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Clinical Application of Decellularized and Lyophilized Human Amnion/Chorion Membrane Grafts for Closing Post-Laryngectomy Pharyngocutaneous Fistulas

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Background and Objectives: Squamous cell carcinoma is the most common pathological type among the cancers of the larynx. Standard treatment for squamous cell carcinoma of the larynx is the combination of chemotherapy, radiotherapy, and laryngectomy. Pharyngocutaneous fistula is a common complication of laryngectomy. We hypothesized that decellularized and lyophilized human amnion/chorion membrane can be an effective, non-invasive method of treating pharyngocutaneous fistula.

Methods: A total of 67 patients with laryngeal squamous cell carcinoma were retrospectively analyzed after treatment in a prospective trial. After preoperative chemotherapy, radiotherapy, and total or extended laryngectomy, primary wound healing occurred in 42 (62.7%) patients. Pharyngocutaneous fistula developed in 8 (11.9%) patients. Decellularized and lyophilized human amnion/chorion membrane grafts were used to reconstruct the fistulas.

Results: The average time for the full healing of the wound in all patients after transplantation of these grafts was 18 days.

Conclusion: The advantages of using these grafts over other existing methods of pharyngocutaneous fistula treatment are that they are non-invasive, prevent donor morbidity, and enable management of the wound without using classical wound gauze.

KEY WORDS: decellularized human amniotic membrane; pharyngocutaneous fistula; squamous cell carcinoma; total laryngectomy

INTRODUCTION

Treatment of laryngeal cancer is complex, and an ideal strategy has not yet been developed. Worldwide, 157,000 new cases of laryngeal cancer were diagnosed in 2012 [1], and according to the United States National Cancer Institute, the incidence of new cases of larynx cancer was 3.2 per 100,000 men and women [2].

Squamous cell carcinoma is the most common cell type among the cancers of the larynx [3–5]. Unfortunately, 40% of patients have late presentations (Stage III or IV) of disease [6,7]. Tumors may be identified in earlier stages, when vocal cords are affected by tumor. But, in most of the cases, tumors develop above or below vocal cords, which results in tumors manifesting at a later stage, as they remain asymptomatic for a longer period of time. In those cases, wide field resections are necessary, including total or extended laryngectomy, with possible resection of the base of tongue, and reconstruction with pharyngostomy, esophageal, or tracheal stomas [8,9].

Early complications of total laryngectomy include wound hematoma and/or infection, along with tracheoesophageal, pharyngotracheal, or pharyngocutaneous fistulas [10–13]. Pharyngocutaneous fistula (PCF) is the most frequent major complication of those mentioned above. Rates of PCF between 8.5% and 24% have been most frequently reported [14–23]. Factors associated with the occurrence of PCF are stage of cancer, extent of resection, the use of preoperative radiation treatment in doses at or exceeding 50 Gy, and the method of repair of the pharyngeal defect [12,24]. A variety of treatments for PCF have been used, including local or regional flaps. Unfortunately, complications rates for these treatments remain high, in part due to impaired wound healing in the face of preoperative radiation treatment [25–36].

Attention has recently been focused on biological and biosynthetic materials, hydrogel membranes, and three-dimensional scaffolds, which have been successfully used for treatment of non-healing wounds of various etiologies [37–42]. Human amniotic membrane has aroused particular interest in treatment of non-healing wounds, as it possesses immunomodulative, anti-microbial, anti-inflammatory, fibrogeneic, and angiogeneic properties, as well as increasing extracellular matrix deposition [43–52].

The hypothesis for this study was that decellularized and lyophilized human amnion/chorion membrane (DLHACM) grafts could be an effective and non-invasive treatment for PCF after total laryngectomy, obviating the need for futher surgical procedures. The aim of the study

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Conflict of interest: The terms of this arrangement have been reviewed and approved by all institutions involved in accordance with their policy on objectivity in research.

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was to develop a method for producing DLHACM grafts and for treating patients with PCF.

METHODS

All patients signed written informed consent for the study, conducted according to guidelines of the 1975 Declaration of Helsinki, and approved by the Ethics Committee of the Georgian National Institute of Medical Research in Tbilisi, Georgia.

Fabrication of DLHACM Grafts

Three placentas were obtained from donors who signed an informed consent form and who gave birth at 38–42 weeks of gestation. All donors had normal pregnancies and delivered healthy newborn babies with weights ranging from 2,300 to 3,900 grams.

Decellularization of placentas was performed according to our previously described method [53]. Briefly, newly acquired placentas were washed with 0.9% saline solution and heparin at 37°C under physiological pressure. For this purpose, polyethylene catheters were inserted into the umbilical artery and vein of the placentas and fixed in place with sutures. The placentas were flushed through the arterial catheter until clear solution was returned through the umbilical vein. After the washing, placentas were frozen at −80°C. Frozen placentas were then thawed to 40°C and washed with Phosphate Buffered Saline (PBS, Sigma) overnight, perfusing PBS through the catheter in the placental artery. Placentas were then perfused with Sodium Dodecyl Sulfate (SDS, Sigma) in distilled water for 72 hr starting with 0.01% SDS for 24 hr, then with 0.1% SDS for 24 hr, and finally with 1% SDS for 24 hr. Subsequently, to remove residual SDS, placentas were flushed with distilled water for 15 min and with 1% Triton X-100 (Sigma) for 30 min. Decellularized placentas were then washed with PBS for 1 hr.

After the decellularization process, amniotic membranes were separated from the placentas and cut into 14 × 14 cm flaps, and fixated on special glass frames. The amniotic membranes were then lyophilized using Power Dry PL 6,000 Freeze Dryers. After lyophilization, the DLHACM grafts were packed in a disposable plastic bag and sterilized with gamma radiation using a dose of 15 kGy. The DLHACM grafts were then stored aseptically at room temperature until use.

DNA Quantification of DLHACM Grafts

DNA was isolated from the grafts with standard method using a commercial extraction kit (G-spin Kit; iNtRON Biotechnology). The total DNA was determined on a spectrophotometer (NanoDrop 1000; Thermo Fisher Scientific) at a wavelength of 260 nm. All samples were normalized to the human amnion dry weight.

The DNA content of human amnion/chorion membrane before treatment was 338 μg/ml. After the decellularization and lyophilization procedure, residual DNA content was less than 2%.

Histology and Fluorescence Immunohistochemistry

Histologic evaluation of human amniotic membranes was done both before and after decellularization and lyophilization. Cryosections (5 mm thick) were routinely processed and examined by light microscopy after H&E and Masson's trichrome staining.

Fluorescence immunohistochemistry was done according to the following methods. To test the Collagen Ia1 and Fibronectin antibodies on DLHACM, formalin-fixed paraffin embedded tissue sections 5 um thick were cut on a rotary microtome, mounted on charged slides, and baked overnight at 50°C in an oven. All staining procedures were performed at a room temperature. The slides were deparaffinized and rehydrated with water. Antigen retrieval was performed using steam and proteinase K digestion methods. After antigen retrieval, the slides were allowed to cool at room temperature for 20 min prior to the next step. Then the slides were rinsed in three PBS cycles for 5 min each and were blocked with 3% H_2O_2. After rinsing in three PBS cycles, the slides were incubated in primary antibody consisting of Collagen Ia1 (sc-25974 at 1:100) and Fibronectin (sc-8422 at 1:200) diluted with IHC-Tek Antibody Diluent for 1 hr at a room temperature. The slides were then washed three times in PBS and incubated with biotinylated secondary antibody for 30 min. The slides were washed in PBS and then incubated with HRP-Streptavidin for 30 min. Afterwards, incubation with DAB chromogen substrate solution was performed for 5–10 min, and then slides were rinsed with PBS and counterstained with Mayer's hematoxylin. The stained slides were examined with regular microscope.

The current histological and immunohistochemical studies demonstrated the five different layers of the normal human amniotic membrane: epithelium; basement membrane; compact layer; fibroblast layer; and intermediate or sponge layer. The basement layer was formed by type III and IV collagens and glycoproteins such as laminin, fibronectin, and nidogen, which are the products of secretion of the epithelial layer cells of the amnion [54,55]. Next was the compact layer, forming the main fiber structure of the amnion, which was represented by types I, III, IV, and V collagen and fibronectin (Fig. 1).

Scanning Electron Microscopy (SEM) of DLHACM Grafts

The DLHACM grafts were dehydrated by processing with ethanol solution and were then dried with a Tousimis Samdri-780 critical point dryer (Tousimis Research Corporation, Rockville, MD). After drying, all tissues were sputter coated lightly with gold (adjustments are shown below) and imaged on a Hitachi Scanning Electron microscope.

Scanning electron micrographs at low magnification demonstrated that types I and III collagen were bundled in the compact layer, and types IV and V collagens were located between the compact layer and basement membrane. These findings confirmed other studies, demonstrating that the process of decellularization and lyophilization used here preserved the unique, and porous structure of human amnion/choirion membrane within the DLHACM grafts [56–58].

Gene Expression Analysis of Human Amnion/Chorion Membrane and DLHACM Grafts

Total RNA from the amnion tissue was purified using miRNeasy mini kit according to the manufacturer's instructions (Qiagen). The cDNA was synthesized using the iScript cDNA synthesis Kit (BioRad). Q-PCR was carried out with iTaq universal SYBR green supermix (BioRad) on a 7,500 Fast Real-Time PCR system (Life Technologies). The 18S rRNA was used as internal control for gene expression normalization.

These studies demonstrated that the DLHACM grafts consisted of a large number of different growth factors, especially BMP7, BMP8a, which enhance the wound healing process (Fig. 2).

RESULTS

A total of 65 male and two female patients (mean age 54 years) with laryngeal squamous cell carcinoma, undergoing surgical treatment from January 2009 to December 2014 in Cancer Research Center of Tbilisi, Georgia, were enrolled into the study. All patients received comprehensive treatment consisting of preoperative chemotherapy and 50 Gy radiotherapy, followed by laryngectomy. Of the 67 patients, 12 (17.9%) were Stage I/II, 34 (50.7%) were Stage III, and 21 (31.4%) were Stage IV. Ten (14.9%) patients had total laryngectomy, 49 (73.1%) had total laryngectomy plus neck dissection, and 8 (12.0%) had extended larygectomy and neck dissection. In all cases, the initial defects after laryngectomy were closed using local and/or regional (delto-pectoral or thoraco-dorsal) flaps.

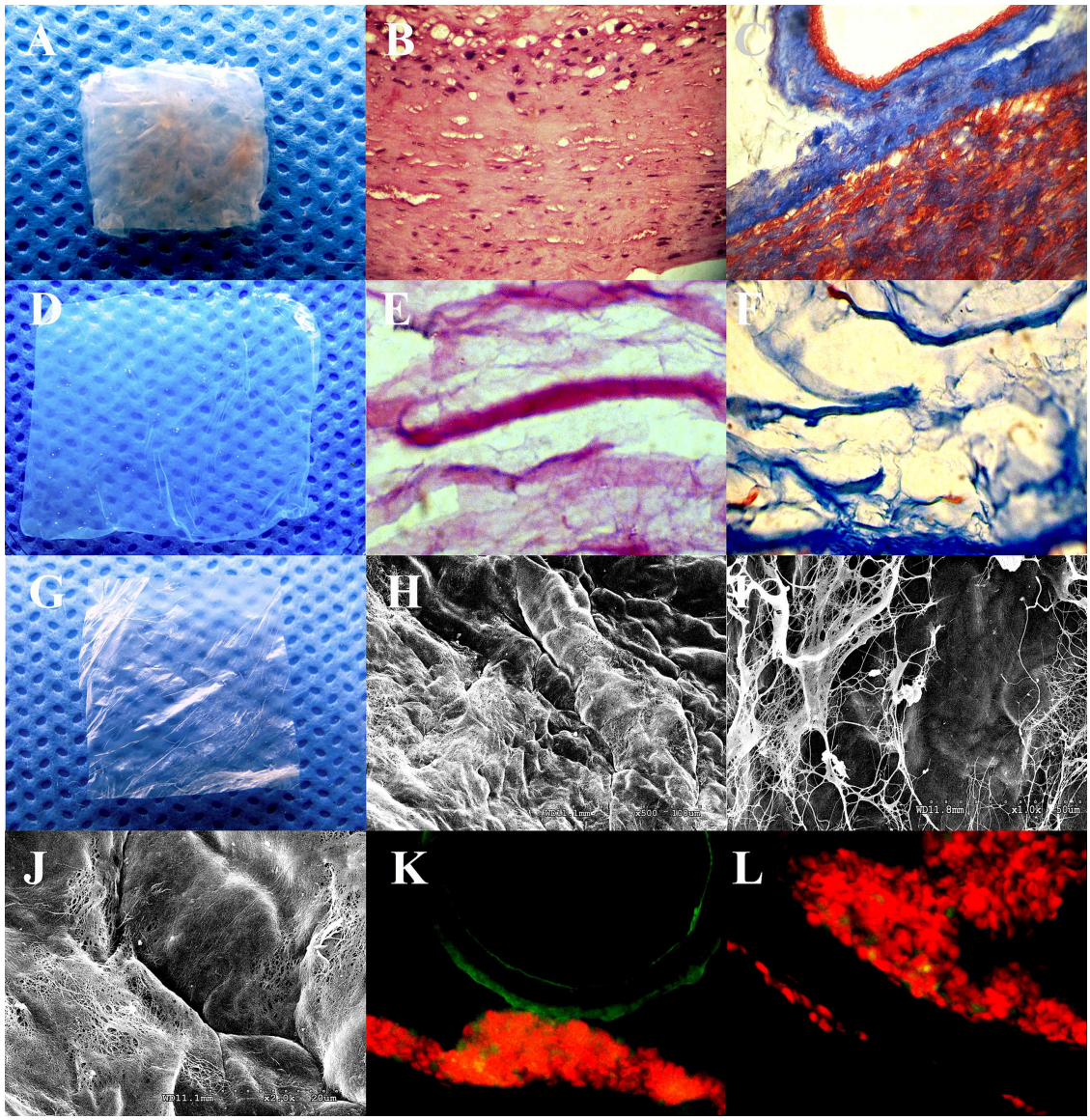

Fig. 1. Native, Decellularized, and lyophilized human amnion/chorion membrane. (**A**) Native human amnion/chorion membrane; (**B**) Hematoxylin and eosin staining of human amnion/chorion membrane; (**C**) Masson-Trichrome staining of human amnion/chorion membrane; (**D**) Decellularized and lyophilized human amnion/chorion membrane; (**E**) Hematoxylin and eosin staining of decellularized and lyophilized human amnion/chorion membrane; (**F**) Masson-Trichrome staining of decellularized and lyophilized human amnion/chorion membrane; (**G**) Decellularized and lyophilized human amnion/chorion membrane; (**H** and **I**) Scanning electron microscopy image of decellularized and lyophilized human amnion/chorion membrane; **J**) Scanning electron microscopy image of decellularized and lyophilized human amnion/chorion membrane after rehydratation; Immunofluorescence staining of decellularized and lyophilized human amnion/chorion membrane on biomatrix scaffolds with specific markers of (**K**) Collagen Ia1 and (**L**) Fibronectin.

Primary wound healing occurred in 42 (62.7%) patients. The remaining 25 (37.3%) patients developed wound complications including hematoma, wound separation, necrosis of the skin edges, and flap necrosis. PCF occurred in various locations of the neck area in 8 (11.9%) patients, and DLHACM grafts were used to close these fistulas.

Application of DLHACM Grafts to Treat PCF

For all eight patients who developed a PCF, the wound was prepared with solution of 10% polyvidone-iodine. After anaesthesia was induced with 1% lidocaine without epinephrine, necrotic tissue was mechanically debrided until viable, and bleeding tissue were encountered. DLHACM was delicately applied to the region, so that there were no air bubbles or blood clots between DLHACM and host tissue. DLHACM was generally tightly fixed to the wound, and only rarely were surgical sutures or fibrin glue applied.

The stages of reconstruction of a large PCF in the submental space are shown in Figure 3. All eight PCF closed, and the average time for the full healing of the wound after using DLHACM grafts was 18 days. Patients were followed every 3 months after closure of PCF, and there have not been any recurrences of PCF.

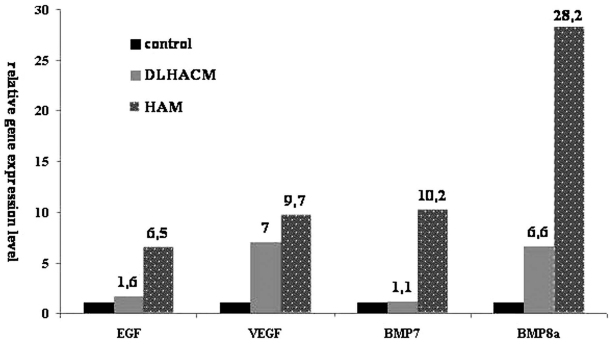

Fig. 2. Relative gene expression levels in native human amniotic membrane and decellularized and lyophilized human amnion/chorion membrane.

DISCUSSION

Historically, the treatment of PCF after total laryngectomy has employed local or regional flaps created from the skin, muscles, and mucous membrane [25–29]. Local flaps may be unreliable, since in most cases they have been affected by irradiation [30–32]. The use of local flaps can lead to recurrence rates for PCF as high as 66.6%, necessitating closure using pectoralis major myocutaneous flaps [33]. Even after primary reconstruction of PCF with pectoralis major myocutaneous flaps, the development of recurrent PCF varies between 16% and19%. The operation can also be accompanied by donor site infection (18%), donor site hematoma (10%), and flap necrosis (10%), with overall wound complications rates as high as 60% [34–36]. These complications occur on the backdrop of operating in a radiated, contaminated space with significant tumors burdens, all of which further complicate wound healing [59–61].

The healing of postoperative PCF after using the DLHACM grafts was possible due to the unique characteristics of the human amnion/chorion membrane, which possesses immunomodulative, anti-microbial, and anti-inflammatory properties, hastens fibrogenesis and angiogenesis, and increases extracellular matrix deposition [62–65]. The amniotic membrane, separated from the chorion by the intermediate or the sponge layer, is mostly composed of type III collagen and glycoproteins. It also contains numerous growth factors, such as EGF, bFGF, KGF, VEGF, TGF-a, TGF-b, PDGF, HGF, and NGF [66–71]. In this study, the DLHACM grafts were tightly attached to the wound surface and efficiently absorbed wound exudates. It has also been previously demonstrated that this tight adherence allows removal of surface debris and bacteria from the wound [72]. The unique physicomechanical and compositional properties of the human amnion/chorion membrane promote the migration of keratinocytes and various epithelial cells. Angiogenic growth factors, which are components of DLHACM grafts, also probably contribute to significant acceleration of neovascularization and formation of granulation tissue. DLHACM grafts also form an early closed physiologic space with the host, forming an adherence barrier with the wound via fibrin and elastin linkages that close the wound and prevent contamination [73].

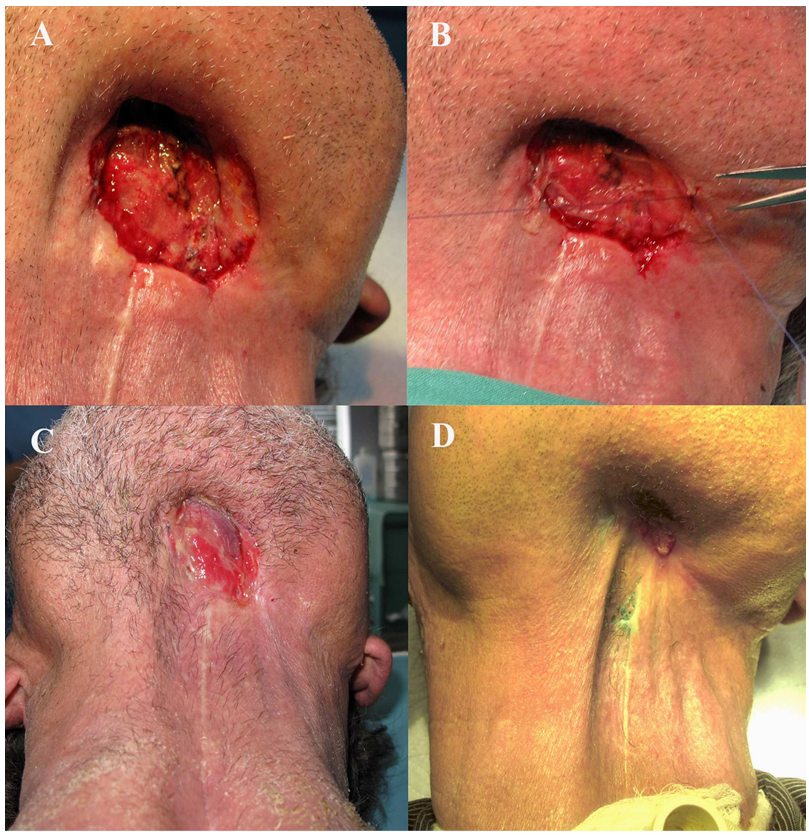

Fig. 3. Application of decellularized and lyophilized human amnion/chorion membrane graft on the pharyngocutaneous fistula. (**A**) Pharyngocutaneous fistula developed after extended laryngectomy in submental region before transplantion; (**B and C**) After transplantation of decellularized and lyophilized human amnion/chorion membrane; (**D**) healing process on day 14.

CONCLUSION

After laryngectomy, the occurrence of a PCF is a severe complication. DLHACM grafts are very good biomaterials for clinical application, especially in patients suffering from this complication. The advantages of using DLHACM grafts over other existing treatment methods for PC is that they are non-invasive, prevent donor morbidity, and are effective in closing PCF without further surgical intervention.

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