rate of this organ and its similar structural features to the human bone make it an interesting xenograft for bone regeneration.⁷⁻⁹ To confirm the efficiency of xenografts, manufacturing factories perform various tests, including cytotoxicity tests and animal studies. Since xenografts are obtained from different species, it is important to evaluate the quality of these materials, including the assessment of organic solvent residues and sterilization process.^{10, 11} Organic solvents are applied during the synthesis of some medical product formulations. Various manufacturing processes and techniques are used to remove them. Nonetheless, after such processes, some solvents may remain, even in small quantities.^{12, 13} Additionally, sterilization of grafting materials is crucial for the prevention of disease transmission.¹⁴

Although deer antler has potential applications as efficient xenografts, it has not been produced commercially. Therefore, the Technology Unit of the Dental Sciences Research Center of Shahid Beheshti University of Medical Sciences produced an antler-derived xenograft, which is safe, efficient, and in accordance with international standards. Considering the lack of laboratory tests on antlers, the present study aimed to assess the sterilization

and organic solvent residuals of a novel Persian gazelle antler-derived xenogeneic graft.

Methods and Materials

This experimental study was conducted at Nikoo Farmed Aria Laboratory, Hesgar Saba Laboratory, Technology Unit of the Dental Sciences Research Center of Shahid Beheshti University of Medical Sciences, and the Department of Periodontics at the School of Dentistry, Shahid Beheshti University of Medical Sciences (Tehran, Iran). It was also registered with the ethics code, IR.SBMU.DRC.REC.1400.127.

Sample preparation

The test sample was extracted from a deer antler (Lotus Biomaterials®, PZMI Co., Iran), whereas the control sample was bovine bone (Bone⁺B®, NovaTeb, Iran), extracted from the femoral region. Both samples were prepared using chemical and thermal techniques. First, both test and control samples were immersed in 4°C deionized water for 24 hours. This process was extended to 48 hours due to the color change of water. Second, the samples were dried in an oven at a temperature of 100°C for six hours to completely remove moisture and then boiled in sterile water for six hours.

The samples were dried again in an oven, and then, chemical treatment with chloroform and methanol (1:1) was performed to remove waste products from digestion of bone blocks. To remove any remaining organic solvents, the final washing was performed 10 times. Subsequently, the thermal process was conducted in a furnace heated to 650°C at a rate of 10°C per minute for three hours (Azar Furnace, Iran). Finally, sterilization was carried out using gamma irradiation at a radiation dose of 25 kGy.

Sterility testing

The sterility test was performed based on the standard ISO 11737-2:2019 method (<https://www.iso.org/standard/70801.html>). In detail, the culture medium was incubated directly (using the direct inoculation technique) for both deer antler and bovine bone samples in sterility testing. In this technique, a small volume of the sample was removed aseptically from the sample unit and inoculated directly into a suitable volume of growth medium before incubation. Tryptic soy broth (TSB) and fluid thioglycollate media (FTM) culture media were used to assess the presence of fungal and bacterial microorganisms in the samples, respectively. The volume of the used broth medium was 400 mL. Next, the

samples were transferred to the culture medium, incubated, and controlled for the bacterial growth at the temperature of 30-35°C and for the fungal growth at the temperature of 20-25°C for 14 days. Finally, the incubated media were observed for the presence (turbidity) or absence (clearness) of microbial growth daily. The procedure was repeated five times for both control and test samples to ensure the accuracy of the results.

The minimum number of items to be tested from each batch of solid medical devices was 10% of the batch or four units (whichever was greater) if the number of items in the batch did not exceed 100. If the number of items in the batch was more than 500, 2% of the batch or 20 units (whichever was less) were tested. The minimum number of items to be tested for each medium was 10 units if the number of items in the batch was between 101 and 500. Additionally, a negative control assessment was performed using the same culture media and incubation periods. Besides, environmental control was performed using a settle plate and viable counting.

Residual solvent testing

Gas chromatography/mass spectrometry (GC/MS) was employed to evaluate the remaining organic solvents. This method is the process of separating compounds in a mixture by injecting a gaseous or liquid sample into a mobile phase and passing the gas through a stationary phase. A capillary column and helium as the carrier gas were employed. The inlet line temperature was 270°C, and the source temperature was 210°C. The temperature program for the column was set at 25°C for five minutes, which was increased to 250°C at a rate of 7.5°C/min and maintained for five minutes. The MS scan was performed at 30-310 m/z. Acetonitrile (C₂H₃N, M=41) was used as a solvent for the GC/MS analysis of both test and control samples.

Results

Sterility testing

In both the test and control samples, no evidence of bacterial or fungal growth was found on any of the incubation days (Table 1). Besides, no evidence of bacterial or fungal growth was found in five repetitions of the procedure. The same result was obtained for the negative control (Table 2).

Table 1- Results of sterility testing of antler xenograft and bovine xenograft

Culture Medium	Incubation days														Microorganisms
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
TSB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Fungal Aerobic Anaerobic
FTM	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

+: Pass; TSB: Tryptic Soy broth; FTM: Fluid Thioglycollate media

Table 2- Results of negative control assessment for sterility testing of both samples

Culture Medium	Incubation days														Microorganisms
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
TSB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Fungal Aerobic Anaerobic
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
FTM	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

+: Pass; TSB: Tryptic Soy broth; FTM: Fluid Thioglycollate media

Residual solvents testing

The results of GC/MS revealed that the test and control samples had no detectable levels of residual organic solvents (Figure 1).

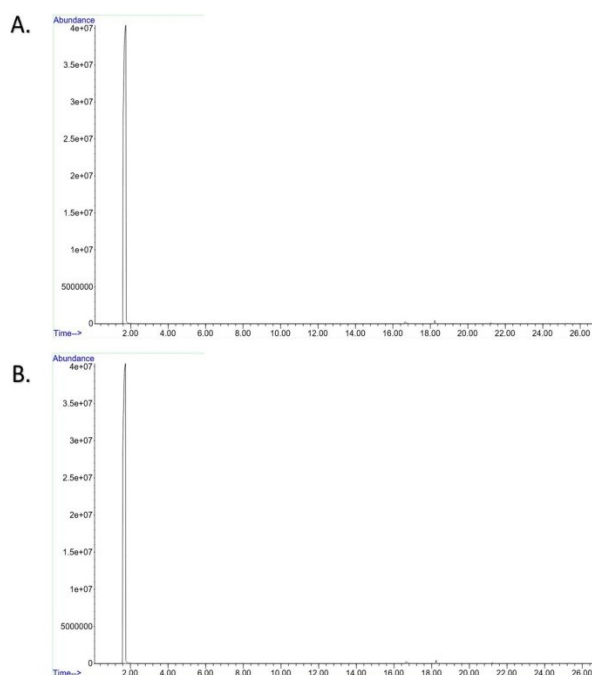


Figure 1: Results of residual solvents testing of a) Antler xenograft b) Bovine xenograft: The peak in the chromatograms is related to acetonitrile that was used as the GC solvent during GC procedure.

Discussion

There are various techniques for the management of localized bone loss. Both autogenous and allogeneic bones have been successfully employed for this purpose. It is known that autogenous bone graft harvesting is risky and that the amount of available bone is constrained.¹⁵ On the other hand, allografts are associated with the possible risk of post-operative infection and disease transmission.¹⁶ Alloplastic materials and xenografts are two other bone replacement grafts, which have been developed to aid in bone formation and defect filling.¹⁷ Because of the similarity of the inorganic type to deproteinized human bone in terms of porous architecture and composition, xenografts are appealing to clinicians.⁵ However, the need for animal sacrifice is the main problem of most available xenografts. Therefore, it is crucial to introduce a xenograft that can be made from the regeneratable parts of an animal. Antler is a remarkable model for xenografts due to its

ability to regenerate, as well as material characteristics.⁸ Xenografts need to be sterilized as they are derived from different animal species. Additionally, chemical treatment is carried out for most xenografts while they are being prepared. These chemicals should not be present in the xenografts at any amount, as they are toxic and non-biocompatible. In the current study, an antler-derived xenograft was compared with a bovine-derived xenograft, and sterilization and removal of organic solvent residues were evaluated. The present findings revealed that sterilization and elimination of organic solvent residues were successfully achieved in the test and control samples. Biomaterials can be sterilized using various methods, including dry heat, ethylene oxide, high-pressure steam, gamma irradiation, and gas plasma; meanwhile, gamma irradiation is the most popular one. Almost all available xenografts were sterilized with gamma irradiation, including Bio-Oss[®], Cerabone[®], Bone Plus[®], and OCB-X[®]. Generally, gamma sterilization is characterized by better penetration, greater sterility assurance, and higher effectiveness, regardless of temperature and pressure.¹⁸ Therefore, gamma irradiation was selected in the current study to sterilize the antler-derived xenograft.

The two recommended techniques for sterility testing of biomaterials are membrane filtration and direct inoculation. Membrane filtration sterility testing is used for filterable products. Consequently, the direct inoculation technique was used in the current study.¹⁹ This technique was also employed in studies by Nguyen et al.²⁰ and Hilmy et al.²¹ to validate the sterilization of allografts. While direct inoculation is a straightforward procedure, it has some limitations. The sensitivity of this test is constrained by the fact that only small amounts of the product can be inoculated into the culture medium. It may be difficult to identify turbidity caused by microbial growth at the end of the incubation period if the sample initially appears cloudy or turbid after inoculation. Additionally, the sample needs to be neutralized if the product has antimicrobial qualities to prevent the inhibition of microbial growth.²² One important factor in direct inoculation is environmental contamination. Therefore, air and surface of the working desk need to be monitored²²; otherwise, it may yield erroneous results. Accordingly, a negative control test was also carried out in this study to make sure that there were no false positive results and to guarantee the environment sterility for sterility testing. Solvents that are used in the production of materials but are not entirely eliminated after

processing are known as residual solvents. To make sure that solvents are not present at levels posing a threat to human health, residual solvent testing is essential. Any organization that uses solvents in manufacturing and production procedures is required to conduct residual solvent testing. Additionally, residual solvent testing can help prevent contamination and product failure and guarantee suitable products for human consumption.²³

Testing for residual solvents in biomaterials is typically performed using one of the following three techniques: thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography (GC). However, due to its advantages, GC/MS analysis is the technique of choice. The advantages of GC/MS include improved sample identification, increased sensitivity, use of small samples, strong separation capability, good selectivity, broad applications, and quicker results.²⁴ However, there are two significant drawbacks to GC/MS analysis. First, the range of volatile, thermally stable compounds that can be analyzed is relatively small, and second, molecular ions are frequently absent or weak in the mass spectra.²³

Additionally, almost all GC separations use GC solvents for the analysis of low-volatile substances. Acetonitrile was used as the GC solvent in this study due to its low chemical reactivity, high miscibility with water mixtures, and low viscosity.²⁵ Acetonitrile, with a retention time of 1.572 minutes, was the only visible peak in the chromatograms of both samples used in this study, suggesting that there were no solvents remaining in the samples. Moreover, any newly developed xenograft needs to meet certain requirements as a bone substitute (e.g., biocompatibility, non-toxicity, immunity to disease and antigens, and similarity to the human bone structure). Each of these requirements should be verified using multiple tests.

To confirm the non-toxicity and absence of microorganisms in the antler-derived xenograft, the sterility and residual solvent tests were performed for the antler-derived xenografts in this study. However, there are other important tests that need to be performed to confirm xenografts in terms of biocompatibility, absence of antigens, and similarity to the natural structure of the human bone, namely, assessment of morphology, porosity, Ca/P ratio, cytotoxicity, and immunogenicity, thermogravimetric analysis, X-ray diffraction analysis, and Fourier-transform infrared spectroscopy.

Limitations

In this study, only two tests were selected from a group of tests that should be performed to confirm the use of xenografts commercially. Another limitation was that in this *in vitro* study, the effects of preparation methods on the clinical outcomes were not examined. Finally, the effects of preparation methods on the stimulation of osteogenic factors (e.g., differences in cell response to bone graft substitutes prepared by different methods) were not investigated.

Conclusion

The results of the current study showed that the sterilized antler-derived xenograft was appropriate in terms of the removal of residual solvents, and there was no evidence of microorganisms or residual solvents in either the study or control samples. Further animal and clinical assessments in future studies, as well as *in vitro* research using other laboratory tests, are suggested.

Conflict of Interest

No Conflict of Interest Declared ■

References

1. Fernandez de Grado G, Keller L, Idoux-Gillet Y, Wagner Q, Musset A-M, Benkirane-Jessel N, et al. Bone substitutes: a review of their characteristics, clinical use, and perspectives for large bone defects management. *J Tissue Eng.* 2018; 9:2041731418776819.
2. Rasch A, Naujokat H, Wang F, Seekamp A, Fuchs S, Klüter T. Evaluation of bone allograft processing methods: impact on decellularization efficacy, biocompatibility and mesenchymal stem cell functionality. *PLoS One.* 2019; 14(6):e0218404.
3. Moussa NT, Dym H. Maxillofacial bone grafting materials. *Dent Clin North Am.* 2020; 64(2):473-90.
4. Markel MD. Bone Grafts and Bone Substitutes. *Equine Fracture Repair, Second Edition* 2019; Chapel1:163-72.
5. Amid R, Kheiri A, Kheiri L, Kadkhodazadeh M, Ekhlasmandkermani M. Structural and chemical features of xenograft bone substitutes: A systematic review of *in vitro* studies. *Biotechnol Appl Biochem.* 2021; 68(6): 1432-52.
6. Titsinides S, Agrogianis G, Karatzas T. Bone grafting materials in dentoalveolar reconstruction: a comprehensive review. *Jpn Dent Sci Rev.* 2019; 55(1):26-32.
7. Meng S, Zhang X, Xu M, Heng BC, Dai X, Mo X, et al. Effects of deer age on the physicochemical properties of deproteinized antler cancellous bone: an approach to optimize osteoconductivity of bone graft. *Biomed Mater.* 2015; 10(3):035006.
8. Zhang X, Cai Q, Liu H, Heng BC, Peng H, Song Y, et al. Osteoconductive effectiveness of bone graft derived from antler cancellous bone: an experimental study in the rabbit mandible defect model. *Int J Oral Maxillofac Surg.* 2012; 41(11):1330-7.
9. Picavet PP, Balligand M, Crigel MH, Antoine N, Claeys S. *In vivo* evaluation of deer antler trabecular bone as a reconstruction material for bone defects. *Res Vet Sci.* 2021; 138:116-24.
10. Gashtasbi F, Hasannia S, Hasannia S, Mahdi Dehghan M, Sarkarat F, Shali A. Comparative study of impact of animal source on physical, structural, and biological properties of bone xenograft. *Xenotransplantation.* 2020; 27(6):e12628.
11. Hua Kc, Feng JT, Yang XG, Wang F, Zhang H, Yang L, et al. Assessment of the defatting efficacy of mechanical and chemical treatment for allograft cancellous bone and its effects

- on biomechanics properties of bone. *Orthop Surg.* 2020; 12(2):617-30.
12. Tran NM-p, Nguyen DT, Dai Luong T, Bui NH, Van Toi V, Nguyen T-H, et al. Decellularization of bovine cancellous bone for bone tissue engineering application. *International Conference on the Development of Biomedical Engineering in Vietnam*; 2018:139-42.
13. Emami A, Talaei-Khozani T, Vojdani Z, Zarei Fard N. Comparative assessment of the efficiency of various decellularization agents for bone tissue engineering. *J. Biomed. Mater. Res. Part B Appl. Biomater.* 2021; 109(1):19-32.
14. Rahman N, Khan R, Hussain T, Ahmed N. Investigation of the mechanism of gamma irradiation effect on bovine bone. *Cell Tissue Bank.* 2020; 21(2):249-56.
15. Mohaghegh S, Hosseini SF, Rad MR, Khojasteh A. 3D printed composite scaffolds in bone tissue engineering: a systematic review. *Curr Stem Cell Res Ther.* 2022; 17(7):648-709.
16. Raouf GA, Gashlan H, Khedr A, Hamedy S, Al-jabbri H. In vitro new biopolymer for bone grafting and bone cement. *Int. J. Curr. Res. Sci. Technol.* 2015; 4(2):46-55.
17. Nasr HF, Aichelmann-Reidy ME, Yukna RA. Bone and bone substitutes. *Periodontol* 2000. 1999; 19:74-86.
18. Harrell CR, Djonov V, Fellabaum C, Volarevic V. Risks of using sterilization by gamma radiation: the other side of the coin. *Int J Med Sci.* 2018; 15(3):274-9.
19. Akers MJ, Wright GE, Carlson KA. Sterility testing of antimicrobial-containing injectable solutions prepared in the pharmacy. *Am J Hosp Pharm.* 1991; 48(11):2414-8.
20. Nguyen H, Morgan DA, Forwood MR. Validation of 11 kGy as a radiation sterilization dose for frozen bone allografts. *J Arthroplasty.* 2011; 26(2):303-8.
21. Hilmy N, Febrida A, Basril A. Validation of radiation sterilization dose for lyophilized amnion and bone grafts. *Cell Tissue Bank.* 2000; 1(2):143-8.
22. Jain A, Jain R, Jain S. Quantitative Analysis of Reducing Sugars by 3, 5-Dinitrosalicylic Acid (DNSA Method). *Basic Techniques in Biochemistry, Microbiology and Molecular Biology.* Part of the Springer Protocols Handbooks, Humana, New York, NY. 2020; 181-3.
23. Rocheleau MJ, Titley M, Bolduc J. Measuring residual solvents in pharmaceutical samples using fast gas chromatography techniques. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2004; 805(1):77-86.
24. Mastovská K, Lehotay SJ. Evaluation of common organic solvents for gas chromatographic analysis and stability of multiclass pesticide residues. *J Chromatogr A.* 2004; 1040(2):259-72.
25. Desai AM, Andreae M, Mullen DG, Holl MM, Baker JR Jr. Acetonitrile shortage: Use of isopropanol as an alternative elution system for ultra/high performance liquid chromatography. *Anal Methods.* 2011; 3(1):56-8.

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