# Cell Removal from Bovine Spongy Bone Used as Bone Replacements: SDS &TritonX-100 Comparison

**Abstract**

**Background:** Preserving the biological structure of cancellous bone in its natural state may enable it to serve as an appropriate scaffold for successful bone tissue engineering. Additionally, it is critical to eliminate cells from its bed to increase biocompatibility and reduce immunological responses.

**Methods:** Chemical methods were used to decellularize three-dimensional scaffolds constructed from spongy calf pelvic bone in this study. To this end, bone samples were degreased, and then their cells were removed using a chemical method (sodium dodecyl sulfate (SDS) and TritonX-100 at varying concentrations). Hematoxylin and eosin staining, trichrome staining, and an optical microscope were used to characterize the samples. Finally, to ensure that the scaffold was free of toxic substances, a cell toxicity test was performed.

**Results:** The results indicate that decellularized samples containing 2% TritonX-100 and a combined solution containing 3% TritonX-100 and 4% SDS (T3S4) can be used to replace damaged cancellous bone tissue. The results indicated that calf pelvic spongy bone tissue could be used as a scaffold for bone tissue engineering when decellularized with SDS and Triton x-100 chemical solutions. Natural bone tissue, which retains collagen fibers and contains porosity, can provide an ideal environment for tissue regeneration.

**Conclusion:** The findings indicate that T3S4-acellular bone tissue could be further evaluated as a natural scaffold for bone tissue engineering and restorative medicine.

**Keywords:** Bone tissue, Xenograft, Tissue Scaffolds, Bone Substitute, Cell Engineering

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## Introduction

Each year, a significant amount of money is spent on hard tissue replacements[1]. A bone graft is one of the most frequently performed procedures. Despite its limitations, including limited resources, chronic pain, and increased surgical time, an autograft is considered the most appropriate tissue alternative[2,3], and autologous bone grafting is still the gold-standard technique for bone filling for various reasons[4]. However, this approach has some drawbacks, including donor site complications, surgical difficulty, and persistent postoperative pain[5].

An allograft is another possibility but has additional drawbacks, including a potentially dangerous, exciting host body immune response and the transmission of microorganisms from the cadaver to transplant recipients[6]. Due to the physiological and biomechanical similarities between large animals, sheep, and bovine with humans, this provides a convenient field of application for Xenograft transplantation[7,8]. Bovine cancellous bone exhibits characteristics similar to human bone, which has attracted researchers as a suitable biomaterial for orthopedic surgery[9]. Recent biomaterials may also be used as scaffolds to provide a suitable environment for cell culture without imposing any restrictions on bone transplantation. Numerous studies have been conducted recently on maintaining the ECM of a biological scaffold[10].

Decellularization is necessary for autogenous cell seeding regardless of bone transplantation restrictions for removing cells from organs without damaging the ECM[11,12].

Thus, the ECM acts as a scaffold for tissue-resident cells to attach, communicate, and interact, thereby regulating cell dynamics and behavior plus contributing to the maintenance of tissue-specific cell phenotypes and functions. The ECM's properties have been extensively investigated in tissue engineering and regenerative medicine research to restore function to damaged or dysfunctional tissues[13].

In the last decade, tissue decellularization has become a standard technique for obtaining a decellularized extracellular matrix (DCEM)[14]. Thus, a biomimetic mineral matrix that increases osteoinductivity and osteoconductivity is required for osteoblast proliferation and differentiation[15]. The osteoconductive properties of these matrixes are conferred by providing a framework for cell proliferation and bone formation following demineralization. Additionally, the osteoinductive property of these matrixes is primarily determined by the remaining growth factors, which are inversely related to the methods of preparation[16]. Among the various protocols for tissue-specific decellularization, chemical and enzymatic techniques are the most effective. Physical techniques may cause damage to the matrix, whereas chemical techniques may alter the ECM's chemical composition[17-19]. As a result, establishing the decellularization protocol is critical within each specific approach.

Detergents are chemical agents that are used to solubilize and dissociate cell membranes. Triton X-100 is the most frequently used of these detergents in decellularization processes. It explicitly targets lipid–lipid and lipid-protein interactions but leaves protein interaction intact[20,21]. It is beneficial in tissues where the primary matrix components are proteins. Although it is an effective detergent for removing cells from various tissues, it is generally avoided in tissues containing glycosaminoglycans (GAGs) as a significant matrix component.

Along with Triton X-100, sodium dodecyl sulfate (SDS) is the other most frequently used decellularization detergent. SDS is another ionic detergent with the $C_{12}H_{25}NaO_4S$ formula. Due to the external and nuclear membranes, proteins are also denatured, resulting in a change in the matrix structure[22,23]. Accordingly, short-term SDS treatment is the most frequently used method to minimize potential damage to proteins and matrix structure[24].

The effect of SDS and TritonX-100 on the decellularization of selected tissues has been previously investigated in several studies[25-27]. Seddon et al.[28] demonstrated that ionic detergents alter the nature of proteins by disrupting protein interactions. In comparison, Schaner et al.[29] concluded that SDS is an appropriate detergent for cell removal from biological vascular structures in the context of vessel tissue engineering. The study concluded that it did not affect the morphology or resistance of the tissue's ECM. Additionally, Lumpkins et al.[30] demonstrated that SDS is more suitable for decellularizing temporomandibular joint disc tissue than TritonX-100 or acetone. In this study, cells were removed from the spongy bone of a bovine hip using chemical ionic (SDS) and nonionic (TritonX-100) materials, as well as a combination of the two. The findings may be used to repair bone defects.

## Methods

*2.1. Fat removal*

From spongy sections of the bovine pelvis, cylindrical bone samples with a diameter of 5mm and a height of 10mm were cut. They were initially washed with distilled water. They were then immersed in a 5% hydrogen peroxide solution for 45 minutes. Afterward, samples were immersed in diethyl ether for 48 hours and then washed with 70% ethanol for 1 hour. After degreasing, bone samples were washed five times with distilled water. They were kept in distilled water for 1 hour until the decellularization stage began.

*2.2. Decellularization*

The fat-removed bone samples were divided into three groups: the first group was immersed in SDS for 1 hour at 25°C with a concentration of 2%, 2.5%, and 3%, designated S2, S2.5, and S3. The second group was immersed in SDS for 1 hour at 25°C with a concentration of 2%, 2.5%, and 3%,

designated S2, S2.5, and S3, respectively. The second group samples were immersed in TritonX-100 at the same concentration for 8 hours at a temperature of 25°C. These samples were designated T2, T2.5, and T3. The third group immersed samples in a solution of SDS and TritonX-100, as indicated in Table 1. Following decellularization, all nine samples were washed with phosphate-buffered solution (PBS) for 30 minutes, followed by 75% ethanol for 1-2 minutes to remove SDS. Following that, sterile distilled water was used to neutralize the ethanol effect. Moreover, the samples were sterilized using a typical saline solution. Figure 1 illustrates the stages of decellularization sample preparation graphically.

**Table 1**. Decellularized samples with SDS and TritonX-100

| Type | Stage 1 | Stage 2 | Stage 3 |
|---|---|---|---|
| S3T2 | Immersion in SDS 3% for 3hours | Washing and sterilizing | Immersion in TritonX-100 2% for 5hours |
| S2,5T4 | Immersion in SDS 2,5% for 3hours | " | Immersion in TritonX-100 4% for 3hours |
| T3S4 | Immersion in TritonX-100 3% for 4hours | " | Immersion in SDS 4% for 2hours |

*2.3. Characterization*

After preparation, the samples were stained with hematoxylin-eosin (H&E) and trichrome. H&E staining and trichrome were used as a specific dye to determine the collagen content of ECM scaffolds to demonstrate the decellularization of spongy bone tissue. Finally, a cytotoxicity test was performed to ensure the scaffold was free of toxic substances. The samples used in the recent test were sterilized with 70% ethanol. They were then placed in a container with six cells (each sample was placed separately in one cell), with one of these cells serving as the control.

After inserting samples into one of these cells, 5mL of L929 fibroblast cell suspension (at a 410 4cell/ml concentration) was purified and placed in an incubator. Samples were then examined under an inverted microscope 24 and 47 hours after incubation (Nikon TE-100).

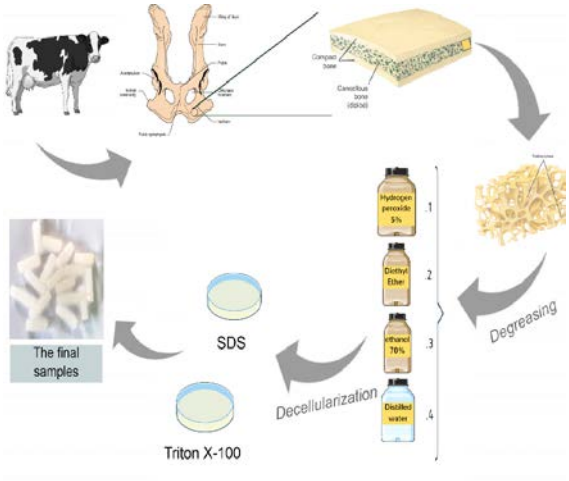


**Fig. 1. Graphical abstract of the preparation stages of decellularized samples**

## Results

Figure 2 illustrates the histopathology and trichrome staining of spongy bone tissue from a bovine hip before decellularization. Concerning the histology study, it was specified that after decellularization (Fig. 3), cell removal with SDS at 2.5% and 3% concentrations was greater than at a 2% concentration. However, this cell removal and purification process destroyed the structure, which is non-optimal for scaffold provision (Fig. 3A-C). Comparing decellularized samples to Triton X-100 solution revealed that a 2% concentration produced the best results (comparing Figures 3D-F). Comparing the consolidated method of the two chemical solutions to Figure (3G-I) revealed that the T3S4 sample contained no cellular debris.

Nevertheless, cells were poorly removed at higher concentrations, and the density of cell nuclei became more apparent. Collagen fibers have been shown to induce cells, and their density affects cellular behavior. As a result, their maintenance within the scaffold is critical. Thus, provided structures are investigated using various colors, including a trichrome and an optical microscope. A suitable environment for stem cell

replacement can be created by retaining collagen fibers and porosity in a decellularized scaffold. In this method, and to investigate the availability and maintenance of collagen fibers, the staining trichrome results indicated that collagen fibers maintained greater abundance in all groups than in the control sample (Fig. 4).

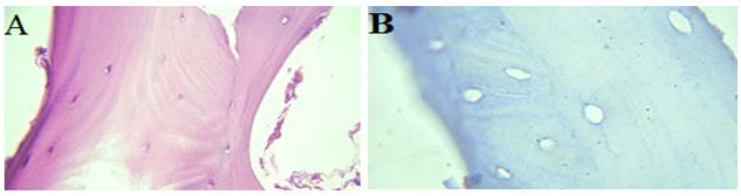


**Fig. 2. H & E staining(A) and Trichrome staining(B) of native calf's bone tissue (40X magnification)**

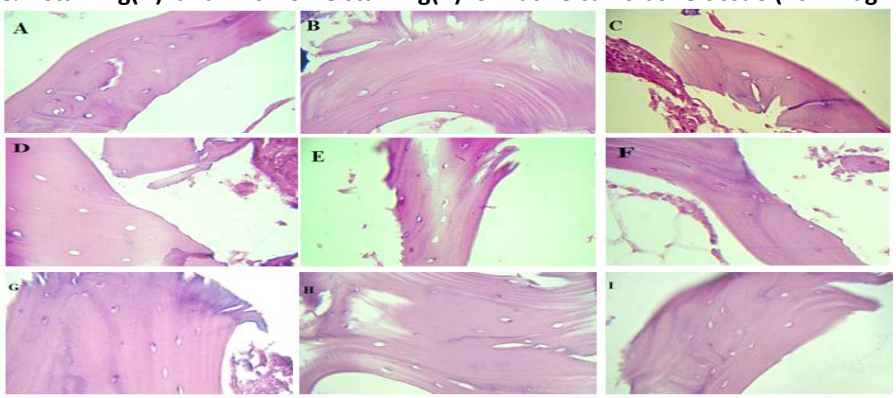


**Fig. 3. H & E staining of S2(A), S2.5(B), S3(C), T2(D), T2.5(E), T3(F), S3T2(G), S2.5T4(H) and T3S4(I)- (40X magnification)**

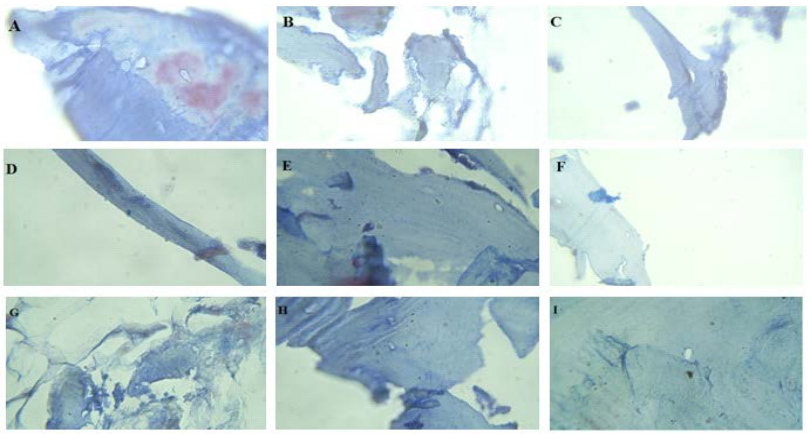


**Fig. 4. Trichrome staining of S2(A), S2.5(B), S3(C), T2(D), T2.5(E), T3(F), S3T2(G), S2.5T4(H) and T3S4(I) - (40X magnification)**

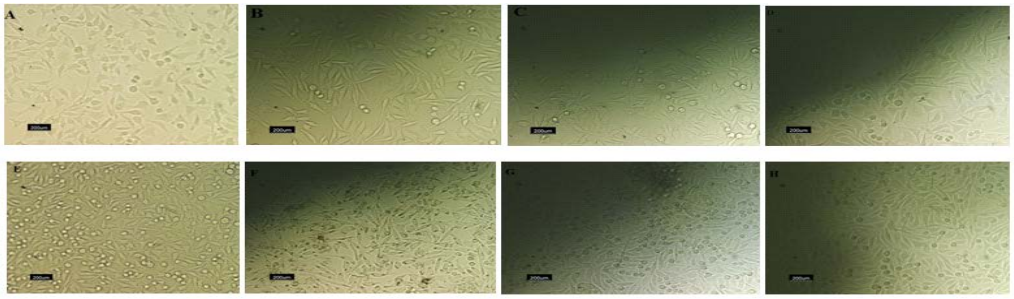


**Fig. 5. Optical microscopy images of fibroblast cells on the samples after 24hrs: control(A), S2(B), T2(C), T3S4(D) and after 48hrs: control(E), S2(F), T2(G), T3S4(H)**

The results of the toxicity test on the samples are depicted in Figure 5. After 24 hours, there was no evidence of toxicity in the samples (Fig. 5 A, B, C, D). After 48 hours, however, the number of cells was increased 24 hours. The images of (5 E-H) demonstrate no toxicity in any three samples compared to the control sample (Fig. 5 E). Nonetheless, there was minor toxicity in sample S2 as the exposure time increased (Fig. 5 F) and evident from the total observation of the three samples. Sample T3S4 was a viable option for constructing a natural bone scaffold as it passed the toxicity test without concern. Additionally, they had a higher decellularization rate than the T2 solution.

## Discussion

Although bone has an excellent ability to heal naturally, in specific clinical situations, such as severe injuries, congenital malformations, or diseases, natural bone restoration may be insufficient or too slow. As a result, bone reconstruction using various types of grafting is recommended in these instances[5,31]. Cancellous bone is used exclusively to fill defects and promote bone formation. There are several advantages to using autologous cancellous bone in clinical settings, including availability and safety. However, this approach has several disadvantages; for example, harvesting autologous tissue can result in morbidity at the donor site, difficulty during the harvesting process, and persistent postoperative pain[5].

In this respect, bone tissue engineering and the use of porous scaffolds may help meet the growing demand for suitable autograft plus allograft tissues for the reconstruction of significant bone defects[32,33]. Decellularization of allogenic tissues is a critical step in minimizing the immune response and minimizing the risk of disease transmission following scaffold transplantation. Due to the encouraging results obtained with xenografts derived from various animal tissues[34-37], decellularization of cancellous bovine bone was sought as a potential xenograft scaffold for studying bone tissue engineering. In comparison to other resources, bovine cancellous bone has been considered an appropriate model for tissue engineering due to its Haversian organization similar to that of human bone, higher metabolic activity than cortical bone, availability in large quantities from the epiphyseal of bovine bone, plus its osteoconductive and osteoinductive properties[38]. Additionally, whole-cell bodies must be removed to create an applicable scaffold while leaving the decellularized matrix's structure and function unaltered[39] as chemical detergents disrupt the cell membrane in this case.

Decellularization has been accomplished using various chemicals, including nonionic (Triton X-100) and ionic detergents (SDS). SDS treatments have met the standard criteria for complete cell removal and elimination of at least 90% of host DNA in a variety of tissues and organs, including a rat forearm[40], the porcine cornea[41], porcine myocardium[42], porcine heart valve[43], porcine small intestine[44], porcine kidney[45], human vein[46], rat, porcine, human lungs[47,48], and the human heart[49]. While SDS effectively removes unwanted native tissue constituents, it can cause damage to structural and signaling proteins. Collagen in SDS-treated heart valves, for example, became compacted[43], and the decellularized ECM of human and porcine lungs appeared more fibrous than the smooth native tissue structure[48]. Because SDS is also cytotoxic, the tissue must be thoroughly cleansed to ensure the viability of reseeded cells[44].

While most surfactant-treated tissues must be washed with phosphate-buffered saline (PBS), SDS is more challenging to remove due to its ionic nature. Another disadvantage of SDS as a decellularizing agent is the lengthy washing process required following treatment[52]. Triton X-100, a nonionic surfactant, is frequently used to remove residual SDS. This practice has been particularly prevalent in the decellularization of whole organs via perfusion[50,47,51,44,40,49]. Not only is Triton X-100 advantageous during the wash process, but also frequently used as a standalone decellularizing agent, and due to its nonionic nature, it proves abrasive than SDS and thus

less damaging to the tissue's structural integrity[52].

In this respect, various solutions and durations were tested to determine the optimal conditions for SDS and Triton X-100 treatment (and their combination). To this end, bone specimens were washed with distilled water first. They were then immersed in a solution of 5% hydrogen peroxide followed by diethyl ether. They were then washed with 70% ethanol and five times with distilled water. Finally, all specimens were treated with various SDS and Triton X-100 solutions and combined with the two over specific durations. H&E staining was used to determine the degree of decellularization. The results indicated that treatments with SDS 2.5% and 3% were more effective than treatments with SDS at 2%, but the cell removal and purification occurred at the expense of structure destruction, deemed non-optimal for scaffolding.

On the other hand, 2% Triton X-100 treatments removed the most cells compared to 2.5% and 3% Triton X-100 treatments. Whereas the T3S4 specimen had the best cell removal result in the consolidated treatments. For confirming the efficiency of decellularization, collagen in the ECM was also stained with trichrome and found to be significantly more abundant in all groups than in the control sample. Specimens were observed for 24 and 48 hours to assess the cytotoxicity and ensure that none of the three specimens exhibited toxicity compared to the control sample (Fig. 5 E). However, as the duration of exposure increased, there was minor toxicity in sample S2 (Fig. 5 F). The total observation indicates that, of the three specimens, sample T3S4 was the most suitable for constructing a natural bone scaffold, as this sample coupled with S2 passed the toxicity test without issues and demonstrated a higher decellularization rate than the T2 solution.

## Conclusion

In this study, various solutions and durations were tested to determine the optimal conditions for SDS and Triton X-100 treatment (and their combination). To this end, bone specimens were treated with varying amounts of SDS and Triton X-100, and a combination of the two H&E stainings was used to determine the degree of decellularization.

The results indicated that treatments with SDS 2.5% and 3% were more effective than treatments with SDS 2%, but the cell removal and purification occurred at the expense of structure destruction, which was deemed non-optimal for scaffolding. On the other hand, 2% Triton X-100 treatments removed the most cells compared to 2.5% and 3% Triton X-100 treatments, whereas the T3S4 specimen had the best cell removal result in consolidated treatments. Collagen in the ECM was stained with trichrome to demonstrate decellularization efficiency and was substantially more abundant in all groups than in the control sample. Specimens were observed for 24 and 48 hours to determine cytotoxicity and to ensure that none of the three specimens exhibited toxicity compared to the control sample.

Nonetheless, there was minor toxicity in sample S2 as the exposure time increased. Overall, the observation demonstrates that sample T3S4 was an appropriate candidate for constructing a natural bone scaffold among the specimens. The findings of this study could be applied to the development of a natural scaffold for tissue engineering and bone regenerative medicine.

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