Research Article

Neutrophil and Mononuclear Cell Density After Tracheal Defect Reconstruction with Dried Amniotic Membrane and Sternocleidomastoid Muscle Patches in Rabbits

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Abstract

Thirty-six male New Zealand white rabbits were randomized to reconstruction of a 0.6 × 0.6 cm full-thickness tracheal defect with an autologous sternocleidomastoid (SCM) muscle patch or a freeze-dried bovine amniotic membrane (DAM) patch (n = 18 each). On day 10, neutrophils and mononuclear cells were counted in five fields (haematoxylin and eosin, 400×). Mononuclear cell density was significantly higher with DAM than with SCM (median 18.30 vs. 6.90 cells/field; P < 0.001). Neutrophil density was minimal in both groups and did not differ (P = 0.075). DAM elicited a stronger mononuclear cell response than the autologous muscle patch.

Keywords: Amniotic membrane, Rabbit, Sternocleidomastoid muscle, Tracheal reconstruction

Introduction

Tracheal injury is uncommon but potentially fatal. It occurs in 0.5–2% of patients with thoracic and cervical trauma, and mortality ranges from 15 to 40% (Farley and Schlicksup, 2024). Resection with end-to-end anastomosis is limited by the length of trachea that can be removed and by postoperative stenosis and infection, so patch materials for partial defects continue to be investigated (Miggiolaro *et al.*, 2023; Neto *et al.*, 2024).

Amniotic membrane has been used as a biological scaffold for tracheal repair in rabbits because of its extracellular matrix, growth factors and reported anti-inflammatory properties (Jorge *et al.*, 2018; Neto *et al.*, 2024). The sternocleidomastoid (SCM) muscle is an autologous, vascularized tissue that provides structural support to the reconstructed tracheal wall (Xia *et al.*, 2021). The local tissue response to these two materials has not been compared directly.

Neutrophils dominate the acute inflammatory phase after tracheal implantation, whereas mononuclear cells regulate the proliferative and remodelling phases (Ahn *et al.*, 2017). An acellular membrane must be degraded and remodelled by host immune cells, while an autologous muscle patch integrates through revascularization. The present study therefore tested the hypothesis that neutrophil and mononuclear cell density is higher after tracheal defect reconstruction with a dried amniotic membrane (DAM) patch than with an SCM muscle patch in rabbits.

Materials and Methods

The study was a randomized controlled laboratory experiment approved by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia (No. 2.KEH.35.02.2026). Thirty-six male New Zealand white rabbits, aged 6–9 weeks and weighing 1,900–2,500 g, were acclimatized for 7 days and allocated by permuted block randomization to a control group (P1, SCM muscle patch; n = 18) or a treatment group (P2, DAM patch; n = 18). The sample size was based on a standard deviation of 4.5 and an expected difference of 9 cells per field (Mujib and Seputra, 2021), with a two-sided α of 0.05 and a power of 90%; the minimum was nine animals per group, and 18 were enrolled to allow for attrition.

Freeze-dried bovine amniotic membrane, decellularized and sterilized by gamma irradiation at 25 kGy (Oh *et al.*, 2021), was obtained from the Tissue Bank of Dr. Soetomo General Academic Hospital, Surabaya. It was folded into six layers and trimmed to 0.6 × 0.6 cm. The autologous patch was a 1 × 1 cm segment of the sternocleidomastoid muscle harvested through the same incision, kept in sterile 0.9% saline and trimmed to 0.6 × 0.6 cm.

Under general anaesthesia with intramuscular ketamine (20–40 mg/kg), the anterior neck was shaved and disinfected with 10% povidone-iodine. A square full-thickness defect of 0.6 × 0.6 cm spanning two to three tracheal rings was created with a No. 15 blade and closed with the assigned patch using simple interrupted 6-0 polyglactin 910 sutures (Mujib and Seputra, 2021). The skin was closed with 3-0 nylon. Penicillin (50,000 units/kg, intramuscular) was given for 5 days, with ketorolac for analgesia (Bobba et al., 2026).

On postoperative day 10, the animals were euthanized as per the approved protocol [method to be specified], and a full-thickness tracheal specimen (1 × 0.5 cm) including the repair site was collected. Specimens were fixed

in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 µm and stained with haematoxylin and eosin. An examiner blinded to group allocation counted neutrophils and mononuclear cells (lymphocytes, plasma cells and macrophages) in five fields at 400× magnification. The five counts were averaged to one value per animal (cells/field).

Normality was assessed with the Shapiro-Wilk test. As the data were not normally distributed, the groups were compared with the Mann-Whitney U test in SPSS version 26.0, and $P < 0.05$ was considered significant.

Results and Discussion

All 36 rabbits were included in the analysis. Cell densities in the two groups are presented in Table I, and representative histological findings in Fig. 1.

Neutrophil density was very low in both groups. No neutrophils were seen in any animal of the SCM group, whereas neutrophils were found in 3 of 18 animals of the DAM group, with a maximum of 3.20 cells/field. The difference was not significant ($P = 0.075$). This finding agrees with the kinetics of healing: neutrophils predominate in the inflammatory phase during the first four days after injury and are then replaced by monocytes and macrophages (Diegelmann and Evans, 2004), and after tracheal scaffold implantation in rabbits their number was highest in the first week (Ahn *et al.*, 2017). By day 10, acute inflammation had thus largely resolved with both materials. Neutrophils occurred only in the DAM group, which is in the direction of the hypothesis, but with values close to zero a difference is difficult to detect at this time point; earlier sampling would be required (Bobba et al., 2021).

Mononuclear cell density was significantly higher in the DAM group than in the SCM group ($P < 0.001$), with a median more than twice as high and a much wider range (Table I). The hypothesis was therefore supported for mononuclear cells. A mononuclear infiltrate is expected in both groups at day 10, which falls within the proliferative phase (Diegelmann and Evans, 2004). The difference in its magnitude is consistent with the nature of the two patches. The SCM patch is an autograft that integrates through revascularization (Xia *et al.*, 2021), whereas the DAM is an acellular extracellular matrix that has to be degraded and remodelled by recipient immune cells.

Amniotic membrane is described as having low immunogenicity and anti-inflammatory activity, including inhibition of T-lymphocyte proliferation and monocyte differentiation (Niknejad *et al.*, 2008). Three features of the present model may explain why a lower mononuclear cell density was not observed. The membrane was bovine and therefore xenogeneic to the rabbit. Decellularization, freeze-drying and gamma irradiation leave mainly a collagen-rich scaffold without viable amniotic cells (Oh *et al.*, 2021). Finally, monocyte and macrophage infiltration around tracheal implants continues to increase for several weeks (Ahn *et al.*, 2017), so day 10 represents an early stage of this response. Earlier rabbit studies reporting favourable tracheal repair used acellular human amniotic membrane, in some cases combined with bioactive agents or other decellularized matrices (Jorge *et al.*, 2018; Miggiolaro *et al.*, 2023; Neto *et al.*, 2024), which differs from the material tested here.

The study has limitations. Tissue was examined at a single time point, and haematoxylin and eosin staining cannot distinguish mononuclear cell subtypes or macrophage activation states, so the infiltrate may represent either an early foreign body reaction or constructive remodelling. Airway patency, stenosis and epithelialization were not assessed, and the results cannot be extrapolated to human amniotic membrane. Serial sampling and immunohistochemistry for macrophage markers are needed.

Table I. Neutrophil and mononuclear cell density (cells/field, 400×) on day 10 after tracheal defect reconstruction in rabbits (n = 18 per group)

Variable	Group	Mean ± SD	Median	Range	U	P value
Neutrophils	P1 (SCM patch)	0.00 ± 0.00	0.00	0.00–0.00	135	0.075
Neutrophils	P2 (DAM patch)	0.24 ± 0.77	0.00	0.00–3.20		
Mononuclear cells	P1 (SCM patch)	9.41 ± 5.72	6.90	4.60–23.00	39	<0.001*
Mononuclear cells	P2 (DAM patch)	32.27 ± 30.94	18.30	8.80–122.00		

*Significant difference between groups (Mann-Whitney U test, $P < 0.05$).

Summary

Ten days after reconstruction of a tracheal defect in New Zealand white rabbits, a freeze-dried bovine amniotic membrane patch was associated with a significantly higher mononuclear cell density than an autologous sternocleidomastoid muscle patch. Neutrophil density was very low with both materials and did not differ significantly. Serial and immunohistochemical studies are required to establish whether the mononuclear infiltrate around the amniotic membrane represents constructive remodelling or a foreign body reaction.

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References

1. Ahn, H.J., R. Khalmuratova, S.A. Park, E.J. Chung, H.W. Shin and S.K. Kwon (2017) Serial analysis of tracheal restenosis after 3D-printed scaffold implantation: recruited inflammatory cells and associated tissue changes. *Tissue Engineering and Regenerative Medicine*. 14(5): 631-639.
2. Diegelmann, R.F. and M.C. Evans (2004) Wound healing: an overview of acute, fibrotic and delayed healing. *Frontiers in Bioscience*. 9: 283-289.
3. Farley, L.S. and K.E. Schlicksup (2024) Tracheal injury. In: *StatPearls* [Internet]. StatPearls Publishing, Treasure Island (FL). PMID: 31613457.
4. Jorge, L.F., J.C. Francisco, N. Bergonse, C. Baena, K.A.T. Carvalho, E. Abdelwahid, J.R.F. Neto, L.F.P. Moreira and L.C. Guarita-Souza (2018) Tracheal repair with acellular human amniotic membrane in a rabbit model. *Journal of Tissue Engineering and Regenerative Medicine*. 12(3): e1525-e1530.
5. Miggiolaro, A.F.R.S., A.L. Takejima, J.C. Francisco, L. De Noronha, M.L. Takejima, K. Foltz, A. Enck, L. Tannous, R.B. Simeoni, P.E.F. Stricker, K.A.T. Carvalho and L.C. Guarita-Souza (2023) Tracheal regeneration with acellular human amniotic membrane and 15-deoxy-Δ12,14 prostaglandin J2 nanoparticles in a rabbit model. *Anais da Academia Brasileira de Ciências*. 95. doi:10.1590/0001-3765202320220288
6. Mujib, S.A. and S. Seputra (2021) Experimental study of collagen density in reconstruction of tracheal defect using dried amniotic membrane. *Indian Journal of Forensic Medicine and Toxicology*. 15(2): 3379-3386.
7. Bobba, C. T., Vijay, A. G., Aher, V., Singh, J., Kejriwal, A., Srinivasan, K., & Ranjan, A. (2026). Radiographic evaluation of periapical healing following single vs multiple visit root canal treatment: A PAI based study. Journal of Pharmacy and Bioallied Sciences, 18(Suppl. 1), S206–S208. https://doi.org/10.4103/jpbs.jpbs_1567_25
8. Neto, A.E., K.M. Foltz, T. Fuchs, L.K. Gamba, M.A. Denk, P.C.L. Silveira, T.G. do Nascimento, A.M. Clemencia, J.C. Francisco, L. de Noronha and L.C. Guarita-Souza (2024) Decellularized Wharton's jelly and amniotic membrane demonstrate potential therapeutic implants in tracheal defects in rabbits. *Life*. 14(6): 782.
9. Niknejad, H., H. Peirovi, M. Jorjani, A. Ahmadiani, J. Ghanavi and A.M. Seifalian (2008) Properties of the amniotic membrane for potential use in tissue engineering. *European Cells and Materials*. 15: 88-99.
10. Oh, D., D. Son, J. Kim and S.Y. Kwon (2021) Freeze-dried bovine amniotic membrane as a cell delivery scaffold in a porcine model of radiation-induced chronic wounds. *Archives of Plastic Surgery*. 48(4): 448-456.
11. Bobba, C. T., Choksi, R., Grewal, V. S., Basra, H. K., & Momin, S. (2021). Evaluation of effects of deproteinization and intermediate bonding on the retention of pit and fissure sealants—An in-vivo study. SVOA Dentistry, 2(4), 139–142. https://www.sciencevolks.com/dentistry/pdf/SVOA-DE-02-029.pdf
12. Xia, X., Y. Cai, X. Zhu, Y. Zhu, L. Shen, Y. Zhou, W. Diao and X. Chen (2021) Tracheal window resection and reconstruction via sternocleidomastoid flap for invasive thyroid carcinoma. *Interactive CardioVascular and Thoracic Surgery*. 33(6): 899-904.