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Original Article

Effect of oxidized regenerated cellulose on the healing of pharyngeal wound: An experimental animal study

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Abstract

Background: This study aimed to investigate the relationship between oxidized regenerated cellulose (ORC) and mucosa healing in an experimental animal model.
Methods: Fifteen adult Sprague-Dawley rats were randomly divided into three groups that underwent different wound treatments. In Group 1, no pharyngeal wound was created. In Group 2, the pharyngeal wound was sutured with Prolene only. In Group 3, the pharyngeal wound was sutured with Prolene, and covered with one layer of ORC before closure of the skin wound. The animals were euthanized either 5 or 10 days after operation, and wound conditions were inspected and recorded. Specimens including sections of larynx and pharynx/upper esophagus were taken for microscopic and molecular biological examination.
Results: The pharyngotomy/esophagotomy wounds achieved good healing outcomes 10 days after operation. Wounds treated with ORC had significantly diminished inflammatory cell infiltration in microscopic examination when compared with that of those without ORC 5 days after operation. The matrix metalloproteinases (MMP) expression level was higher in wounds of Group 2 and Group 3, when compared with that of group 1. In addition, the MMP expression level was lower in the ORC-treated wounds when compared with that of those without ORC. There was no significant difference in fibroblast proliferation, collagen deposition, endothelin-1, alpha-smooth muscle actin, and transforming growth factor beta 1 expression level between wounds treated with ORC and those without ORC.
Conclusion: Reduced inflammatory response and decreased MMP expression level was observed in ORC-treated wounds. Whether ORC facilitates mucosa healing requires further investigation.
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Keywords: animal model; matrix metalloproteinases; oxidized regenerated cellulose; pharyngocutaneous fistula; wound healing

1. Introduction

Pharyngocutaneous fistula (PCF) can be a serious complication after ablative surgery of the aerodigestive tract, including the pharynx and larynx.[1] The predisposing factors of PCF formation are linked to poor nutritional status, infection and, most importantly, radiation therapy.[2,3] Once the fistula has formed, saliva leakage, protracted difficulties in deglutition leading to malnutrition, and delay in postoperative radiation may ensue. Therefore, several methods for preventing PCF formation have been proposed, including suture materials, soft tissue flap coverage, and meticulous suture technique.

Oxidized regenerated cellulose (ORC) has been used clinically, where its hemostatic effect is particularly advantageous.

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This biomaterial was also designed to modify the chronic wound environment by significantly reducing harmful factors such as proteases, oxygen free radicals and excess metal ions, while simultaneously protecting growth factors and redelivering them to the wound.[4] Previous studies found that ORC/collagen was beneficial for optimal healing in chronic wound repair because it minimizes infection while providing a moist healing environment.[5] However, the relationship between PCF and ORC has never been reported.

One previous study found that elevated proteolytic activity and matrix metalloproteinases (MMPs) contribute to their chronicity.[6] Although controlled degradation is necessary for normal wound repair, excess proteolysis is considered detrimental, and it may result in the degradation of the extracellular matrix (ECM) and key functional molecules such as growth factors, their cell surface receptors and integrins.[7,8]

Growth factors such as transforming growth factor-beta (TGF-β1) are pivotal in normal wound repair, driving cell migration, proliferation, protein synthesis, matrix formation, and generally controlling the repair process.[9] Endothelin-1 (ET-1) is one of a cascade of inflammatory mediators released during epithelial injury, and it initiates early contractile activity along with mechanical tension of myofibroblast.[10] Therefore, the degradation of either growth factors or the ECM by excess proteases in the wound could lead to diminished growth factors activity and delayed healing.

This study describes the evaluation of a new proprietary wound treatment, which here assesses the efficacy of ORC in the pharyngotomy/esophagotomy animal model. We aimed to investigate the relationship between wound healing after pharyngotomy/ esophagotomy and the application of ORC. We also examined the proteases and growth factor in the wound tissue.

2. Methods

2.1. Animal model

This study was conducted at the animal center of Taichung Veterans General Hospital, Taichung, Taiwan, ROC. The Institutional Animal Care and Use Committee of Taichung Veterans General Hospital approved this protocol. Fifteen adult Sprague-Dawley rats weighing 400–560 g were divided randomly into three groups. All the animals were maintained in accordance with the hospital's guidelines.

The rats were anesthetized by 2%–3% isoflurane using a face mask. Maintenance of anesthesia was achieved through a veterinary anesthesia delivery system. Then pharyngotomy/esophagotomy procedures were carried out in studied animals under anesthesia and the detailed procedures were the same as in our previous publication.[1] We also observed the normal pharyngotomy/esophagostomy wounds healing process on 16 rats before conducting of this study. Four animals were euthanized 1 day, 3 days, 7 days, and 14 days after operation, respectively. Based on histopathologic examination of the animal subjects, the inflammatory response reached its highest level after 3 days and diminished after 7 days. Detailed data are presented in Table 1.

The rats were divided into three groups. In Group 1, the pharynx/esophagus was exposed and no further pharyngotomy/esophagotomy was done. This group was the control group. In Group 2, the pharyngotomy/esophagotomy was closed with 5-O Prolene suture (Ethicon Inc. Somerville, New Jersey, USA). In Group 3, the pharyngotomy/esophagotomy was closed with 5-O Prolene followed by topical application of one layer of ORC (Surgicel, Johnson & Johnson Medical Ltd, Gargrave, North Yorkshire, BD23 3RX, UK). The applied thickness of ORC was less than 1 mm, and the neck skin of the rat was closed with 5-O nylon suture. All the animals had unrestricted access to food and water on the following day. The rats were euthanized either 5 or 10 days after operation with carbon dioxide chamber asphyxiation. The larynx of each animal was obtained, along with the pharynx and upper esophagus, where after the specimens were placed in a 10% neutralized formalin solution for histopathologic evaluation. One-half of each specimen was frozen immediately in liquid nitrogen, and stored at −80°C for subsequent molecular assay.

2.2. Histologic examination

The primary study outcome involved the presence or absence of PCF, which was characterized as purulent discharge after operation. The secondary study outcome was the characteristics of histopathologic findings using hematoxylin and eosin (H&E) staining. The artificial pharyngotomy site was scored by an animal pathologist using a standard process-blinded technique, relying on the 0–4 Ehrlich and Hunt numerical scale as modified by Phillips and colleagues[11] and adopted in another study.[12] Inflammatory cell infiltration, blood vessel neodevelopment, fibroblast proliferation, and collagen deposition were graded from 0 to 4 as follows: 0, no evidence; 1, occasional evidence; 2, light scattering; 3, abundant evidence; and 4, confluent cells or fibers. Another serial section was stained with picrosirius red stain for conformation of extracellular collagen matrix deposition.[13]

Table 1
Histopathologic results of the normal pharyngotomy/esophagostomy wound healing process.

Day after operation	Day 1, $n = 4$	Day 3, $n = 4$	Day 7, $n = 4$	Day 14, $n = 4$
Inflammatory cell infiltration	2.00 ± 0.41	3.50 ± 0.29	2.25 ± 0.48	0.50 ± 0.29
Neovasculization	0.75 ± 0.48	1.75 ± 0.48	3.75 ± 0.48	2.50 ± 0.29
Fibroblast activity	0.50 ± 0.29	2.50 ± 0.48	2.50 ± 0.48	2.75 ± 0.41
Collagen deposition	1.00 ± 0.41	2.00 ± 0.41	3.25 ± 0.25	4.00 ± 0.41

Data expressed as mean ± standard error.

2.3. Immunofluorescence staining for expression of endothelin (ET)-1 in myofibroblast (alpha smooth muscle actin positive)

ET-1 was assessed using a specific mouse anti-ET1 which was kept overnight at 4°C and then incubated with rhodamine-conjugated donkey anti-mouse immunoglobulin G (IgG) antibody, and evaluated individually by indirect immunofluorescence staining method. Then, alpha smooth muscle actin (α-SMA) was directly incubated with Fluorescein isothiocyanate (FITC)-conjugated mouse anti-α-SMA antibody. Sections were washed, counterstained with 4′,6-diamidino-2-phenylindole (DAPI), mounted and then examined by laser confocal microscopy.

2.4. Measurement of pharyngeal tissues MMPs activities by zymography

A total of 20 μg of protein from each tissue sample was diluted with Phosphate buffered saline (PBS), and electrophoresed on 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels containing 1 mg/ml gelatin. The gels were washed twice with 2.5% Triton X-100 (The Dow Chemical Company, Midland, Michigan, USA) solution for 30 minutes, then incubated with a reaction buffer at 37°C for 16 hours. The gels were stained with a solution containing 50% methanol, 10% acetic acid, and 0.1% Coomassie Brilliant Blue (Imperial Chemical Industries, London, England, UK) for 10 minutes, then destained in 10% methanol with 10% acetic acid.[14] The gelatin-lytic bands were quantified by Image Analysis with a Gel-Pro Analyzer (version 4.5; Media Cybernetics, Medical Lake, WA, USA).

2.5. Western blot and densitometric analysis

All tissues were sonicated in a radioimmunoreactive protein extraction assay lysis buffer. The homogenate was centrifuged at 10,000 rpm at 4°C for 10 minutes, and the supernatant was collected and stored at −80°C. A total of 25 μg of protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide) and transferred to a polyvinylidene difluoride membrane that was incubated with mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α-SMA, ET-1 antibody, washed, and then incubated with a 1:10,000 dilution of rabbit anti-mouse IgG conjugated to horseradish peroxidase. Bands were visualized by development with the Amersham ECL-Plus (GE Healthcare Bio-Sciences Corp. Piscataway, New Jersey, USA) detection reagents for one-dimensional gel electrophoresis, and normalized with the internal control protein, GAPDH.

2.6. Analysis of transcripts of TGF-β1 genes by quantitative reverse transcription polymerase chain reaction

Total cellular RNA was extracted from pharyngoesophageal tissue using the RNeasy Mini kit (Qiagen GmbH, Hilden, Germany). An aliquot of 1.0 μg of total RNA from each sample was subjected to assay using TaqMan One-Step RT-PCR Master Mix Reagent kit (Invitrogen Corporation, Carlsbad, California, USA), and Applied Biosystems TaqMan Gene Expression Assays (Life Technologies Corporation, Carlsbad, California, USA) pre-designed primers and probes were used. The expression levels of the target gene TGF-β1 (Assay ID: Rn_00572010_ml) were normalized with the control housekeeping gene, β-Actin (Assay ID: Rn01759928_g1) according to the manufacturer's instructions. Real-Time Quantitative RT-PCR was performed on a ABI StepOne Real-Time PCR (Applied Biosystems, Foster City, California, USA) System (Applied Biosystems, Foster City, CA, USA).[15]

2.7. Statistical analysis

We used descriptive statistics for general data presentation. In addition, comparisons of nominal or ordinal variables among the three groups were analyzed by the chi-square test. Furthermore, nonparametric statistics was applied to compare the continuous variables among the three groups. All statistical analyses were performed using IBM SPSS software (version 10.1; SPSS Inc., Chicago, IL, USA) for Microsoft Windows (Microsoft Corporation, Redmond, Washington, USA). Statistical significance was considered as $p < 0.05$.

3. Results

3.1. Descriptive statistics

Fifteen rats were separated randomly and equally into three groups. In each group, three rats were euthanized after 5 days while the other two rats were euthanized after 10 days. No rat developed PCF in the current study. Wound healing in study groups (Groups 2 and 3) was compared with the control group (Group 1). Outcomes in the control group were regarded as normal, noncompromised wound healing. On Day 5 postoperation, Group 3 had larger esophageal tumidity compared with Group 2. However, after 10 days, Group 3 had lower tumidity compared with Group 2. The tumidity of wounds was smaller 10 days after operation when compared with the wounds assessed at Day 5 postoperation in both groups. Samples were taken from the wounds for further examination.

3.2. Histologic assessment

Examination of wound tissue was collected in the tumidity part of the wound. In the H&E stain, the control group had normal muscle and proper connective tissues at the wound site. By contrast, the tumidity parts were infiltrated with light to abundant angiogenesis, inflammatory cells and fibroblasts in the study groups. Less inflammatory cells infiltration was noted in Group 3 when compared with Group 2 (Ehrlich and Hunt numerical scale: 2.67 ± 0.33 vs. 1.33 ± 0.33, $p = 0.047$, Mann-Whitney test) 5 days after operation. However, the differences between Group 2 and Group 3 were not

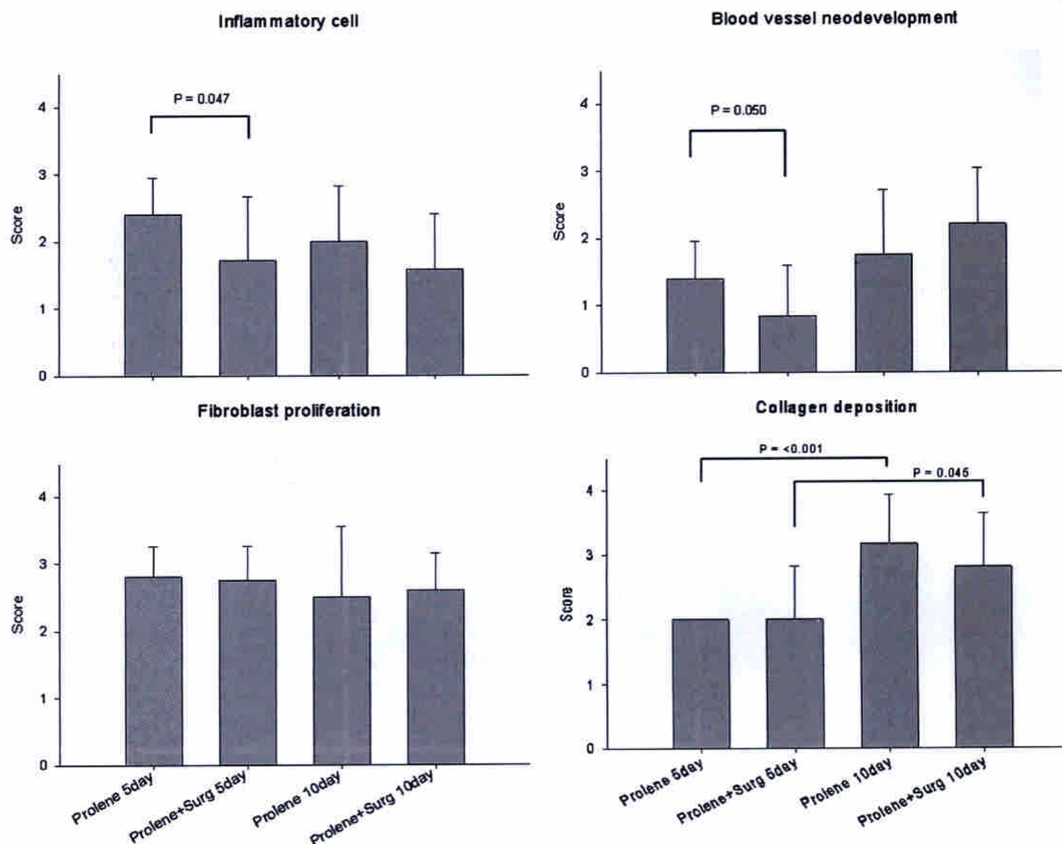

Fig. 1. Ehrlich and Hunt numerical scale of histopathological results in different groups. Less inflammatory cells infiltration was noted in Group 3 when compared with that of Group 2 ($p = 0.047$) 5 days after operation. In addition, collagen deposition was abundant 10 days after operation when compared with that of specimens 5 days after operation ($p < 0.05$).

statistically significant in neovascular development (Fig. 1). It appeared that more collagen deposits and fewer inflammatory cells were found in the wounds 10 days after the operation (Fig. 1).

The collagen deposition was reconfirmed by picrosirius red staining. Collagen was stained red and mesenchymal cells were stained green. In the study groups, collagen and connective tissue separated muscles because of the healing process. However, the collagen expression around the wound was not significantly different between Group 2 and Group 3 (Fig. 1). Moreover, collagen tended to be abundant in the subgroup evaluated 10 days after operation when compared with specimens obtained 5 days after operation ($p < 0.05$).

3.3. Immunofluorescence staining for expression of α-SMA and ET-1

The protein expression of α-SMA and ET-1 were weak in the control group and only positive in the vessels (Fig. 2). α-SMA expression was remarkable in Groups 2 and 3. ET-1 expression revealed a heterogenous distribution, and it mostly coexisted with α-SMA positive myofibroblast in the peripheral area of wounds in the study groups (Fig. 2).

3.4. Western blot

Based on the protein expression level, α-SMA was used to evaluate the amount of activated fibroblasts. In the control group, the tissue contained a certain amount of fibroblasts to balance the ECM synthesis and degradation. After 5 days healing, the level of α-SMA expression was increased in both Group 2 and Group 3. However, there was no significant difference between these two groups. The ET-1 expression level was elevated in the study groups and there was also no significant difference between Group 2 and Group 3.

3.5. Zymography

The control group showed normal activity of MMPs in the wound tissues. After 5 days, the MMPs activity was significantly elevated in Group 2 and Group 3 (MMP-9: $H = 7.26$, $p = 0.027$; MMP-2: $H = 6.71$, $p = 0.035$, Kruskal-Wallis test). This is shown in Fig. 3. MMP-2 and MMP-9 expressions in Group 2 were higher than those in Group 3. However, the difference was not significant. We did not collect relevant data from the 10-day group. Therefore, we could not show the MMPs activity trend in this study.

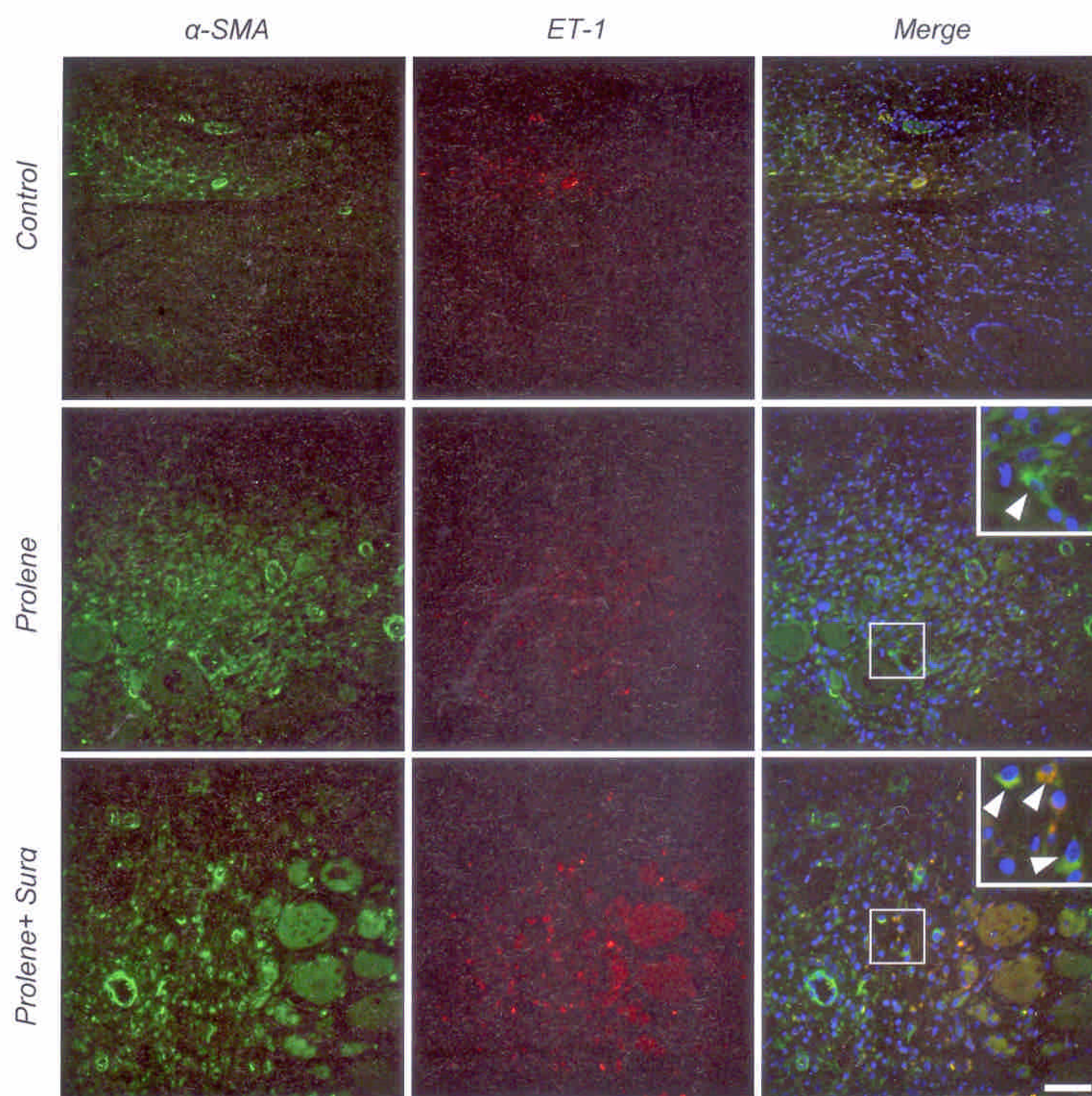

Fig. 2. Immunofluorescence staining for expression of α-SMA and ET-1 in pharyngotomy/esophagotomy wound healing after 5 days in different groups. The level of α-SMA and ET-1 expression was increased in both group 2 and group 3 when compared with those of group 1. However, there was no significant difference between group 2 and group 3. ET-1 = endothelin-1; α-SMA = alpha smooth muscle actin.

3.6. Analysis of transcripts of TGF-β1 genes by quantitative reverse-transcript polymerase chain reaction

The level of TGF-β1 was increased in Group 2 and Group 3 when compared with that of Group 1. Although the amount of increase was two to fourfold, the difference between Groups 2 and 3 was not remarkable. The aforementioned data were only from the 5-day group. Therefore, we could not show trend of level of TGF-β1 in current study.

4. Discussion

Wound healing is a dynamic and complex process involving blood cells, extracellular matrix, growth factors/cytokines, and parenchymal cells. It begins with hemostasis, followed by inflammation, proliferation, and tissue remodeling.[16] The mucosa healing process consists of the same fundamental phases as cutaneous wounds; however, the distinctive composition of the mucosa suggests that an altered sequence of procedures occurs in response to mucosa injury.[17]

To the best of our knowledge, this is the first study concerning ORC and pharyngeal/esophageal mucosa healing in the literature. In our study, we found that excellent pharyngeal/esophageal wound healing was achieved 10 days after operation in an animal model. However, there was no significant difference between Group 2 and Group 3 in gross wound healing. Microscopic examination showed that wounds treated with ORC had reduced inflammatory cell infiltration after 5 days. This could be explained by the antibacterial capability of ORC that was reported by previous experimental and clinical studies.[5,18] Although blood vessel neodevelopment was decreased in ORC-treated wounds 5 days after operation in this study, this change did not reach statistical significance. A previous study found that an ORC/collagen matrix could accelerate re-epithelialization in acute wound healing in an

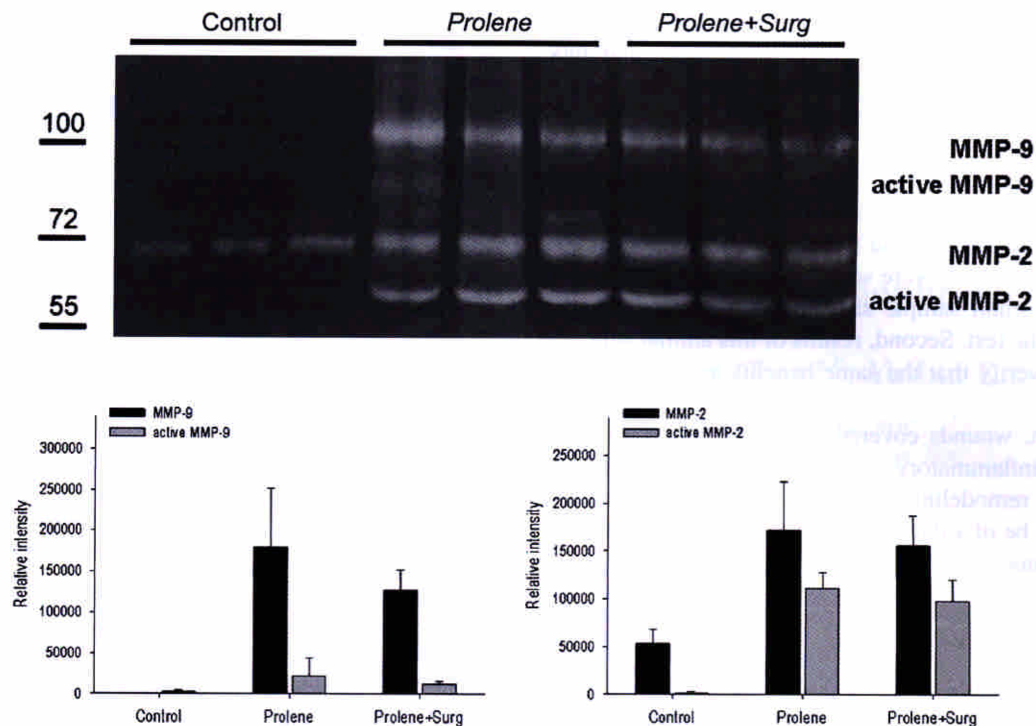

Fig. 3. Activity of MMP 5 days after wounding by zymography. The MMPs activity was significantly elevated in Groups 2 and 3 when compared with that of group 1 ($p < 0.05$). MMP = matrix metalloproteinase.

animal model. It also revealed no significant differences in collagen deposition and blood vessel neodevelopment between the ORC/collagen-treated wounds and controls.[5] The discrepancy could be explained by the different biomaterials used and different treatment methodologies, as our study used ORC only and focused on pharyngeal/esophageal mucosa healing.

ET-1 was reported to influence ECM composition and promote the contractility of fibroblasts. Also, the activated fibroblast could be evaluated by the expression of α-SMA.[19] Our study failed to demonstrate any differences in ET-1 and α-SMA between wounds treated with and without ORC. There is no study to date in the literature concerning the relationship between ET-1 and ORC, or between α-SMA and ORC in wound healing. In the current study we found that MMP-2 and MMP-9 expression was reduced in ORC-treated wounds. A previous study[20] found a significant reduction in MMP-2 and MMP-9 when fluid samples from wounds were incubated in the presence of ORC/collagen. Although the MMPs were reduced in ORC-treated wounds in this study, the difference was not significant.[20] This might be due to the small sample size of our study and the dissimilarity between *in vitro* and *in vivo* studies.

Normal wound healing involves interactions among the ECM, growth factors, and cells. ECM constitutes components synthesized and deposited outside the cell surface, providing structural and functional integrity to connective tissues and organs. Degradation and remodeling of the ECM by proteases, especially MMPs, is a crucial feature of re-epithelialization and tissue remodeling. MMPs can also degrade growth factors and their receptors.[8] Therefore, reduction in MMPs might facilitate wound healing. Smeets and colleagues,[4] in their study on wound exudates of patients with chronic venous ulceration, found no significant differences in MMP-2 concentrations between patients treated with ORC/collagen and the control group. They proposed that reduced gelatinase and elastase activity in ORC/collagen-treated wound exudates may restore the equilibrium of the hostile chronic wound microenvironment, thereby facilitating wound repair.[4] However, elastase was not evaluated in the current study so it is not possible to compare our data with the findings of Smeets and coworkers.[4]

There was no significant difference in TGF-β1 between wounds treated with and without ORC in our study. During the wound repair process, TGF-β1 is activated and promotes inflammatory cell recruitment. In addition, TGF-β1 enhances matrix protein synthesis while decreasing matrix protein degradation and results in fibrotic tissue formation.[17] Gago and colleagues[21] found that ORC increased the expression of TGF-β1 in mesothelial cells. However, they discussed the ability of ORC to reduce postoperative adhesion rather than its effects on wound healing.[21]

From the results of this study, we speculate that the decreased inflammatory response and possible suppressed MMPs activity with the application of ORC might facilitate the healing process in human wounds. Therefore, placement of ORC between the pharyngeal wound and the skin flap in laryngectomized patient might diminish inflammatory cell infiltration so that a reduction in wound infections and a decrease in PCF rate might be expected. However, the results of this animal study do not necessarily verify that the same benefits may be found in human wounds, due to the different

mechanisms of wound formation and the genetic polymorphisms influencing wound healing in humans and rats. Nevertheless, our findings do indicate that further study on the effects of ORC in human pharyngeal/esophageal mucosa healing is warranted.

The strength of this study is its status as a prospective animal study discussing the relationship between pharyngeal/esophageal mucosa healing and ORC, which was not seen in literature. However, there were some limitations in the present study. First, the small sample size could reduce the broader applicability of the test. Second, results of this animal study do not necessarily verify that the same benefits may be found in human wounds.

In conclusion, wounds covered with ORC were found to have reduced inflammatory cell infiltration and possibly enhanced ECM remodeling. Therefore, our findings suggest that ORC might be of value in promoting the mucosa healing process in humans.

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