ORIGINAL ARTICLE

The Effects of Decellularization on Mechanical Properties of The Bone Using Sonication Method

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ABSTRACT

Introduction: Bone tissue engineering generated implantable bone substitutes for non-healing fractures. Decellularization procedures were utilized to create implantable bone substitutes by removing cellular material and retaining the extracellular matrix (ECM). However, traditional chemical decellularization methods could negatively impact bone mechanical properties. This study aimed to investigate the effects of sonication-based decellularization on bone mechanical characteristics. **Materials and Methods:** To decellularize bovine bone, the technology of sonication (20 kHz & 40 kHz) was utilized. The compression test of the bone was then carried out to evaluate the stiffness of the bone samples using the Instron 8874 to investigate the effect of sonication-assisted decellularization on bone mechanical characteristics. The combination of Scanning Electron Microscopy (SEM) and Water Contact Angle (WCA) provided important insights into the success of decellularization and its impact on the surface structure and contact angle of decellularized bone. **Results:** The findings showed that sonication assisted decellularization significantly increased bone mechanical properties, particularly its stiffness. The stiffness of the decellularized bone (754.069 ± 367.580 MPa) group was significantly higher than that of the control bone (176.951 ± 65.272 MPa) group. Additionally, the surface characteristics of the decellularized bone became more hydrophobic by sonication-based decellularization, possibly due to surface lipid residues that may cause the blockage of the bone's porous surface. **Conclusion:** The findings of this study may offer insights into potential applications for regenerative medicine and tissue engineering, as well as contributes to the development of safer and more effective bone grafts by understanding how this method affects bone tissue.
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INTRODUCTION

Bone loss can be due to a variety of causes, including trauma, diseases, and aging, resulting in deterioration of its mechanical properties. Severe cases of bone defects have poor healing capability, hence why obtaining biomaterials that mimic natural bone's mechanical properties via tissue engineering is critical for this application (1, 2). Nonetheless, bone is a complex tissue made up of collagens and minerals, which resides within the structurally organized network called the extracellular matrix (ECM). It contains functional structures, which involves the complex establishment of proteins and matrix components such as collagen, proteoglycans, fibronectin, laminin, elastin, and other glycoproteins (3-6). Decellularization is a promising method for removing cells from tissues while retaining the ECM's structure and components (1). To obtain decellularized bone, various decellularization methods, including chemical and physical methods, have been developed. Chemical agents, especially detergents such as sodium dodecyl sulfate (SDS) and Triton X-100, were commonly utilized to decellularize tissues (7, 8). However, the use of SDS at various concentrations (0.1 to 1.0%) have been shown to have detrimental effects on the collagen fibers of the extracellular matrix, in addition to the increased SDS residues with higher concentration of the detergent (9). Therefore, it is important to minimize the effects of these residual chemicals within the tissue structure, through the implementation of physical methods. Physical methods comprise of perfusion and agitation, pressure, supercritical fluid, and sonication (10). Nonetheless, despite its potential, the influence of sonication-assisted decellularization on the mechanical characteristics of bone has received insufficient attention (11).

Sonication-assisted decellularization employs sound waves to generate shear stresses, hence enabling decellularization (12). The use of sonication in bone decellularization is a novel method, but its effects on the critical mechanical characteristics of bone tissue are not entirely understood. Previous studies where decellularization using sonication was employed also applied non-ionic detergent such as sodium dodecyl sulfate (SDS), with varying sonicator power and frequencies. As a result, there is a knowledge gap on the effects of sonication towards the structural integrity, surface quality, and mechanical behavior of the bone. Hence, elucidating the changes in mechanical properties caused by sonication is critical to ensuring that the resulting decellularized bone retains the structural and functional properties required for successful bone replacements in tissue engineering and regenerative medicine applications (13). Furthermore, understanding the effects of sonication on bone decellularization will help to improve the decellularization process itself, ensuring that the ECM scaffold is ideally prepared for subsequent cell repopulation and tissue regeneration (13). In light of the lack of research, this study implemented sonication to evaluate the impact of decellularization on the mechanical characteristics of bone. The study aims to analyze changes in the mechanical behavior of bovine bone samples subjected to a highly controlled sonication method, namely its ultimate strength. The findings of this work will offer insight on the viability of sonication-assisted decellularization as a method for producing functional bone substitutes, as well as provide important insights into the fields of bone tissue engineering and regenerative medicine (14).

MATERIALS AND METHODS

Samples preparation

The samples of cow femoral were obtained from a carcass acquired from a butcher at the NSK Grocer, U Mall, Skudai, Johor, Malaysia. To prepare the bone samples for compression testing, the samples were sliced into 4 mm by 4 mm pieces. The specimen geometry is considered suitable for mechanical testing, as long as the aspect ratio between height and width is withing 1 and 2 (15). The cutting was done with a Stanley 300 mm Hacksaw and a 24 Teeth Per Inch (TPI) blade. Following preparation, the bone pieces were cleaned in PBS, pH 7.4, at room temperature until the bone samples were visibly whiter. This process guaranteed that any pollutants or impurities in the form of blood and fat residues on the bone surface and within the trabecula were removed properly, allowing for a clean and standardised testing environment. Compression tests required smaller components. To provide reliable and consistent results during the subsequent experimental procedures, the bone samples were properly cleaned in PBS.

Control groups

In this study, a specific area of the bone was examined. The bone specimens were kept at -20°C until the samples were processed two days later. The mechanical properties of the bone in its natural form were studied prior to using the decellularization process. Before any alterations were done, this process created a baseline understanding of the bone's original features. The processed sample was derived from a bone sample that had previously been treated with PBS. Decellularized bone were compared to its initial state by using the PBS-treated sample for any alterations.

Decellularization Protocols

The method begins by defrosting the bone samples, which were kept at -20°C. This step made handling easier and prepared the grafts for further cleaning treatments. The defrosted bone grafts were then submerged in 10 mL of distilled water at room temperature. To begin decellularization, the bone samples and distilled water were sonicated at a frequency of 40 kHz for 30 minutes using a Sonicator (Q700, QSonica, United States of America (USA)) with touch screen control (16, 17).

Following the initial sonication, the fluid containing the bone samples was rinsed with 10 ml of PBS and 5 ml of distilled water until clear. This rinsing step was critical for washing away any remaining cellular remains, sonication fluid, and other debris, which improved the decellularization process. The bone samples were sonicated twice more to guarantee complete removal of DNA and cellular debris. Each cycle consists of 20 minutes of sonication at a frequency of 20 kHz. Following sonication cycles support the decellularization process by gradually removing any remaining cellular components and achieving a totally decellularized matrix (16, 17).

Bone samples were immersed in the washing solution and gently stirred, by shaking or spinning, to allow the washing fluid to come into touch with the surface of the bone and remove any leftover debris or detergents. The bone samples were then moved to a new solution for additional rinsing, guaranteeing thorough cleanup of dirt and contaminants. Several washes were carried out, ranging in quantity and length according to the particular decellularization method and desired level of cleanliness. In order to completely remove cellular debris and produce a clean, acellular bone scaffold suited for various applications in tissue engineering and regenerative medicine, the sonication-assisted washing procedure is essential (18).

Mechanical Testing

The mechanical properties of bone tissue were investigated in this study using the Instron 8874 (Illinois Tool Works Inc., USA) testing equipment. Compression testing is critical for measuring material strength and ductility, and it was employed in this study to assess the

mechanical properties of bone tissue before and after decellularization. Treated bone samples were placed in the Instron 8874 testing machine to study the influence of decellularization on the mechanical properties of bone tissue. The bone tissue sample was squeezed between two plates during the compression test, and a constant rate of deformation of 1 mm per minute was used. The machine generated data by measuring the ensuing deformation, allowing crucial mechanical qualities, which involves compressive strength, modulus of elasticity, and stiffness. Stiffness was obtained via the resulting load-displacement curve of tested bone samples. Understanding how decellularization influences the mechanical properties of bone tissue is critical for optimizing the decellularization process and ensuring that the resulting tissue scaffold has the mechanical properties required for tissue engineering applications.

Decellularization Assessments

Furthermore, this study aimed to perform a thorough evaluation of the decellularization process by utilising two critical assessment techniques such as SEM and WCA testing. These analyses aimed to confirm the success of the decellularization method and provided critical insights into the surface features and structural alterations of the resultant bone scaffold.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy (Hitachi TM3000 SEM, Japan) is a sophisticated imaging technology that enables for the visualization of the microstructure of the bone sample. The bone samples were cleaned and fixed on to a coin. Then, the samples were gradually dehydrated by immersing it in increasing concentrations of 70% (v/v) ethanol (Th. Geyer GmbH & Co. KG, Renningen, Germany) before dried for 4 hours in room temperature. To improve imaging quality in SEM, a small layer of platinum or another conductive substance can be applied to the bone sample. Platinum coating increases sample conductivity, decreases charging effects, and improves image sharpness. Using conductive adhesive or carbon tape on topic of a coin, the bone sample was attached to a sample holder or stub. It is critical to ensure that the sample and the holder have good electrical conductivity. The sample holder has been inserted into the SEM chamber and the image parameters were adjusted (x100, x250, x1k, x2k) magnifications, according to the SEM equipment (19, 20).

Water Contact Angle (WCA)

A measurement of how a water droplet spreads or beads up on a surface is the WCA. It can shed light on a material's wettability and other surface characteristics. The sonication technique was utilized for decellularization to create both the decellularized sample and the control sample. Upon completion of the process, the samples were cleaned and thoroughly dried. The bone sample was placed inside a drying oven at a designated temperature (60°C) for a suitable period (24 hours). This step aids in eliminating moisture from the sample. Employing a small needle, a tiny droplet of distilled water was placed onto both the decellularized bone and control sample's surface. An image of the droplet on the sample surface was captured using a high-resolution camera or a specialized device like a contact angle goniometer. The picture was clear and well-focused. To enhance accuracy, it was recommended to replicate the process and multiple water droplets were measured on different areas of the samples. This accounts for any variations or uniformity concerns on the surface. On the decellularized bone and control bone, one could measure the water contact angle to learn more about the surface properties and wettability of the substance. Understanding how the decellularization process has impacted the sample's surface qualities using this information could be helpful (21-23).

Statistical Analysis

The paired t-test is used when comparing measurements using the Microsoft Excel taken from the control and decellularized bone samples, before and after sonication process. The paired t-test produce a t-value and a corresponding p-value. The t-value represents the magnitude of the difference relative to the variation within the pairs, while the p-value indicates the probability of obtaining such a difference if the null hypothesis of no difference is true. A small p-value (usually less than 0.05) will indicate statistical significance, suggesting that there is a significant difference between the paired measurements.

ETHICAL CLEARANCE

This study was exempted from ethical review for animal research by the UTM Research Ethics Committee, Approval No.: UTMREC-2024-E2.

RESULTS

Decellularization using Sonication method

The decellularization process entails submerging the bone in a solution and sonicating it, as seen in Figure 1 (a) & (b). Small bubbles form and burst as a result of the sonicator's sound waves, causing considerable local pressure fluctuations that make it easier to separate the cells from the bone matrix. Red blood cells and other biological elements separate from the bone matrix as a result of this process, reducing the redness of the ECM. Finally, cellular contents were released, and cell membranes were broken down during sonication, which helps to clean bone tissue. Figure 2 (a) & (b) represents the effect of the decellularization technique on bone samples. The control image on the left illustrates the bone's natural state just before decellularization, which is crucial to the study's goals. The decellularized samples are shown on the right side of the image, where the samples were decellularized and became white since

the ECM has been eliminated. The ECM, which is made up of proteins and other elements that give the tissue structural support and organization, affected the colour of the bone naturally. However, cellular elements and deoxyribonucleic acid (DNA), including those from the cow from whom the samples were taken, were effectively eliminated by successful decellularization (19).

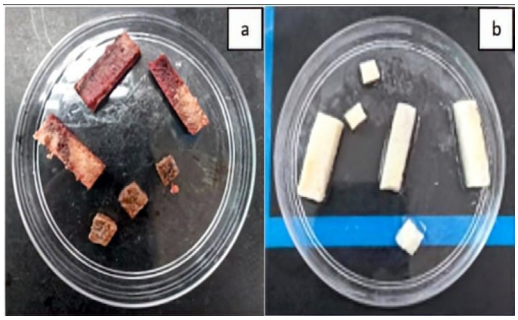

Fig. 1: Images of (a) Control and (b) Decellularized samples.

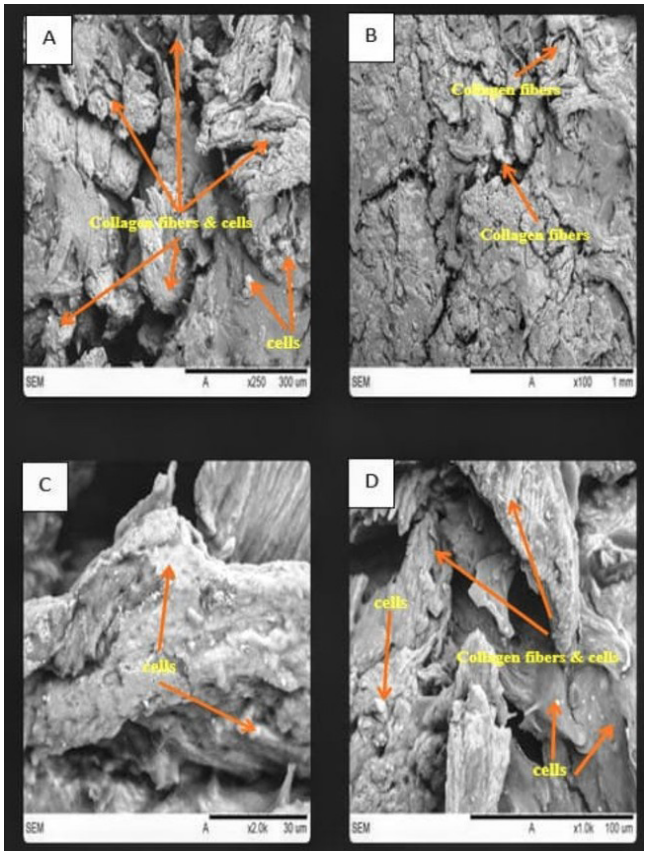

Fig. 2: SEM analysis of control bone samples [A: x250, B: x100, C: x2.0k, D: x1.0k] magnifications

Mechanical testing

The stiffness of the decellularized bone samples (in MPa) were compared to those of the control bone samples as shown in Table I. Notably, across all four tested samples (n = 4), the decellularized bone consistently demonstrated greater stiffness values than the control bone samples. This finding implies that the sonicator-assisted decellularization procedure may have altered the mechanical structure of the bone. Furthermore, from the results, it is evident that all decellularized bone samples (754.069 ± 367.580 MPa) exhibit significantly greater stiffness compared to the average value of the control bone samples (176.951 ± 65.272 MPa. When comparing the average stiffness findings of the decellularized bone samples using a sonicator with the control bone samples, the decellularized bone consistently exhibits higher stiffness (24, 25). This result indicated sonication procedure could lead to changes in the collagen and elastin structure in the bone samples. This is because collagen and elastin largely responsible for the mechanical properties of the bone (6). However, it is crucial to recognize that these conclusions are based on the individual samples provided, and further research and testing are necessary to develop more generalized and robust results (24, 25).

Table I: Stiffness and contact angle measurements of control and decellularized bone

Parameters	Control bone, n = 4	Decellularized bone, n = 4
Stiffness (MPa)	176.951 ± 65.272	754.069 ± 367.580
Contact angle	91.97 ± 16.42°	105.16 ± 22.96°

Scanning Electron Microscopy (SEM)

The control bone sample's microstructure was examined using the SEM images, as shown by the arrows in Figure 2. The structure and make-up of the bone matrix can be understood better thanks to these images. A more thorough examination of the SEM images reveals a well arranged and connected structure within the bone matrix. Collagen fibres that have been mineralized and are hierarchically densely packed make up the majority of the matrix (26). The distinctive lamellar structure of bone tissue is a result of this structural configuration. The surface of the control bone samples has a distinctively rough texture, which is an intriguing finding from the images obtained from the SEM. Collagen fibres, mineralized nodules, and surface flaws are a few examples of the microscale and nanoscale properties that contribute to this roughness (26). These characteristics contribute to the image' overall rough appearance.

Additionally, compared to the decellularized bone, the SEM images show the presence of a meat-like structure with several layers which is the collection of cells, indicating a more complex organisation. The control bone sample's integrity and intricate composition are further demonstrated by its complexity. Overall, the SEM images showed the microstructure of the control bone sample. These images presented the interconnection and organization of the bone matrix, as well as how its surface was layered and had rough texture. These results help us comprehend the complexity and composition of the control bone sample (26).

The decellularized bone sample is shown with arrows in Figure 3, and these images from the SEM shed light

on the bone's microstructure. These images show how the decellularization procedure successfully eliminated cellular elements, leaving a distinct structure with fewer cellular components visible than in the control bone samples. Upon closer inspection, the decellularized bone samples' SEM photos reveal a clearer, less thick appearance. This is because cells' internal parts are eliminated during the decellularization procedure. Due to the absence of cell borders or nuclei, the surface of the decellularized bone samples could appear smoother (27). The voids between collagen fibrils may also be more noticeable in the decellularized bone samples, pointing to a less densely packed structure. The absence of the cellular material that would typically fill these holes is responsible for this. The SEM images might also demonstrate a more uniform and homogeneous look, pointing to the efficient removal of cellular trash and leftovers (28).

In conclusion, the SEM images of the decellularized bone samples confirm that cells and cellular components were successfully removed by the decellularization procedure. The images demonstrate structural alterations that took place during decellularization, including a more uniform look, a decrease in cellular density, an increase in the visibility of collagen fibril spacing, and a cleaner appearance (29). Regarding to a previous study, after the decellularization procedure, neither the structural integrity nor the pore wall damage changed. The middle portion had larger and more directed pores than the periphery, with pore sizes ranging from 20 to 150 m (29). These results show that decellularization is effective in producing a bone matrix devoid of cells, which is essential for later tissue engineering or regenerative medicine applications (30).

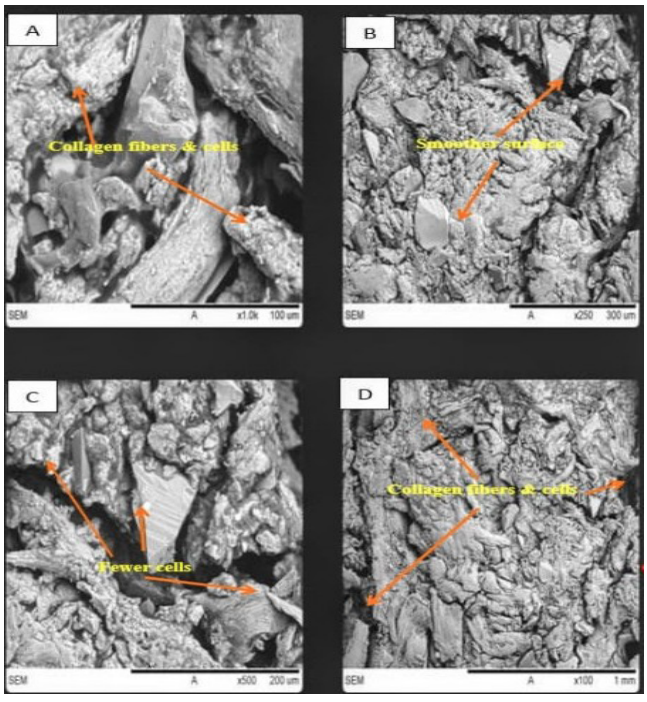

Fig. 3: SEM analysis of decellularized bone samples [A: x1.0k, B: x250, C: x500, D: x100] magnifications.

Water Contact Angle (WCA)

The decellularized bone sample in Table I displays the water contact angle of the decellularized bone sample's mean WCA temperature is 105.16°, with a standard deviation of 22.96°. Generally speaking, a surface is called hydrophilic if its water contact angle is less than 90°, indicating a strong affinity for water and effective wetting (31). A surface that has a higher water contact angle (more than 90° is said to be hydrophobic, which denotes a poorer affinity for water and poor wetting. A mean water contact angle of 91.97° is seen in the control bone sample, which is near to the hydrophilic-hydrophobic barrier (31). The control bone sample may be slightly hydrophilic, according to the comparatively lower mean angle. On the other hand, the decellularized bone sample has a greater mean water contact angle of 105.16°. This shows that, in comparison to the control bone sample, the decellularization procedure has enhanced the hydrophobicity of the bone surface (31).

In comparison to the control bone sample, the decellularized bone sample had a greater mean water contact angle and a wider range of angles. The decellularized bone sample's higher mean angle and greater variability point to a surface that is more hydrophobic. According to the data, the decellularized bone samples were relatively more hydrophobic than the control bone sample. The decellularization procedure might have changed the surface characteristics, which might have an impact on how well the bone interacts with water or other liquids (32). These results provide important insight into the hydrophilicity or hydrophobicity of decellularized bone samples, as well as the behaviour and prospective uses of such samples (32).

DISCUSSION

A further possibility for a new decellularization treatment is a study on the sonication decellularization approach. The study investigated the occurrence of cavitation caused by ultrasonic power, detergent with or without saline. Previous investigations have revealed sonication treatment as an alternate decellularization approach. Sonication treatment is distinguished by its disruption of cell membranes, homogeneous treatment, and quick treatment period (33). The effectiveness of sonication decellularization and its connections with ultrasonic intensity have been the focus of earlier research. The ideal circumstances for producing biological scaffolds with preserved ECM and improved decellularization efficiency were discovered in this study (12). Decellularization's major objective is to create a scaffold that preserves tissue structure while removing cellular material by removing the ECM (19). The decellularized samples in the SEM images appear white, indicating that the ECM has been successfully removed, highlighting the substantial difference that develops during the decellularization process. The

method was successful in the removal of native cells and other cellular components. The blood vessels that supply oxygen and nourishment to the living cells found within bone's tissue are what give bone its characteristic red colour. Red and white blood cells can both be found in bone marrow, which is located in the spongy regions of bone (34).

Previous studies have shown no significant changes to the bone's stiffness, where sonication was applied to the meniscus and aorta samples, with added SDS (12, 35). Additionally, Nie et al. contended that the mechanical properties of the matrix were not considerably changed by the decellularization procedure, and the bone's mechanical strength was adequately preserved (36). However, the stiffness of samples in this study increased, which suggest changes in the bone architecture after sonication. The mechanical properties of native arteries are mainly determined from ECM proteins, where collagen provides tensile stiffness, and elastin contributes to elasticity and compliance. The decellularized tissue prepared using sonication treatment performed at 15 W had increased stiffness, which could be attributed to the loosening of tissues due to cell removal and the voids created by absence of collagen fibers. This can be associated with an increase in stiffness, as also suggested by a previous study (12). Nonetheless, it's crucial to note that the effects of decellularization on bone mechanical properties might vary depending on a variety of parameters, including decellularization method, time, and the different parts of the bone tissue being examined (37-39).

The SEM images for control and sonicated bone samples revealed differences in the presence of cells and surface of the collagen fibres. The control bone samples exhibited higher coincidences of cells within the bone structure (Figure 2). Meanwhile, the sonicated bone samples elicited visibly less cells with smoother collagen fibre's surface. These results could indicate the disruptive effect of sonication to the cellular membranes, which lead to cell removal from the structure (12). Similarly, in a prior study using bone allografts as samples, sonication-processed allografts shown a more effective decellularization than chemically processed bone allografts. It was observed that the SEM images of the sonicated bone grafts revealed rough surface and clearer trabecular network with hollow marrow cavities (40).

As was previously studied, the contact angle reveals the material's surface's wettability and hence its hydrophilic or hydrophobic properties. A hydrophobic surface is generally indicated by a contact angle more than 90°, while a hydrophilic surface is indicated by a contact angle value less than 90° (39). Hence, the sonicated bone samples with contact angle of 105.16 ± 22.96° indicate more hydrophobic surface, compared to the control bone samples at 91.97 ± 16.42° (Table I). However, prior studies reported that hydrophilic surface of the tissue led to better cell attachment and adhesion (41, 42). The increased hydrophobicity of the samples might be due to lipid residues from incomplete delipidation. Delipidation of the bone samples might not occur properly due to the lack of delipidation protocol, leaving fat residues on the bone surface. The hydrophobic fat residues also resulted to the blockage of the porous surface, which led to increased hydrophobicity (43, 44). Therefore, further study in improving the removal of surface lipids for sonicated samples could be developed, such as using ethanol treatment or lipase (36).

While current study is concerned with the decellularization of bone via sonication and its consequences on mechanical characteristics. The ECM was successfully retained when cellular components were removed. Compression experiments revealed that decellularized bone had higher stiffness and compression strengths than control bone samples. Sonication have been shown to remove cells effectively and form voids on the surface, which may improve cell adhesion (45). When compared to the control bone, the decellularized bone was more hydrophobic. The findings on hydrophilicity differ from those found in prior studies on mechanical strength and ECM preservation (46). Overall, the findings of this study have the potential to have a substantial impact on the development of decellularized bone for tissue engineering and regenerative medicine applications, eventually leading to better treatment choices for patients with non-healing fractures and bone abnormalities (47).

CONCLUSION

The findings of the study could have implications for tissue engineering because understanding the mechanical properties of decellularized bone can aid in the development of biomaterials with similar properties. The sonication method considerably improved the mechanical properties of decellularized bone. Higher compression test findings in decellularized samples offered evidence of this. SEM examinations showed additional evidence of effective removal of cellular components and changes to the microstructure and surface characteristics of the decellularized bone. The findings of this study may offer insights into potential applications for regenerative medicine and tissue engineering, as well as contributes to the development of safer and more effective bone grafts by understanding how this method affects bone tissue.

Future research should aim to improve the decellularization process parameters, include a wider variety of mechanical tests, increase the sample size, assess in vivo performance, and evaluate the applicability of the findings to various tissue types and decellularization techniques in order to address these limitations. This will advance the creation of superior

tissue scaffolds for a variety of regenerative medicine applications and contribute to a more thorough understanding of decellularization methods.

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