Original Article

Rapid Decellularization of Bovine Amniotic Membrane Using EDTA Pretreatment and Low Concentration SDS

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ABSTRACT

Objective: To identify a cost-effective protocol that achieves effective decellularization while preserving the key biological properties of bovine amniotic membrane.

Study Design: Experimental laboratory study

Place and Duration of Study: This was conducted at the Tropical Disease Center Laboratory, Universitas Airlangga, Surabaya, Indonesia; the Histology Laboratory, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia; and the Pathology Laboratory, R.T. Notopuro Regional General Hospital, Sidoarjo, Indonesia, between December 2025 and April 2026.

Methods: BAM samples from six healthy donor cows underwent 0.2% EDTA pretreatment followed by varying SDS concentrations and exposure durations. Decellularization efficacy was assessed by residual double stranded DNA (dsDNA) quantification and histological evaluation of residual nuclei. Collagen density, transforming growth factor beta 1 (TGFβ1), and BHK 21 fibroblast viability were evaluated to assess scaffold preservation and cytocompatibility.

Results: Decellularization significantly reduced residual dsDNA levels ($p = 0.001$). Treatment with 0.2% EDTA for 30 minutes followed by 0.1% SDS for 30 minutes reduced dsDNA below the accepted decellularization threshold and eliminated detectable cellular nuclei. Increasing SDS concentration or exposure duration provided no additional decellularization benefit. Collagen density differed significantly among groups ($p = 0.009$), with higher SDS exposure associated with greater extracellular matrix disruption. TGFβ1 levels were not significantly different between groups ($p = 0.157$). All groups demonstrated cell viability above 70%, indicating acceptable cytocompatibility.

Conclusions: Pretreatment with 0.2% EDTA for 30 minutes followed by 0.1% SDS for 30 minutes provided effective decellularization while preserving extracellular matrix structure, TGFβ1 retention, and cytocompatibility. This simple protocol may represent a practical approach for producing BAM derived scaffolds for regenerative medicine.

Key Words: Amniotic Membrane, Decellularization, Extracellular Matrix, Tissue Engineering, Regenerative Medicine

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INTRODUCTION

Amniotic membrane is widely used as a biological scaffold because its extracellular matrix (ECM) architecture and bioactive molecules support tissue repair, angiogenesis, and matrix remodeling.[1,2] These properties have enabled broad applications in regenerative medicine and tissue engineering. However, clinical use of human amniotic membrane is limited by donor availability, ethical concerns, and stringent donor screening requirements.[2] As a result, alternative tissue sources suitable for scalable biomaterial production are increasingly being explored.[3]

Bovine amniotic membrane (BAM) is a promising alternative because it shares key structural and biological features with human amniotic membrane, including a collagen-rich ECM and regenerative growth factors.[4] Larger tissue availability and favorable biomechanical properties further support its potential use in regenerative medicine.[5,6] However, as a xenogeneic tissue, BAM contains cellular and nuclear components that may trigger host immune responses

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after implantation. Effective decellularization is therefore necessary to improve biocompatibility while preserving ECM structure and function.[3]

Among decellularization methods, sodium dodecyl sulfate (SDS) is widely used for its ability to remove cellular and nuclear material.[7] However, excessive exposure may damage the ECM and impair scaffold performance.[7] Ethylenediaminetetraacetic acid (EDTA) disrupts calcium-dependent cell–matrix interactions and may enhance detergent-based decellularization.[7] Previous studies have achieved amniotic membrane decellularization using detergent combinations, enzymatic digestion, prolonged processing, or extensive tissue manipulation.[8-11] Although effective, these approaches may increase cost and processing complexity.

This study evaluated a simplified decellularization protocol using EDTA pretreatment followed by varying SDS concentrations and exposure durations. Decellularization efficacy was assessed by residual double-stranded DNA quantification and histological evaluation, while scaffold preservation was examined through collagen density, transforming growth factor beta 1 (TGF-β1) retention, and cytocompatibility analyses. The aim was to identify a cost-effective protocol that achieves effective decellularization while preserving the key biological properties of bovine amniotic membrane.

METHODS

Sample preparation and decellularization: This experimental laboratory study was conducted at the Tropical Disease Center Laboratory, Universitas Airlangga, Surabaya, Indonesia; the Histology Laboratory, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia; and the Pathology Laboratory, R.T. Notopuro Regional General Hospital, Sidoarjo, Indonesia, between December 2025 and April 2026. The study protocol was approved by the Animal Care and Use Committee (ACUC), Universitas Airlangga (Approval No. 2.KEH.187.012.2025).

Bovine amniotic membranes (BAM) were obtained from six healthy cows immediately after normal delivery. Following removal of blood contaminants and separation from the chorion, membranes were assigned to seven groups: native BAM without treatment (K0), EDTA 0.2% for 30 minutes (K1), EDTA 0.2% for 30 minutes followed by SDS 0.1% for 30 minutes (K2), EDTA 0.2% for 30 minutes followed by SDS 0.1% for 60 minutes (K3), EDTA 0.2% for 30 minutes followed by SDS 0.3% for 30 minutes (K4), EDTA 0.2% for 30 minutes followed by SDS 0.5% for 60 minutes (K5), and EDTA 0.2% for 30 minutes followed by SDS 0.5% for 90 minutes (K6).

Following decellularization, membranes were washed with distilled water, freeze dried, sterilized by beta irradiation (25 kGy), and stored at 4°C until analysis.

Evaluation of decellularization efficiency: Decellularization efficacy was assessed by residual double stranded DNA (dsDNA) quantification and histological evaluation of residual nuclei. Residual dsDNA was measured using the QuantiFluor® dsDNA System (Promega, USA) and expressed as ng/mg dry tissue weight. Histological assessment was performed using hematoxylin and eosin staining, and residual nuclei were quantified from five randomly selected non overlapping high power fields per specimen.

Evaluation of scaffold preservation: Scaffold preservation was assessed through collagen density and transforming growth factor beta 1 (TGFβ1) retention. Collagen density was evaluated using Mallory Azan staining and quantified with ImageJ software (National Institutes of Health, USA). TGFβ1 concentrations were measured using a bovine specific ELISA kit (ELK Biotechnology, China) according to the manufacturer's instructions.

Cytocompatibility assessment: Cytocompatibility was evaluated using an indirect MTT assay in BHK 21 fibroblasts. Cell viability was expressed as a percentage relative to untreated controls. Materials with viability ≥70% were considered non cytotoxic according to ISO 10993.[12]

Statistical analysis: Data normality was assessed using the Shapiro Wilk test. Comparisons among groups were performed using the Friedman test followed by the Durbin Conover post hoc test when appropriate. Statistical significance was defined as $p < 0.05$.

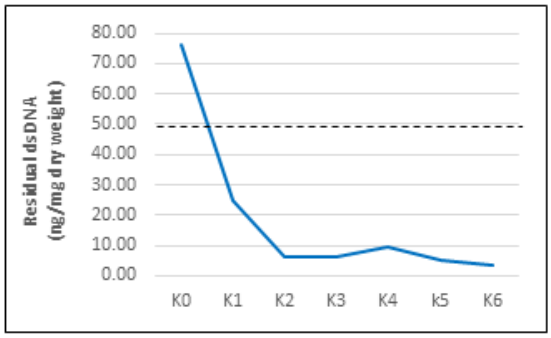
RESULTS

Decellularization efficiency: Residual dsDNA levels decreased significantly following decellularization ($p = 0.001$). Native BAM demonstrated a median dsDNA content of 76.10 ng/mg dry weight, which decreased to 24.78 ng/mg after EDTA treatment alone (K1) and to 6.36 ng/mg following treatment with EDTA 0.2% and SDS 0.1% for 30 minutes (K2). Further increases in SDS concentration or exposure duration did not result in meaningful additional reductions in dsDNA levels (Figure 1). Post hoc analysis demonstrated a significant difference between K0 and K2 ($p < 0.01$), whereas no significant difference was observed between K2 and the remaining SDS treated groups.

Histological analysis supported the dsDNA findings (Figure 2). Native BAM contained abundant cellular nuclei, whereas progressive cellular removal was observed following decellularization. No residual nuclei were detected in groups K2 through K6. Quantitative analysis demonstrated a significant difference among groups ($p < 0.01$), with complete removal of visible nuclei achieved following treatment with EDTA 0.2% and SDS 0.1% for 30 minutes.

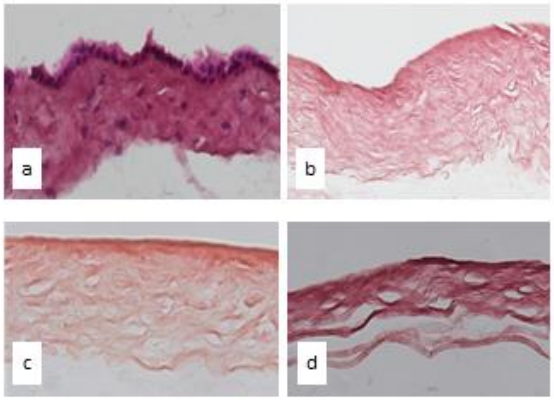
Scaffold preservation: Collagen architecture was progressively affected by increasing SDS concentration and exposure duration (Figure 3). Native BAM

exhibited densely organized collagen fibers, whereas higher intensity SDS treatments resulted in a more dispersed collagen structure.



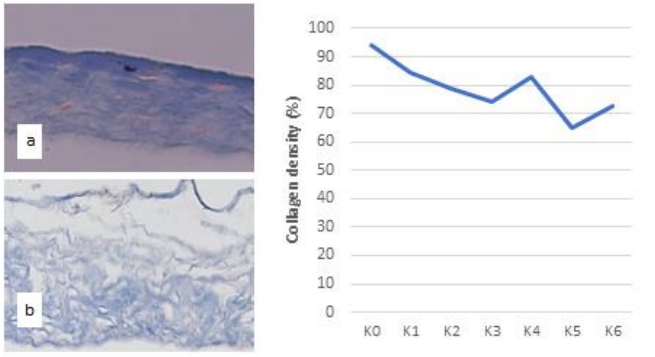
	Median	Min	Max
K0	76.10	8.02	500.00
K1	24.78	16.45	185.00
K2	6.36	0.93	18.10
K3	6.09	0.27	32.75
K4	9.46	6.14	27.65
K5	5.30	2.21	26.90
K6	3.66	0.43	41.00

Figure No.1: Residual double stranded DNA (dsDNA) content of bovine amniotic membrane following decellularization. Treatment groups included native BAM (K0), EDTA 0.2% for 30 minutes (K1), EDTA 0.2% for 30 minutes followed by SDS 0.1% for 30 minutes (K2), EDTA 0.2% for 30 minutes followed by SDS 0.1% for 60 minutes (K3), EDTA 0.2% for 30 minutes followed by SDS 0.3% for 30 minutes (K4), EDTA 0.2% for 30 minutes followed by SDS 0.5% for 60 minutes (K5), and EDTA 0.2% for 30 minutes followed by SDS 0.5% for 90 minutes (K6). The dashed line indicates the recommended decellularization threshold of 50 ng/mg dry tissue.



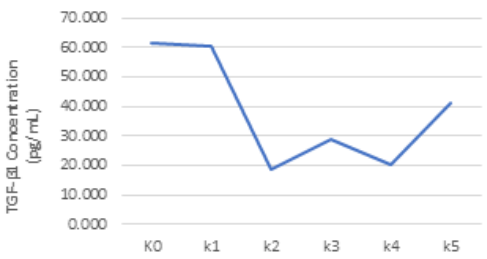
	Median	Min	Max
K0	21.1	1	61.6
K1	0	0	37.2
K2	0	0	0
K3	0	0	0
K4	0	0	0
K5	0	0	0
K6	0	0	0

Figure No.2: Histological evaluation of cellular removal following decellularization. Representative hematoxylin and eosin-stained sections of (a) native BAM (K0), (b) K1, (c) K2, and (d) K6. Progressive reduction in cellular nuclei was observed following treatment, with complete removal of detectable nuclei in groups K2 through K6. Original magnification ×400.



	K0	K1	K2	K3	K4	K5	K6
Median	93.865	84.347	78.506	74.025	82.697	65.028	72.436
Min	81.388	76.326	51.952	56.657	41.366	39.626	60.031
Max	96.931	96.077	87.632	83.570	89.780	77.223	95.437

Figure No.3: Assessment of extracellular matrix preservation following decellularization. Representative Mallory Azan-stained sections of (A) native BAM (K0) and (B) K5, demonstrating progressive alteration of collagen architecture with increasing SDS exposure. Quantitative analysis of collagen density is presented for all treatment groups. Original magnification ×400.



	Median	Min	Max
K0	61.771	19.018	1004.612
K1	60.408	17.991	941.095
K2	18.757	5.886	23.056
K3	28.640	22.557	74.783
K4	20.286	13.794	42.202
K5	41.021	23.056	51.403

Figure No.4: Transforming growth factor beta 1 (TGFβ1) concentrations in native and decellularized bovine amniotic membrane. Data are presented as median, minimum, and maximum values. TGFβ1 analysis was performed in groups K0 through K5.

Table No.1: Comparison of published amniotic membrane decellularization protocols and the protocol identified in the present study

Study	Tissue	Decellularization protocol	Duration	Additional processing
Taghiabadi et al.[8]	HAM	EDTA 0.1% + SDS 0.03%	40 h	TBS
Gholipourmalekabadi et al.[9]	HAM	EDTA 0.2%	30 min	NaOH, ammonium chloride, scraping
Yang et al.[10]	BAM	SDS 1%	12–24 h	Trypsin
Leonel et al.[11]	CAM	SDS 1%	48 h	TRIS, Triton
Sang et al.[23]	HAM	EDTA 0.05%	2 h	Scraping
Asgari et al.[16]	h-HAM	SDS 0.5%	Variable	Triton
Ignatov et al.[17]	HAM	SDS 0.5%	5 h	Triton X-100
Ballesteros et al.[24]	BAM	SDS 0.1%	4 h	PAA, NaOH
Present study	BAM	EDTA 0.2% + SDS 0.1%	60 min	None

Quantitative analysis demonstrated significant differences in collagen density among groups ($p = 0.009$). However, collagen density in K2 did not differ significantly from native BAM, indicating preservation of extracellular matrix structure despite effective decellularization.

TGFβ1 remained detectable in all evaluated groups (Figure 4). Median concentrations were 61.77 pg/mL in K0, 60.41 pg/mL in K1, 18.76 pg/mL in K2, 28.64 pg/mL in K3, 20.29 pg/mL in K4, and 41.02 pg/mL in K5. Although lower median values were observed in several decellularized groups, no significant differences were identified among groups ($p = 0.157$).

Cytocompatibility assessment: All treatment groups demonstrated cell viability values above the 70% threshold for non-cytotoxic materials. Median viability ranged from 81.74% to 92.60% across experimental groups. No reduction in cell viability was observed with increasing SDS exposure, indicating that the decellularization and washing procedures produced scaffolds with acceptable cytocompatibility.

DISCUSSION

The present study demonstrated that pretreatment with 0.2% EDTA followed by 0.1% SDS for 30 minutes provided the best balance between effective decellularization and preservation of scaffold properties. This protocol reduced residual dsDNA below the accepted threshold for decellularized tissues and completely eliminated detectable cellular nuclei, indicating effective removal of potentially immunogenic cellular components.[3] Increasing the SDS concentration or extending the exposure time did not further improve decellularization, suggesting that low concentration SDS was sufficient when combined with EDTA pretreatment. The complementary actions of EDTA and SDS likely contributed to this effect, as EDTA disrupts calcium dependent cell matrix interactions and facilitates cellular detachment, whereas SDS solubilizes cellular membranes and nuclear material.[7,13]

Preservation of the extracellular matrix is equally important during decellularization. In the present study, collagen density progressively decreased with increasing SDS concentration and exposure time, consistent with previous reports that excessive detergent exposure disrupts extracellular matrix organization and compromises scaffold integrity.[16-18] Importantly, collagen density in K2 did not differ significantly from native BAM, indicating that effective decellularization can be achieved with minimal matrix disruption when detergent exposure is carefully controlled. These findings suggest that increasing detergent exposure beyond the K2 protocol offers little additional benefit while increasing the risk of extracellular matrix damage.

TGFβ1 remained detectable in all treatment groups without significant differences after decellularization. As TGFβ1 regulates cell proliferation, differentiation, and matrix remodeling during tissue repair,[19,20] its preservation may reflect continued binding to extracellular matrix components throughout processing.[21,22] Although only TGFβ1 was evaluated, these findings suggest that the selected protocol preserved important biological characteristics of BAM.

All treatment groups demonstrated cell viability above the accepted threshold for non cytotoxic biomaterials, indicating that the washing procedure effectively minimized residual detergent toxicity. Residual SDS is a recognized concern in detergent based decellularization because incomplete removal can impair cell attachment, reduce cell viability, and adversely affect host tissue responses.[18] The favorable cytocompatibility observed in this study further supports the suitability of the selected protocol for biomaterial applications.

Comparison with previously published protocols highlights the practical advantages of the present approach (Table 1). Previous studies have achieved successful amniotic membrane decellularization using prolonged detergent exposure, enzymatic digestion, multiple reagents, or mechanical manipulation.[8-11,16,17,23,24] In contrast, the protocol identified in this study achieved effective cellular removal using only EDTA and low concentration SDS within a total treatment time of 60 minutes. This simplified approach may improve reproducibility, reduce processing costs, and facilitate larger scale production, particularly in resource limited settings.

Several limitations should be acknowledged. Bioactivity was assessed only by measuring TGFβ1 despite the presence of multiple regenerative growth factors in amniotic membrane. Biomechanical testing was not performed, precluding evaluation of functional changes in the extracellular matrix after decellularization. In addition, this study was limited to in vitro analyses. Future studies should investigate mechanical properties, host response, tissue integration, and regenerative efficacy in vivo.

CONCLUSION

Pretreatment with 0.2% EDTA followed by 0.1% SDS for 30 minutes effectively decellularized bovine amniotic membrane, reducing residual dsDNA below accepted thresholds and eliminating detectable nuclei. This protocol preserved collagen density, retained detectable TGF-β1, and demonstrated acceptable cytocompatibility. Higher SDS concentrations or longer exposure did not enhance decellularization and resulted in greater extracellular matrix disruption. These findings support a simple, low-concentration SDS protocol for producing biologically preserved bovine amniotic membrane scaffolds for regenerative medicine. Further studies should evaluate biomechanical properties and in vivo performance.

Author's Contribution:

Concept & Design or acquisition of analysis or interpretation of data:	Erfan Nasrullah, Heri Suroto
Drafting or Revising Critically:	Erfan Nasrullah, Heri Suroto
Final Approval of version:	All the above authors
Agreement to accountable for all aspects of work:	All the above authors

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Conflict of Interest: The study has no conflict of interest to declare by any author.

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