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COMPARASION OF HUMAN MEMBRANE AMNION USING SODIUM DODECYL SULFATE (SDS) DECELLULARIZATION OUTCOME AND COMBINATION OF SDS WITH DNase ENZYME

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Abstract

Introduction. Decellularized human amnion membrane (dHAM) has a lower risk of immunogenic inflammatory response than human amnion membrane (HAM) without decellularization. Decellularized human amnion membrane can accelerates the wound healing process by preserving skin structure, making wound recovery using stem cells faster than without dHAM. This research aims to prove that dHAM can be made using 0.5% SDS alone or in combination with DNase while maintaining the collagen component of HAM.

Methods. This research is an in vitro experimental study that uses HAM from the birthing process in a hospital. This study consisted of one control group of freeze-dried HAM which was not decellularized, as well as four treatment groups underwent the decellularization process using SDS 0.5% for 15 minutes (dHAM 1) and SDS 0.5% for 30 minutes (dHAM 2), SDS 0.5 % for 15 minutes and DNase for 15 minutes (dHAM 3), SDS 0.5% for 30 minutes and DNase for 15 minutes (dHAM 4). The variables assessed were differences in cellular and collagen components, between the control and the treatment group, which were evaluated using an optical microscope.

Results. Histological examination per field of view at 400x magnification of optical microscope showed that the decellularization process carried out was effective in reducing the number of cells in HAM ($p < 0.0001$; CI = 95%), the control group still appeared hypercellular (41.57 ± 6.1 cells) , dHAM 1 appeared medium cellular (24.86 ± 7.08 cells), dHAM 2 and dHAM 3 appeared hypocellular with almost the same effectiveness (dHAM 2 = 1 ± 1 cells; dHAM 3 = 1.28 ± 0.49 cells) , dHAM showed hypocellular results close to zero (0.28 ± 0.49 cells). The collagen component showed almost the same results in the control group, dHAM 1, dHAM 2, dHAM 3, but the collagen component decreased in dHAM 4 ($p < 0.0001$; CI = 95%).

Conclusion. Making dHAM with just 0.5% SDS takes a minimum of 30 minutes. The duration of SDS 0.5% can be reduced when combined with DNase. Increasing the duration of 0.5% SDS does not reduce the density of the HAM collagen component. However, increasing the duration of 0.5% SDS combined with DNase can reduce the density of HAM collagen.

Keywords: decellularization; *sodium dodecyl sulfat* ; DNase ; *human amnion membrane* ; *scaffold* ; dHAM

1. INTRODUCTION

The prevalence of chronic wounds is estimated at 2.21 per 1,000 population. The estimated prevalence of chronic wounds in developing countries reaches 1 – 2% of the population. Deep dermal or full-thickness dermal wounds will be difficult or unable to heal spontaneously, they will become chronic wounds if not treated adequately[1,2,3].

A natural scaffold can be formed by collagen in superficial wounds, which crosses the surface of the wound bed and facilitates epithelial cells crossing the extracellular matrix. This wound recovery pattern is sometimes unsuccessful in wounds that are wide, deep, and have vital structures exposed[1].

Wound treatment using scaffolds has been proven to be effective in accelerating the process of granulation, epithelialization, and wound healing in deep and full thickness dermal wounds compared to standard treatment and does not result in rejection reactions. HAM scaffold products have been published since the beginning of the 20th century, but product safety and packaging are still being significantly improved in various studies. The use of dHAM scaffolds in wounds provides for and stimulates the formation of extracellular matrix (ECM) components[4,5].

The dHAM scaffold, which is tolerant, cheap, feasible, and has no antigenicity, can be a promising product for wound healing. dHAM scaffolds can be made through a decellularization process using physical, biological, and chemical methods. The effective method used is the chemical method, with the frequently used detergent, i.e Sodium Dodecyl Sulfate (SDS). The use of SDS is often combined with other ingredients, such as DNase and RNase enzymes. Scaffold creation is carried out while maintaining the natural structure of the ECM[6,7,8].

Dr. Soetomo General Hospital Tissue Bank has made bovine cartilage acellular scaffolds using 5% SDS for 72 hours and 0.5 mg/ml DNase for 10 minutes. The thickness of bovine cartilage in this study was 2–5 mm, which means it was 100x thicker than HAM, which was only 0.02–0.05 mm[9,10]. These differences indicate that the decellularization process in HAM requires SDS and the DNase enzyme at different doses and times compared to previous studies. We hope that this research will yield a dHAM scaffold, a promising modality for wound treatment. We will evaluate the created dHAM scaffold using microscopic examination.

2. METHODOLOGY

This research is an in vitro experimental study that uses HAM from 1 mother donor who gave birth in a hospital, which was adjusted to the 2017 Asia Pacific Association of Surgical Tissue Banks (APASTB) standards.

2.1. Storage and Washing Process for Fresh Human Amnion Membrane (fHAM)

Donor amniotic membranes were stored at -20 °C for less than one month in the Cell and Tissue Bank of Dr. Soetomo General Hospital Surabaya. Preparation of the amniotic membrane begins by moving it to a 4°C refrigerator. Evaluation of the amniotic membrane was carried out by assessing the presence or absence of color changes, debris, contaminants, changes in odor, and other signs of damage. Amniotic membranes that were in good condition were washed using a sterile water-bath shaker in 400 ml of sterile distilled water four times. The next step was using 400 ml of sterile 0.05% NaCl once, then 400 ml of sterile distilled water four times. Fluid changes were carried out every 10 minutes nine times[9].

2.2. Decellularization Process

Four samples of fresh human amnion membrane (fHAM) measuring 15 x 30 cm were decellularized with 0.5% SDS, which was rinsed with aquadest in a sterile water bath shaker for 5–10 minutes at room temperature. Decellularize with 50 U/ml DNase using a

pipette slowly until all surfaces were wet, and incubate at room temperature for 15 minutes. The DNase reaction was stopped by washing with 0.1% SDS for 15 minutes, then rinsing with distilled water in a sterile water bath shaker for 5 x 10 minutes. This research consisted of 4 treatment groups :

1. **dHAM 1**: Decellularize 15x30 cm fHAM using 0.5% SDS for 15 minutes.
2. **dHAM 2**: Decellularize 15x30 cm fHAM using 0.5% SDS for 30 minutes.
3. **dHAM 3**: Decellularize 15x30 cm fHAM using 0.5% SDS for 15 minutes and 50U/ml DNase for 15 minutes.
4. **dHAM 4**: Decellularize 15x30 cm fHAM using 0.5% SDS for 15 minutes and 50U/ml DNase for 15 minutes.

The control group in this study used a fHAM measuring 15 x 30 cm, which only went through the washing process.

2.3. Preservation Process

The four groups of dHAM 1-4 and control samples were preserved using a lyophilization process at the Cell and Tissue Bank of Dr. Soetomo Hospital by:

a. The freezer was cleaned with 70% alcohol and cooled to -80°C.

b. All amniotic membrane samples that have been processed were stretched in a sterile container, then placed in the freezer for 24 hours[11].

c. The amnion membrane was dried using an AMSCO brand lyophilizer until the water content in the amnion membrane was 5 – 7%.

2.4. Cellular test with hematoxylin and eosin (HE) staining and collagen test with Masson trichrome (MT) staining

Each dHAM 1-4 and control sample was cut into 7 samples measuring 2x 4 cm. All samples were fixed using 10% neutral buffered formalin (NBF) for 24 hours at room temperature. The samples were dehydrated and stored in paraffin wax. Paraffin wax was cut to a thickness of 4 mm and stained with HE and MT, then examined using a light microscope [12,13].

Evaluation of cellular components was carried out by staining using Hematoxylin and Eosin (HE), to count the number of bluish cell nuclei in 1 field of view at 400x magnification. Evaluation of collagen components is carried out using Masson Trichrome (MT) staining, which is shown as a bluish color on optical microscope examination. These two stainings were carried out on the control group and the four treatment groups (dHAM 1, dHAM 2, dHAM 3, dHAM 4).

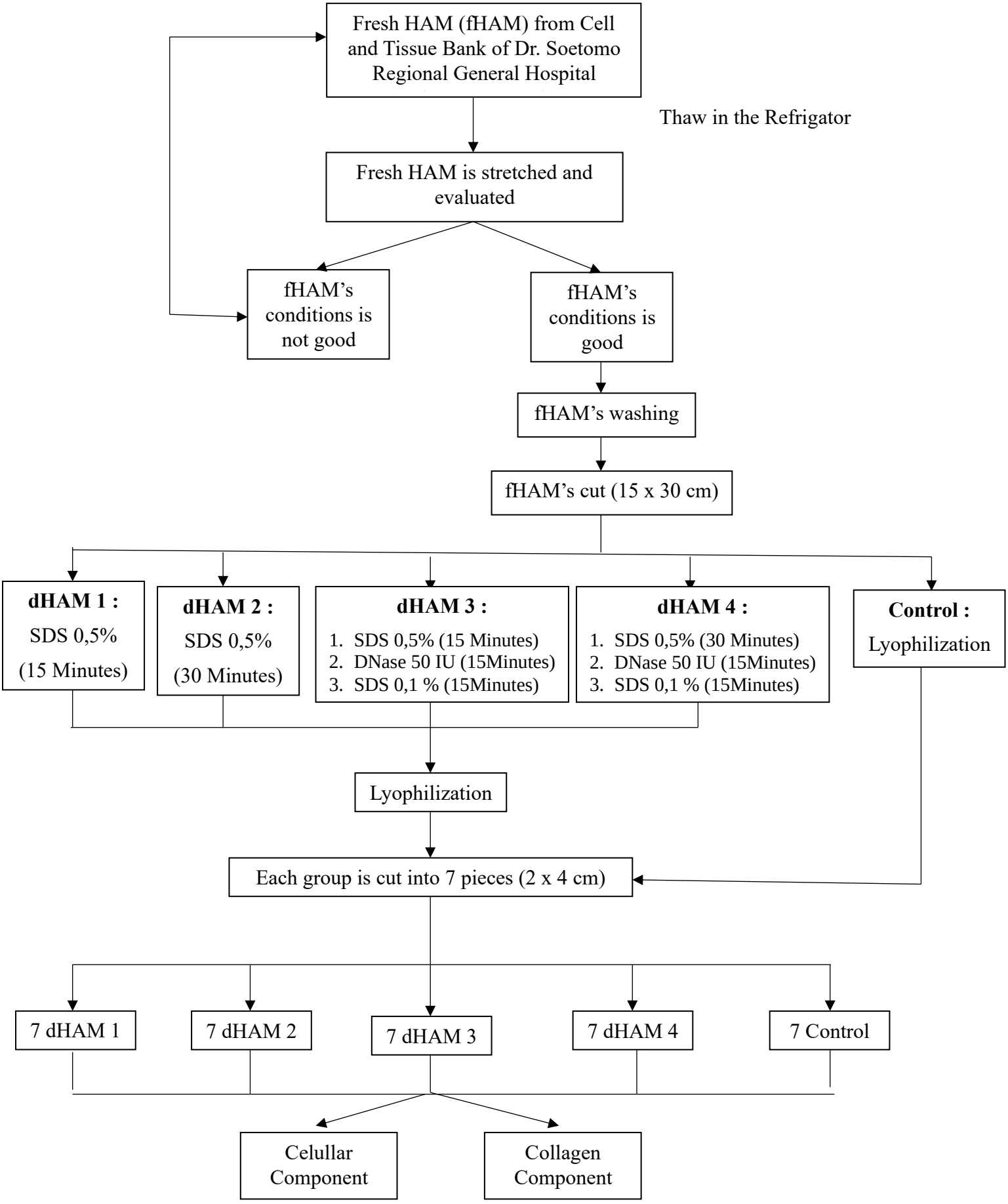


Figure 4.1. Research framework

3. RESULTS

This study compares the cellular and collagen components in dHAM 1-4 group and control group, processing the data using SPSS.

3.1 Cell Components

The control group showed an image of an abundant cell component with a seemingly intact nuclear wall, i.e 41.57 ± 6.1 cells in one field of view at a 400x magnification of the seven control preparations.

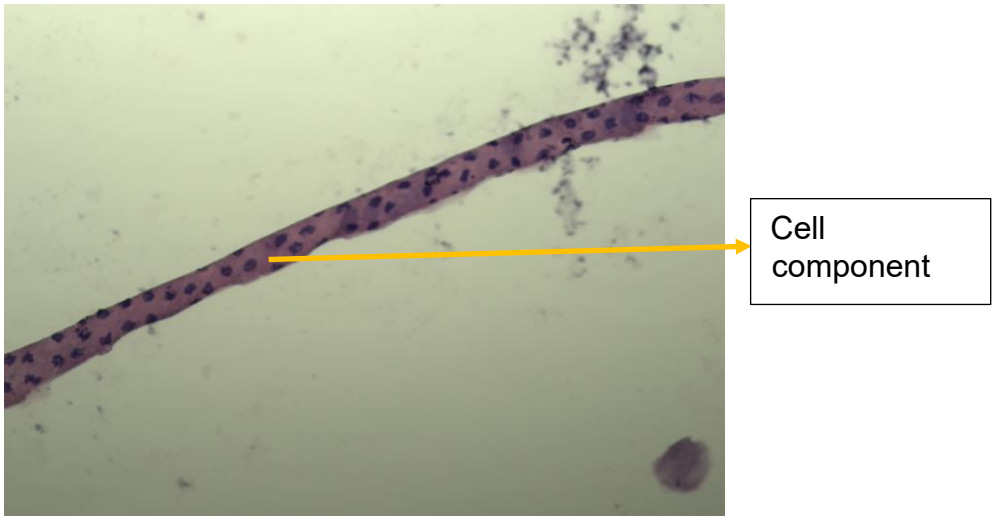


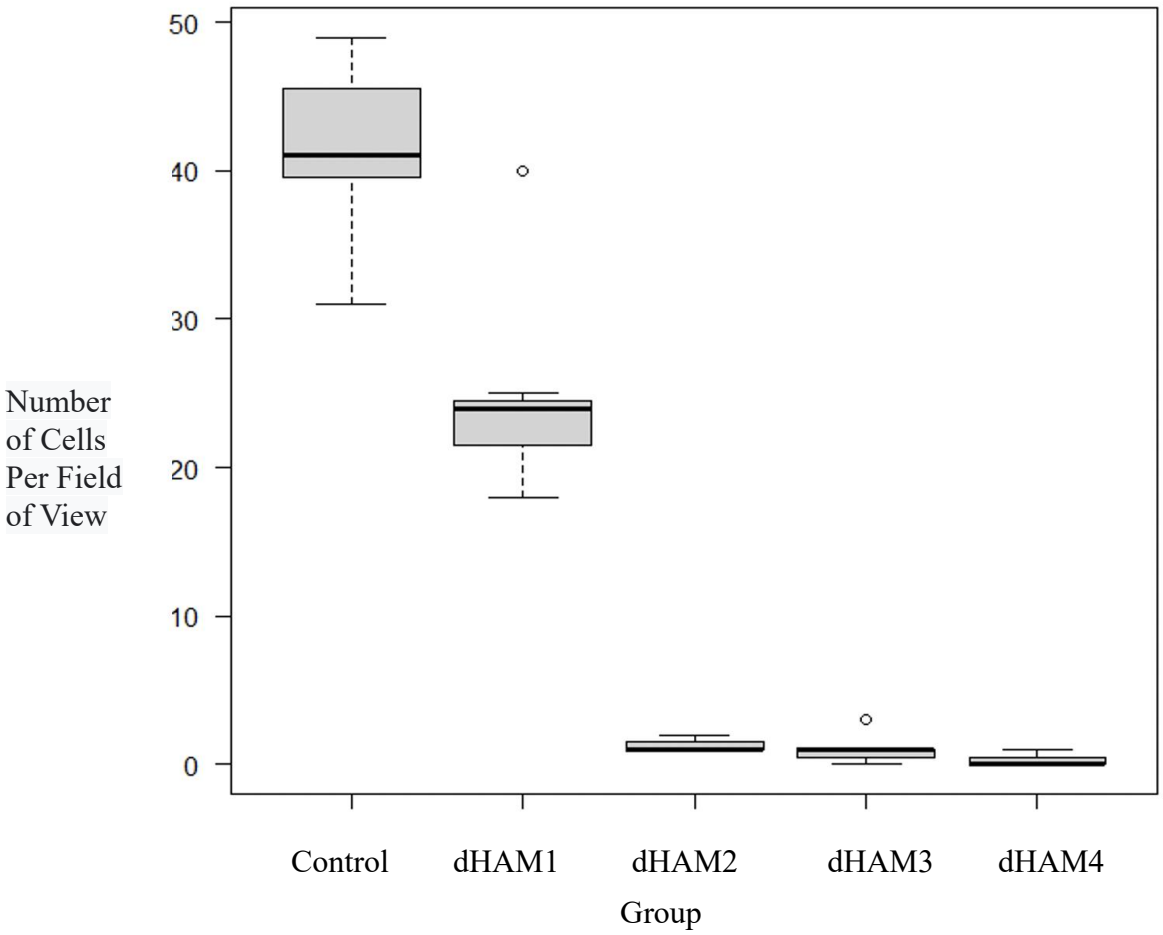
Figure 5.1. The histopathological image in the control group shows that there are still many cell components and the nuclear wall appears intact

The four decellularization treatments in this study provided significant results on the HAM decellularization process (P <0.001) (table 5.1).

Table 5.1. Statistical analysis between control and treatment of cellular component research variables

Variable	Group	N	Mean	CI 95 %	P
The number of cells in 1 field of view is 400x	Control	7	41,57 ± 6,1	7,82 – 19,78	**<0.0001**
	dHAM 1	7	24,86 ± 7,08		
	dHAM 2	7	1 ± 1		
	dHAM 3	7	1,28 ± 0,49		
	dHAM 4	7	0,28 ± 0,49		

The number of cells in HAM that had gone through the decellularization process using SDS 0.5% and SDS 0.5% in combination with DNase 50 IU experienced a significant decrease in cell number (graph 5.1).



Graph 5.1. Statistical analysis between control and treatment of cellular component research variables

The evaluation results of the dHAM 1 treatment group showed a picture of the cellular medium with many cellular components still visible, but with some of the nuclear walls appearing damaged or incomplete (figure 5.2). The dHAM 2 and dHAM 3 groups showed hypocellular results (figure 5.3). The dHAM 4 group showed the most acellular features compared to the other groups (figure 5.4).

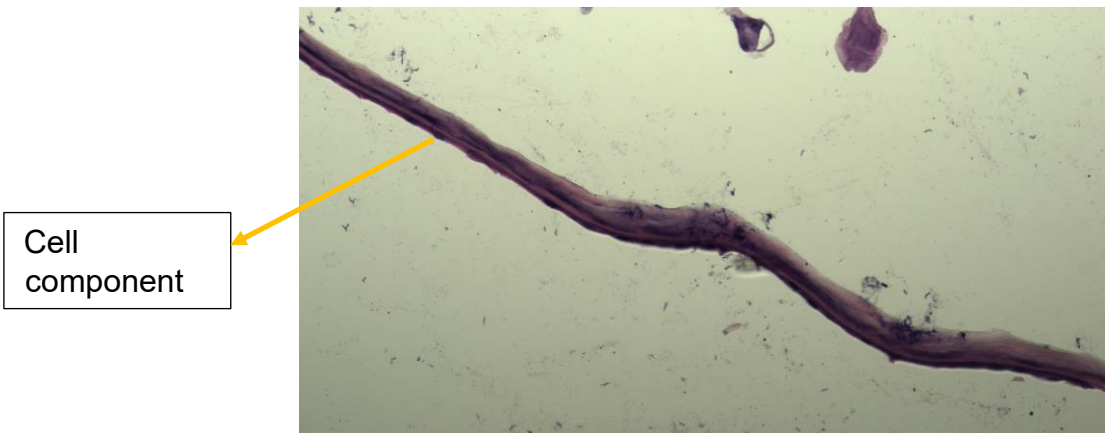


Figure 5.2. The histopathological image in dHAM 1 group still shows scattered cells.

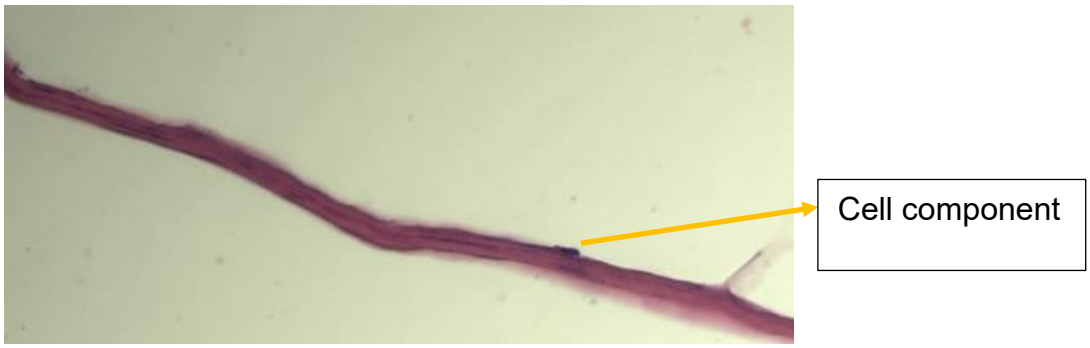


Figure 5.3. The histopathological image in the dHAM 2 group appeared hypocellular with few cells

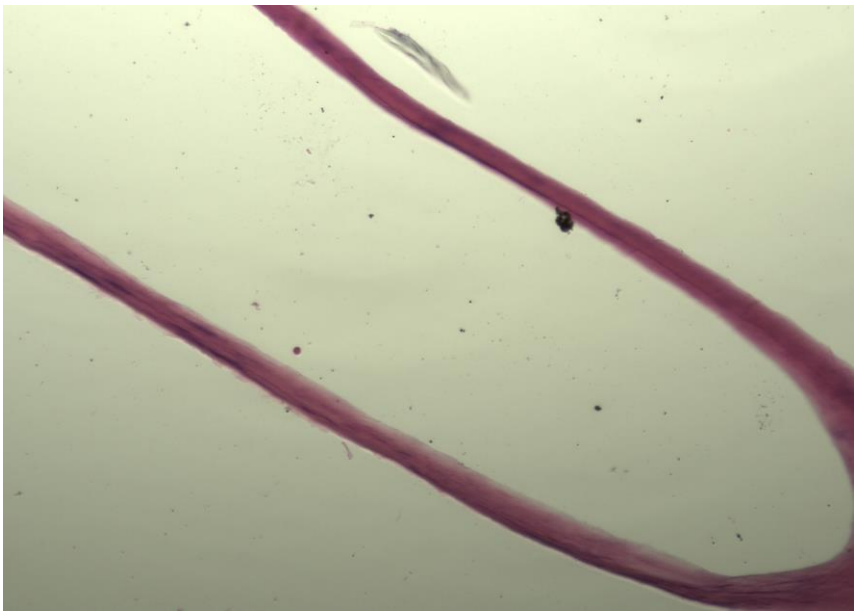


Figure 5.4. The histopathological image in the dHAM 4 treatment group was the most effective in eliminating cell components in the tissue

3.2 Collagen Components

The collagen component in this study was categorized into 3 degrees (0, 1, 2), based on staining using Masson trichrome (MT) which was examined microscopically using 400x magnification in one field of view. Grade 2 shows the dark blue color which is most visible in the dHAM 3 treatment group (figure 5.4).

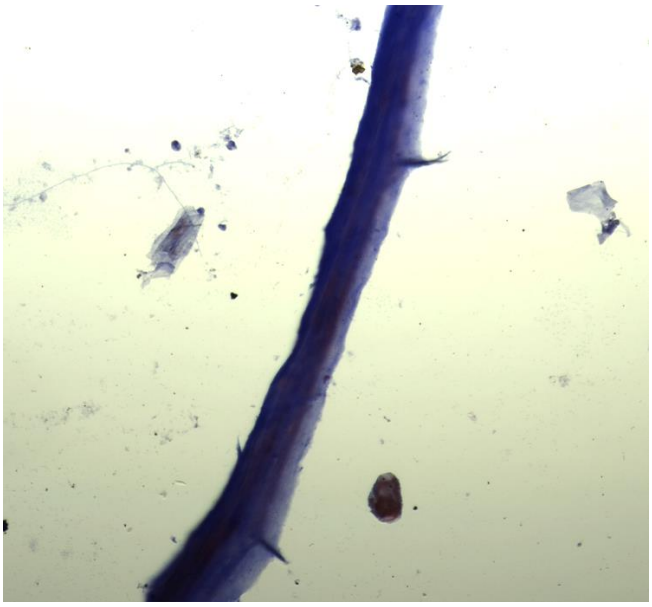


Figure 5.5. Image of grade 2 collagen components with MT staining

Grade 1 shows a lighter blue color which is found in all samples in the control group and most samples in the dHAM 1 and dHAM 2 treatment groups (figure 5.5).

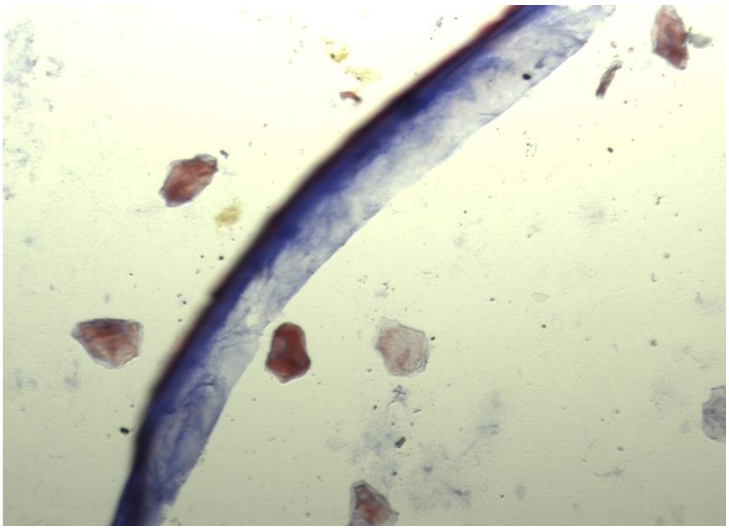


Figure 5.6. Image of grade 1 collagen components with MT staining

Grade 0 does not show blue. Grade 0 was commonly found in dHAM 4, which received the longest treatment with 0.5% SDS (30 minutes) with a combination of DNase 50 IU for 30 minutes (figure 5.6).

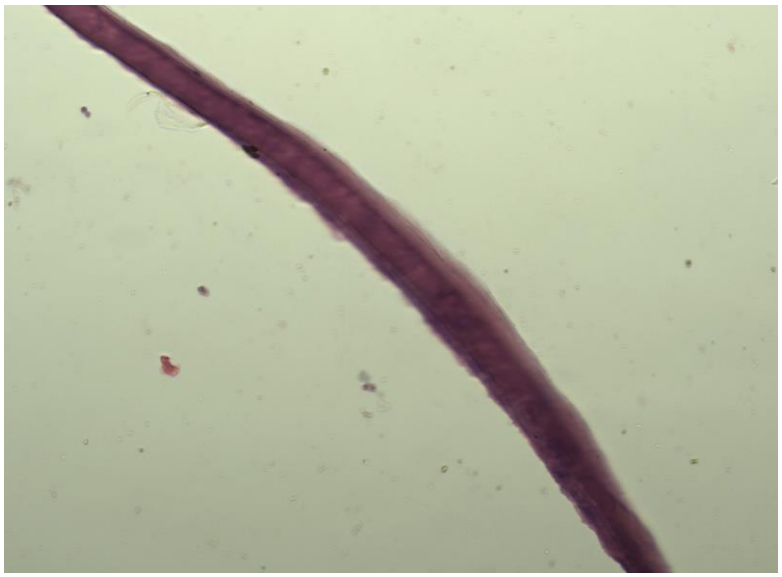


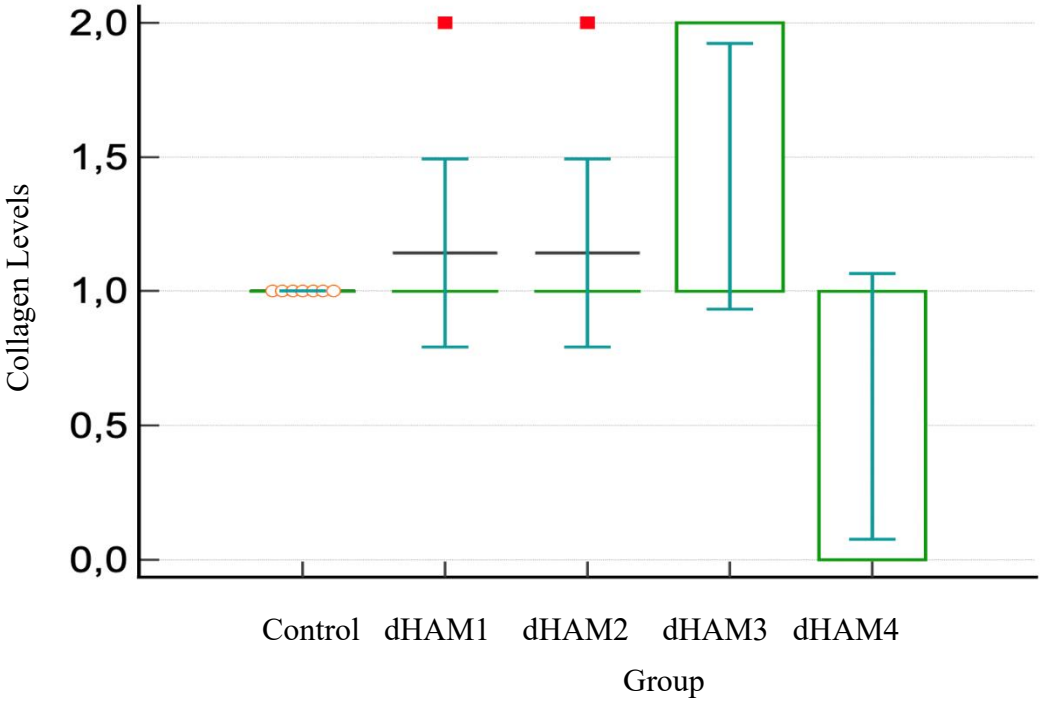
Figure 5.7. Image of grade 0 collagen components with MT staining

Significant differences in collagen components were seen in the treatment groups, especially in dHAM 3 and dHAM 4 (P <0.001) (table 5.2).

Tabel 5.2. Statistical analysis between control and treatment of collagen component variables

Variable	**Group**	**N**	**Mean**	**CI 95 %**	**P**
Collagen components in 1 field of view 400x	Control	7	1	0,89 – 1,22	**<0.0001**
	dHAM 1	7	1,14 ± 0,38		
	dHAM 2	7	1,14 ± 0,38		
	dHAM 3	7	1,43 ± 0,53		
	dHAM 4	7	0,57 ± 0,53		

The control group had stable collagen components in all seven samples. The dHAM 1 and dHAM 2 groups that do not use DNase have the same collagen components. The collagen components found in the dHAM 3 group were the most abundant compared to the other groups. The collagen component found in the dHAM 4 group was the least compared to the other groups (graph 5.2).



Graph 5.2. Statistical analysis between control and treatment of collagen component research variables

4. DISCUSSION

The dose and duration of SDS to create dHAM differed in various studies. Wilshaw et al used 0.03% SDS for 24 hours, combined with several other enzymes, namely calbiochem, aprotinin, peracetic acid, and DNase (50U/ml)[14]. Frazao et al used a longer duration of SDS 0.5% for 24 hours with a combination of Triton X-100 and 0,001 mg/ ml DNase[15]. Luo et al made acellular HAM using 0.5% SDS for 24 hours and combined with 0.25% Trypsin for 4 hours[16]. The decellularization technique used was successful in creating acellular HAM which can support the proliferation of implanted fibroblast cells[14,15,16]. However, none of these studies examined the collagen levels of the dHAM produced.

Freeze dried undecellularized HAM was the control group in this study. The control group had a still dense number of cells, namely 31 – 49 cells/field of view (mean 41.57) at 400x magnification. This research shows that a dose of 0.5% SDS for 30 minutes succeeded in eliminating HAM cells significantly with the remaining cellular components only 0 – 3 cells/field of view (mean 1.14) at 400x magnification. Almost the same results were shown at doses of 0.5% SDS for 15 minutes, 50U DNase/ml for 15 minutes, and DNase inhibitors (0.1% SDS for 15 minutes), with only 1 – 2 cells/field remaining cellular components. view (mean 1.43) at 400x magnification. Both groups of samples provided significant results in the HAM decellularization process while maintaining the same composition and density of HAM collagen as the control group. The sample group given a dose of 0.5% SDS for 30 minutes with a combination of DNase 50U/ml for 15 minutes, and a DNase inhibitor (SDS 0.1% for 15 minutes) gave better decellularization results with remaining cells approaching 0 cells/ visual field (mean 0.28) at 400x magnification but resulted in decreased HAM collagen levels compared to the control group.

Based on the evaluation results of the collagen components in the four control groups, it was found that increasing the SDS duration by 0.5% alone did not result in changes in the

composition of the HAM collagen components. However, increasing the duration of 0.5% SDS in combination with DNase can result in a reduction in the collagen component of HAM.

5. CONCLUSION

A single decellularization technique using 0.5% sodium dodecyl sulfate (SDS) with a duration of 30 minutes can produce dHAM with the same collagen structure and density as freeze-dried HAM. Making dHAM using 0.5% SDS and 50 U/ml DNase can be done while maintaining collagen structure and density, namely with a combination of 0.5% SDS for 15 minutes, 50 U/ml DNase for 15 minutes, and a DNase inhibitor (SDS 0.1% for 15 minutes). Increasing the duration of 0.5% SDS does not reduce the density of the HAM collagen component. However, increasing the duration of 0.5% SDS combined with DNase can reduce the density of HAM collagen. However, increasing the duration of 0.5% SDS combined with the DNase enzyme can result in reduced collagen structure and density.

Statement of Ethics : The Authors have no ethical conflicts to disclose

Ethical Approval number 0519/KEPK/XI/2022 from Ethical Comitte of Medical Research Dr. Soetomo Regional General Hospital, Surabaya, Indonesia

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Author Contribution : Jilvien is responsible for the designed the research, data analysis, and preparation of the manuscript. Iswinarno and Rizaliyana critically revised the manuscript for important intellectual content and extracted the compund used in this study. Priangga responsible for interpretation the sample and control with optic microscope, and Budi responsible for statistic analysis. All authors approved the final content for journal submission and publication.

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References

1. Flood, M.S., Weeks, B., Anaeme, K.O., Aguirre, H., Hobizal, K.B., Jiongco, S.E., and Klein, R.J., et al, 2020. ‘Treatment of deep full-thickness wounds containing exposed muscle, tendon, and/or bone using a bioactive human skin allograft: a large cohort case series’. *Wounds,* 32(6). PMID: 32804658.

2. Jabrink, K., Ni, G., Sonnergren, H., Schmidtchen, A., Pang, C., Bajpai, R., and Car, J., 2016. ‘Prevalence and incidence of chronic wounds and related complications: a protocol for a systematic review’. *Systematic Reviews,* 5(1). PMID: 27609108. https://doi.org/10.1186/213643-016-0329-y.

3. Martinego, L., Olsson, M., Bajpai, R., Soljak, M., Upton, Z., Schmidtchen, A., and Car, J., et al, 2018. 'Prevalence of chronic wounds in the general population: systematic review and meta-analysis of observational studies'. *Annals of Epidemiology*, 29.PMID: 30497932. https://doi.org/10.1016/j.annepidem. 2018.10.005.

4. Demling, R.H., Niezgoda, J.A., Haraway, G.D., and Mostow, E.N., 2004. 'Small intestinal submucosa wound matrix and full-thickness venous ulcers: preliminary results'. *Wounds*, 16(1).

5. McKenna, B. and Summers, N.J., 2017. 'Amnion: the ideal scaffold for treating full-thickness wounds of the lower extremity'. *Clinics in Pediatric Medicine and Surgery*, 35(1).PMID: 29156159. https://doi.org/10.1016/j.cpm.2017. 08.007.

6. Bush, K. and Gertzman, A.A., 2016. 'Process development and manufacturing of human and animal acellular dermal matrices'. In: M.Z. Albanna, dan J.H. Holmes, eds., *Skin Tissue Engineering and Regenerative Medicine*, USA: Academic Press.

7. Choudhury, D., Yee, M., Sheng, Z.L.J., Amirul, A., and Naing, M.W., 2020. 'Decellularization systems and devices: state-of-the-art'. *Acta Biomaterialia*, 115. PMID: 32771593. https://doi.org/10.1016/j.actbio.2020. 07.060.

8. Crapo, P.M., Gilbert, T.W., and Badylak, S.F., 2011. 'An overview of tissue and whole organ decellularization processes'. *Biomaterials*, 32(12). PMID: 21296410. https://doi.org/10.1016/j.biomaterials.2011.01.057.

9. Hilmy, N., Yusof, N., and Nather, A., eds., 2018. *Human amniotic membrane: basic science and clinical application*. Singapore: World Scientific Publishing Co. Ple. Ltd. https://doi.org/10.1142/10607.

10. Sari, I.B.K., 2018. Pengaruh teknik deselularisasi terhadap kadar kolage tipe II dan porositas matriks *cartilage bovine scaffold*. *Thesis.* Fakultas Kedokteran. Surabaya: Universitas Airlangga.

11. Ferdiansyah, Noer, S., Martanto, T.W., Rizaliyana, S., and Wardhana, T.H., 2005. *Buku pedoman kerja bank jaringan*. Surabaya: Instalasi Pusat Biomaterial Bank Jaringan RSUD Dr. Soetomo.

12. Ferdiansyah, Noer, S., Martanto, T.W., Rizaliyana, S., and Wardhana, T.H., 2005. *Buku pedoman kerja bank jaringan*. Surabaya: Instalasi Pusat Biomaterial Bank Jaringan RSUD Dr. Soetomo.

13. Suvarna, S.K., Layton, C., and Bancroft, J.D., eds., 2019. *Bancroft's: theory and practice of histological techniques, 8th ed*. London: Elsevier.

14. Wilshaw, S.P., Kearney, J.N., Fisher, J., and Ingham, E., 2006. 'Production of an acellular amniotic membrane matrix for use in tissue engineering'. *Tissue Engineering*, 12(8). PMID: 16968153. https://doi.org/10.1089/ten.2006.12. 2117.

15. Frazao, P.L., Castro, J.V., Silva, C.N., and Neves, N.M., 2019. 'Method to decellularize the human chorion membrane'. *Methods in Cell Biology*, 157(2). https://doi.org/10.1016/bs.mcb.2019.11.002

16. Luo, J.C., Li X.Q., and Yang, Z.M., 2004. [Preparation of human acellular amniotic membrane and its cytocompatibility and biocompatibility]. Chinese journal of reparative and reconstructive surgery, 18(2). PMID: 15065408.