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ORIGINAL RESEARCH ARTICLE

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Use of autologous epithelium transplantation on various scaffolds to cover tissue loss in oral cavity: long-term observation

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ABSTRACT

Background: The aim of this study was to investigate the application of mucous membrane keratinocyte cultures on amniotic membrane and on poly(L-lactic acid) (PLLA) Purasorb PL38 to cover tissue loss in the oral cavity. Developments in molecular biology techniques and tissue engineering allow the culturing and identification of cells that can be anchored in the wound to achieve integrity of the tissue. Transplantation of tissues obtained from the patient's own cells is superior to allogenous transplantation where there is a possibility of transfection, rejection and the need for long-term immunosuppression.
Methods: In 9 patients (15 procedures) keratinocytes cultured on amniotic membrane and PLLA were transplanted to cover antro-oral fistulas and bone loss after osteoradionecrosis.
Results: In all 6 patients with outlasting antro-oral fistulas, the defects were healed. In 3 patients with 5 cases of tissue loss after osteoradionecrosis, we obtained healing of the wound in only 1 case. Histological examination of the cultures indicated that cultured cells formed well-differentiated layers, very similar to the keratinocytes of mucous membranes, although those seeded on amniotic membrane formed a single layer of cells, while those seeded on the PLLA scaffold were arranged on 2 or more layers: these differences were shown to be statistically significant with a morphometric analysis.
Conclusions: Autologous transplants of epithelium cultured on amniotic membrane and PLLA constitute a new and effective way of covering nonhealing tissue loss in the oral cavity in chosen cases, using modern methods of tissue engineering.

Keywords: Amniotic membrane, Lactic acid, Mucous membrane, Tissue engineering

Introduction

The rapid development of bioengineering in recent years has resulted in a search for new therapies involving the use of tissue cultures (1, 2). Autologous transplants are used in many areas of medicine, of which the most advanced is ophthalmology (2-4). In Stevens-Johnson syndrome, ocular cicatricial pemphigoid and chemical corneal injuries, it is not possible to obtain stem cells from the limbal cornea, due to its defective nature. In such cases, the best solution appears to be to take a small section of mucous membrane from the oral cavity of the patient and establish a tissue culture to graft it onto the damaged cornea (2, 5). Mucous membrane keratinocytes cultured on various organic and inorganic bases can be applied, such as amniotic membranes, which are used as biomaterials in surgery (2, 3, 5). Their thin placental membranes consist of a single-layer cylindrical epithelium placed on a basement membrane with a specific layer of stroma. Several growth factors, such as epidermal growth factor (EGF), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF) and basic fibroblast growth factor (βbFGF), are present in the epithelium. The basement membrane contains collagen types III, IV and VII; laminin and fibronectin. The

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marrow contains nerve growth factor, antiinflammatory proteins, protease and angiogenesis inhibitors. The basement membrane has antibacterial and antifibroblastic properties, and it supports the migration of cells (6, 7). The present study was conducted to examine the feasibility of using an amniotic membrane (AM) and a membrane prepared for poly(L-lactic acid) (PLLA) as the carriers for this application. The tissues were cultured on amniotic and resorbable membranes with PLLA. The purpose of transplantation is to cover tissue loss in the oral cavity due to persistent antro-oral fistulas, osteoradionecrosis, bone inflammation, injuries, postimplantation and postdental dehiscence and cicatrization removal. Thus, it would appear reasonable to search for new solutions using the patient's own tissues to cover such losses.

Methods

Patients who exhibited traumatic or postinflammatory loss of mucous membranes in the mouth, scars after burns, persistent antro-oral fistulas that could not be treated conventionally, loss of the hard palate after cancer treatment, a healed cancer lesion, tissue loss after osteoradionecrosis, postimplantation loss or dehiscences around the roots qualified for treatment with the described method. Patients with active cancer lesions, active infections near the operation field, reduced immunity to disease, autoimmune disease or active tuberculosis, as well as children, pregnant women and post–organ transplantation patients, were excluded from the treatment.

The surgical procedure was performed on 9 patients (15 procedures) – 7 men and 2 women with a mean age of 52 years (range 28-66 years). The mean observation period was 17 months. Six of the patients had persistent antro-oral fistulas, and 3 had 5 cases of tissue loss after osteoradionecrosis.

All of the medical procedures and experiments were approved by the Bioethics Commission at the Medical University of Silesia on 22 June 2010 and were conducted in accordance with the tenets of the Declaration of Helsinki. Informed consent was obtained from the patients after an explanation of the nature and possible consequences of the study.

Preparation of amniotic membrane

AMs were obtained from a tissue bank (FRK Homograft, Zabrze, Poland). The membranes were taken during caesarean sections, washed with saline containing phosphate-buffered saline (PBS) and 50 IU/mL penicillin, and stored at -80°C in Dulbecco's modified Eagle's medium (DMEM) and glycerol (1:1). Immediately before use, the tissues were defrosted, washed 3 times in PBS and cut into 4 × 4 cm fragments under aseptic conditions. They were then immersed in 0.02% ethylenediaminetetraacetic acid (EDTA) at 37°C for 2 hours to remove the epithelium and reduce cellular adhesion. After this process, the loose epithelial cell layer was gently scraped.

Preparation of nano-based PLLA

The procedure for preparation of nano-based PLLA was performed at the Faculty of Chemistry of Jagiellonian University in Krakow.

Mobile scaffoldings were prepared from PLLA Purasorb PL38. Dry polymer was dissolved in chloroform, stands for high-performance liquid chromatography (HPLC) grade with water as the main pollutant (25 cm^3 of chloroform per 1 g of polymer) and then shaped by pouring the polymer solution onto a mold with channels and removing almost all of the chloroform with a jet of hot air. The channels were parallel lines 1 mm apart, with triangular cross-section and 0.3-mm depth and 0.2-mm width. The polymer was squeezed between the sides of the mold in a vice (the second part of the mold was flat), at a temperature above the glass transition temperature of the polymer (65°C), and then the scaffolding, which stayed on the side of the mold with the channels, was placed in hot water for 30 minutes to facilitate its removal. The scaffolding was placed in a vacuum for half an hour to remove any trace of the chloroform. The thickness of the scaffolding, apart from crests, was less then 0.4 mm. The surface was rough (with 20- to 50-μm features) and without any prominent porosity (Fig. 1).

Manufacture of mucous membranes

Patients who qualify for transplantation of mucous membranes require a healthy oral cavity. Therefore, the patients in this study were not allowed to smoke tobacco or drink alcohol, and they were required to brush their teeth and rinse their mouths with iodine after every meal. The treatment was carried out under local anesthesia as outpatient surgery. Biopsies of the mucous membrane were obtained from the lower lip or cheek; the biopsy specimen size was approximately 3 × 3 mm. The biopsy specimen was immersed in 18 mL of transport medium (DMEM Nutrient Mixture F-12 HAM) mixed with 2 mL of fetal bovine serum (FBS; Gibco-BRL) and 100 mL of antibiotics (penicillin, 10,000 IU/mL streptomycin and Fungizone).

The submucosal membranes of the biopsies were removed with scissors. The samples were cut into small fragments and immersed 3 times (10 minutes each time) at room temperature in the saline of the PBS with a mixture of antibiotics, penicillin, 50 IU/mL streptomycin and 5 μg/mL amphotericin B. After incubation under standard conditions (37°C; atmosphere 5% CO_2 and 95% air) for 1 hour in 1.2 IU diastase, the samples were added to a 0.25% solution of trypsin and EDTA for 10 minutes at room temperature to separate the cells. The suspension, with a density of 2×10^4 to 4×10^4 cells, was placed in 2 mL of medium. The medium was an equal 1:1 mixture of DMEM and HAM's F12 with 10% FBS, 5 μg/mL of 0.5% dimethyl sulfoxide (DMSO) bovine insulin, 0.1 nM/L cholera toxin, 0.18 nM adenine, 2 nM triiodothyronine, 4 nM glutamine, 0.4 mg hydrocortisone, 10 ng/mL mouse EGF, 50 IU/mL penicillin-streptomycin and 5 μg/mL antifungal amphotericin B. The cells were cultured in 35-mm-diameter plastic Petri dishes; the medium was changed every 48 hours. The AM was extended and laid epithelial side up on a Petri dish. The 3T3 fibroblasts were incubated for 2 hours in 4 μg/mL stands for mitomycin C (MMC) to inactivate their proliferation, after which they were washed in saliva with PBS to remove the MMC after trypsinization (0.1% trypsin and 0.01 EDTA for 80 minutes). The suspension of epithelial cells, at a concentration of

Fig. 1 - Scanning electron micrograph of poly(L-lactic acid) (PLLA). Scale bars: 1.00 mm.

2×10^4 to 4×10^4/mL, was seeded on the AM coated with nutritionally inactivated MMC 3T3 fibroblasts. The material was ready for transplantation after 14 days of uninterrupted production.

The same procedure was applied to the PLLA nanomembranes.

Histology of semithin sections

The epithelia seeded on both the AM and the PLLA were immediately fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4) at +4°C for 4 hours, washed with 0.2 M phosphate buffer (pH 7.4), trimmed into small slices, and postfixed in 1% OsO_4 in 0.2 M phosphate buffer (pH 7.4) at +4°C for 1 hour. The specimens were then dehydrated in graded ethanol and acetone and flat-embedded in Durcupan for a better orientation. Semithin sections (1 µm) were cut with an LKB Ultratome V ultramicrotome, stained with an aqueous solution of 1% toluidine blue in 1% borax and 1% pyronin Y (8), and viewed and photographed with a Zeiss Primo Star light microscope.

Morphometric analysis

Light microscopy was used on the epithelial layers to carry out a morphometric analysis of the oral epithelia of both groups of scaffolds. Light microscopy data were obtained from 10 semithin sections/specimen, collecting 1 semithin section out of every 100. All micrographs were obtained with a Zeiss Primo Star light microscope at the same magnification of ×450 and acquired (ratio 1:1) with an Epson Perfection 4180 scanner and an Apple MacBook, using Adobe Photoshop 8.0.1 software. On each micrograph, the epithelial thickness was measured using Photoshop software by tracing a straight line perpendicular to the epithelium and calculating the epithelial thickness, expressed in micrometers along each line. Furthermore, the number of cells grown on the AM and the PLLA membranes was measured on the same semithin sections used for the evaluation of the epithelial thickness, by counting the number of cells per millimeter of epithelial surface.

Statistical analysis

Statistical analysis of the results was performed using Student's t-test with SAS/Sta 6.0.3 software. A p value ≤0.05 was considered statistically significant.

Results

We obtained total wound closure in all 6 patients with persistent antro-oral fistulas (Fig. 2). In the 3 patients with 5 cases of tissue loss after osteoradionecrosis, we only achieved wound healing in 1 case.

Histological examination

Oral epithelial cells seeded on AMs and PLLA nanomembranes started to form colonies on day 3. After 10 days, both scaffolds were covered by the epithelial tissue; in week 3, the cultured cells formed distinct layers, similar to the keratinocytes of the mucous membranes. However, the epithelial cells seeded on the AMs formed a thin, single layer of flat cells (Fig. 3), while those seeded on the PLLA scaffold were higher and often arranged in 2 layers (Fig. 4).

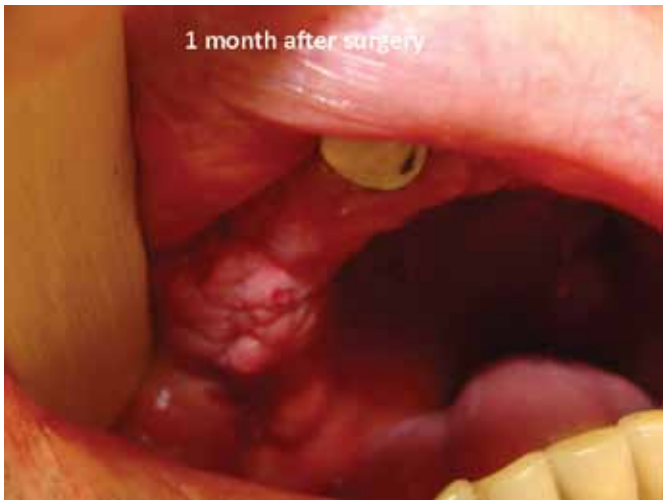

Fig. 2 - Photograph of patient's condition 1 month after operation; the wound had healed completely.

Morphometric analysis

When the mean heights of the oral epithelia on the different supports were evaluated, the mean height of the epithelia grown on the AMs was 21.35 ± 3.01 µm, while that of the epithelia grown on the PLLA was 38.85 ± 6.37 µm. The difference between the 2 groups was found to be statistically significant ($p<0.001$) (Fig. 5). As to the quantitative evaluation of the cells per millimeter of epithelial surface, the mean number of the cells on the AMs was 22.4 ± 2.3/mm, while that of the cells on PLLA scaffold was 43.2 ± 4.1/mm. The difference between the 2 groups was also statistically significant ($p<0.001$) (Fig. 6).

Discussion

The promising positive results of the research conducted by ophthalmologists (2, 3) encouraged the authors of this paper to attempt to graft cultured tissue into loss of soft and/or hard tissues in the oral cavity. This process was carried out in 9 patients with nonhealing antro-oral fistulas and tissue loss

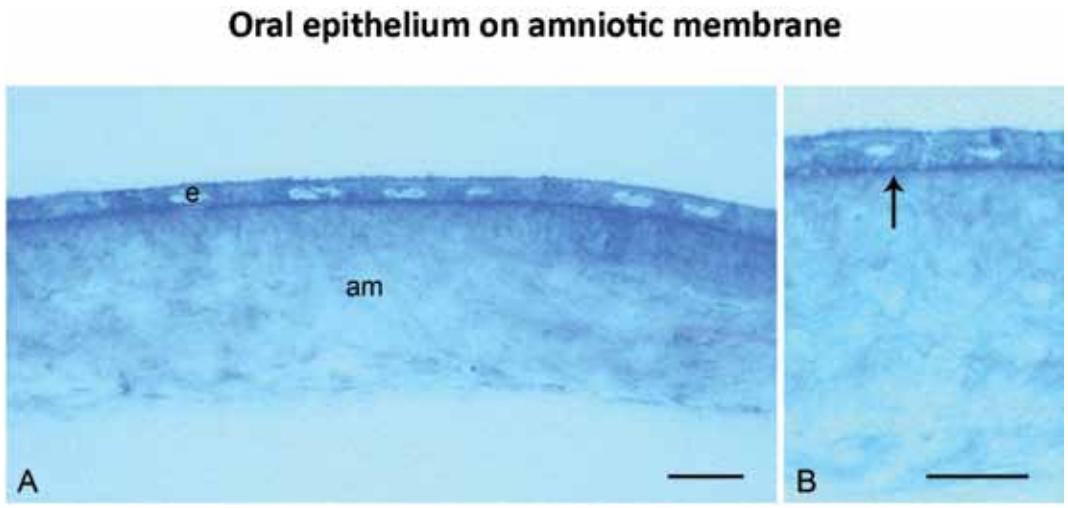

Fig. 3 - (**A**) Semithin section of the oral epithelium (e) arranged in a single, thin layer of flat cells on the amniotic membrane (am). (**B**) At higher magnification, note the presence of a well-defined basement membrane (arrow). Scale bar: 50 µm.

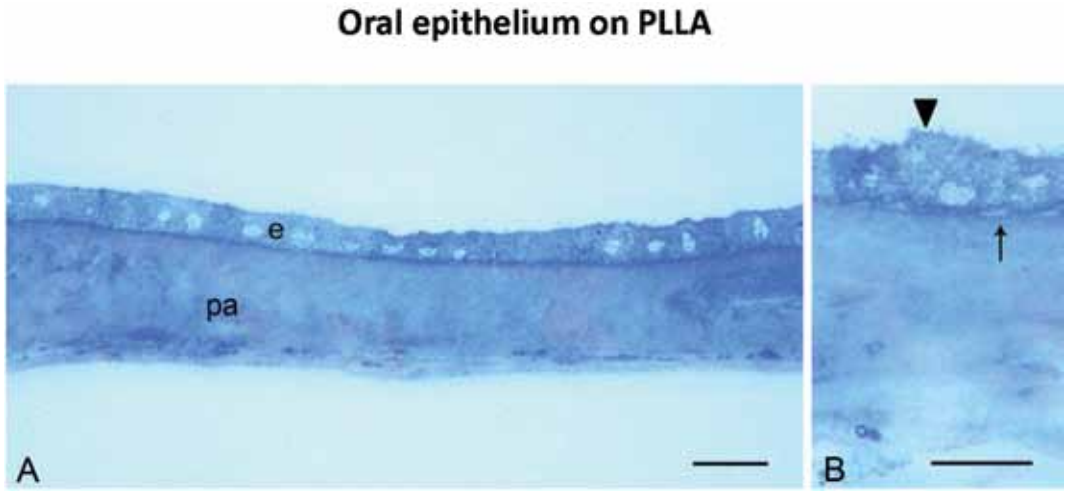

Fig. 4 - (**A**) Semithin section of the oral epithelium (e) arranged in a higher layer of cuboidal cells on poly(L-lactic acid) (PLLA) membrane (pa). (**B**) Note a well-defined basement membrane (arrow) and the presence of islets of double-layered epithelial cells (arrowhead). Scale bar: 50 µm.

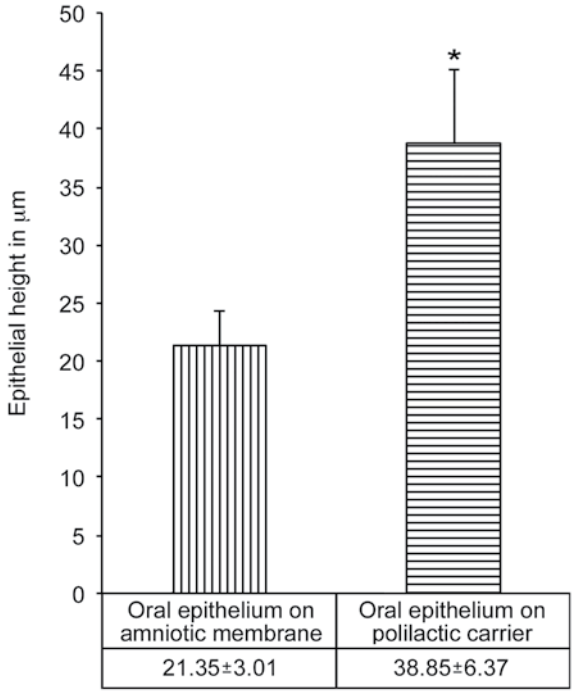

Fig. 5 - Mean heights of oral epithelia on the different supports used. *p<0.001 vs. amniotic membrane.

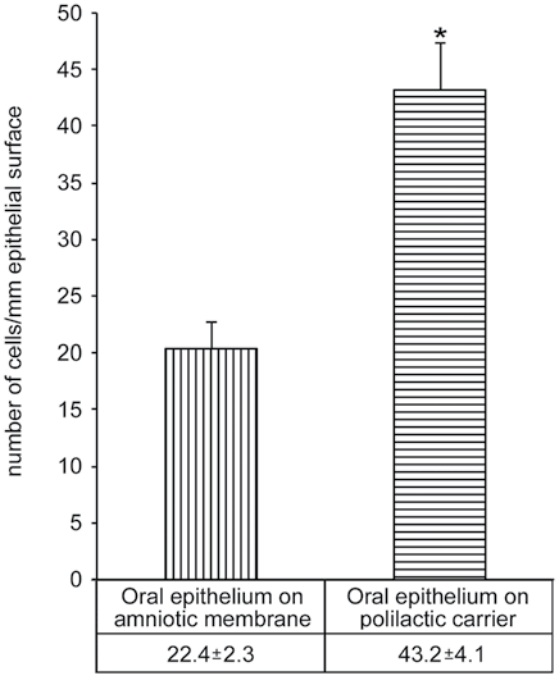

Fig. 6 - Number of cells per millimeter of epithelial surface. *p<0.001 vs. amniotic membrane.

related to osteoradionecrosis, in whom the known methods of treatment had not been successful. For that reason, innovative transplantation of cultured epithelium keratinocytes was performed to cover the tissue loss, using AMs and PLLA nanomembranes as carriers. The defects were healed in all of the patients with nonhealing antro-oral fistulas. However, it must be noted that this was a small group of patients, and this is only an introduction to further research. Obtaining a patient's own tissue and transplanting it after culturing offers many advantages; the only disadvantage is the need for a second procedure. The mucous membrane can be obtained from such donor locations as the nasal cavity, mouth, trachea, esophagus, and in women, the vagina. Most authors choose the oral cavity due to easy access, very quick healing and minimal scarring. According to some authors, it is sufficient to take 2-3 mm^2 of mucosal tissue (9, 10). Because of the brittleness and delicacy of the epithelial layers, scaffolds are needed to support the tissue culture and help in transplanting it to the recipient area. The ideal environment for culturing and autotransplantation appears to be the AM, due to its characteristics. As a biomaterial, it is successfully used in dermatology, ophthalmology and plastic and reconstructive surgery, with very significant results (7, 11, 12). The innovative aspect of this present work was the attempt to use preserved human AMs and PLLA nanomembranes as cell carriers. In addition to this function, the AM has antiinflammatory, antifibrosis and immunosuppressive properties, and it is not immunogenic. Its basilar membrane, due to growth factors, stimulates the migration and proliferation of epithelial cells and inhibits apoptosis. Thus, it is an appropriate environment for the regeneration of epithelia and an ideal carrier for cells grown in vitro. Nakamura et al described the reconstruction of epithelium keratinocytes on AMs in the mouths of rabbits. They explored the basic components of the hemidesmosomes of the basilar membrane, integrin-α6, and laminin-α5, which were present in the reconstructed oral epithelia of the rabbits. The transplanted epithelia displayed structural integration (13). The greatest benefit from the application of AMs for culturing is a rapid increase in keratinocytes and their correct differentiation, which makes the culture a very stable one. It also prevents postoperative inflammation. A short turnover rate and a very rapid growth rate in culture are characteristic of these cells, and they can be stored in a culture for an extended period of time without apparent keratinization (12). The presence of keratin 3 is a significant marker for epithelial cells in vitro and is confirmed in reconstructed tissues. Its ability to generate epithelial cells in culture decreases with the age of the patient. In elderly patients, the number of stem cells contained in obtained transplants is lower, and their culturing is slower as well. The more stem cells there are in a culture, the more long-lived it is. According to some authors, the sign of a sufficient quantity of cells in transplanted tissues is when an area of oral epithelium is visible in slit-lamp examination after administering fluorescein. Culture samples should be subjected to immunohistochemical examinations specifying the characteristic markers for oral mucosal tissues, such as keratin 4 and 13, which then appear in transplanted tissues, such as on the surface of the cornea and oral mucous membranes. Keratin 3, which is specific to the cornea, is also present in the epithelial cells of the mouth. AMs in autotransplantations are biodegradable within several weeks, demonstrated by a gradual decrease in a characteristic marker, collagen III, in the

transplanted tissues (6). Autotransplantation integrates perfectly with the main and surrounding tissues. PLLA scaffoldings are intended to replace bone loss in the oral cavity, and cultured epithelia are intended to activate and support growth of the patient's own mucous membranes. Biodegradable plates and pins made of PLLA (produced by KLS Martin, Tuttlingen, Germany) are often used in treating premature fusing of the cranial seams in children. Their advantage is slow resorption (within 6 weeks), so they do not require another surgery to remove them (14). The selection of scaffolding for epithelial culture depends on the type of loss. When only mucous membranes are lost, a culture on AM is enough. However, when bone and mucous tissues are lost, it seems to be necessary to reconstruct the bone layer using PLLA scaffolding and a nonbiodegradable t grid coated with PLLA. Despite controversy regarding the use of animal-derived products, 3T3 fibroblasts are still the main substrate to support epithelial proliferation. However, presence in culture medium on the bottom of the plates, MMC inactivation and washing out are not a guarantee of an absence of alive cells in the transplant. In our observations, we did not note any adverse reactions related to animal-derived supplements. Autologous serum became an alternative to FBS, now it is widely used (12). The application of autologous transplantation of epithelia obtained by tissue culture is a safe and effective way to cover persistent loss of tissue in the oral cavity in select cases. The transplant integrates well with the main and surrounding tissues and provides continuity of the epithelium of the mouth. Autologous transplants of epithelia on AMs and PLLA constitute a new and effective way of covering nonhealing tissue losses in the oral cavity using modern methods of tissue engineering.

Disclosures

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Conflict of interest: None of the authors has any financial interest related to this study to disclose.

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