

INITIATION OF PROCESS STANDARDIZATION TO FABRICATE DEMINERALIZED BONE DIRECTED TOWARDS MAKING MATERIALS FOR BONE GRAFTING

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ABSTRACT

Background: Bone tissue, a vital component for self-healing. This inherent regenerative capacity enables bones to repair themselves following injury or damage. Understanding the mechanisms behind bone tissue self-healing is of great significance in the field of medical research and has implications for the development of novel therapeutic approaches. In this article, we explore the fundamental characteristics and mechanisms underlying the self-healing properties of bone tissue. In certain instances of trauma or pathological conditions resulting in bone defects, the natural healing process may be insufficient. Consequently, the implementation of bone transplant interventions becomes imperative for successful bone regeneration. The predominant origins of bone grafts encompass autologous, homologous, and heterologous sources. The allograft, a widely utilized bone grafting technique, has gained significant popularity, and is currently in high demand within the medical field. The current state of allograft utilization in our nation has proven insufficient in meeting the demands of patients. Henceforth, a method has been devised and standardized for the procurement of demineralized osseous tissue, a crucial investigation in light of the insufficiency of allografts in meeting the requirements of patients. **Objectives:** Initially, standardizing an efficient procedure to obtain demineralized bone sources from human skull fragments from three different inclusion methods. **Materials and Methods:** Using three different types of solutions to obtain demineralized bone from human skull tissue including solutions of Formic Acid (10%), Hydrochloric Acid (5%), a mixture of solution: Formic Acid (10%) - Acid Hydrochloric (5%) (ratio 1:1,v/v). Demineralized bones were evaluated by comparing the mineral content extracted from bone samples, demineralization time, and analysis of ions concentration in bone samples (including Ca and P) before and after demineralization. Besides, demineralized bones were histologically stained before and after demineralization to assess the bone structure. **Results:** The Ca and P extraction rates of this method are high and standard, about 98% and 93%, respectively. The demineralization process only takes about five days. Relative bone tissue retains most of the structure of compact bone tissue. **Conclusion:** The procedure that our team has established is the right one for obtaining demineralized bone as bone replacement material and applies to a wide variety of human bone tissue.

Keywords: Demineralized bone, demineralized solution, skull bone, bone grafting material.

I. INTRODUCTION

Bone replacement grafting is a frequently employed therapeutic approach to address instances of bone nonunion resulting from diseases or injuries that cause bone defects. Present-day grafting bones are also extremely diverse, including autologous human bone products, allogeneic bones (derived from animals), and allogeneic bones (derived from humans). While there exists a wide variety of bone types which are suitable for bone grafting, each possessing

distinct merits and demerits, a prevalent attribute is that the supply fails to satisfy the patient's requirements. As a result, a strategy under investigation and implementation is the production and integration of numerous varieties of replacement bones derived from diverse sample sources. Demineralized bone, which is obtained from allogeneic bone sources, is regarded as a highly effective alternative bone graft material, particularly well-suited for use in filling applications involving defects. Cavities of the bone in orthopedics and dentistry [1], [2]. One benefit of demineralized bone is that it originates from humans and contains biological factors, including cytokines, growth factors, and bone morphogenetic proteins, which can stimulate bone formation and accelerate the healing process [3], [4].

As a result, it is essential to conduct research and establish a standardized procedure for producing demineralized bone units in order to facilitate the development of numerous bone transplantation products and inspire greater confidence in clinicians. Selected for application as bone graft and infill materials to treat bone defects and restrict the use of patients' autologous bone grafts [5].

II. MATERIALS AND METHODS

2.1. Study design: descriptive experimental study

2.2. Research subjects

Skull bone fragments obtained from the Tissue Bank, Department of Histology – Embryology – Genetics, Pham Ngoc Thach University of Medicine, are deemed unsuitable for reattachment purposes as they have passed the regulatory preservation period. Preservation process at the Tissue Bank or fragments of skull bone from patients who have undergone re-surgery using alternative bone graft materials (these fragments of skull bone are utilized by the patient and are not intended for re-transplantation). consent to donating tissue for the benefit of scientific research). Ensuring adherence to regulations and processing protocols are integral components of the bone tissue collection and processing procedures at the Tissue Bank.

2.3. Research methods

Collect and assemble cranium fragments

At the Tissue Bank, human cranium bone samples are gathered and processed in accordance with established protocols, thereby guaranteeing the maintenance of contemporary standards. After defrosting the chosen bone fragments and scraping them free from soft tissue and tendons, they will be subjected to multiple washes with sterile physiological saline. Finally, they will be cleansed with sterile cool distilled water.

For demineralization, a total of 45 bone units were extracted from 5 standard cranium fragments. Each cranium fragment comprises nine bone units, with each bone unit measuring approximately 2x2 cm.



Figure 1. Bone samples are collected for demineralization. Bone samples were finely diced into bone units with an approximate dimensions of 2x2 cm

Implement demineralization

Three experimental groups comprised the experiment. The bone units were demineralized in a sequential manner using a 5% solution of hydrochloric acid, a 10% solution of formic acid, and a mixture of 5% hydrochloric acid and 10% formic acid in a 1:1 volume-to-volume ratio. Each experimental set was replicated five times, with three bone unit samples per experiment.

The demineralization procedure consists of the following steps:

- The bone units are submerged in a demineralization solution comprising formic acid, hydrochloric acid, and a mixture of hydrochloric acid and formic acid. The volume ratio of the solution to the sample is 20 times.

- The demineralization solution will be replaced in 24-hour intervals until the procedure reaches its conclusion.

- Before proceeding with each subsequent demineralization solution, a volume of 5 ml of the previous solution should be sucked in order to assess the demineralization process using a 1% v/v solution mixture of ammonium hydroxide and ammonium oxalate.

- To the solution mixture above, incorporate 5 ml of demineralization solution at a volumetric ratio of 1:2 (v/v). Demineralization is deemed to be accomplished when the appearance of the white precipitate ceases.

Subsequently, the bone samples will be rinsed once more with distilled water. Bone fragments should be neutralized for 15 minutes with a 5% ammonia solution.

- After being rinsed with distilled water until the pH approaches 7.0, the bone will be let naturally dry at room temperature.

- Units of bone will be freeze-dried, encapsulated, and sterilized by the use of 25 kGy of gamma radiation.

- Products containing demineralized bone are kept at ambient temperature.

Assess outcomes subsequent to demineralization.

Time of demineralization

The duration, measured in days, from the initiation to the conclusion of demineralization will be recorded for each sample. In addition, demineralization time is a criterion for determining which of the three demineralization methods is optimal.

Ca and P elemental content analysis.

Bone samples will be analyzed for the percentage content of the Ca element using the AOAC 999.11 method, and for the P element using the UV-Vis (P) analysis method, both prior to and following demineralization.

Histological assessment (H&E)

Bone samples should be histologically stained with H&E prior to demineralization. Conduct three additional histological examinations of three bone samples that have undergone demineralization using three distinct methods: demineralization with a 5% solution of hydrochloric acid, demineralization with a 5% solution of hydrochloric acid compounded with 10% formic acid in order to compare and contrast the histological structures of bone samples prior to and subsequent to demineralization across the three demineralization methods, these specimens are utilized.

III. RESULTS

3.1. Demineralization time

Following the completion of the investigation, the demineralization times of the experimental samples were determined and are presented in Table 1.

Table 1. Summary of demineralization time of 3 demineralization methods

	Sample-batch	Demineralization time (days)		
		Hydrochloric acid 5%	Hydrochloric acid 5% –	Formic acid 10%
1	S1-B1	5.0	7.0	12.0
2	S1-B2	5.0	7.0	12.0
3	S1-B3	4.0	6.0	12.0
4	S2-B1	3.0	4.0	9.0
5	S2-B2	4.0	6.0	10.0
6	S2-B3	4.0	5.0	9.0
7	S3-B1	3.0	4.0	9.0
8	S3-B2	4.0	5.0	12.0
9	S3-B3	3.0	4.0	8.0
10	S4-B1	3.0	5.0	12.0
11	S4-B2	4.0	5.0	12.0
12	S4-B3	4.0	5.0	10.0
13	S5-B1	3.0	5.0	12.0
14	S5-B2	4.0	5.0	12.0
15	S5-B3	4.0	5.0	12.0
Average		3.8	5.2	10.9

The findings indicate that the reduction technique utilizing 5% hydrochloric acid yields the most rapid reduction period, approximately four days. In contrast, the reduction process utilizing a combination of demineralization solutions necessitates approximately two and a half times the time as the reduction method utilizing formic acid (5.2 days versus 10.9 days).

3.2. Analysis of calcium and phosphorus content

Table 2. The results of the analysis of the content of elements Ca and P before and after demineralization

Sequence number	Samples/Analyses indicators	Ca (%)	P (%)	Ca (%)	P (%)	Ca (%)	P (%)
1	Undemineralized S1	10.70	2.32	10.70	2.32	10.70	2.32
Demineralization methods		Hydrochloric acid (5%)		Hydrochloric acid (5%) + Formic acid (10%)		Formic acid (10%)	
2	S1-B1	0.13	0.18	0.15	0.09	0.15	0.20
3	S1-B2	0.11	0.12	0.15	0.25	0.11	0.18
4	S1-B3	0.16	0.13	0.17	0.08	0.15	0.09
Average		0.13	0.14	0.16	0.14	0.14	0.16
% extract		98.75	93.82	98.54	93.97	98.72	93.25

The results of elemental content analyses for Ca and P indicate that extraction rates are both quite high. The two primary structural components that comprise bone tissue are as follows. Analysis reveals that all three techniques yield remarkably high Ca (approximately 98%) and P (approximately 93%) extraction rates. The outcomes of this

extraction demonstrate the efficacy of the demineralization which compounds in demineralizing bone tissue.

3.3. Evaluation of histological results

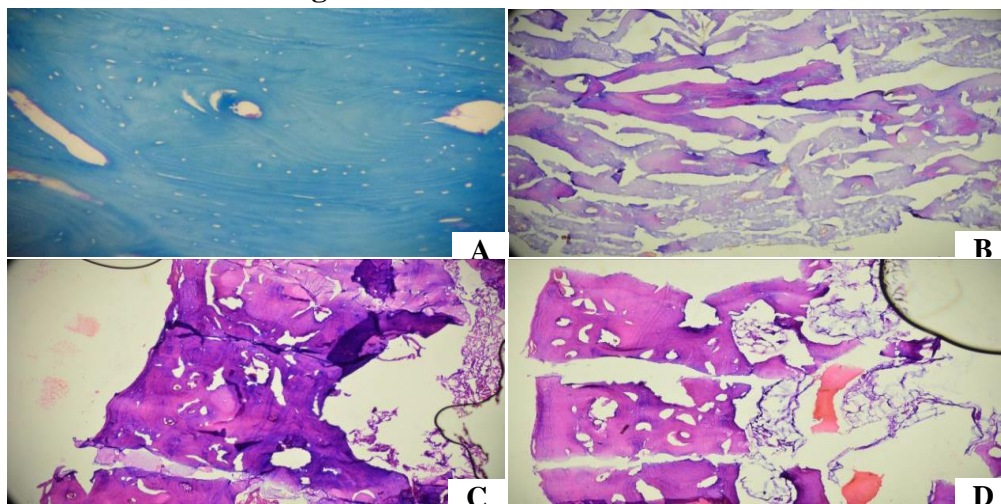


Figure 2. Results of histological staining. (A). Unmineralized bone sample (10X). (B). Bone samples were demineralized with hydrochloric acid solution (5%) (10X). (C). Bone samples were demineralized with a mixture of hydrochloric acid (5%) – formic acid (10%) solution (10X). (D). Bone samples were demineralized with formic acid (10%) (10X)

Based on the histological evaluation results, bone samples demineralized with hydrochloric acid did not preserve the structure the best among the three methods. Bone tissue was separated into discrete bone trabeculae, no longer having the structure of compact bone tissue when compared to the control sample (Figure 2.A). Meanwhile, demineralization with a solution mixture gives the best results in preserving tissue structure when compared among the three methods (Figure 2.C). Relative bone tissue retained most of the structure of compact bone tissue when compared to reduction with formic acid (10%) (Figure 2.D) and hydrochloric acid (5%) (Figure 2.B).

IV. DISCUSSION

Presently, a wide array of techniques exists for demineralizing bone tissue, encompassing biological, chemical, and mechanical approaches [6], [7]. While numerous techniques exist, they all share the common goal of achieving efficient demineralization (primarily by extracting Ca and P content) and preserving the structure of bone tissue without causing decomposition once demineralization is finished.

Chemical demineralization techniques, such as nitric acid (5%-10%) and hydrochloric acid (5%-10%), have the benefit of a short demineralization duration but the following drawbacks: Frequently, the structure of bone tissue is unstable. remains unaltered despite demineralization-induced structural degradation [3], [6], [7], [8]. Additionally, chemical demineralization techniques, including 5%-10% formic acid, 5% trichloroacetic acid, 10% Morse solution of sodium citrate and 20% formic acid, 10% EDTA (pH 7.4, 10%), 10% EDTA/TRIS-HCl solution (pH 7.4), and 10% EDTA/Glycerol solution (pH 7.4, 0.07%), preserve the structure of healthy bone tissue; however, they require a longer period of time to demineralize [3], [5], [6], [7], [8].

As a result, the research team combined hydrochloric acid and formic acid [3], two

chemicals frequently employed for the rapid demineralization of bone tissue [3], [6], in order to aid in the preservation of bone structure. bone tissue structure in the course of demineralization. Our team's research indicates that demineralization can be accomplished more efficiently by combining the chemicals hydrochloric acid and formic acid, as opposed to using each chemical individually. This result is also comparable to a number of studies [3], [6], [9] and preliminary research conducted by our group [10], [11].

The Ca and P extraction rates of the combination of two demineralizing chemicals—hydrochloric acid and formic acid—are quite high and standard, about 98% and 93%, respectively. Additionally, the demineralization process only takes about five days. Relative bone tissue retains most of the structure of compact bone tissue. Therefore, our group chose this combination as the standardized procedure for bone tissue demineralization.

V. CONCLUSION

The study concluded that employing a mixture of hydrochloric acid (5%) + formic acid (10%) is a significant method in establishing a standardized protocol for bone demineralization. It is vital to prioritize research on bone tissue and its suitability for the prevailing conditions in Vietnam. This research will aid in the development of demineralized bone products that can effectively meet the increasing demands of patients. This endeavor holds great promise in addressing the evolving healthcare needs of the population.

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