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


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.

Key words: amniotic membrane; gingival; healing; rabbit; wound.

Accepted for publication 15 September 2005
membrane. May be enhanced by transplantation of this membrane, whether gingival wound healing in rabbits may be enhanced by transplantation of this membrane. Since amniotic membrane may induce rapid physiological wound healing, the aim of the present study was to determine whether gingival wound healing in humans and the latter sites. Attempts to produce materials for enhancement of periodontal wound healing and reconstruction of periodontal tissue damage have been made. For example, enamel matrix proteins and acellular dermal matrix allograft 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 epithelialization and tissue vascularization and reduce inflammation. 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. 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. 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 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...
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 and experimental herpetic keratitis may reduce PMN infiltration, thereby inhibiting inflammation. The exact mechanism by which
amniotic membrane reduced the number of gingival PMN seen in the present study is unclear, but may be via the induction of cell apoptosis\(^1\), 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\(^8\).

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\(^10\) 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-\(\beta\) 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 \textit{in vitro} and \textit{in vivo} suppressed neovascularization\(^10,18\). The exact reason to explain this discrepancy is not clear. \textsc{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-\(\beta\) and tissue inhibitors of metalloproteinase polypeptides (TIMPs), which up-regulate the production of collagen fibres by fibroblasts in the wound healing\(^9,22\). 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-\(\beta\) 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\(^18\). 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.

Acknowledgements. This work was part of the thesis for Masters degree (to M.R.) in Gadjah Mada University and supported by the Postgraduate Scholarship (to M.R.) from the Ministry of National Education, the Indonesian Government. The authors gratefully thank Dr Abdurrahman (School of Medicine, Airlangga University) for providing human amniotic membranes and Mrs Ika Rahutami for statistical analysis.

References

1. \textsc{Altavilla} D, \textsc{Safta} A, \textsc{Cucinotta} D, \textsc{Galeano} M, \textsc{Deodato} B, \textsc{Colonna} M, \textsc{Torre} V, \textsc{Russo} G, \textsc{Sardella} A, \textsc{Urna} G, \textsc{Campo} GM, \textsc{Cavallari} V, \textsc{Squadrito} G, \textsc{Squadrito} F. Inhibition of lipid peroxidation restores impaired vascular endothelial growth factor expression and stimulates wound healing and angio-

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{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\(\times\)). Panel (C) represents mean and standard deviation of collagen density. Arrow indicates collagen fibres.}
\end{figure}

Address:
Wihas Sosroseno
Department of Oral Biology
School of Dental Sciences
Universiti Sains Malaysia
Kota Bharu 16150 Malaysia
Fax: +60 9 7642026.
E-mail: wihaskoro@kb.usm.my