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HEAD AND NECK

Early effects of irradiation on laryngeal mucosa in a gastroesophageal reflux model: an experimental study

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Abstract

Objective The aim of this study was to evaluate the early histopathological changes of gastroesophageal reflux and irradiation on laryngeal mucosa in rats.

Study design Animal study.

Setting Experimental animal laboratory, tertiary referral center.

Subject and method Twenty-four adult female Wistar Albino rats were grouped as: control ($n=6$), reflux and irradiation ($n=10$), and irradiation ($n=8$). Rats were operated to create a reflux model 30 days before irradiation. Ionizing radiation was administered in a single fraction of a 20 Gy to the larynx. Laryngeal tissue samples were taken at the 4th day of irradiation and all specimens underwent histopathological examination.

Results Edema and vascular dilation in lamina propria were higher in the reflux and irradiation, and irradiation groups than control group. Inflammation was higher in the reflux and irradiation group than the control group. Inflammation in squamous epithelium was higher in the reflux and irradiation and irradiation groups compared to the control group. Inflammation in the squamous epithelium of the irradiation group was higher than the reflux and irradiation group. In the respiratory tract epithelium, inflammation was higher in the reflux and irradiation group; additionally, a significant loss of cilia was present in the reflux and irradiation and irradiation groups while pseudostratification was higher in the reflux and irradiation group.

Conclusion Ionizing radiation-induced inflammation may increase on previously inflammated area due to gastroesophageal reflux. Therefore, it may be helpful to investigate and treat the reflux in laryngeal cancer patients that will receive ionizing radiation.

Keywords Gastroesophageal reflux · Inflammation · Larynx · Ionizing radiation · Animal study

Introduction

Gastroesophageal reflux (GER) is a spontaneous and effortless regurgitation of gastric contents into the esophagus. Laryngopharyngeal reflux (LPR) is an atypical form of GER in which gastric contents severely regurgitate up to the upper esophageal sphincter without vomiting.

Patients with GER may present to the clinic with nonspecific complaints; they are often given a diagnosis of pharyngitis, and treatment is initiated. Radiotherapy is used as a treatment option in the organ-preserving treatment strategies of early-stage or advanced stage laryngeal cancers. There are different studies investigating the inflammatory effects of GER on irradiated laryngeal epithelium [1–8]. Although ionizing radiation and GER are known to precipitate chronic inflammation, there is insufficient knowledge about the

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cumulative or additive effects of gastric content on irradiated laryngeal tissue.

This experimental study aims to analyze whether there are early histopathological changes of ionizing radiation on laryngeal mucosa which was subjugated acidic content by a LPR model.

Subject and methods

All experimental protocols were approved by local animal care and use committee. The research took place at the animal experiment laboratory. Irradiation procedure was done in Radiation Oncology Clinic. Twenty-four adult female Wistar Albino rats (160–230 g) were housed in the temperature-controlled room (22 ± 2 °C) with standardized light/dark (12/12 h) cycles that the relative humidity (65–70%) was kept constant. Animals were divided into three groups; control (C, $n = 6$), irradiation (IR, $n = 8$) and reflux and irradiation (RIR, $n = 10$). The experimental model of LPR with a surgical method described by Gaia Filho et al. [9] was used. According to this method, the animals underwent a 2-cm midline laparotomy starting at the xiphoid process following preoperative preparation and anesthesia. After completion of abdominal exploration, the line called "limiting ridge line" that determines the border between the fundus and the corpus of the stomach and the pylorus of the stomach were found. A ligation was performed at the fundus–corpus border from the lateral part of the esophagus continuing along the entire stomach. The volume of the stomach was decreased by one-third. A 3-mm ring was cut from 16 F Nelaton silicone catheter to be used at the pyloric region later. The pyloric region with high-vascularity was separated from its serosa carefully and elevated. The ring was placed to the pylorus by cutting from one end, and the cut edges were sutured again to prevent migration. Therefore, gastric emptying was delayed and forced by narrowing the stomach outlet at the pyloric region (Fig. 1). In the control animals, a median laparotomy was performed and the stomach was mobilized. This procedure was done 1 month prior to irradiation for each animal.

Irradiation was performed with a linear accelerator (Trilogy, Varian Medical Systems, Inc., Palo Alto, Calif., USA) producing 6 MV photons at a focus 100 cm distant from skin. Under ketamine (100 mg/kg, ip) anesthesia, each animal receives 20 Gy ionizing radiation to laryngeal area (Fig. 2). Following the irradiation rats were returned to their home cages. The current dose was chosen due to our previous studies that examined the early irradiation effects on gastrointestinal mucosa [10]. Therefore, on the 4th day of irradiation animals were killed and before the kill the laryngeal samples from animals were taken under anesthesia. Specimens were fixed with a 10% formaldehyde solution. Preparations were cut in about 4–6-μm thick sections. Sections were stained with hematoxylin–eosin. The specimens were examined at a magnification of 40×, 100× and 400× under a light microscope. The semi-quantification of changes in lamina propria (edema, vascular dilation and inflammation) and changes in mucosa inflammation in squamous epithelium and, inflammation, loss of cilia and pseudostratification in the respiratory tract epithelium were performed according the criteria outlined in Table 1. Histopathological score of each sample was given separately by the same pathologist (PG) blinded to all experimental groups. Each parameter was given from 0 to 3 at a magnification of 100× [11].

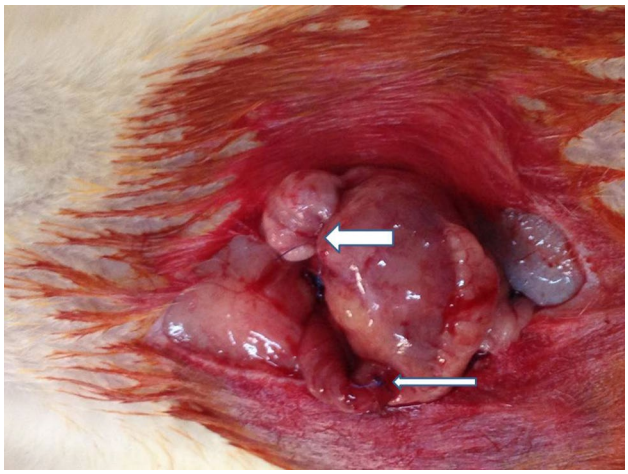


Fig. 1 Surgical method. Surgical technique used to develop experimental model of gastroesophageal reflux. Fundus–corpus border performed a ligation (thick arrow), application of the ring prepared from nasogastric catheter and applied to the pyloric region of the stomach (thin arrow) are seen

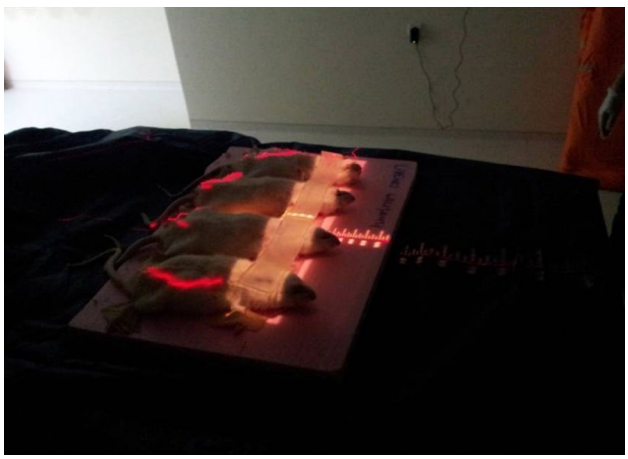


Fig. 2 Administration irradiation to larynx and cervical esophagus

Table 1 Semiquantitative grading system for histopathological changes

Parameters	Grade			
	0	1	2	3
Edema	No differences	Mild	Moderate	Severe
Vascular dilatation	No differences	Mild	Moderate	Severe
Pseudostratification	No differences	Mild	Moderate	Severe
Inflammation[a]	1–20 lymphocytes, no PMNL	21–50 lymphocytes, 1–2 PMNLs	51–80 lymphocytes, 3–10 PMNLs	81–120 lymphocytes, > 10 PMNLs
Cilia loss	No differences	Less than normal area	Severe loss than normal area	Total loss of cilia

[a]Polymorphonuclear leucocytes (PMNL) and lymphocytes account

Statistical analysis

Statistical analysis was performed using NCSS (Number Cruncher Statistical System) 2007 & PASS (Power Analysis and Sample Size) 2008 Statistical Software (Utah, USA). All data are expressed as median values ± SEM. Groups of data were compared with an analysis of variance (ANOVA) followed by Mann–Whitney *U* test or Kruskal–Wallis test. Values of $p < 0.05$ were considered as significant.

Results

All results were summarized in Tables 2 and 3.

Lamina propria

Edema

The scores for the edema in the IR ($p = 0.028$) and RIR ($p = 0.014$) groups were significantly higher compared to the control group scores. No significant difference was found between IR and RIR groups ($p = 0.388$) (Fig. 3).

Vascular dilation

The scores for vascular dilation in IR ($p = 0.011$) and RIR ($p = 0.001$) groups were significantly higher compared to the control group. No significant difference was determined between IR and RIR groups ($p = 0.167$).

Inflammation

Scores for inflammation in RIR groups were significantly higher compared to the control group ($p = 0.005$). No significant difference was determined between the control and IR ($p = 0.194$) or IR and RIR ($p = 0.217$) groups (Fig. 3).

Squamous epithelium

Inflammation

In experimental groups (IR and RIR) scores were significantly higher compared to the control group ($p = 0.001$). However, the score of IR was significantly higher than RIR score ($p = 0.014$).

Respiratory tract epithelium

Inflammation

There was no significant difference determined between the scores of IR and control groups ($p = 0.725$). or in terms of inflammation. Meanwhile, RIR was significantly higher than IR ($p = 0.014$) and control group scores ($p = 0.010$).

Cilia loss

Both IR ($p = 0.012$) and RIR ($p = 0.010$) groups of animals were significantly lost their respiratory tract cilia than control group animals. No significant difference was observed in IR and RIR groups ($p = 0.914$) (Fig. 3).

Pseudostratification

No significant difference was found between the control group and IR group ($p = 0.215$). No significant difference was determined between IR and RIR ($p = 0.067$). However, RIR group score was significantly higher than the control group score ($p = 0.005$).

Table 2 Results of histopathological analysis

Change	C ($n=6$) (%)	IR ($n=8$) (%)	RIR ($n=10$) (%)	p[a]
LPE				
No differences	3 (50)	0 (0)	0 (0)	0.018*
Mild	3 (50)	7 (87.5)	7 (70)	
Moderate	0 (0)	1 (12.5)	3 (30)	
Min–max (median)	0–1 (0.50)	1–2 (1.0)	1–2 (1)	
VD				
No differences	5 (83.3)	1 (12.5)	0 (0)	0.001**
Mild	1 (16.7)	7 (87.5)	9 (90)	
Moderate	0 (0)	0 (0)	1 (10)	
Min–max (median)	0–1 (0)	0–1 (1)	1–2 (1)	
LPI				
Mild	5(83.3)	4(50.0)	1(10)	0.031*
Moderate	1 (16.7)	3 (37.5)	9 (90)	
Severe	0 (0)	1 (12.5)	0 (0)	
Min–max (median)	1–2 (1.0)	1–3 (1.5)	1–2 (2)	
SEI				
No differences	6 (100)	0 (0)	0 (0)	0.001*
Mild	0 (0)	4 (50)	10 (100)	
Moderate	0 (0)	4 (50)	0 (0)	
Min–max (median)	0–0 (0)	1–2 (1.5)	1–1 (1.0)	
RTEI				
Mild	4 (66.7)	6 (75.0)	2 (20.0)	0.048*
Moderate	2 (33.3)	2 (25)	8 (80)	
Min–max (median)	1–2 (1)	1–2 (1)	1–2 (2)	
RTEC				
No differences	4 (66.7)	0 (0)	0 (0)	0.012*
Mild	1 (16.7)	2 (25)	3 (30)	
Moderate	1 (16.7)	6 (75)	6 (60)	
Severe	0 (0)	0	1 (10)	
Min–max (median)	0–2 (0)	1–2 (2)	1–3 (2)	
RTEP				
Mild	5 (83.3)	4 (50)	1 (10)	0.016*
Moderate	1 (16.7)	4 (50)	9 (90)	
Min–max (median)	0–2 (1.0)	1–2 (1.5)	1–2 (2)	

LPE edema in lamina propria, *VD* vascular dilation in lamina propria, *LPI* inflammation in lamina propria, *SEI* inflammation in squamous epithelia, *RTEI* inflammation in respiratory tract epithelia, *RTEC* cilia loss in respiratory tract epithelia, *RTEP* pseudostratification in respiratory tract epithelia, *C* control, *IR* irradiation, *RIR* reflux and irradiation

[a]Kruskal–Wallis test

*$p<0.05$, **$p<0.01$

Table 3 Post hoc analysis for histophatological results

p value	C–IR	C–RIR	IR–RIR
LPE	0.028*	0.014*	0.388
VD	0.011*	0.001**	0.167
LPI	0.194	0.005**	0.217
SEI	0.001**	0.001**	0.014*
RTEI	0.742	0.071	0.023*
RTEC	0.012*	0.010*	0.914
RTEP	0.215	0.005**	0.067

C control, *IR* irradiation, *RIR* reflux and irradiation

Mann–Whitney *U* test *$p<0.05$, **$p<0.001$

Discussion

In previous studies reflux was found a possible cause of inflammatory disease and mucosal damage in laryngeal mucosa [12–16]. In some animal studies, it has been shown that the destructive effect of reflux acid on laryngeal mucosa occurred just in a few days [2]. Gastric pepsin with higher protease activity is also responsible from this destructive effect in addition to the direct effect of gastric acid on the cells. Pepsin has an optimal pH value between 2 and 3.5, and it exerts a rapid destructive activity within this range [3].

In the animal study of experimental reflux performed by Ling et al. [4] determined an increase of polymorphonuclear leukocytes infiltration in the larynx after the 10th week and proliferation in the glands of the lamina propria after the 20th week.

Shimazu et al. [5] investigated pharynx, larynx, trachea and lung histopathologically changes of reflux in an animal model. They determined the mucosal thickening and inflammatory cell infiltration in the hypopharynx at the 8th week, postoperatively. Fibroblasts and collagen fibers increased and capillary dilation occurred as time progressed. They also observed inflammatory cell infiltration in the thoracic esophagus, arytenoid region, and lung at the postoperative 16th week.

Ionizing radiation is used as a treatment option in the early-stage or, organ-preserving treatment strategies of advanced stage laryngeal cancers [17]. Particles and reactive oxygen species (ROS) charged secondarily in the nucleus due to radiation cause a series of damage in DNA which is basic target. However, the most important part of the damage is as a result of indirect effect which is done by free radicals.

Early radiation effects were described in literature previously. In a study performed by Lidegran et al. [7] rats were irradiated in the head and neck region and the larynx were studied by light and electron-microscopy and immunohistochemistry 10 days after irradiation. No change was observed in the ultrastructural appearance of the granules of the subglottic glands, on the other hand, substance P, bombesin

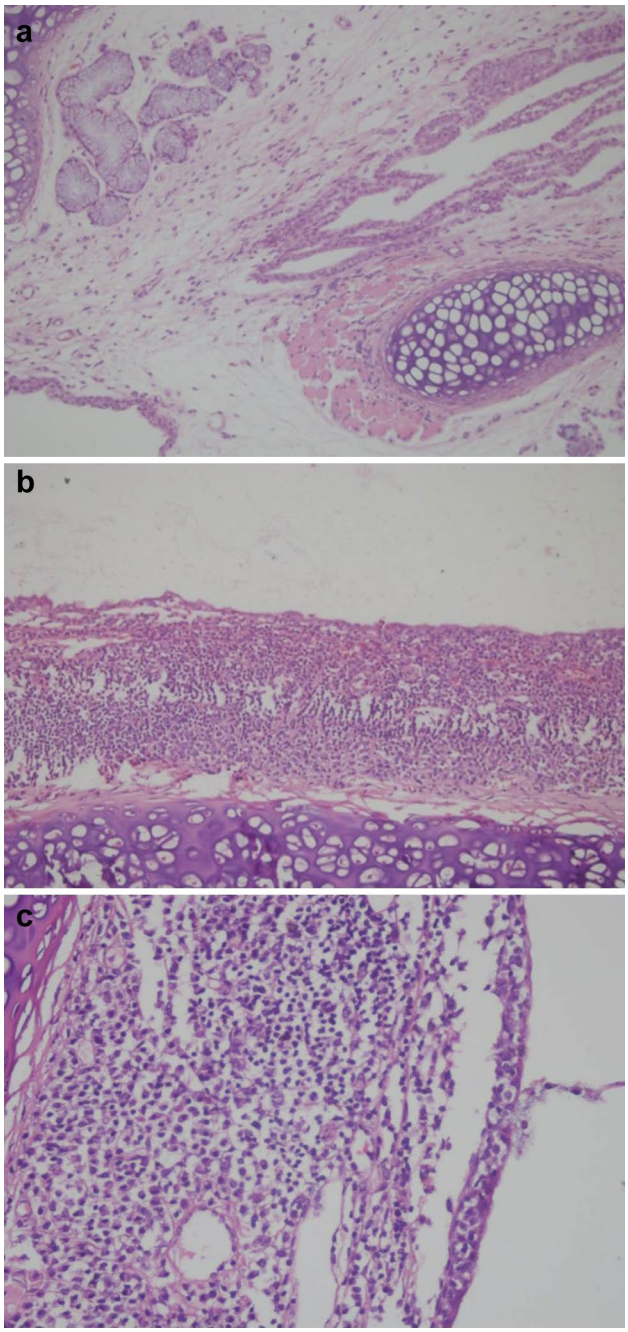


Fig. 3 Histopathological appearance of larynx tissue in irradiation (IR) (**a**), reflux and irradiation (RIR) (**b**) and (**c**) groups (H&E). **a** Moderate edema in lamina propria and mild cilia loss. **b** Moderate inflammation in respiratory tract epithelia. **c** Moderate inflammation and severe cilia loss in respiratory tract epithelia

and enkephalin like immunoreactivity was increased in local ganglionic cells and glandular nerve fibers. The mast cells were reduced in number. In another study performed by Saltman et al. [8] larynx of the rats exposed to 2 fractionation schedules of 40 Gy dose of ionizing radiation at the level of the vocal cords were analyzed histologically and increased ROS synthesis and inflammatory and profibrotic gene expression in the fibroblasts were determined.

In our study, the results of histopathological evaluation from the lamina propria showed that both irradiation groups (IR and RIR) showed worse scores than control group. However, there was no significant additive effect of gastric acid and pepsin found on destructive effects of irradiation. On the other hand, squamous epithelium of the laryngeal specimens of the rats was much more affected in the IR group than reflux added group. Marginal borders of the epiglottis and the vocal cords in the larynx are lined by squamous epithelium, and the remaining part of the laryngeal mucosa is lined by pseudostratified ciliated columnar epithelium. Therefore, it may be considered that dryness of the vocal folds induced by irradiation augments the mucosal surface trauma on vocal fold. Despite its acidic content, reflux may reduce the traumatic effect of irradiation by providing humidity with contact with mucosa. We think that the effect in the squamous epithelium may be caused by dryness and traumatic effect due to irradiation.

Respiratory tract epithelium was much more affected in the RIR group than irradiation alone group. This result can support that radiation-induced damage may enhance in previously damaged mucosa by acidic content of stomach. Surface of respiratory tract is larger than squamous-lined area; hence this may be explained by the volume effect caused by multi factors. In other words, other than vocal cord and edges of the epiglottis, larynx is covered by respiratory epithelium. That is why respiratory epithelia may be exposed to acid reflux more than squamous epithelia. In addition, the amount of reflux may be another factor which effects the changes. According to reflux level exposed surface may chance. We think that due to this exposure respiratory epithelia may be more affected than squamous epithelia in RIR group.

Clinical findings showed that acute effects may exacerbate the late effects of irradiation [18, 19]. Hence, the ionizing radiation is administered in the presence of reflux, clinical morbidity may increase and medical treatment for reflux administered either previously or simultaneously may have a potential role to reduce the symptomatic changes in the laryngeal epithelium in patients receive radiotherapy.

Conclusion

Our study showed that, acute inflammation due to gastroesophageal reflux may have an additive destructive effect on laryngeal mucosa on irradiated animals. Thus, it may be helpful to investigate and treat the inflammatory conditions before the treatment of laryngeal cancer with radiation. Further studies are required to understand the exact mechanism

and consequences of gastric acid-induced inflammation on laryngeal mucosa that will receive ionizing radiation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Research involving human participants and animal All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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