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Bovine nucleus pulposus decellularization using freeze drying technique to form a biological scaffold

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ABSTRACT

Introduction: As much as 40% of low back pain patients are caused by degenerative disc disease. The current treatments are fusion stabilization and total disc replacement, which have a risk for adjacent segment disease. These suboptimal results have become the beginning of the development of regenerative therapy. The success of this therapy will increase if the scaffold meets the ideal conditions. Biological scaffolds provide a cell growth environment that resembles the original tissue. Bovine intervertebral nucleus pulposus can become promising scaffolds.
Objective: This research describes the proper freeze-drying approach for decellularizing bovine nucleus pulposus to create a biological scaffold.
Methods: We conducted an experimental post-test control design study using bovine coccygeal nucleus pulposus material. All treatment groups underwent freeze-drying with Buchi Lyovapor™ L-200/L-200 Pro. All groups were evaluated for the level of decellularization using the Quick-DNA™ Miniprep Plus Kit (Zymo Research®) and remaining glycosaminoglycan levels by Alcian Blue staining.
Results: Comparison of DNA concentrations obtained p-values respectively <0.0001 (p <0.05), which means that all the treatments showed a decrease in DNA concentration compared to the control group. The comparison of the glycosaminoglycan percentage between the P1 vs. Control group obtained a value of p=0.381 (p>0.05), which means that the glycosaminoglycan percentage results for the P1 group were not significantly different from the control group.
Conclusion: This study of group P1 showed that decellularization of the bovine nucleus pulposus by freeze-drying technique can form an excellent biological scaffold.

Keywords: Biocompatible Materials, DNA, Freeze Drying, Glycosaminoglycans, Low Back Pain.
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INTRODUCTION

Low back pain (LBP) is a complaint experienced by 80% of adults[1]. In Indonesia, one-third of the population has LBP; the age group is 50-59 years[2]. As much as 40% of the total LBP patients are caused by DDD[3,4,5]. Generally, patients with severe pain or neurological disorders will be treated with surgery. Surgical therapy itself has many options. One of the treatments is fusion stabilization[6]. However, within a certain period, fusion will be a risk for adjacent segment disease (ASD)[5]. Fusion stabilization will also reduce the spine's range of motion. Even a total disc replacement does not rule out the possibility of ASD[7].

The suboptimal results from existing treatment modalities have become the beginning of the development of regenerative therapy with the hope that its application in DDD patients can restore the structure and function of the intervertebral disc (IVD)[8]. There are three main components in regenerative therapy: stem cells, growth factors, and scaffold[9,10,11,12,13,14]. The possibility of success of regenerative therapy will increase if the scaffold meets the ideal conditions and is carried out using appropriate tissue engineering techniques[15,16]. The scaffold must have good biocompatibility, sufficient porosity, and be similar to natural IVD, both in structure, biological and mechanical properties[17,18].

There are two types of scaffolds: biological and synthetic. The ideal biological scaffold has a similar extracellular matrix (ECM) content to the original tissue, an advantage over synthetic ones. Another condition is that the biological scaffold must have no cells or optimal decellularization. This avoids toxicity to the stem cell culture and prevents host immune rejection during implantation[15,17]. Bovine intervertebral discs are believed to have promising potential in regenerative therapy due to their biological similarity to human IVD. The disc also has a higher glycosaminoglycan (GAG) content than humans, so after decellularization, the matrix component of the NP can maintain GAG at its critical value[18].

This research describes the proper freeze-drying approach for decellularizing bovine NP to create a biological scaffold.

Table 1. Comparison of previous research and current research regarding the formation of the intervertebral disc scaffold

No	Study	Results	Difference To Current Research
1	Elder et al. 2009[19]	1. Formation of the scaffold from bovine joint cartilage 2. Chemical decellularization 3. DNA in control 6405ng/mg, decellularization yield 4642ng/mg 4. GAG in control 0.17mg/mg, GAG in control 0.17mg/mg	The scaffold is formed from NP bovine Decellularization is done by freeze drying (physical)
2	Mercuri et al. 2011[20]	1. Scaffold formation from porcine NPs 2. Decellularization chemicallyand physically 3. Succeeded in reducing DNA by (97.55-98.44)% 4. There was a reduction in GAG of 41–49%	The scaffold is formed from NP bovine Decellularization is done by freeze drying (physical)
3	Chan et al. 2013[21]	1. Scaffold formation of bovine DIV 2. Scaffold in the form of endplate-to-endplate 3. Chemical decellularization 4. Successfully reduced 71% of cells 5. GAG composition reduced by 3% (NP) and 15.6% (AF)	Consists only of bovine NP components Decellularization is done by freeze drying (physical)
4	Fernandez et al. 2016[15]	1. Scaffold formation from bovine NP 2. Decellularization chemicallyand physically 3. DNA reduced by 73.2%, 73.6%, and 92.77% 4. GAG reduced by 67.9%, 69.1%, and 69.6%	Decellularization is done by freeze drying (physical)
5	Norbertczak et al. 2020[22]	1. Scaffold formation of bovine DIV 2. Scaffold in the form of endplate-to-endplate 3. Chemical decellularization 4. There was no significant difference in the amount of DNA and GAG in NPs before and after decellularization	It consists only of NP components Decellularization is done by freeze drying (physical)

It provides an overview of bovine intervertebral disc NP using the freeze-drying method after decellularization. The table below compares previous and current research regarding the formation of the intervertebral disc scaffold.

MATERIALS AND METHODS

Bovine Intervertebral Disc

This experimental post-test control design uses bovine intervertebral disc NP taken at the coccygeus level, especially at coccygeus discs (Cc) 1-2, 2-3, and 3-4. The bovines used are ≤ four years old, have no morbidity, have no history of injury, and were taken from official slaughterhouses[23,24,25]. This step begins with separating the disc from the bone using a sharp scalpel to avoid damaging the disc structure too much. Then, the NP sample will be separated from the disk material. A total of 24 bovine IVDs were used, determined based on the Federer formula.

Specimens will be divided into four groups, namely: control group; treatment group 1 (P1), freeze-drying NP; treatment group 2 (P2), NP were subjected to freeze drying and washing with Phosphate Buffered Saline (PBS) solution; lastly, treatment group 3 (P3), NP without isolation from discs material undergo freeze drying and washing with PBS. The control group will be stored at 20^{0}C, while the treatment group will be fixed with ethanol liquid. See Figure 1.

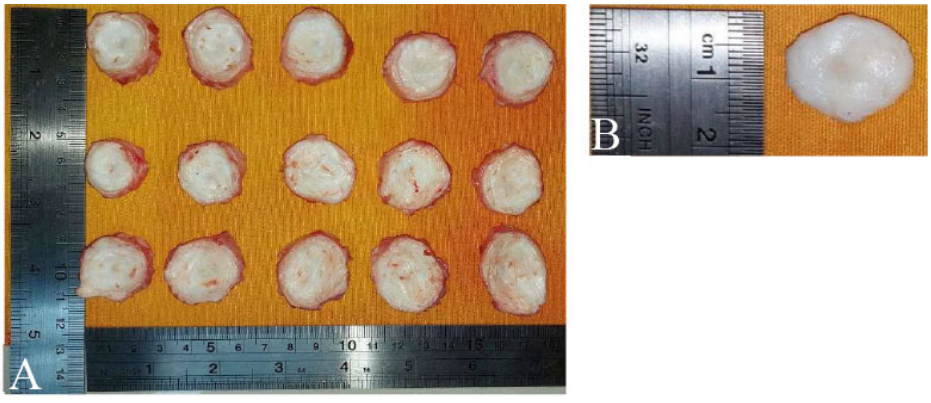

Figure 1. Structure the intervertebral discs Cc 1, 2, and 3 separated from the cow's tail (A). The NP structure maintains its integrity after being separated from the disc (B).

Freeze Drying

The freezing process begins with the materials entering the freezer at -80C for 60 minutes until the sample is completely frozen, indicated by the appearance of shiny and iced flowers on the NP structures (Figure 2). The sample will undergo a drying stage (sublimation and evaporation) shortly after. The frozen material is put into the Buchi Lyovapor™ L-200/L- 200 Pro. It is set at a temperature

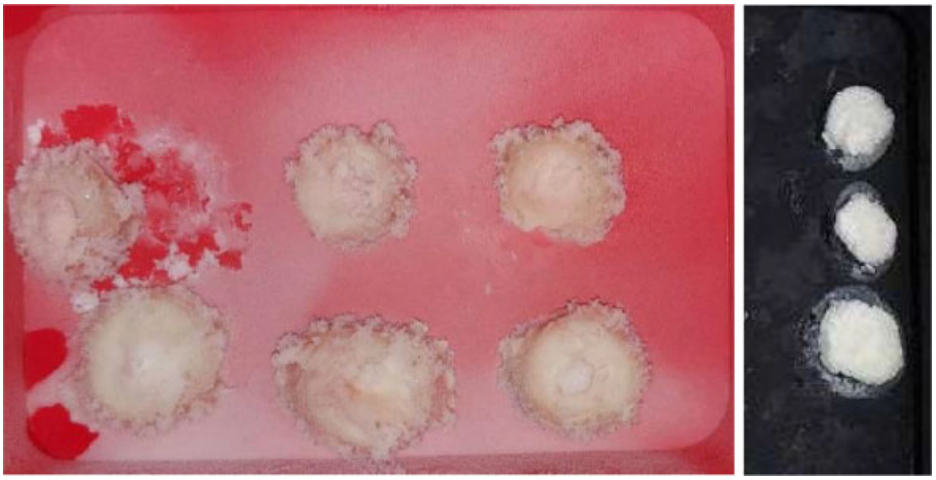

Figure 2. shows the sparkling structure and the appearance of frost on the material.

Figure 3. The final result of freeze-drying is that the material is dry and resembles a freeze- dried cake.

of -5C. The pressure used is 0.15 mBar. This process was maintained for 12 hours to obtain dry materials resembling freeze-dried cake (Figure 3).

Twelve samples for groups P2 and P3 will undergo a washing step with PBS solution. The sample is put into a cylindrical container and immersed in PBS solution. Then, it was put into a shaker with a temperature setting of 4C and a rotation of 100 rpm. This step was carried out for 48 hours with the PBS solution replaced every 3rd, 9th, 24th, 27th, and 33rd hour[21].

Evaluation of Decellularization

DNA isolation was carried out using the Quick-DNA™ Miniprep Plus Kit (Zymo Research®). DNA solution can be stored at temperatures ≤ -20C. The DNA isolation results will be measured quantitatively using UV-Vis Spectrophotometry. Next, the computer software will calculate the results of DNA purity at wavelengths 260/230, 260/280, and DNA concentrations.

Evaluation of GAG Content

After AB staining, all preparations were observed under an Olympus CX23® microscope with 200x magnification. Quantitative data was obtained with Image J® software, which can convert image quality into a percentage of the observed material content of GAG. The percentage of GAG density in each field of view can be obtained from the AB staining gradient representing GAG in the preparation.

Data Processing

The data obtained was subjected to the Shapiro-Wilk test to determine the normality of the data distribution. Furthermore, the Levene test was carried out to determine the homogeneity of the data. However, the data distribution is not homogeneous; a Brown-Forsythe test was conducted. Next, a post hoc test using the Tamhane test was carried out to determine the relationship between one treatment and another. This data processing step is done for both dependent variables using SPSS 26 software. This research hypothesizes that decellularizing bovine nucleus pulposus using freeze-drying techniques can form a biological scaffold.

RESULTS

DNA levels in the NP decellularization group with freeze drying (P1) action obtained the highest average value of 3.85 ± 0.70 ng/ul, then in the NP decellularization group with freeze drying + washing action PBS solution (P2) got the lowest average value of 1.05 ± 0.35 ng/ul, and in the NP decellularization treatment group without isolation from discs by freeze drying + washing action PBS solution (P3) got an average value of 1.30 ± 0.21 ng /ul.

The Brown-Forsythe test results obtained a p-value <0.0001, which was significant at p<0.05. These results indicated a significant effect between the treatments on DNA levels. The P2 action group eliminated most of the DNA from the matrix, followed by the P3 treatment group. Look at Table 2.

In order to determine partial differences between treatment groups, Tamhane's Post Hoc Multiple Comparisons test was carried out. A comparison of DNA concentration between groups P1 and P2 yielded a value of p=0.116 (p>0.05), which means there was no significant difference in DNA concentration levels between groups P1 and P2. Group P1 vs. P3 got a value of p=0.176 (p>0.05), which means that there is no significant difference in DNA concentration levels between groups P1 and P3, and the comparison of groups P2 vs. P3 obtained a value of p = 0.993 (p> 0.05), which means there is no significant difference in DNA concentration levels between groups P2 and P3.

Table 2. Simultaneous different tests (Brown-Forsythe) of DNA concentration based on treatment preparation groups

Variable	Group				p
	Control	P1	P2	P3	
DNA Concentration	10.69±0.38	3.85±0.70	1.05±0.35	1.30±0.21	<0.0001*

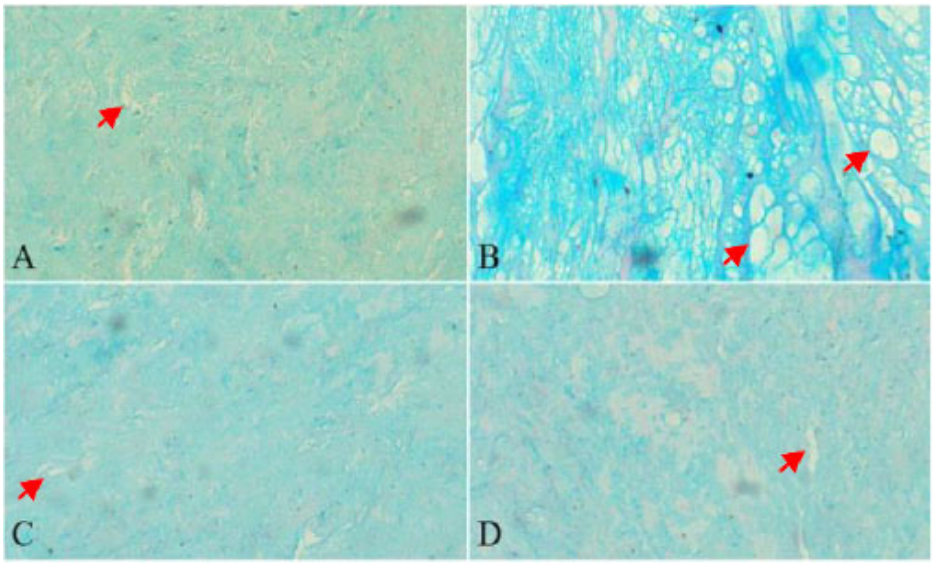

Figure 4. An example is a photo of the preparation under a microscope with 200x magnification after AB staining. (A) Control group. (B) P1 group. (C) P2 group. (D) P3 group. Red arrows indicate micropores. The P1 group has more prominent and more pores. The blue color that spreads represents the distribution of GAG for each preparation. The darker blue color indicates the focus of the GAG.

Table 3. A simultaneous difference test (Brown-Forsythe) of GAG percentage was performed based on treatment preparation groups

Variable	Group				p
	Control	P1	P2	P3	
Percentage GAG	90.98 ±2.78	84.44±5.06	74.28±4.52	74.63±2.72	<0.001*

The percentage of GAG density in each field of view can be obtained from the AB staining gradient representing GAG in the object. The following is a photo of the preparation under the microscope (Figure 4).

Examining the percentage of GAG in the control group obtained the highest average value, namely 90.98 ± 2.78. The NP decellularization group with freeze drying (P1) action obtained an average value of 84.44 ± 5.06. Then, the P2 group got the lowest average value of 74.28 ± 4.52; in the P3 treatment group, the average value was 74.63 ± 2.72. The statistical test results obtained a value of p=<0.001 (p<0.05), which means there was a significant difference in the percentage levels of GAG between the control group, P1, P2, and P3 simultaneously (Table 3).

A comparison of the percentage of GAG between groups P1 and P2 showed a value of p=0.183 (p>0.05), which means there is no significant difference in the percentage level of GAG between groups P1 and P2. Then P1 vs. P3 got a value of p=0.123 (p>0.05), which means There was no significant difference in GAG percentage levels between groups P1 and P3. The P2 vs. P3 comparison obtained a value of p=1,000 (p>0.05), which means there was no significant difference in GAG percentage levels between groups P2 and P3.

DISCUSSION

Intervertebral disc (IVD) degeneration is one of the primary causes of low back pain (LBP). IVD degeneration is characterized by changes in cell populations and subsequent loss of extracellular matrix (ECM) from the nucleus pulposus (NP), which results in dehydration. Finally, degenerative disc disease (DDD) occurs. Until now, the therapy that can be given is only a therapy that eliminates the symptoms caused by the DDD condition[1].

Alternative therapies for spinal fusion are increasingly needed. The current artificial discs are not intended to produce tissue remodeling. Tissue engineering offers a method for designing biomaterials to aid biological IVD tissue regeneration. Because current surgical procedures focus solely on the symptoms associated with IVD degeneration, tissue engineering offers many strategies to prevent and possibly cure degenerated discs by promoting tissue repair. Tissue engineering promotes tissue regeneration using scaffold biomaterials, stem cells, and growth factors[21,22].

In regenerative therapy, especially in tissue engineering, a scaffold is essential to help cell proliferation, differentiation, and biosynthesis[26]. Scaffolds also help maintain cells in desired locations, providing mechanical properties and biochemical signals to surrounding tissues to facilitate and guide cell growth[27]. A scaffold must meet several criteria, including being biocompatible, biodegradable, having a three-dimensional structure, and having mechanical properties resembling the place of implantation[9,28]. In addition, the scaffold must allow cells to associate, differentiate, and proliferate. More specifically, the scaffold can be injected to allow minimally invasive surgery aimed at regenerative intervertebral disc therapy, thereby preventing damage to the annulus fibrosus[29].

These biomaterial structures should not elicit rejection from the immune response, have a structure similar to native tissue, be biocompatible and biodegradable, and exhibit mechanical properties similar to biological tissue after successful regeneration. Many existing biomaterials

have demonstrated biocompatibility, but the newly developed materials at least meet the <50 ng dsDNA requirement per mg ECM[30]. The ideal scaffold has pores to provide cell attachment and tissue growth while allowing the diffusion of nutrients and metabolic waste. The porous structure should allow space for the ECM to be ejected and eventually form a network similar to normal, original IVD tissue.

The results of this study are that decellularization of the bovine nucleus pulposus by freeze drying technique can form a biological scaffold. The freeze-drying technique can reduce DNA to the most minor 0.20 ug/mg matrix concentration. This technique can also maintain GAG in the matrix until it is only reduced by ± 16% from the control group. The following describes a comparison of techniques and results of decellularization from previous studies.

The partial test stated no significant difference between the P1, P2, and P3 groups. The three treatment groups effectively eliminated DNA. Partial tests on the percentage of GAG showed no significant difference between groups P2 and P3 compared to group P1, so it can be concluded from this statistical calculation that in the P1 treatment group, NP decellularization by freeze drying action is the most optimal decellularization technique in eliminating DNA and maintaining GAG levels.

CONCLUSION

This study of group P1 showed that decellularization of the bovine nucleus pulposus by freeze-drying technique can form an excellent biological scaffold.

ETHICAL APPROVAL

This study got ethical approval from our institution.

DECLARATIONS OF INTEREST

None. All authors state that there are no financial and personal relationships with other people or organizations that could inappropriately influence (bias) for this study.

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In this study, we use our resources without any financial assistance from other parties.

RESEARCH REGISTRATION

'N/A'

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