Histological evaluation of rabbit gingival wound healing transplanted with human amniotic membrane

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Abstract. Human amniotic membrane has been used as a material to accelerate wound healing and reconstruct damaged organs. The aim of the present study was to assess histologically human amniotic membrane transplantation on rabbit's gingival wound. Three- to 4-month-old male rabbits were divided into 2 groups, i.e., control (group I) and amniotic membrane-transplanted animals (group II). Buccal gingival wounds were created by a punch-biopsy instrument and covered by a 5-layered human amniotic membrane for group II or left uncovered for group I. Gingival biopsies were taken at days 1, 3, 5, 7 and 10, processed for paraffin sections and stained with haematoxylin–eosin or von Gieson. Thickness of epithelial layer, the number of polymorphonuclear cells (PMN), fibroblasts and new blood vessels as well as density of collagen fibres were assessed. The results showed that the number of fibroblasts and new blood vessels, but not PMN, from group II was higher than that from group I ($P < 0.05$). Similarly, the epithelial thickness and density of collagen fibres from group II were significantly higher than those from group I ($P < 0.05$). The results of the present study indicate that amniotic membrane transplantation may induce rapid epithelialization and both granulation tissue and collagen formation but suppress inflammation, suggesting that amniotic membrane transplantation may promote rapid gingival wound healing in rabbits compared to secondary healing.

The amniotic membrane, the innermost layer of the foetal membrane, contains basement membrane components and both growth factors and proteinase inhibitors. The use of this material in surgical procedures such as a biological bandage for dressing burns[9], and skin ulcers[14] and as an aid to physiological wound healing[5] has long been known. The precise mechanism by which the amniotic membrane accelerates wound healing remains to be further investigated. Studies on corneal transplantation indicate that this membrane may enhance epithelialization, inhibit both inflammation and scarring, regulate angiogenesis and posse anti-bactericidal activity[3].

The biological events of wound healing in gingival tissues are similar to those in the skin, but healing at the former site seems to be much faster and with less scar

formation as compared with that at the latter sites[6]. Attempts to produce materials for enhancement of periodontal wound healing and reconstruction of periodontal tissue damage have been made. For example, enamel matrix proteins[17] and acellular dermal matrix allograft[21] were applied on the gingival wound in humans and the results showed that these materials promote both epithelialization and tissue vascularization and reduce inflammation. Since amniotic membrane may induce rapid physiological wound healing, the aim of the present study was to determine whether gingival wound healing in rabbits may be enhanced by transplantation of this membrane.

Materials and methods

Gingival wound preparation and amniotic membrane transplantation

Three- to 4-month-old male rabbits (weight 1–2 kg) were divided into 2 groups, each consisted of 15 animals. Groups I and II were a control and an amniotic membrane-treated group, respectively. All animals were anaesthetized intramuscularly with Ketamine HCl (50 μg kg^{-1} body weight) 1 h before surgical procedures. Immediately before surgical procedure, animals were anaesthetized intramuscularly with Ketamin HCl (80 mg kg^{-1} body weight). Gingival wound was generated by using a punch-biopsy instrument (diameter 4 mm) at the buccal site of gingiva between maxillary incisor and premolar. The wound was left uncovered for group I or was covered by a sterile 5-layered human amniotic membrane (5 mm × 5 mm in size) for group II. The freeze-dried human amniotic membranes were kindly donated by The Biomaterial Center and Tissue Bank, Dr Soetomo's Hospital, School of Medicine, Airlangga University, Surabaya, Indonesia. The basement membrane of the first layer was attached by using sterile saline onto the epithelial surface of the second layer. This procedure was repeated until all 5 layers were bound. The amniotic membrane was transferred to the gingiva with the basement membrane attaching the gingival wound and fitted to cover the whole wound by #5-0 vicryl suturing, followed by trimming off the excess portion. The analgesic medication was orally given 3 times a day for 2 days after surgical procedure. The experimental protocol was approved by the ethical committee, School of Medicine, Gadjah Mada University, Yogyakarta, Indonesia.

Histological assessment

Animals were sacrificed at days 1, 3, 5, 7 and 10. Three animals of each group were sacrificed at the indicated day. The buccal gingival tissue was resected, fixed in 10% neutral formalin buffer, embedded in paraffin and sectioned serially at 6 μm. The sections were stained with either haematoxylin–eosin (HE) or von Gieson staining and viewed under a light microscope. The number of polymorphonuclear (PMN) cells, fibroblasts and new blood vessels were then accounted. Fibroblasts were morphologically characterized as pink-stained stellate or spindle-shaped cells with blue-stained nucleus. Blood vessels were characterized as endothelial cell-assembled capillary vessels that contained erythrocytes[1]. The epithelial thickness was determined by using visopan (Merck, Reichert) and the measurement was then converted to micrometer (1 cm = 0.075 μm). Density of collagen fibres was scored using scales modified from a study by TALAS et al.[19] as follows: 1 = few collagen fibres; 2 = few and partially spread collagen fibres; 3 = few and fully spread collagen fibres and 4 = dense collagen fibres. The

observers other than the authors were blinded to the treatment groups.

Statistical analysis

The differences between groups I and II and within each group on the number of PMN cells, fibroblasts and blood vessels were analysed by '*t*' test and multi-analysis of variance, respectively. The density of collagen fibres between groups I and II and within each group were analysed by Mann–Whitney *U*-test and Kruskal–Wallis, respectively. Data were calculated by using a statistical package (SPSS Inc., Chicago, IL, USA).

Results

For the sake of brevity and clarity, photomicrographs are only shown from both the control and amniotic membrane-treated animals at day 10, otherwise indicated. Histological sections taken at day 1 showed that necrotic tissues with many infiltrated inflammatory cells, particularly PMN, in the wounded site of group I were much prominent as compared with those in group II (Fig. 1A and B). Gingival PMN from both groups at day 10 could hardly be
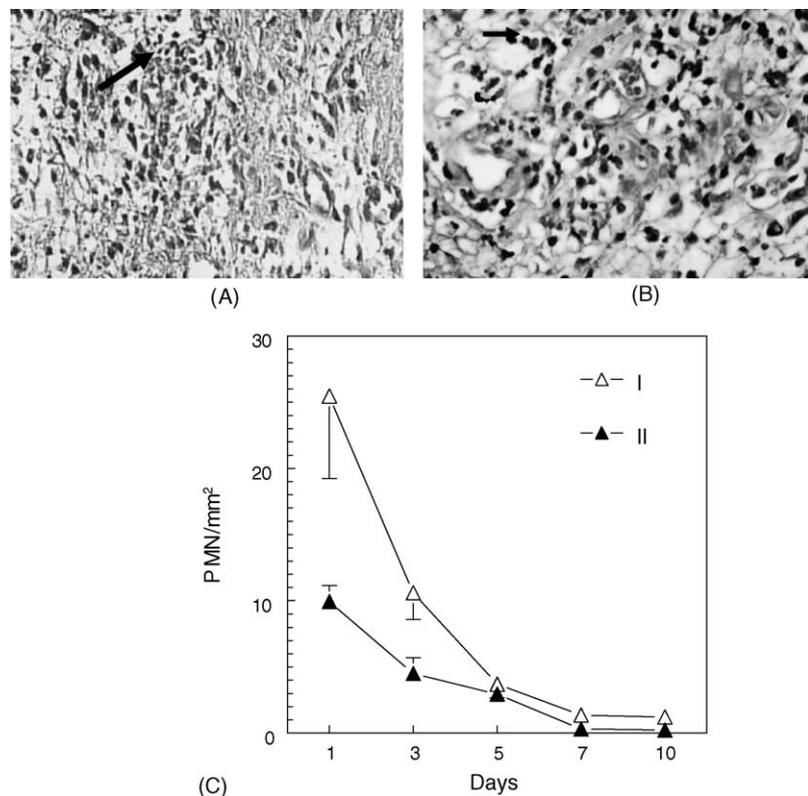

Fig. 1. Large necrotic tissue with polymorphonuclear cells (PMN) infiltration in the gingival wounded site from group I (A, left panel) at day 1 is shown as compared with that from amniotic membrane-transplanted animals (group II) (B, right panel) (HE, 400×). Panel (C) represents mean and standard deviation of the number of PMN. Arrows indicate PMN.
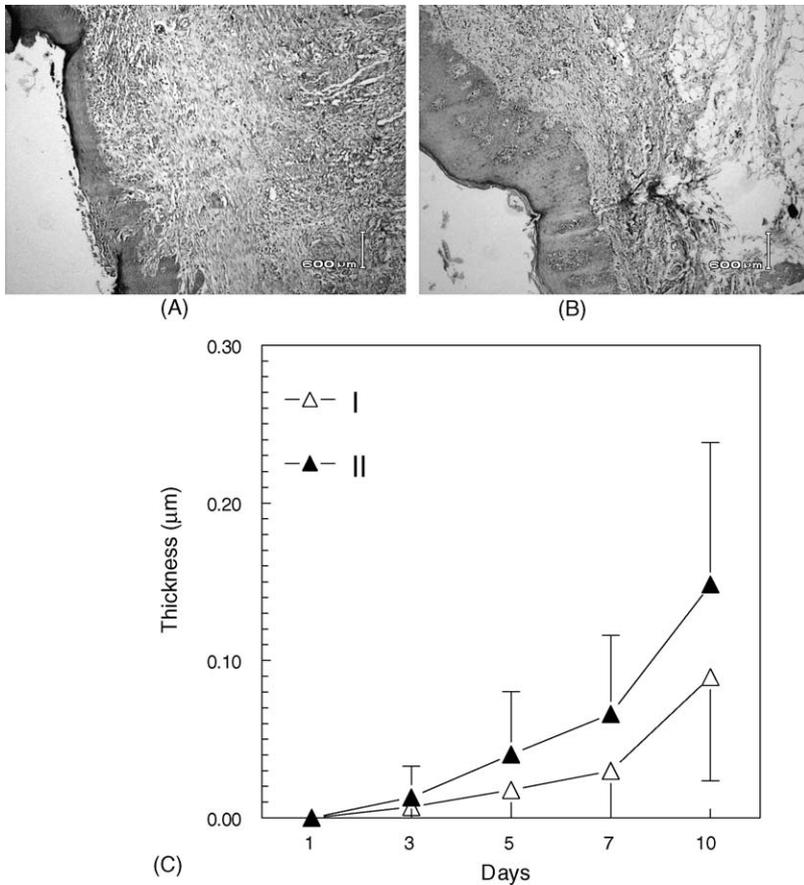
Fig. 2. The epithelial thickness at the gingival wounded site from the control (group I) (A, left panel) at day 10 is less as compared with that from amniotic membrane-transplanted animals (group II) (B, right panel) (HE, 100×). Panel (C) represents mean and standard deviation of the epithelial thickness.
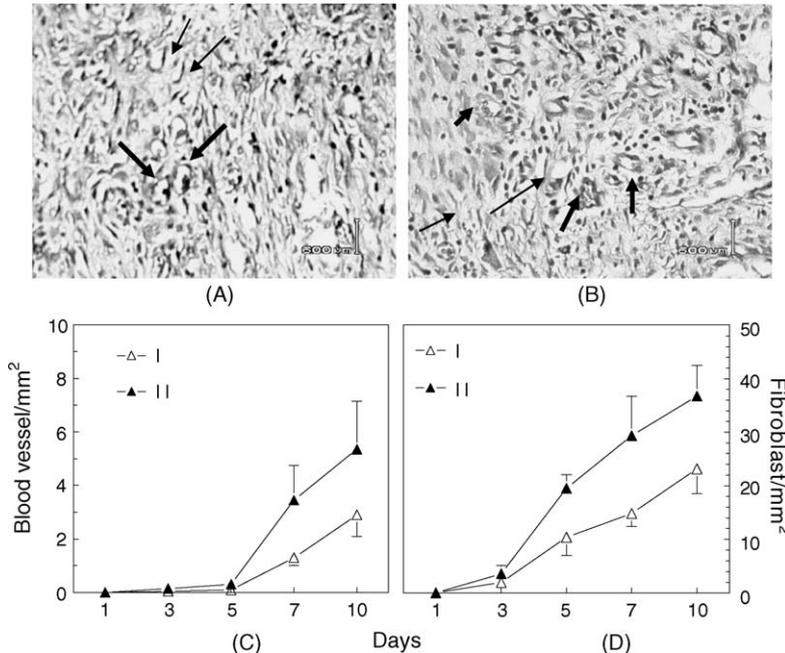

Fig. 3. Increased number of new blood vessels and fibroblasts in the gingival wounded site from amniotic membrane-transplanted animals (group II) (B, right panel) is seen as compared with that from group I (A, left panel) at day 10 (HE, 400×). Panel (C) represents mean and standard deviation of the number of blood vessels and fibroblasts. Thick and thin arrows indicate blood vessels and fibroblasts, respectively.

observed (see Fig. 2A and B). Statistically, the number of gingival PMN from amniotic membrane-treated animals (group II) was significantly lower than that from group I throughout the experiments ($P < 0.05$) (Fig. 1C).

Photomicrographs taken at day 10 showed that stratified squamous epithelium from group II was thicker than that from group I and rete peg formation was seen only in group II (Fig. 2A and B). The thickness of gingival epithelial layer in the wound site from both groups was steadily increased until day 10 (Fig. 2C). Statistical analysis revealed that gingival epithelial layer from group II was thicker than that from group I at days 5–10 ($P < 0.05$) (Fig. 2C).

Histological appearance of wounded gingival tissues from both groups of animals at day 10 showed that granulation tissues, which consisted of new blood vessels and large number of fibroblasts, from group II were much denser than those from group I (Fig. 3A and B). Blood vessels from group II were observed as early as day 3, whereas those from group I were seen at day 5 ($P < 0.05$) (Fig. 3C). The number of new blood vessels from group II at days 7 and 10 was significantly higher than that from group I ($P < 0.05$) and fibroblasts at the wounded gingiva from both groups started to appear at day 3 (Fig. 3D). Further analysis revealed that the number of fibroblasts from group II at days 3–10 was higher than that from group I ($P < 0.05$) (Fig. 3D).

Histological sections taken at day 10 showed that immature and unorganized gingival collagen fibres from group I at day 10 were seen (Fig. 4A). However, dense and well-organized collagen fibres beneath gingival epithelial layer of amniotic membrane-treated animals (group II) could be observed at the same period of experiment (Fig. 4B). Collagen fibre density at the wounded gingival site of both groups gradually increased from days 3 to 10. The density of collagen fibres from group II at days 3 to 10 were much higher than that from group I ($P < 0.05$) (Fig. 4C).

Discussion

The results of present study showed that amniotic membrane may suppress the migration of PMN at the wounded gingival site. Similar results have also been previously documented that human amniotic membrane transplantation in acute corneal alkali burn[9] and experimental herpetic keratitis[8] may reduce PMN infiltration, thereby inhibiting inflammation. The exact mechanism by which
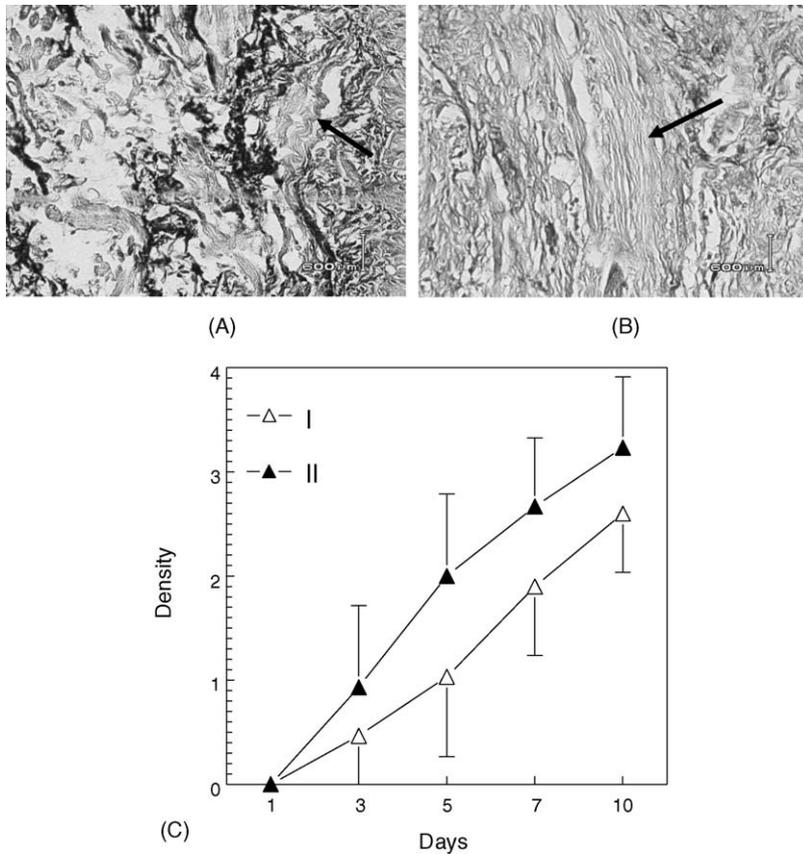
(A)   (B)

(C)

Fig. 4. Collagen fibres at the gingival wounded site from amniotic membrane-transplanted animals (group II) (B, right panel) at day 10 are much denser than those from the control (group I) (A, left panel) (von Gieson, 400×). Panel (C) represents mean and standard deviation of collagen density. Arrow indicates collagen fibres.

amniotic membrane reduced the number of gingival PMN seen in the present study is unclear, but may be via the induction of cell apoptosis[15], inhibition of cell migration due to the suppression of chemokine and anti-inflammatory cytokine (e.g., IL-10) synthesis[2,7] and the prevention of microbial contamination due to its ability to tightly attach on the wound[20].

The induction of rapid epithelialization of the gingival wound by amniotic membrane was demonstrated in the present study. These results support the previous studies showing increased epithelialization in alkali-burned corneal surface[9] and experimental herpetic keratitis[8] transplanted with amniotic membrane. Amniotic membrane contains growth factors, necessary for epithelialization[11], and provides substrates such as laminins for rapid epithelial cell attachment which, in turn, up-regulates the expression of growth factor receptors such as EGF receptor on epithelial cells[13,22]. Therefore, one may assume that these growth factors might induce rapid gingival epithelial cell migration and attachment to the wound area, thereby stimulating rapid cell proliferation and differentiation. This contention is, however, speculative and further studies are needed to clarify.

Formation of granulation tissue marks the proliferative stage of wound healing and is characterized by dense fibroblast-derived cellular matrix and neovascularization. Indeed, the present study showed that amniotic membrane may stimulate rapid formation of granulation tissue in the gingival wound by inducing rapidly increased number of fibroblasts and vascularization. Since amniotic membrane contains bFGF, EGF, TGF-β and interleukin-1 (IL-1)[11,16], one may assume that these growth factors may stimulate fibroblast growth and neovascularization in the gingival wound. In contrast, previous studies demonstrated that amniotic membrane transplanted on the cornea *in vitro* and *in vivo* suppressed neovascularization[10,18]. The exact reason to explain this discrepancy is not clear. FAULK et al.[4] showed that neovascularization in human leg ulcers could be induced by human amnion membrane transplantation. Therefore, one may assume that neovascularization of the wound healing following

transplantation with human amniotic membrane is dependent on the anatomical site of the wound, being that amniotic membrane-induced suppression of neovascularization may solely occur in the wounded cornea. Further studies are needed to delineate this contention.

The ability of amniotic membrane to stimulate rapid production of collagen fibres in the gingival wound was also suggested in the present study. This membrane contains TGF-β and tissue inhibitors of metalloproteinase polypeptides (TIMPs), which up-regulate the production of collagen fibres by fibroblasts in the wound healing[7,22]. If so, that increased density of collagen fibres in the amniotic membrane-treated gingival wound seen in the present study may be augmented by TIMPs and TGF-β cannot be ruled out and requires further studies.

The histological findings of rabbit gingival wound healing transplanted with amniotic membrane seen in the present study seem to be in consistence with those of human periodontal wound healing guided with enamel matrix proteins[17] or acellular dermal matrix allograft[21]. Moreover, cryopreserved human amniotic membrane transplantation stimulates very minimal allo and xenograft rejection due to the fact that this membrane expresses low major histocompatibility complex class II molecules but high immunoregulatory molecules such as Fas ligand and HLA-G[12]. Therefore, the use of amniotic membrane transplantation to accelerate gingival wound healing in humans, regardless of the genetic background, is promising.

References

1. ALTAVILLA D, SAITTA A, CUCINOTTA D, GALEANO M, DEODATO B, COLONNA M, TORRE V, RUSSO G, SARDELLA A, URNA G, CAMPO GM, CAVALLARI V, SQUADRITO G, SQUADRITO F. Inhibition of lipid peroxidation restores impaired vascular endothelial growth factor expression and stimulates wound healing and angio-

genesis in the genetically diabetic mouse. Diabetes 2001: **50**: 667–674.

2. BÜLTMAN S, YOU L, SPANDAU U, ROHRSCHNEIDER K, VÖLCKER HE, KRUSE FE. Amniotic membrane downregulates chemokine expression in human keratinocytes. Invest Opthalmol Vis Sci 1999: **40**(Suppl.):S578.

3. DUA HS, GOMES JAP, KING AJ, MAHARAJAN VS. The amniotic membrane in ophthalmology. Surv Ophthalmol 2004: **49**: 51–77.

4. FAULK WP, MATTHEWS R, STEVENS PJ, BENNETT JP, BURGOS H, HIS BL. Human amnion as an adjunct in wound healing. Lancet 1980: **1**: 1156–1158.

5. GOMES MF, DOS ANJOS MJ, NOGUEIRA TO, GUIMARAES SA. Histologic evaluation of the osteoinductive property of autogenous demineralized dentin matrix on surgical bone defects in rabbit skull using human amniotic membrane for guided bone regeneration. Int J Oral Maxillofac Implant 2001: **16**: 563–571.

6. HÄKKINEN L, UITTO V-J, LARJAVA H. Cell biology of gingival wound healing. Periodontol 2000 2000: **24**: 127–152.

7. HAO Y, MA DH, HWANG DG. Identification of anti-angiogenic and anti-inflammatory proteins in human amniotic membrane. Cornea 2000: **19**: 348–352.

8. HEILIGENHAUS A, BAUER D, MELLER D, STEUBL K-P, TSENG SCG. Improvement of HSV-1 necrotizing keratitis with amniotic membrane transplantation. Invest Opthalmol Vis Sci 2001: **42**: 1969–1974.

9. KIM JS, KIM JC, NA BK, JEONG JM, SONG CY. Amniotic membrane patching promotes healing and inhibits proteinase activity on wound healing following acute corneal alkali burn. Exp Eye Res 2000: **70**: 329–337.

10. KOBAYASHI N, KABUYAMA Y, SASAKI S, KATO K, HOMMA Y. Suppression of corneal neovascularization by culture supernatant of human amniotic cells. Cornea 2002: **21**: 62–67.

11. KOIZUMI N, INAMOTO T, SOTOZONO T, FULLWOOD NJ, QUANTOCK AJ, KINOSITA S. Growth factors mRNA and proteins in preserved human amniotic membrane. Curr Eye Res 1997: **20**: 173–177.

12. KUBO M, SONODA Y, MURAMATSU R, USUI M. Immunogenicity of human amniotic membrane in experimental xenotransplantation. Invest Opthalmol Vis Sci 2001: **42**: 1539–1546.

13. KURPAKUS MA, DANESHVAR C, DAVENPORT J, KIM A. Human corneal epithelial cell adhesion to laminins. Curr Eye Res 1999: **19**: 106–114.

14. MARTINEZ-PARDO ME, REYES FRIAS ML, RAMOS DURON LE, GUTIERREZ SALGADO E, GOMEZ JC, MARIN MA, LUNA ZARAGOZA D. Clinical application of amniotic membranes on a patient with epidermolysis bullosa. Ann Transplant 1999: **4**: 68–73.

15. PARK WC, TSENG SCG. Modulation of acute inflammation and keratinocyte death by suturing, blood and amniotic membrane in PRK. Invest Opthalmol Vis Sci 2000: **41**: 2906–2914.

16. ROTE NS, MENON R, SWAN KF, LYNDEN TW. Expression of IL-1 and IL-6 protein and mRNA in amniochorionic membrane. Placenta 1993: **14**: A63.

17. SALLUM EA, CASATI MZ, CAFFESSE RG, FUNIS LP, NOCITI Jr FH, SALLUM AW. Coronally positioned flap with or without enamel matrix protein derivative for the treatment of gingival recessions. Am J Dent 2003: **16**: 287–291.

18. SHAO C, SIMA J, ZHANG SX, JIN J, REINACH P, WANG Z, MA JX. Suppression of corneal neovascularization by PEDF release from human amniotic membranes. Invest Opthalmol Vis Sci 2004: **45**: 1758–1762.

19. TALAS DU, NAYCI A, ATIS S, POLAT A, COMELEKOGLU U, BAGDATOGLU C, RENDA N. The effects of corticosteroids on the healing of tracheal anastomoses in a rat model. Pharmacol Res 2002: **45**: 299–304.

20. TALMI YP, SIGLER L, INGE E. Antibacterial properties of human amniotic membrane. Placenta 1991: **12**: 285–288.

21. WAGSHALL E, LEWIS Z, BABICH SB, SINENSKY MC, HOCHBERG M. Acellular dermal matrix allograft in the treatment of mucogingival defects in children: illustrative case report. ASDC J Dent Child 2002: **69**: 39–43.

22. YAM H-K, PANG C-P, FAN DS, FAN B-J, YU EY-W, LAM DS-C. Growth factor changes in ex vivo human expansion of human limbal epithelial cells on human amniotic membrane. Cornea 2002: **21**: 101–105.

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