

The esophagus survives non-thermal irreversible electroporation ablation and gradually rehabilitates

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Research

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Abstract

Background When using ablation technology to treat esophageal cancer or tumors adjacent to the esophagus, the damage and recovery of the esophagus itself is of particular concern. Non-thermal irreversible electroporation (NTIRE) is a novel minimally invasive ablation method that uses microsecond electric fields to create nanoscale defects on cell membranes and induce cell death, while keeping all other molecules (including the extracellular matrix) intact. In this study, we aimed to study the effect of NTIRE on the esophagus wall and its subsequent repair process.

Methods A typical NTIRE electrical protocol was applied to the rabbit esophagus and histological analysis was used to analyze subsequent changes in esophageal tissue after ablation.

Results The application of NTIRE resulted in complete cell inactivation, but the experimental animals did not show severe pathological discomfort after in situ ablation. After ablation, the entire layer of the esophageal wall gradually showed signs of recovery: regeneration and creep replacement of epithelial basal cells, regeneration of muscle cells and structural remodeling of the muscle layer, and finally the restoration of clear anatomical structures in each layer.

Conclusions The esophagus can survive the direct application of NTIRE, and the whole layer of the entire wall can be gradually regenerated and repaired. NTIRE can be used for in situ ablation of local tumors, including esophageal, intrathoracic and mediastinal tumors, and upper abdominal tumors, while minimizing collateral damage to adjacent tissues because of the unique ability of the NTIRE ablation method to target the cell membrane.

Introduction

Performing thermal ablation in a soft tissue or an organ has been reported to potentially cause protein denaturation and tissue coagulation necrosis [1], resulting in organ damage and partial loss of function. Collateral injury to the esophagus can be caused by all presently used thermal ablation modalities, including radiofrequency ablation, cryoablation and microwave ablation, etc.[2–4]. For instance, thermal damage to the esophagus often occurs after in situ radiofrequency ablation of atrial fibrillation or hepatic tumors, resulting in vomiting, thoracalgia, dysphagia, or even esophageal ulceration and stenosis [5, 6].

In recent years, non-thermal irreversible electroporation (NTIRE) has been considered to be a promising ablation tool with several potential advantages compared with thermal ablation. NTIRE is an advanced new technology that induces cell death by creating permanent nanopores in the cellular membrane via the application of short intervals of high-voltage direct electrical current [7]. It increases therapeutic effects while avoiding thermal effects [8]. Although NTIRE is thought to be effective in destroying all cells in the ablation area, its non-thermal nature preserves extracellular matrix (ECM), connective tissue and collagen [9, 10]. NTIRE ablation has been tested in lung, prostate, kidney, liver and lymph node tumors in preclinical studies and clinical trials, demonstrating that it can inactivate normal cells and tumor cells while retaining tissue scaffolds [9, 11, 12]. The structural integrity of vessels and nerves remains intact,

and the retention of collagen scaffold allows subsequent tissue repair and regeneration [7, 9]. Possible applications of NTIRE involve treating esophageal cancer and local space-occupying lesions of the mediastinum, chest or upper abdomen, while avoiding thermal damage to nearby organs. NTIRE may become a promising ablation tool in patients with esophageal cancer or other tumors that may be adjacent to or invade the esophagus, such as lung cancer, mediastinal cancer, and tumors of upper abdominal organs.

The esophagus may be particularly sensitive to the treatment of NTIRE because it has a high cell turnover rate, especially the rapid division rate of epithelial basal cells. However, little is known about the mid- and long-term healing process after full-layer electroporation ablation of the esophagus wall. The risk of NTIRE to esophageal damage also needs to be assessed before clinical implementation. In this work, we chose to study the effect of NTIRE on the esophagus, which is often collaterally damaged during minimally invasive ablation procedures. Our hypothesis is that, due to the ability of NTIRE to spare the ECM, the esophagus will remain structurally intact after ablation, survive the treatment, and recover. It can be assumed that because NTIRE can keep the ECM from being damaged, the esophagus will remain intact on the tissue structure, and repair and regeneration can be gradually completed after ablation.

Materials And Methods

In vivo irreversible electroporation ablation procedure

Thirty-six male, 6-month-old New Zealand rabbits with an average weight of 2.6 ± 0.5 kg were used in this study. All animals received humane care and were conducted under a protocol approved by the Ethics Committee of General Hospital of Northern Theater Command. All animal manipulations were conducted in accordance with national and international guidelines to minimize animal suffering. Animals were anesthetized by intramuscular injection with xylazine hydrochloride (5 mg/kg) and diazepam (0.5 mg/kg). The anterior approach of the neck was selected, and the middle of the incision was at the level of the second annular cartilage. A specially designed hand-held clamp, containing two parallel plate electrodes (Platinum Tweezertrode, 45-0486, BTX, U.S.), was applied across the targeted esophagus. The measured distance between the two electrodes was approximately 3.5 mm, which was consistent for all animals tested. Nine trains of 10 direct current square pulses of 572 V (generating an approximate electrical field of 2000 V/cm), each 70 microsecond long, with a pulse frequency of 4 Hz was applied between the electrodes using a high voltage pulse generator (TP3032, Teslaman, Dalian, China). The electrical parameters used in this study are typical to those used in clinical procedures to produce irreversible electroporation without causing additional thermal damage. The length of the ablation segment is approximately 2 cm. The location of treatment was noted based on two suture knots which were placed in the esophageal adventitia to mark the NTIRE-treatment region.

Animals were divided into six groups, and each group of six animals survived for 1, 3 days and 1, 2, 4, and 16 weeks before being euthanized. In each group, one animal was selected as the random control and the electrode was only applied directly on its esophageal adventitia without pulse output. During the

first 24 hours after surgery, the animals were given two additional doses of meperidine hydrochloride (1 mg/kg), spaced out over 8 hour increments. The animals were kept separately and carefully checked daily to ensure that they were not experiencing pain, stayed healthy, and recovered. Symptoms that were monitored and made effort to relieve included fever, reduced food intake and drinking, lack of locomotion and swelling around the incision, etc.

Finite element modelling of electrical parameters to predict the range of effective field strength and thermal damage

In order to choose electrical parameters for experimental use that would not cause extensive heating and thermal damage to the tissue, a transient finite element analysis was performed, modelling the effect of Joule heating on the temperature distribution as described by Phillips [8]. A commercial finite element package (COMSOL Multiphysics 5.4) was used to develop the model and analyze the electrical treatment parameters. The cross section of the esophagus was two-dimensionally modelled as a runway shape (one $10 \times 3.5\text{-mm}^2$ rectangle and two semicircles with a diameter of 3.5 mm) between two stainless steel electrodes (a circular plate with a diameter of 10 mm). The dimension of esophagus was based on experimental measurement. The thermal and electrical properties of the esophagus were assumed to be both homogeneous and isotropic in cross-section. By using a signal analyzer (N9030A PXA, Agilent, U.S.), the conductivity values were 4.0×10^6 [S/m] for the electrode, 1×10^{-17} [S/m] for the insulating layer, and 0.97 [S/m] for the esophagus; the dielectric constants were 4.5 for the insulating layer and 4.0 for the esophagus.

Histological examinations

The animals were weighed and euthanized with a mixture consisting of ketamine (150 mg/kg) and xylazine (20 mg/kg). The treated section together with untreated section of about 1cm long at both ends were selected. Samples were immediately fixed with 10% buffered formalin, embedded in paraffin and sectioned with a microtome (5 μm -thick). Each sample was stained with haematoxylin and eosin (H&E) to examine the basic morphological changes and Masson's trichrome to examine the structure of the ECM. In Masson's trichrome stain, collagen fibers were stained blue, muscle cells were stained reddish purple, and epithelial cells were stained light red or lilac.

Result

Clinical observations

One animal was lost during surgery due to an overdose of xylazine. Seven animals experienced mild anorexia after surgery and resolved on their own within 72 hours. Otherwise, the animals did not show any symptoms of typical pain, vomiting, weight loss or melena.

Distribution of the electric field intensity and prediction of thermal damage

All recorded voltage and current waveforms were smooth, demonstrating the absence of aeroionization. Field strength modeling showed the electric field distribution between the two electrodes was almost uniform when direct-current pulses of 2000 V/cm were applied. The highest field intensity of 10401 V/cm emerged at the edge of the electrode, and the lowest field intensity of 1150 V/cm emerged at the midpoint of each semicircular arc (Figure 1).

The maximum tissue temperature obtained throughout the entire procedure was 40.21 °C. This model showed very little temperature increase to the esophagus tissue during the ablation procedure because of the relatively large surface area of the stainless steel electrodes. Thus, most of the heat was quickly conducted to the electrodes and dissipated. As this model predicts very little damage while incorporating assumptions that would actually over predict tissue temperature (over predictions include two-dimensional model, ignoring heat loss due to natural convection, and using the maximum tissue temperature to obtain the damage parameter), it can be considered that the parameters modelled could be used experimentally without causing thermal damage to the esophagus in vivo.

General observation

Direct visual inspection of the ablation area after NTIRE revealed no blood clots, and the electrodes never showed signs of charring. Within a short time after ablation, the electrode plate caused slight congestion of small blood vessels and local vasodilation, which can last for 3 days. From the third day to one week after ablation, no obvious stenosis, epithelial erythema, erosions, or ulcerations were observed. From 4 to 16 weeks after ablation, there were no macroscopic lesions on the esophageal epithelium and adventitia, and all esophagus appeared normal.

Histopathological assessment

Histological analysis of the esophagus was performed at six time points from day 1 to week 16 after ablation to examine the effects of NTIRE on the esophagus.

On day 1, the boundary between the ablated and non-ablated areas was clearly demarcated (Figure 2). The selected irreversible electroporation protocol is strong enough to affect all layers of the esophagus. Acute inflammatory infiltration was observed throughout the entire layer of the esophagus wall. The superficial part of the epithelium exfoliated, but the basal epithelial layer was intact. The submucosa showed severe edema with a large number of inflammatory cell infiltrations, but the arterioles and veins were intact. The muscle layer was completely ablated, manifested by lysis of muscle cells and inflammatory infiltration of lymphocytes. It should be noted that there were no signs of thermal damage throughout the ablation process, and the basic tissue framework (such as collagen fibers) and structural details were well preserved throughout the esophageal wall. The connective tissues surrounding the striated muscle, bundles and fibers are called the epimysium, the perimysium, and the endomysium respectively, which remained intact at this sampling time.

On day 3, the structure of the esophagus remained intact and there were no signs of lumen stenosis. The blood vessels, Meissner plexus and regenerated muscle cells were clearly visible (Figure 3). The structural details of the epithelium are preserved. The plica of esophagus, composed of the mucosa and submucosa, still maintained its original tissue structure and form. As indicated by the appearance of immature epithelial cells in the basal layer of the epithelium, there were clear signs of mucosal regeneration and repair in the treated area. Meanwhile, immature muscle cells began to appear in the muscle layer.

On week 1, signs of tissue repair were evident (Figure 4). The most important features were an increase in the number of blood vessels in the submucosa, an expansion of the blood vessel lumen, and a rich blood supply. The esophagus appeared to have restored most of its structure and exhibited distinct layers of tissue, including: a mucosal layer with non-keratinized squamous epithelium, a submucosal layer containing mucus glands, blood vessels, lymphatic vessels and the Meissner plexus, a muscular layer containing regular muscle fibers and rich Auerbach plexus, and a well-structured adventitia.

From week 2, the epithelial surface began to keratinize, the epithelial basal cells were still vigorously proliferating, the submucosal vasodilatation was reduced, the muscle layer was clear, and the sarcoplasm of regenerated muscle cells tended to be full (Figure 5 A).

From week 4 to week 16, esophageal repair and regeneration continued to improve, and its micromorphology was similar to the normal control group (Figure 5 B, C). Esophagus was not completely devascularized after NTIRE ablation. Instead, they reached or even exceeded the levels of the normal control group at week 4, indicating that the vasculature is well preserved and blood vessel reconstruction is accelerated.

Discussion

In this study, we perform complete ablation of the esophagus through electroporation. Using finite element modeling, the electrical parameters are selected so that it is higher than the threshold of NTIRE in target tissue to ensure the effectiveness of inactivation, but does not cause unnecessary thermal damage to the esophagus due to Joule heat. The whole layer of the esophagus wall were completely ablated at 1 day after ablation, and signs of recovery began to appear after 3 days post ablation, which indicates that the applied electrical parameters are strong enough to cause irreversible electroporation in all layers of tissue without generating excessive thermal damage.

We observe that the esophagus undergoes three characteristic stages after NTIRE, each of which has its own unique histological manifestations and biological significance. The first stage is the period of tissue inactivation, including cell death, tissue edema, inflammatory infiltration and cell clearance. Due to complete ablation of the epithelial and muscular layers, NTIRE-induced cell death occurs quickly. Partial exfoliation occurred in the epithelial layer, and the cellular structural details in the muscle layer were completely lost. The second stage is the cell regeneration phase, including the phase of cell regeneration, proliferation, and tissue revascularization. NTIRE specifically targets the cell membrane, allowing for the

preservation of tissue structural components such as the ECM, blood vessels, and nerve fibers [13, 14]. It can be seen that this holds true for the esophagus as well. The necessary microenvironment after NTIRE ablation offers the possibility of rapid regeneration and proliferation of tissue cells. On days 1 and 3 post ablation, Masson's trichrome staining showed that the ECM was still intact, and the nerves, lymph-vessels and blood-vessels were still functioning, providing a necessary functional framework for epithelialization and muscle cell regeneration. Although the observed ECM edema may cause tissue ischemia in the ablation segment on day 3, subsequent observations showed that blood supply was fully restored or even increased. One week after ablation, signs of tissue repair were already evident. The epithelial lamina propria of the esophagus contains multipotent stem cells, which differentiate and mature gradually, replacing cells that slough off in normal, healthy tissue every 1 to 3 days [15, 16]. Though the epithelial cells within the treated region were ablated, it appeared that immature epithelial cells or epithelial-derived stem cells were being produced from the edges of the treated regions, and were able to migrate inward to the treated region and produce a new epithelial cell layer. The third stage is the period of tissue remodeling, including remodeling of ablated tissue and complete recovery of the blood supply. NTIRE's ability to retain the tissue framework will greatly aid in the overall recovery of the esophagus. The framework structure like the epimysium and the perimysium, which are mainly composed of reticular collagen fibers and elastic fibers, remained intact after NTIRE. Thermal coagulation and thrombosis to the blood vessels has not occurred, and the capillaries are open and blood is flowing. Although long-term studies are needed to determine what effect will NTIRE have on the esophagus years after treatment, it is believed that the unique ability of NTIRE to retain blood vessels and the ECM not only helps tissue recover in the short term after ablation, but could also protect the tissue from developing long-term complications in thermal ablation treatments.

Therefore, NTIRE is viewed as a promising modality for tumor inactivation therapy. When using thermal ablations to treat esophageal mass, or a mass adjacent to esophagus, like in the thyroid, lung, mediastinum, or the bottom of the stomach, may cause direct or collateral irreversible damage to the wall of the esophagus. For example, some patients with esophageal cancer have locally advanced tumors which may be closely related to the surrounding important blood vessels, nerves and organs and is not amenable to resection or ablation with conventional thermal methods. However, NTIRE can naturally overcome these limitations because of its ability to preserve important framework of the tissue by using the cell membrane as a specific target. NTIRE may be a promising alternative to treat benign or malignant tumors near sensitive organs or in important organs themselves.

Several unique characteristics distinguish NTIRE from current tumor thermal ablation techniques. Firstly, NTIRE is not associated with a temperature increase or with protein denaturation if not in a sufficiently strong field [17]; rather, it effectively retains the protein activity which plays an inductive role during tissue regeneration [18, 19]. Secondly, NTIRE causes complete tissue ablation partially through apoptosis or "apoptosis-mimetic" necrosis [20], which has many beneficial effects. There is an emerging evidence that apoptotic cells release growth signals, stimulate the proliferation of progenitor or stem cells and promote tissue regeneration [21, 22]. These newly formed cells replace the dead cells, initiate the remodeling of the surrounding matrix, and enable the extremely rapid regeneration of ablated tissue [23]. And thirdly, NTIRE

might have a unique ability to spare critical structures, including collagen fibers and the blood vessels [9, 17, 24]. It can be predicted that if some thermal ablation methods were applied to the esophagus, they would cause severe lumen stenosis. Instead, effective maintenance of overall framework tissue integrity and reduced fibrosis have been reported as favorable side-effects of NTIRE in comparison to other thermal ablation methods [25]. It is evident that the intact vascular preservation is fundamental for both necrotic tissue resorption and in situ tissue regeneration.

The purpose of this study was to assess the viability of the esophagus after direct application of NTIRE. This preliminary study indicates that NTIRE has the ability to preserve the ECM, important blood vessels and nerve bundles. This feature allows for a quick recovery of the esophagus after electroporation ablation, and provide an important guarantee for the recovery of peristalsis and secretion of the esophagus.

Conclusions

The purpose of the study is to offer a translational bridge from the laboratory to the clinic to open new avenues for the understanding and treatment of mediastinal tumor. This study carries out a feasibility research for the technique of in situ ablation of esophageal tumors or paraesophageal organ tumors. Although the distribution of electric field in tumor tissue is very complex and requires a lot of preliminary basic research work to assess peristalsis and secretion function of the esophagus over a longer time course after electroporation ablation, this study predicts that, should the esophagus be within the electric field generated by NTIRE, which is mainly applied to treating a tumor in the chest, in the mediastinum, or even in the esophagus itself, the ablated esophagus will be able to heal and regenerate.

Abbreviations

NTIRE: Non-thermal irreversible electroporation; ECM: Extracellular matrix; H&E: Haematoxylin and eosin.

Declarations

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Authors' contributions

YS supervised the study and designed experiments, YS and ZZJ executed experiments and analyzed data.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All the protocols concerning animal experiments were approved by the Ethics Committee of General Hospital of Northern Theater Command.

Consent for publication

We have obtained consents to publish this paper from all the participants of this study.

Competing interests

The authors declare that they have no competing interests.

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Figures

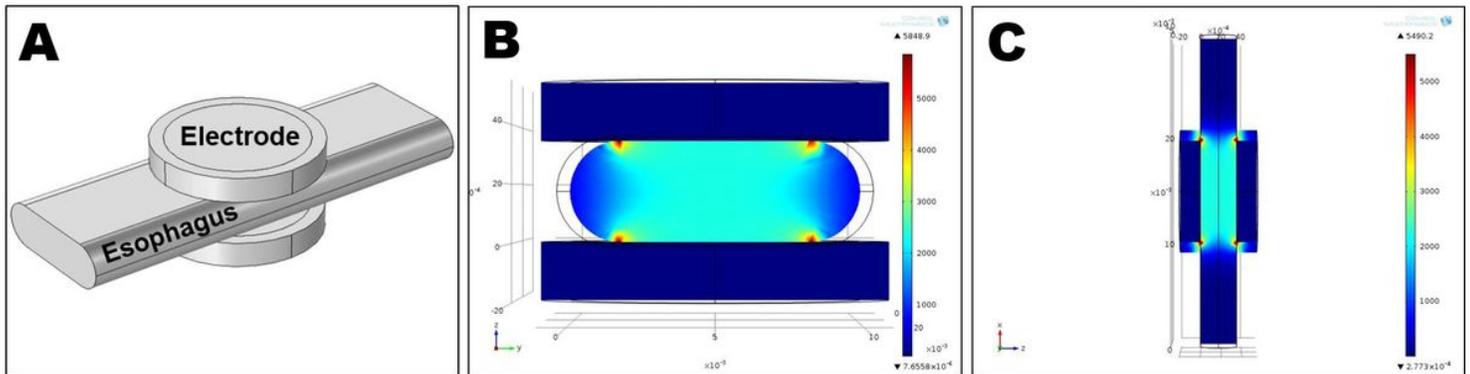


Figure 1

Simulation of electric field intensity distribution in esophageal electroporation. (A) Three-dimensional simulation of esophagus clamped by two plate electrodes. (B) Cross section of the ablation model. The electric field distribution between the two electrodes is substantially uniform. The highest electric field strengths appear at the edges of the electrodes (red areas), and the lowest strength appears at the midpoint of each semi-circular arc (dark blue areas). (C) Longitudinal section of the ablation model.

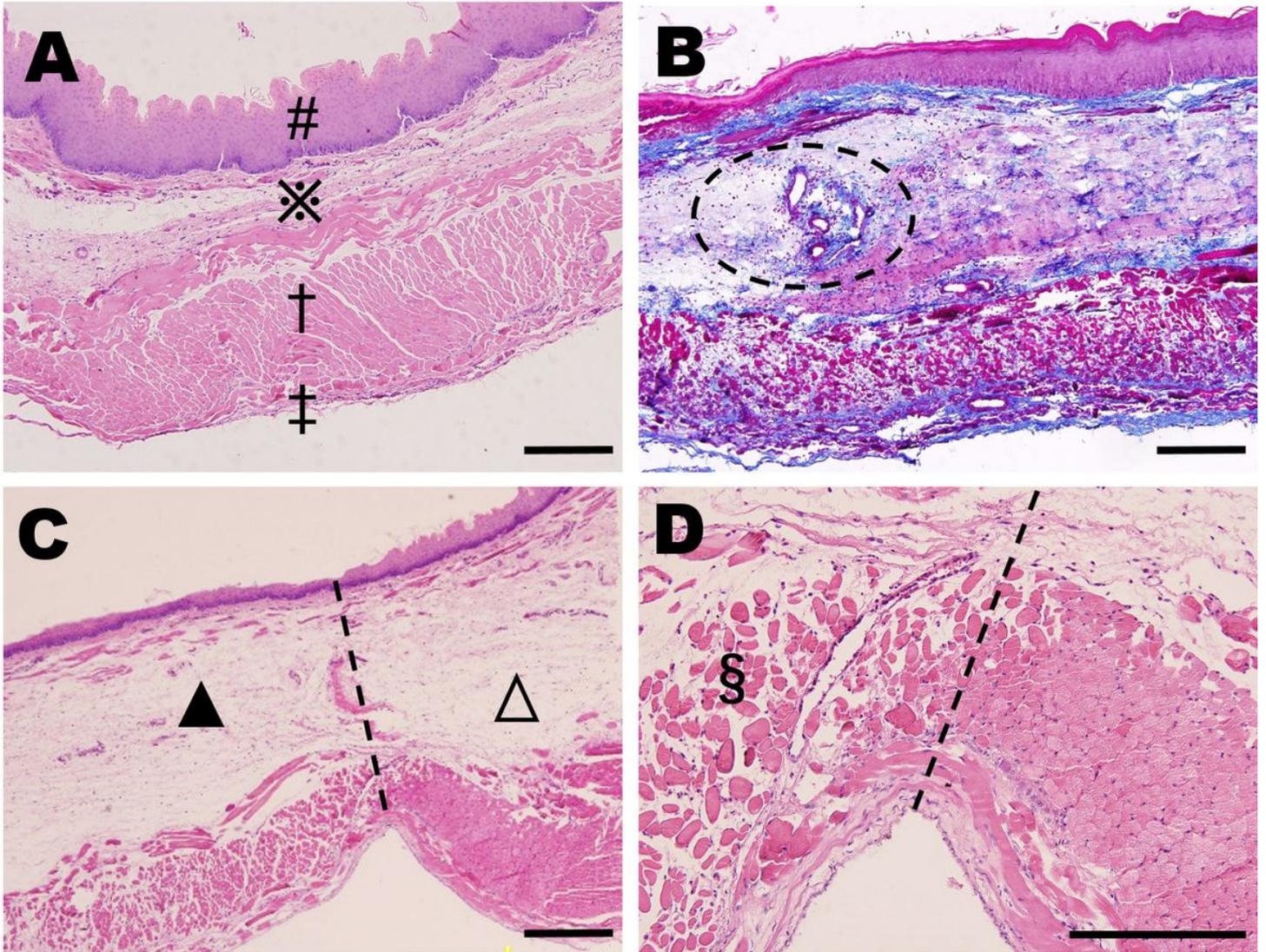


Figure 2

One day after esophagus ablation. Bars represent 50 μ m. (A) Untreated control. A typical healthy esophagus with mucosa (#), submucosa (*), muscular layer (†) and adventitia (‡). (B) Ablation group with Masson's trichrome staining. The fully retained collagen fiber framework is stained blue. Significant edema appears in the submucosa with a large number of inflammatory cell infiltrations. Small arteries and veins appears to be intact (dashed oval). (C) Ablation group with H&E staining. A clear boundary line between the NTIRE treated area (▲) and the untreated area (△). The outer surface of the epithelial layer is partially exfoliated. (D) A higher magnification of (C) shows completely ablated muscle layer, the dissolution and absorption of muscle cells, and the intact epimysium, perimysium, and endomysium (§).

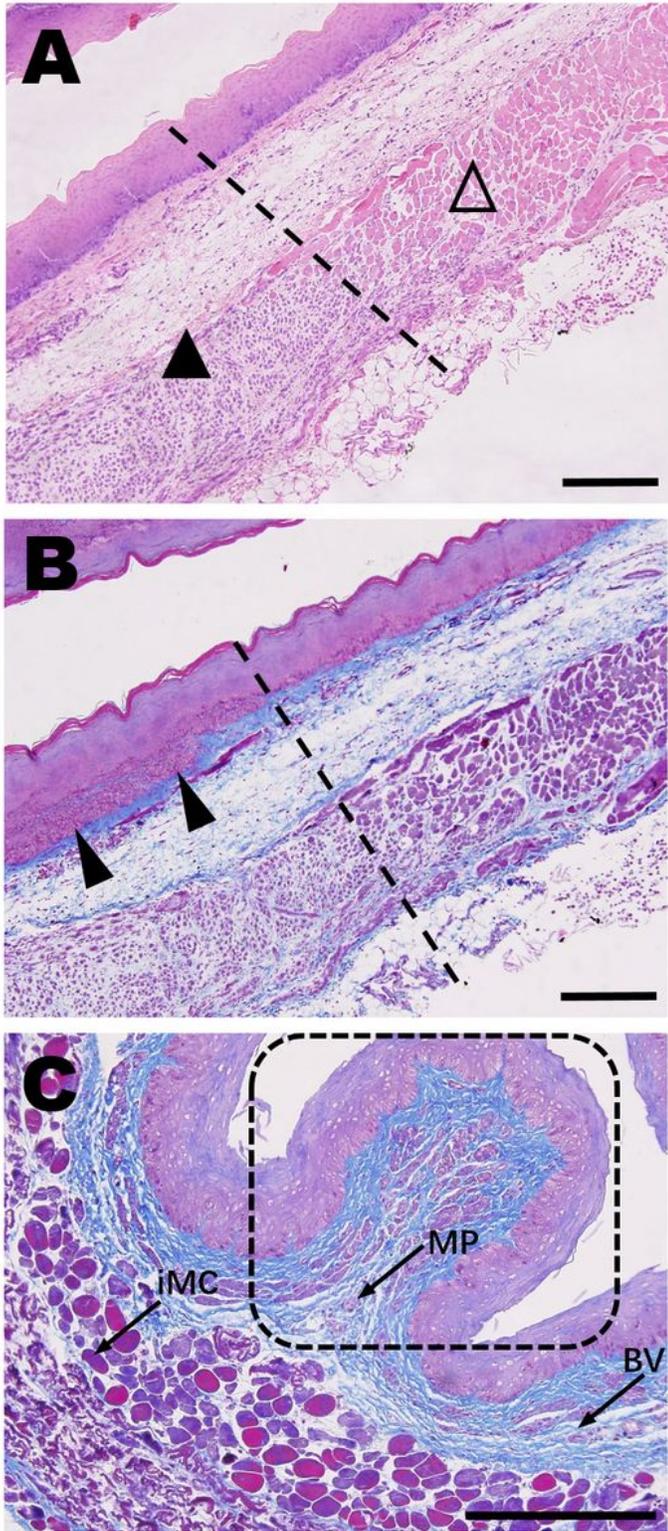


Figure 3

Three days after esophagus ablation. (A) The interface between the NTIRE-treated region (▲) and the untreated region (△). (B) Immature epithelial cells appear in the basal layer of the mucosa (arrows heads), and regeneration and repair of the mucosa are vigorous. (C) Masson trichrome staining shows the plica consisting of the mucosa and submucosa still retains its primitive framework structure (dashed

rectangle). The presence of blood vessels (BV), the Meissner plexus (MP), and immature muscle cells (iMC) can also be seen.

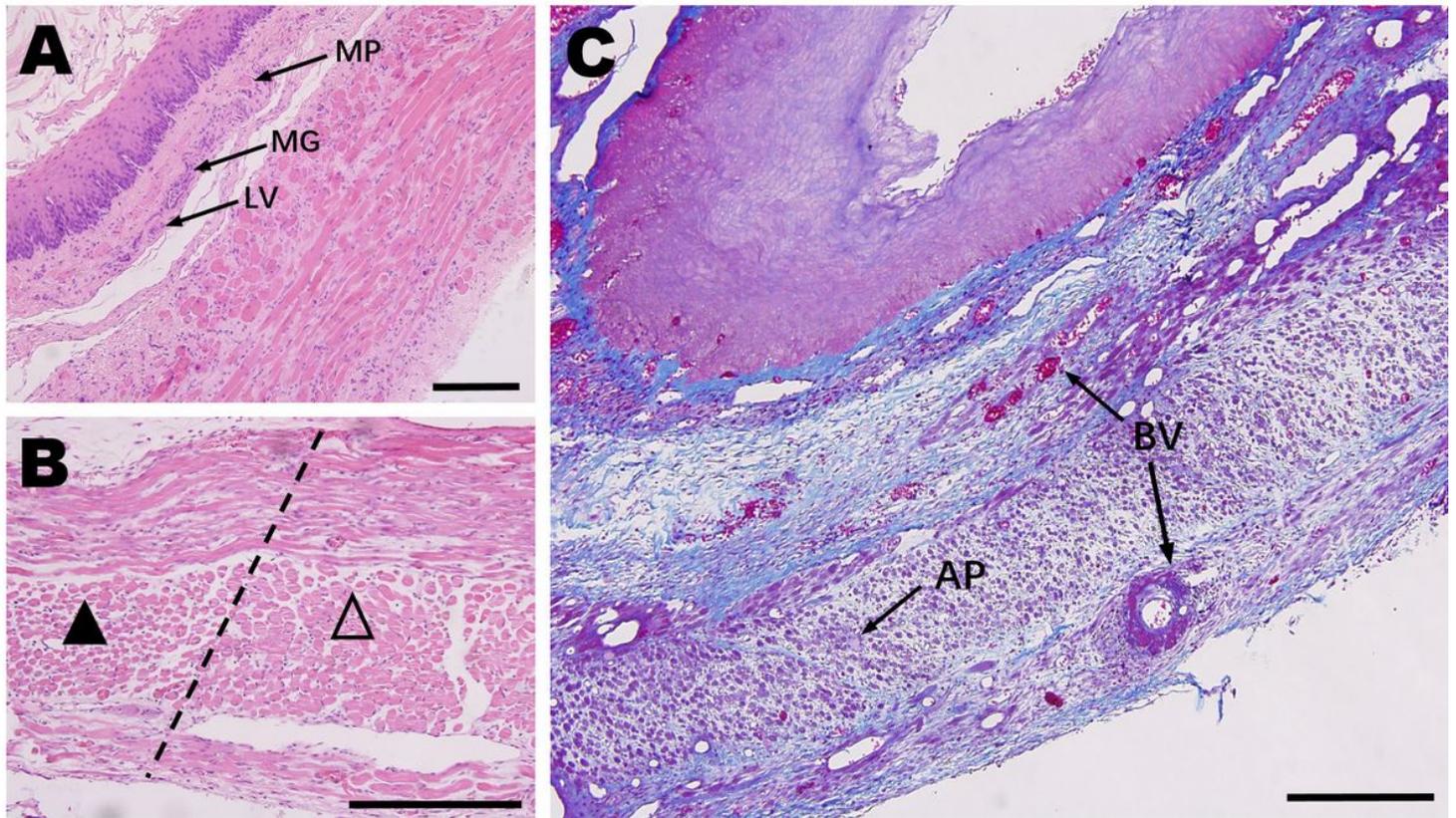


Figure 4

One week after esophagus ablation. (A) Distinct layers of tissue includes a mucosal layer with non-keratinized squamous epithelium, a submucosal layer containing mucus glands (MG), blood vessels, lymphatic vessels (LV) and the Meissner plexus (MP), a muscular layer containing regular muscle fibers and rich Auerbach plexus, and a well-structured adventitia. (B) In muscular layer, the interface between the NTIRE-treated region (\blacktriangle) and the untreated region (\triangle). (C) Cross section of the esophagus stained with Masson trichrome. Signs of regeneration and repair appear: increased number of blood vessels (BV) in the submucosa, dilation of the vascular lumen, and abundant blood supply, a well-ordered muscle fibers mixing with numerous Auerbach plexus (AP) and blood vessels.

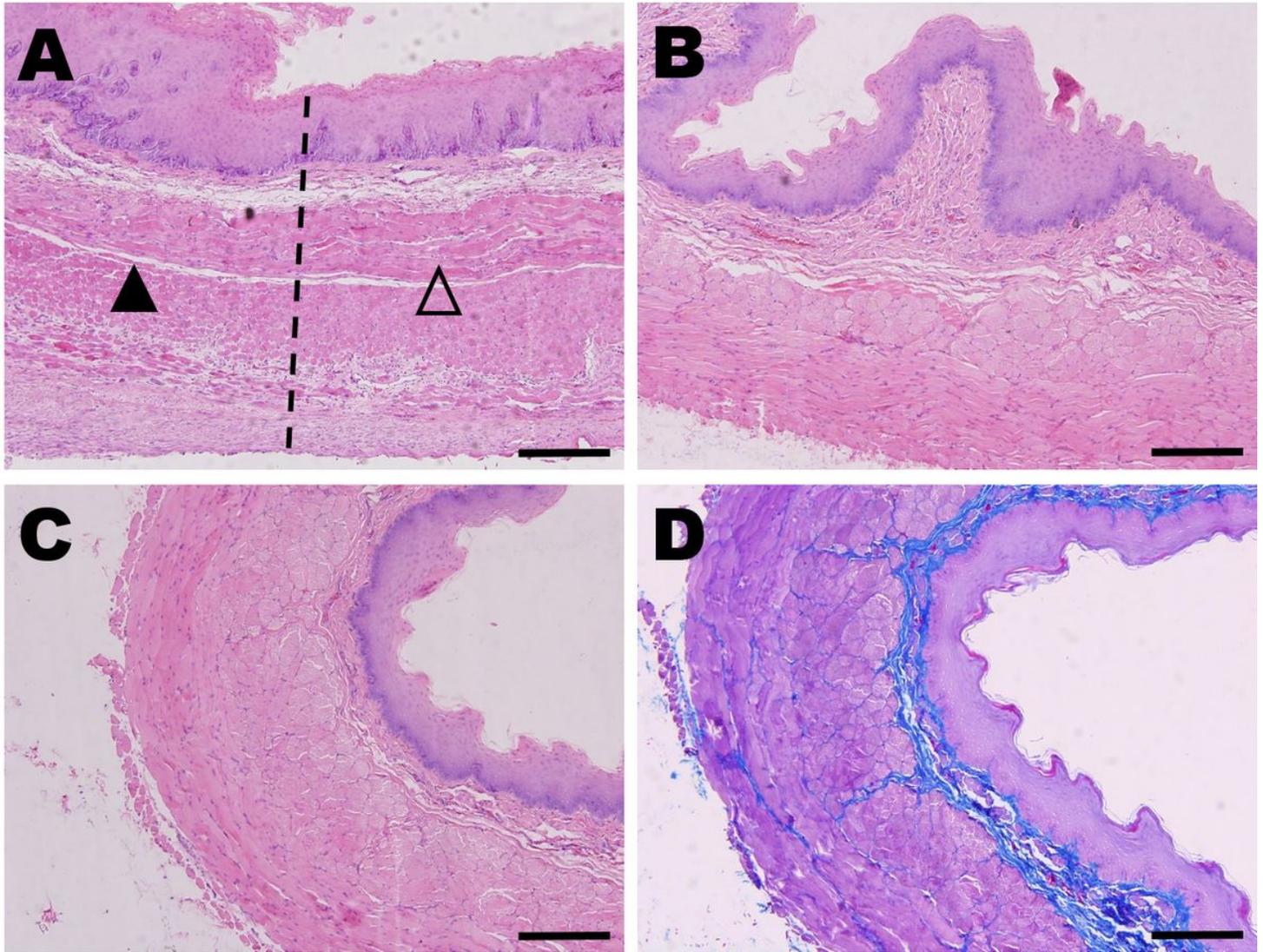


Figure 5

Mid-term observation after NTIRE ablation (A) Two weeks post NTIRE. The interface between the treated region (▲) and the untreated region (△). The surface of the epithelium begins to keratinize, the epithelial basal cells still actively proliferate, the submucosal vasodilatation is reduced, the muscle layer is clear, and the sarcoplasm of regenerated muscle cells increases. (B) Four weeks post NTIRE. A continuous improvement in repair and regeneration. (C) Sixteen weeks post NTIRE. Microscopic morphology is close to that of the normal control group. (D) Masson trichrome staining. A clear, continuous, and (blue stain). Blue staining shows a clear, continuous and intact extracellular framework.