Pathological changes of larynx in inhalational thermal injury causing laryngeal stenosis based on a canine model

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Research

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Abstract

Objective

This study aimed to explore the endoscopic and histologic feature of the larynx in inhalational thermal injury and its correlation with delayed laryngeal stenosis.

Methods

A total of 18 healthy, male adult Beagle dogs were randomly divided into six groups: group C (control group), and groups 1–5. All experimental dogs inhaled 100℃ saturated steam for five seconds to induce laryngeal injury. Endoscopic and histologic examinations were conducted immediately (group 1), and at two weeks (group 2), four weeks (group 3), six weeks (group 4) and eight weeks (group 5) after injury. Endoscopic morphologies of the larynx and histologic morphologies of epiglottis, glottis and cricoid cartilage were observed and analyzed.

Results

Granulation tissue in larynx was observed in group 2, and this shrank to 2/3 and 1/3 of the size in groups 3 and 4, respectively. Microscopically, declined severity of epithelial exfoliations in different regions and patial repair in the glottis were observed. In the epiglottis, cartilage cells appeared degenerative and necrotic and had partial repair. Muscle fibers and cartilage cells were severely injured and had no repair in the glottis, but in the cricoid cartilage, they were totally normal.

Conclusion

The granulation tissue formation, uncompletely repaired tissues in the larynx found after inhalational thermal injury may indicate the process of laryngeal stenosis. The cricoid cartilage that appeared slight pathology in the present study may due to the thermal absorption and protective effect of the upper parts.

1. Introduction

Inhalational injury accounts for up to 90% of all burn-related mortality, although this presents in up to one-third of all burn injuries [1]. Sufferers who survive almost immediate death in burn accidents can develop sequelae in the upper airway and lungs. Complication in the lungs caused by inhalational injury has drawn increasing attention. However, studies on the upper airway, including the larynx, remains limited [2].

Laryngeal stenosis refers to the narrowing of the larynx, which often results from the obstruction of foreign bodies or endogenous substances, such as mucus and neoplasm, or from the constriction of

laryngeal structures. This can be divided into supraglottic, glottic and subglottic stenosis, according to its location [3], and this may cause symptoms of dysphonia, and difficulties in ventilation or swallowing. At present, intubation is widely known as the most common observed etiology for acquired laryngeal stenosis, Henning A. *et al*. [4] found in their study that three inhalational injury patients without intubation history developed symptoms of airway obstruction. In addition, Zhang Guoan *et al*. [5] classified early inhalational injury into three clinical types (congestive, oedematous and obstructive) mainly according to the fibrolaryngoscopic demonstrations of patients without intubation history in their study, proving the presentation of early laryngeal stenosis. In another study conducted by Zhang [6], which aimed to examine the pathological changes of these three types using a canine model, three dogs were observed to die of acute laryngeal stenosis caused by oedema. Gregory R *et al*. [7] also reported a rabbit model, in which vocal fold obstruction was directly observed from endoscopic imaging at 24 hours after inhalational injury. However, to date, all animal model studies on inhalational injury have focused on acute laryngeal stenosis.

In fact, in order to study the interventions to laryngeal stenosis, a series of animal models have been previously established. In 1998, Msrk S. *et al*. [8] reported a New Zealand rabbit model, which developed posterior glottic stenosis by injury of CO_2 laser ablating laryngeal tissues in different depths. In addition, in the study conducted by Cláudia Schweiger [9], with the use of the New Zealand rabbit model, it was observed that the subglottic stenosis was caused by intubation, nylon brush and Bugbee cautery, in which most animals survived at two weeks after injury for further observation of laryngotracheal stenosis. Other methods, such as silver nitrate or hydrochloric acid, have also been reported to be used for inducing laryngeal stenosis [10,11]. Nevertheless, there have been no animal models, in which inhalational thermal injury was used to induce laryngeal stenosis that could be observed in the long term.

In the present study, a canine model was established using 100℃ saturated steam to induce laryngeal injury, and the endoscopic and histologic demonstrations of the larynx were observed for a relatively long survival time, in order to explore the forming process of delayed laryngeal stenosis.

2. Materials And Methods

2.1 Materials

A total of 18 healthy, male adult Beagle dogs, weighing approximately 10.0 kg, were provided by Beijing Vital River Laboratory Animal Technology Co. Ltd. Dogs with hoarseness or neck trauma were excluded. The instruments included a custom-made steam generator, an embedding machine (Tissue-Tek®TEC 5, Sakura Medical Group, Japan), an automatic staining machine (DRS-2000, Sakura Medical Group, Japan), an ultrathin microtome (PowerTome-PC, RMC, USA), an optical microscope (CX 31, Olympus, China) and a video endoscope (4ucam, USA). The custom-made steam generator included a ceramic container, a heating rod, an inlet air pipe, a T shape tube, and a wire [12]. The endoscope included a flexible endoscope (50 cm in length, 9 mm in diameter, and 10 times magnification) and an image indicator.

2.2 Animal grouping and preparation

The present study was reviewed and approved by the Animal Ethics Committee of Beijing Jishuitan Hospital. All experiments were performed in accordance with institutional animal care research protocols. These 18 dogs were randomly divided into six groups, with three dogs in each group: group C (control group) and groups 1–5 (experimental groups). Each dog in the experimental group was anaesthetized with 15 ml of pentobarbital sodium (20 g/L) *via* the vein, and 1 ml of atropin was subcutaneously injected. Then, the experimental animals were immobilized in the supine position. Group C was only used as a control for observational intention.

2.3 Experimental procedures

The temperature in the laboratory was maintained at 26.0 ± 2.0℃ with a humidity of 40.0 ± 2.0%. Tongue of each experimental dog was extended out to avoid glossoptosis. The custom-made steam generator was used to heat the water and generate the 100℃ saturated steam, which was allowed to steadily expel from an inlet air pipe. This was placed deep in the oral cavity, with a distance of 4 cm from the epiglottis, abruptly at the end of the dog's exhalation. Then, the saturated steam was inhaled by the dog through autonomous respiration, thereby inducing injury in the larynx. The inhalation of saturated steam would persist for five seconds. Immediately after the injury, an endoscope was used to observe the laryngeal morphology of the dogs in group 1 and images were taken. Then, dogs in group 1 were sacrificed using 5 ml of KCl (100 g/L) *via* the vein, and the throats were harvested and maintained in 10% formaldehyde solution before the tissues of the epiglottis, glottis and cricoid cartilage were obtained and made into whole layer tissue slices (5–7 μm in thickness). Afterwards, the tissue slices were stained with hematoxylin and eosin (H&E), and observed under a microscope (× 100). The dogs in groups 2–5 were treated in same manner as those in group 1 at 2, 4, 6 and 8 weeks after inhalational thermal injury. Two rubber plugs were inserted to the dog's nostrils to compel it to breathe through the mouth. All experimental dogs immediately underwent tracheotomy after injury to keep the airway fluent, and the tracheal tube was removed at week one after injury. Penicillin (0.4 million U/d) was used *via* intravenous drip for one week after injury.

2.4 Observation index

Eating and drinking, body movement, and phonation were observed in dogs in all groups until sacrifice. The morphological features of the larynx and the pathological changes of tissues from the epiglottis, glottis and cricoid cartilage were examined under a microscope (H&E, × 100).

3. Results

3.1 Vital signs

All dogs survived the whole experiment. Dogs in groups 1–5 could not stand, could only perform certain body movements in the supine positon, and significantly decreased eating and drinking, when compared

with dogs in the control group, after recovering from the anesthesia. The body movement and diet of dogs in groups 2–5 gradually recovered, and returned to normal levels at week two. As to the phonation, dogs in groups 1–5 could not phonate after initially recovering from anesthesia, while dogs in groups 2–5 produced sounds with less frequency and lower strength, when compared to dogs in the control group, at week one, after decannulation. In addition, a slight respiratory stridor could be heard at this time. In week two, the frequency and strength of the sounds produced by dogs in groups 2–5 were remained weaker than that of dogs in the control group, while these remained unchanged until all dogs in the experiment were sacrificed.

3.2 Endoscopic observations

The morphological features of the larynx could be observed using an endoscope. Dogs in group C presented with intact, smooth and pink mucosa, while dogs in group 1 presented with severe damage in the mucosa, which demonstrated as pale, swollen and obviously exudated, with ulceration, necrosis and dilated microvessels in part of the areas. Signs of alleviation could be observed in group 2, in which mucosa paleness, swelling and exudation decreased, but continued to have dilated microvessels in some areas. In group 3, further alleviation of appearance in the mucosa could be observed, which presented with obviously decreased paleness and swelling, and the dilated microvessels vanished. In groups 4 and 5, the appearance of the laryngeal mucosa had no changes, when compared to group 3.

In addition, a neoformation in the glottis, which caused obstruction, could be observed in group 2. This had a granuloma-like, irregular, light-pink color and moist appearance, with no clear surrounding boundary. In group 3, the neoformation was observed to shrink to 2/3 of the size, and had an oval shape and less moist appearance. In group 4, the neoformation continuously shrank to 1/3 of the size, while in group 5, this remained unchanged (Table 1, Fig. 1).

Table 1
Laryngeal morphologies under an endoscope after inhalational thermal injury

Morphologies of the mucosa	Groups					
	Group C	Group 1	Group 2	Group 3	Group 4	Group 5
Paleness in color	Pink	+++[a]	++	+	+	+
Oedema	-	+++	++	+	+	+
Exudation	-	+++	++	+	+	+
Ulceration and necrosis	-	+++	++	+	+	+
Dilated microvessels	-	+++	++	-	-	-
Neoformation in the glottis	-	-	Obvious, granuloma-like, irregular, light pink, moist, no boundary with surroundings	2/3 of the size in group 3, oval-like, less moist	1/3 of the size in group 3	1/3 of the size in group 3

[a] The number of "+" indicates the severity of the paleness in color, edema, exudation, ulcer and necrosis in epithelium and numbers of microvessels; "+++" means obvious damage, "++" lighter damage, and "-" normal.

3.3 Histological observations

3.3.1 Epiglottis

Compared to the control group, which presented with a normal epiglottis, the epiglottis epithelium immediately observed after injury in group 1 was observed to be exfoliated in large areas, with swollen and necrotic residual epithelial cells. Furthermore, the lamina propria and submucosa were observed to be congestive and swollen, had atrophic and necrotic glands, and presented with a large amount of micro-arterial thrombosis in the submucosa, while cartilage cells were degenerative necrotic with stained and ruptured nuclei. Group 2 had repair demonstrations, in which the epithelium completely regenerated, glands in the submucosa partly regenerated, and the lamina propria and submucosa presented with slight oedema. Meanwhile, old necrotic and newly regenerated cartilage cells could be observed. In group 3, slight oedema of the lamina propria and submucosa was observed, and the glands further regenerated. However, in groups 4 and 5, the pathological morphologies exhibited no changes, when compared to group 3 (Table 2, Fig. 2).

Table 2
Histologic demonstrations under a microscope (H&E, × 100)

Epiglottis	**Groups**					
	Group C	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Group 5**
Epithelial exfoliation	-	++++[a]	-	-	-	-
Epithelial cell swelling, necrosis	-	++++[b]	-	-	-	-
Lamina propria, congestion, edema	-	++++	++	+	+	+
Submucosal congestion, edema	-	++++	++	+	+	+
Gland atrophy, necrosis	-	++++	++	+	+	+
Cartilage cell degeneration, necrosis	-	++++, nuclei stained, ruptured	++, New born cartilage cells	++, New born cartilage cells	++, New born cartilage cells	++, New born cartilage cells
Glottis	Groups					
	Group C	Group 1	Group 2	Group 3	Group 4	Group 5
Epithelial exfoliation	-	+++	++	+	+	+
Epithelial cell swelling, necrosis	-	+++	++	+	+	+
Lamina propria, congestion, oedema	-	++++	++	-	-	-
submucosal congestion, oedema	-	++++	++	-	-	-
Gland atrophy, necrosis	-	++++	++	-	-	-
Muscle fiber atrophy, breakage, disarrangement	-	++++	++++	++++	++++	++++
cartilage cells	-	Vesicular cartilage cells	No change	No change	No change	No change
Cricoid cartilage	Groups					

Epiglottis	Groups					
	Group C	Group 1	Group 2	Group 3	Group 4	Group 5
	Group C	Group 1	Group 2	Group 3	Group 4	Group 5
Epithelial exfoliation	-	++	-	-	-	-
Epithelial cell swelling, necrosis	-	++	-	-	-	-
Lamina propria, congestion, oedema	-	+	-	-	-	-
submucosal congestion, oedema	-	+	-	-*	-	-
Gland damages	-	-	-	-	-	-
Cartilage cell damages	-	-	-	-	-	-
Muscle fiber damages	-	-	-	-	-	-

[a] The number of “+” indicates the areas of epithelial exfoliation, while “++++” means epithelial exfoliated in large areas, “+++” means epithelial exfoliated in some areas, “++”epithelial exfoliated in local areas, “-” means intact or totally regenerated epithelium.

[b] The number of “+” indicates the severity of swelling, necrosis in epithelial cells, congestion, oedema in the lamina propria, atrophy, necrosis in the glands and damages in cartilage cells, and muscle fibers, “++++” means severe damage, “-”means normal or regenerated to normal.

* New born cartilage cells observed in the submucosa of the glottis in one of the three dogs in group 3

3.3.2 Glottis

Compared to the control group, which presented with a normal glottis, the epithelium of the glottis observed immediately after injury in group 1 partly exfoliated, and some of the residual epithelial cells were swollen and necrotic. In addition, obvious congestion and oedema were observed in the lamina propria and submucosa, while glands in submucosa were atrophic and necrotic. Furthermore, the muscle fibers presented with atrophy, breakage and disarrangement, while cartilage cells that appeared as vesicular- like were found. In group 2, inflammatory reactions and repair demonstrations could be observed in the glottis, which appeared as a formation of granulation tissues that comprised of numerous fibroblasts, new born capillaries and infiltrated inflammatory cells, and the regeneration of part of the epithelium and glands. In addition, the alleviation of congestion and swelling in the lamina propria and submucosa were observed. However, the muscle fibers and cartilage cells did not present any repair demonstrations. In group 3, the lamina propria, submucosa and glands in the submucosa appeared to be normal, and no further repair or repair was observed in the epithelium, muscle fibers and cartilage cells,

when compared to group 2. New born cartilage cells were found in the submucosa of the glottis in one of three dogs in group 3. In groups 4 and 5, no pathological changes were found, when compared to group 3 (Table 2, Fig. 3).

3.3.3 Cricoid cartilage

Compared to the control group, which presented with a normal cricoid cartilage, the epithelium that covered the cricoid cartilage in group 1 exfoliated in local size, and some of the residual epithelial cells were swollen, while a small part was necrotic. Furthermore, slight congestion and oedema could be observed in the lamina propria and submucosa. However, the glands, cartilage cells and muscle fibers that covered the cartilage appeared to be same with that in the control group. In group 2, the cricoid cartilage epithelium was observed to be completely regenerated with normal epithelial cells, and the congestion and oedema in the lamina propria and submucosa vanished. Tissues from the cricoid cartilage were found to be normal in groups 3, 4 and 5 (Table 2, Fig. 4).

3.3.4 Granulation tissue

Neoformation formed in the glottis area, as observed under a microscope. Numerous fibroblasts, new born capillaries and inflammatory cell infiltrations could be observed, which was in accordance with the endoscopic manifestation of granulation tissues.

4. Discussion

4.1 Animal models surviving long after inhalational thermal injury

In order to explore the effects of heat in inhalational injury on the larynx, several studies have been previously conducted [6,12,13,14], and hot dry air at temperatures of 80, 160 and 320℃ were produced and inhaled by the model animals to burn the upper airway mucosa and structures beneath. Then, the endoscopic and histologic morphologies of the burned larynx were observed. However, during the experimental time of 24 or 36 hours in these studies, neither the healing response of the laryngeal tissues, nor the forming process of the delayed laryngeal stenosis could be demonstrated. Thus, an animal model of inhalational thermal injury must be developed, in which the experimental animals can survive longer. First, the death of animals in previous studies may result from exposing to high temperature air for long durations (10 minutes, 20 minutes, etc.), thus a shorter exposed time may be helpful. Meanwhile, it must be ensured that the animal's larynx is severely injured, in order to have a positive observation. According to Phillip B [15], saturated steam has 4,000 times the heat-carrying capacity of hot air or gas. Thus, it can be assumed that saturated steam can induce severe injury of the larynx in a shorter time. In fact, in a pilot animal experiment where dogs inhaled saturated steam at a temperature of 100℃ for five seconds, severe damages of the larynx were observed as exfoliated epithelium or necrotic tissues. In the present study, all experimental animals survive until sacrifice for observation, which was much longer than that in previous experiments. Furthermore, the signs of forming laryngeal stenosis could be observed under an

endoscope and microscope, testifying that the present canine model may be suitable for studying the delayed pathological process of laryngeal stenosis formation after inhalational thermal injury.

4.2 Correlations with delayed laryngeal stenosis

4.2.1 Granulation tissue in glottis observed under an endoscope

Steven M. *et al*. [16] reported that intubation is the most commonly observed etiology of acquired laryngeal stenosis at present, and for inhalational thermal injury, Allison Reid *et al*. [17] suggested that it is difficult to distinguish laryngeal stenosis that arise from intubation or inhalational injury. The study conducted by Bruce Benjamin *et al*. [18] revealed that granuloma on the vocal process of the posterior glottis, which proceeds from granulation tissues, is one of the main sequelae of intubation. In the present study, it was observed that the neoformation was located in the posterior glottis in group 2, and that its histologic demonstration was compatible with the manifestation of granulation tissues that comprised of fibroblasts, new born capillaries and inflammatory cells. This finding indicates that inhalational thermal injury could separately induce the formation of laryngeal granulation tissues from intubation. However, few cases of inhalational thermal injury have been found with the existence of granulation tissues in the glottis in ordinary clinical work, and reports on these are rarely found. In order to explain this, three reasons were considered: (1) In most cases of inhalational injury, the epithelium of laryngeal structures completely regenerated, while the epithelium would only heal in the manner of forming granulation tissues when the injury was severe. On the other hand, when the injury was excessively severe, the symptoms of laryngeal obstruction appeared, and immediate intubation would conducted so that it would be hard to detect the granulation tissues in the posterior glottis due to the inhalational injury. For sufferers who rapidly died during burn accidents of airway obstruction or gas exchange disability, lesions such as granulation tissues could also not be detected. (2) In the present study, the granulation tissues were first detected at week two after the injury, while merely oedema, exudation, ulceration and necrosis could be observed on the surface of the mucosa when immediately examined after injury. However, recurrent laryngoscopy examinations have been rarely performed after the acute stage of inhalational injury, unless symptoms of voice change or difficult breathing emerges. Thus, in some cases without obvious symptoms, the formation of granulation tissues would escape the examination. (3) Sometimes, the formation of granulation tissues in the posterior glottis would be contributed to intubation or reflux diseases in clinic. Rintaro *et at*. [19] found through an experiment that more rats in the gastroesophageal reflux disease (GERD) group developed granuloma in the vocal process, when compared to the control group, while Petros D *et al*. [20] suggested that antireflux treatment was the main treatment strategy for vocal process granuloma. In the present study, it was found that inhalational thermal injury is one factor that could induce the formation of granulation tissues in the larynx, which might have been previously underestimated. Hence, the molecular mechanisms behind this might need further studies.

It was also found in the present study that the granulation tissue shrank at week four and six after injury in groups 3 and 4 to a size of 2/3 and 1/3, respectively, and this was considered a consequence of the

decrease in oedema and the possible granulation tissue organization. Nevertheless, the disappearance or size change of the granulation tissues in group 5 were not observed. Thus, it was inferred that the granulation tissues in the posterior glottis would not gradually resolve. Conversely, granuloma would occur in this place, or in some cases, this would undergo further organization, fibrosis and scarring. Phillip B *et al*. [15] once reported 11 cases of laryngeal stenosis caused by inhalational thermal injury, and most of the cases had posterior glottic webs developing from the granulation tissue. This was somehow in accordance with what was found in the present experiment. In addition, for the granulation tissue or granuloma obstructive effect, interarytenoid adhesion from the granulation tissue proceeded when this formed, and the subsequent scarring contracture, different extents of laryngeal stenosis would also occur [18].

The behavior of experimental animals in the present study was in accordance with endoscopic morphologies, since the diet and body movements were observed to return to normal at week two after injury, while the voice change persisted up to the end of the experiment.

4.2.2 Histologic demonstrations related to laryngeal stenosis

For the histologic examinations, the glottis epithelium exhibited obvious repair in groups 3 and 4. However, the epithelium did not appear to completely regenerate, even at the end of the present experiment. Instead, the appearance of numerous fibroblasts, new born capillaries, and inflammatory cells indicated the formation of granulation tissues. This healing process involved granulation tissue forming, as observed under a microscope, and this was consistent with what was observed under an endoscope. Another impressive histologic examination in the glottis was the severe damage in muscle fibers and cartilage cells, and the high severitymight be attributable to the special structures - the thin mucosa and poor blood supply in vocal process of the posterior glottis [21], while the protective effects might be attributable to the narrow space and reflex closure that holds the saturated steam for a longer period of time in the glottis. More impressively, muscle fibers and cartilage cells were not observed to be repaired during the experiment at eight weeks, and the reason might be the complex structures and reflex closure of the glottis. However, since the regeneration ability of muscle fiber and cartilage cells was weaker than that of the epithelium, in such a severe condition, fibrosis and scarring in glottis might occur with lack of repair, which might result in abnormity of vocal cords mobility, or collapse of cartilage or muscle framework, thereby leading to glottic stenosis.

Henning A *et al*. [4] reported more subglottic than glottic stenosis cases in their research. Furthermore, they found that subglottic stenosis was more common than supraglottic or glottic stenosis in children [22]. Nevertheless, the histologic demonstrations of cricoid cartilage damage in the present study was much slighter, with local epithelium exfoliation, few epithelial cell necrosis, slight oedema, congestion in the lamina propria and submucosa, when observed immediately after injury in group 1, while in group 2, all these pathological changes returned to normal. In addition, cartilage cells and muscle fibers appeared totally normal, even when observed immediately after injury. This may suggest that the model animals in

the present experiment would not develop subglottic stenosis in the cricoid cartilage where this usually observed, because the totally normal tissues appeared at week two after the injury. Previously, in a rabbit model established to study inhalational injury in the larynx [7], the researcher found that elevated experimental temperature was followed by increased severity of injury or depth in injured tissues. In the present study, 100℃ saturated steam was used to induce injury in the larynx, and it could be inferred that when given a larger quantity of heat by extending the time of inducing injury or using other methods to induce injury, the cricoid cartilage should be observed to have severer damage, which is possibly deep to the cartilage or muscle lamina. Thus, stenosis in this portion might occur due to the framework collapse. In some cases, the granulation tissue would form from the injured subglottic mucosa, and led to stenosis from the obstruction or scar contracture. On the other hand, it was considered that burn accidents that fail to conduct the reflex closure of the glottis might lead to severe injury in the subglottis, or possibly in the lower airway.

The epiglottis of animals in the present study appeared to have severe damages in the mucosa, submucosa and cartilage cells, while complete epithelium repair and some new born cartilage cells could be observed in group 2. It was considered that the reason for such severe damages was the first and unceasing exposure to saturated steam [13]. As this was injured deeply into cartilage, the healing process might result in epiglottis abnormality, which is one manifestation of laryngeal stenosis.

4.3 Pathological and repair demonstrations in different regions

The histologic demonstrations in different regions of the larynx under a microscope in the present study were in accordance with previous studies, and for the first time, the healing process of laryngeal tissues after inhalational injury was observed. The severity of epithelium and epithelial cell injuries gradually decreased in the epiglottis, glottis and cricoid cartilage, when the large, part and local size of exfoliated epithelium had lesser swelling, and the necrotic epithelial cells in these three regions declined. The epithelium in the epiglottis and cricoid cartilage completely regenerated in group 2, indicating the powerful regenerative ability of the epithelium, while the glottis epithelium was observed to heal in the manner of forming granulation tissues, and this was probably due to the special anatomic features in the glottis, such as the thin mucosa or poor blood supply in the region of the vocal process [21]. The lamina propria and submucosa that similarly appeared under the microscope in the present study also revealed the pathological change trends in three different regions, while the pathological changes of congestion and oedema were obvious in the epiglottis and glottis, and slightly obvious in the cricoid cartilage. Glands in the submucosa also revealed obvious atrophy, and necrosis in epiglottis and glottis, while this appeared to be normal in the cricoid cartilage. Pathological changes in the lamina propria and submucosa alleviated to normal in group 2 in the cricoid cartilage, and in group 3 in the glottis. However, in the epiglottis, total recovery of structures in the lamina propria and submucosa was not observed, which might have resulted from the excessively severe damage. For the muscle fibers and cartilage cells, the epiglottis presented with severe damages in cartilage cells, which appeared partly repair in group 2. Furthermore, the glottis presented with severe damages in muscle fibers and cartilage cells, with no signs

of repair, while the muscle fibers and cartilage cells were normal in the cricoid cartilage. The decreased severity of damage in the laryngeal tissues presented above could be explained by the fact that temperatures in lower parts of the larynx were lower than that in the upper parts, which have been proven in the previous study conducted by the investigators [13], and the downward decrease in temperature in the larynx in inhalational injury was considered the result of blood circulation and airway tissue absorption in the upper airway [12]. Meanwhile, the abnormal healing of the epithelium, and no sign of repair of muscle fibers and cartilage cells in the glottis might be explained by the complex anatomic structures at this region.

4.4 Chondrometaplasia of the glottis in one dog in group 3

In one of the three dogs examined at four weeks after inhalational injury in group 3, cartilage cells were detected in the submucosa of the glottis, and this is called chondrometaplasia, which refers to the transformation of cartilage cells from connective tissues. There are only few reports on chondrometaplasia of the larynx at present. Recently, Abdulmohsen Kamel AlBader [23] reported a case of chondrometaplasia of the vocal cords in a 62-year old man without trauma history. Early in 1981, Lyer and Rajagopalan [24] reported a case of chondrometaplasia of the ventricle in a 66-year old patient who had a history of occupational vocal abuse. In 1985, Ferlito A *et al*. [25] reported a case of chondrometaplasia of the ventricular bands in a patient who underwent an excision of epiglottic cyst in the right glossoepiglottic vallecula two years ago. A Orlandi *et al*. [26] also reported a chondrometaplasia case of ventricular bands with a history of intubation after a traffic accident. All sufferers in the above reports, who were examined with chondrometaplasia of the glottis, had a history of trauma, had surgery in the glottis, or were in old age, while inhalational injury that induced chondrometaplasia of the larynx has not been previously reported. In addition, all previous reports involved the symptoms of dysphonia or dyspnea. However, the determination of whether the metaplastic cartilage cells in the present study would proliferate to cause laryngeal stenosis need further observation.

4.5 Experimental limitations

First, dogs were used as the experimental animals in present study to achieve the endoscopic and histologic observational results, since could be misleading or biased when applied in people. Second, the noses of these dogs were plugged with rubber, resulting in that the protective function of structures in the nose were left out of consideration during the burn accidents, thus bias could exist when analyzing the formation process of laryngeal stenosis. Lastly, it was reported that there could be a long time period, which may vary from months to years, before the symptoms of laryngotracheal stenosis onset occur [4,15,27]. In the present study, it could be inferred that there was a long duration of development of laryngeal stenosis after inhalational injury based on what was observed in the experimental time of eight weeks. Thus, in future studies, attention should be given to the mechanisms or pathological progression of laryngeal stenosis that occur long after the inhalational injury.

5. Conclusion

In contrast to the previous studies of the investigators, which focused on acute laryngeal obstruction after inhalational thermal injury, the present study explored the morphological features of the larynx that led to the delayed laryngeal stenosis. Granulation tissue formation, and the unrepaired muscle fibers and cartilage cells in the glottis may cause subsequent laryngeal stenosis, as well as partly repaired cartilage cells in the epiglottis. The relatively severer damage and lower repair in the glottis, compared to the epiglottis, may have resulted from the complex anatomic structures at this region. This present severely injured and long survival canine model can be used for further studies on the progression, prognosis and treatment of delayed laryngeal stenosis after inhalational thermal injury.

Abbreviations

H&E
hematoxylin and eosin.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Informed consent was required and obtained from the participants.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author’s contributions

WC and ZGA designed the trial and analyzed the data. WC drafted the manuscript. WJB, LB and WC collected the data. All the authors had full access to all the data and take responsibility for the integrity of the data and the accuracy of the data analysis. All the authors read and approved the final manuscript.

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Figures

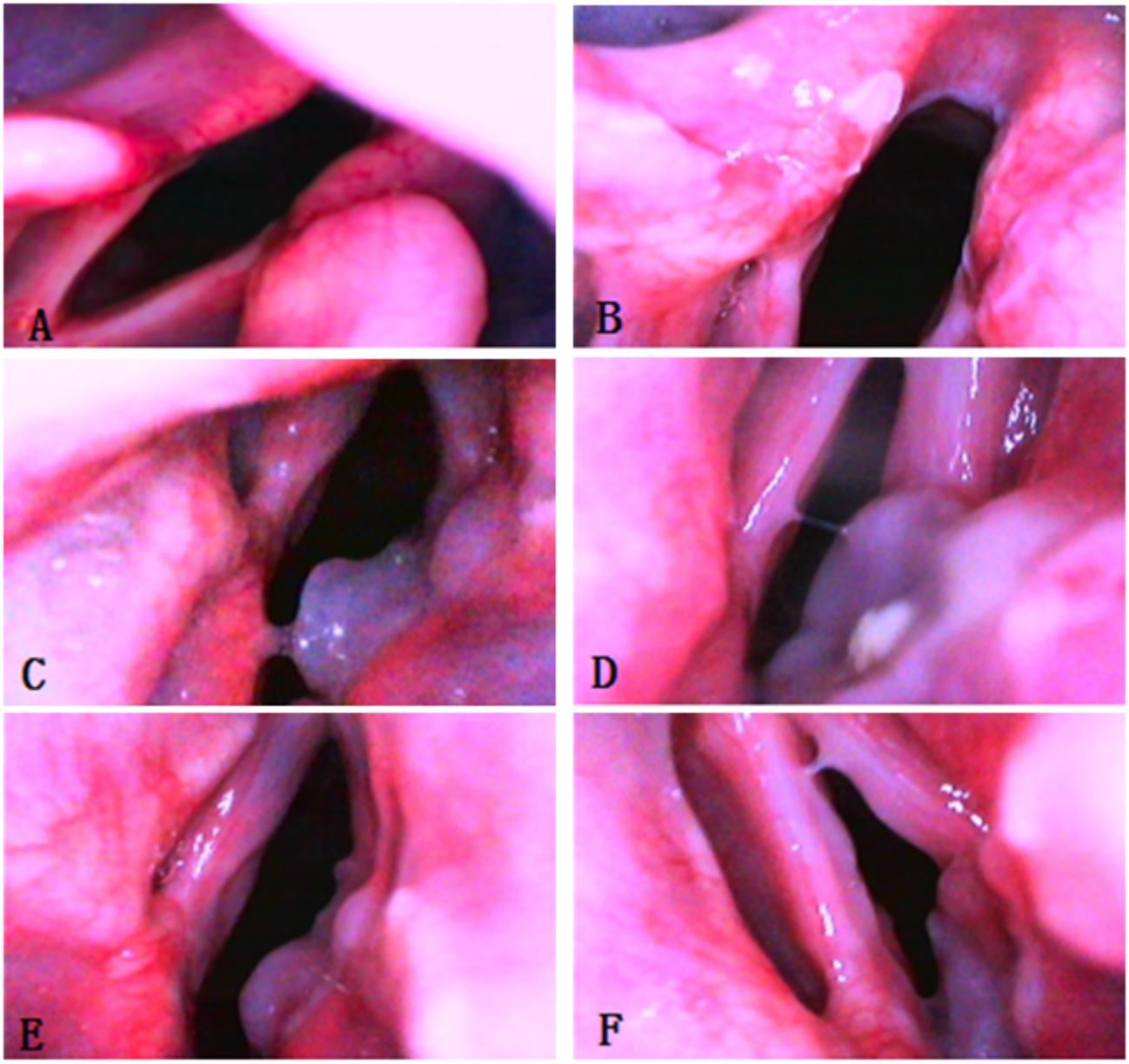

Figure 1

Endoscopic examination. (A) Group C (control group), normal laryngeal mucosa. (B) Group 1 (immediately after injury), pale, oedematous laryngeal mucosa, obvious exudation, ulceration and necrosis in some areas, dilated microvessels. (C) Group 2 (two weeks after injury), alleviation demonstrations, less paleness and edema of the laryngeal mucosa; irregular, light pink, moist neoformation in the glottis, with no clear boundery with the surroundings. (D) Group 3 (four week after injury), paleness and oedema of the laryngeal mucosa further alleviation, the neoformation shrank to 2/3 of the size in group 2. (E) Group 4 (six weeks after injury), no changes in the laryngeal mucosa in group 3, the neoformation shrank to 1/3 of the size in group 2. (F) Group 5 (eight weeks after surgery), no changes in the laryngeal mucosa and neoformation size in group 4.

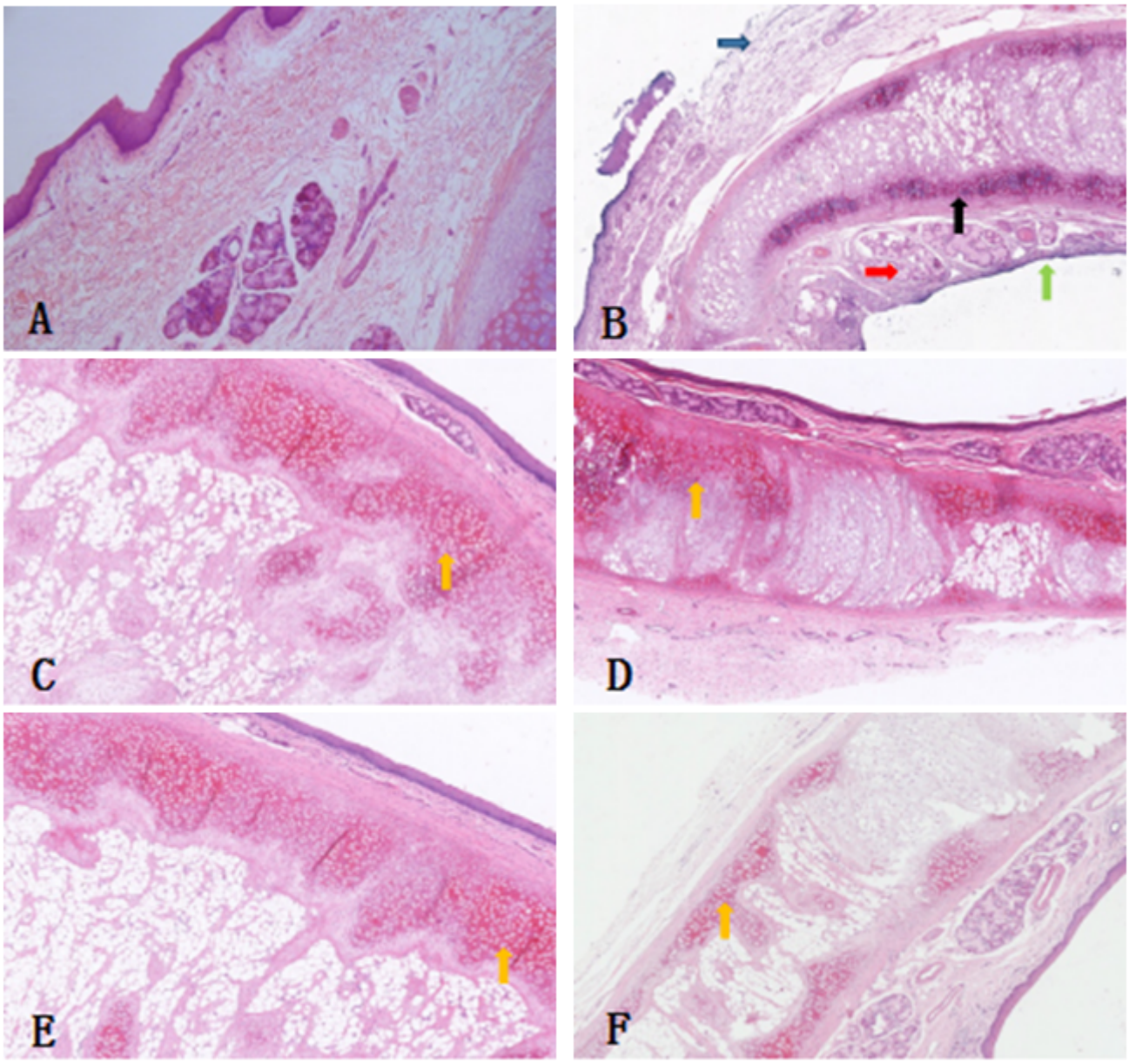

Figure 2

Histopathological examination of epiglottis (H&E, ×100). (A) Group C (control group), normal tissue. (B) Group 1 (immediately after injury), exfoliated epithelium in the large area, swollen and necrotic epithelial cells, congestion and oedema of the lamina propria and submucosa, atrophy necrosis glands and microvessel thrombosis in the submucosa, degeneration and necrosis of cartilage cells. (C) Group 2 (two week after injury), total regeneration of the epithelium, slight edema in the lamina propria and submucosa, the repair of less glands, old necrotic and new born cartilage cells. (D) Group 3 (four weeks after injury), further repair of the glands, normal epithelium, slight oedema in the lamina propria and submucosa, repair demonstrations in cartilage cells similar as those in group 2. (E) Group 4 (six weeks after injury), same demonstrations as in group 3. (F) Group 5 (eight weeks after injury), same demonstrations as in group 3. (blue arrow is for the exfoliated epithelium, red arrow is for the atrophy and

necrosis of glands, green arrow is for microvessel thrombosis, black arrow is for the deformation and necrosis of cartilage cells, and yellow arrow is for new born cartilage cells.)

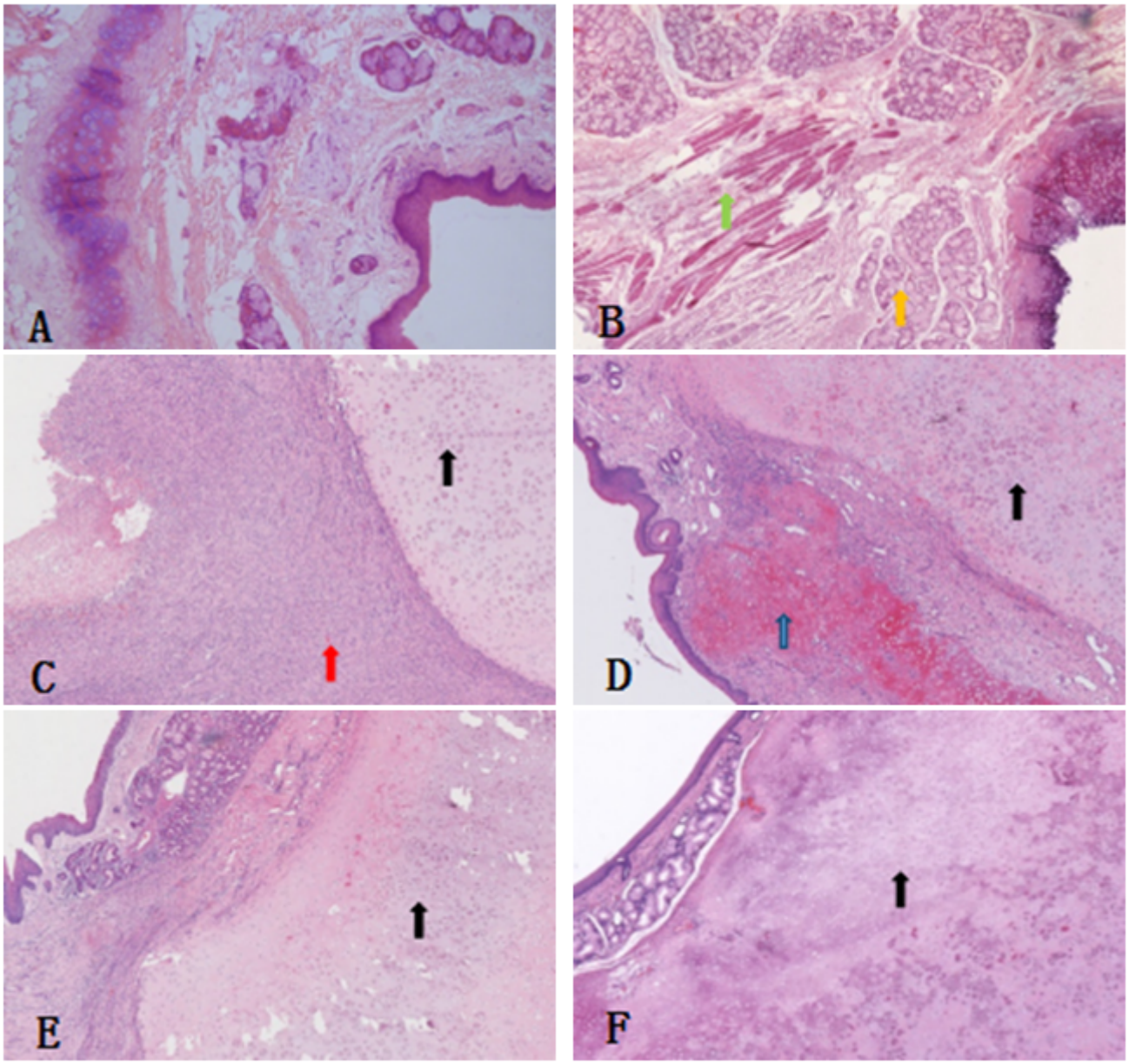

Figure 3

Histopathological examination of the glottis (H&E, ×100). (A) Group C (control group), normal tissue. (B) Group 1 (immediately after injury), partly exfoliated epithelium, swollen necrotic epithelial cells, congestion and oedema of the lamina propria and submucosa, atrophy and necrosis of the glands, atrophy, rupture and disarrangement of muscle fibers, vesicular cartilage cells. (C) Group 2 (two weeks after injury), inflammatory reactions as numerous fibroblasts, new born capillaries and infiltrated inflammatory cells in the epithelium; repair demonstrations as partly regenerated epithelium and glands, obvious less oedema and congestion in the lamina propria and submucosa, no repair demonstrations in muscle fibers and cartilage. (D) Group 3 (four weeks after injury), glands in the submucosa regenerated to normal, the oedema in the lamina propria and submucosa vanished, no further or no repair

demonstrations in the epithelium, muscle fibers and cartilage. One of the three dogs were observed to have new born cartilage cells. (E) Group 4 (six weeks after injury), same demonstrations as in group 3. (F) Group 5 (eight weeks after injury), same demonstrations as in group 3. (yellow arrow is for atrophy and necrosis of the glands, green arrow is for the rupture and disarrangement of muscle cells, red arrow is for fibroblasts, new born capillaries and infiltrated inflammatory cells, blue arrow is for new born cartilage cells, and black arrow is for necrotic cartilage cells.)

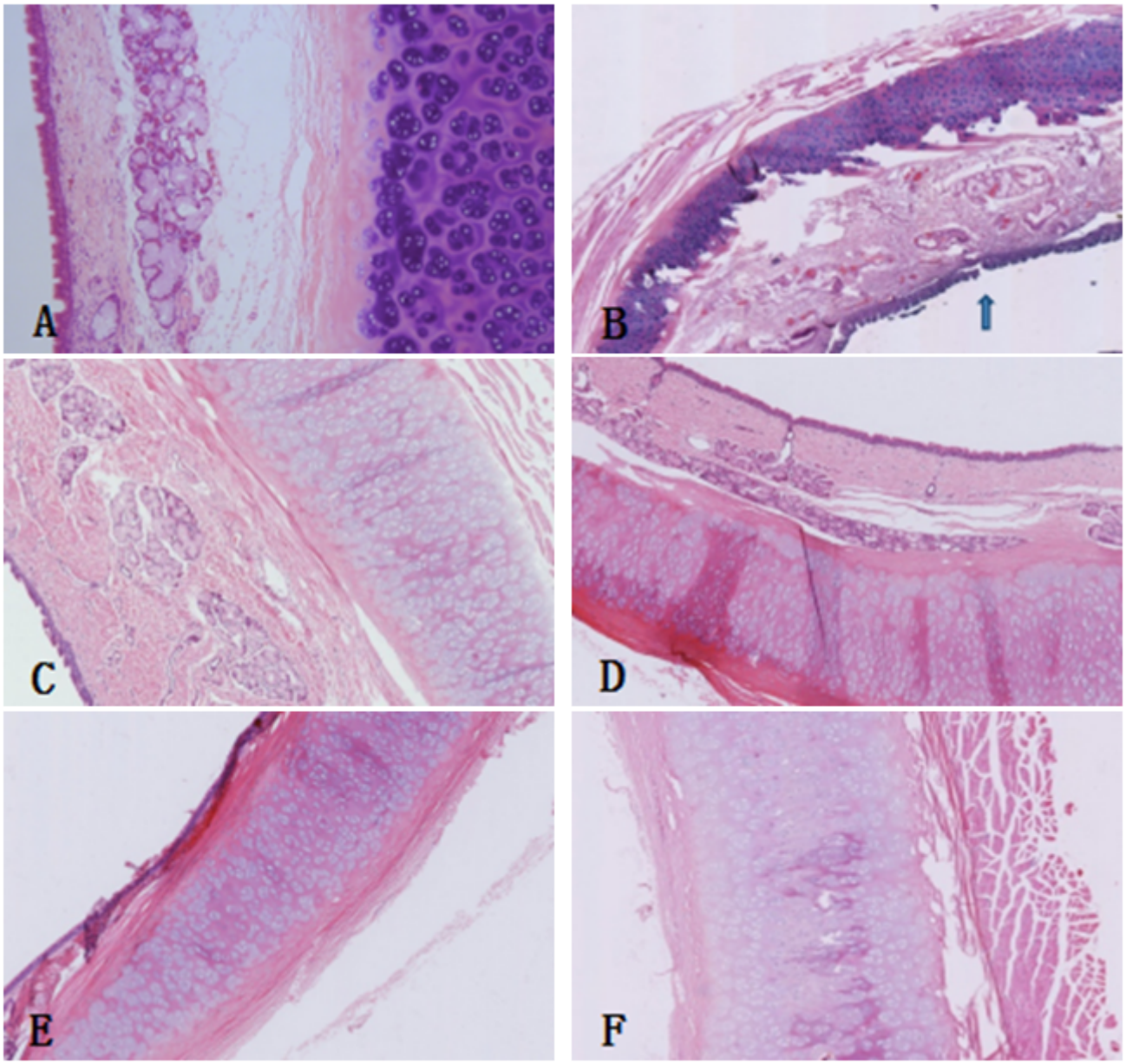

Figure 4

Histopathological examination of the cricoid cartilage. (A) Group C (control group), normal tissue. (B) Group 1 (immediately after injury), exfoliated epithelium in local size, swollen epithelial cells, slight congestion and oedema in the lamina propria and submucosa, normal glands, cartilage cells and muscle fibers. (C) Group 2 (two weeks after injury), normal tissues with totally regenerated epithelium and no congestion or oedema in the lamina propria or submucosa. (D) Group 3 (four weeks after injury), normal

tissues similar to the control group. (E) Group 4 (six weeks after injury), normal tissues similar to the control group. (F) Group 5 (eight weeks after injury), normal tissues similar to the control group. (blue arrow is for the exfoliated epithelium in local areas.)