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Inflamatory Changes in Laryngeal Mucosa in Rats Due to Acid, Nitrite and Pepsin Exposition

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Author's contribution

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Smoking and alcoholism are risk factors for head and neck malignancies. Other possible risk factors for squamous cell carcinomas (SCC) are gastroesophageal (GER) and pharyngolaryngeal (PLR) refluxes. Objectives: Demonstrate the carcinogenic action of hydrochloric acid on the laryngeal mucosa of Wistar rats, potentialized by pepsin, associated with nitrate applied in the rat laryngeal mucosa, simulating the reflux of the gastric contents to the laryngopharynx. Method: Eighty-two Wistar rats were divided in seven groups and submitted to 2 or 3 weekly applications of hydrochloric acid, pepsin and sodium nitrate to the laryngeal mucosa during 6 months. Results: No dysplasia, intra-epithelial neoplasia or invasive carcinomas were seen. Inflammatory changes were observed in varying degrees. Discussion: Several authors demonstrated the co-carcinogenic action of GERD, whose mucosal irritating agent is hydrochloric acid. Conclusion: It is possible that GERD and PLR are co-carcinogenic due to the inflammatory action of hydrochloric acid potentialized by pepsin.

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1. INTRODUCTION

The association of smoking and alcoholism with malignant neoplasms of the upper aero digestive tract has been well known for many years [1-3]. Recently, other risk factors have been associated with the study of ethiopathogenesis of squamous cell carcinomas in this region, especially the gastroesophageal (GER) and pharyngolaryngeal refluxes (PLR), hydrochloric acid as the irritating agents of the mucosa. Due to the unmistakable presence of head and neck squamous cell carcinoma in non-alcoholics and non-smokers [30]. It is clear that the study of other causal factors of the disease may contribute to a better understanding of the pathogenesis and advance in its treatment.

In laryngeal squamous cell carcinoma, several authors have shown the relationship existing between the gastroesophageal reflux disease (GERD) and the development of this neoplasia [4-19]. Some animal studies have confirmed this hypothesis [20-26], whereas other authors have not [27].

The objectives of this study are to show the inflammatory changes on the laryngeal mucosa of Wistar rats secondary to exposure of hydrochloric acid, potentialized by pepsin, associated or not with sodium nitrate, for a limited period of time, simulating the injury caused by gastroesophageal and pharyngolaryngeal reflux on the mucosa of the upper airway and digestive tracts in humans.

2. METHOD

Eighty-two young randomly chosen male Wistar rats weighing between 300g and 400g were studied. The animals were placed in cages lined with sawdust and kept at the Experimental Surgical Center of the Department of Surgery. The committee on ethics in animal experimentation gave favorable opinion to the experiment.

Water was available *ad libitum* to all animals, including those treated with the sodium nitrate solution. Each animal was given a specific number marked with a hydrographic pen on its tail, and the rats were divided into seven groups.

Hydrochloric acid was used in a 0.1N solution, the same concentration found in the human stomach. Pepsin was added to simulate the gastric contents during the digestive process, based on the study by Adams and others [20].

Group I consisted of twelve animals submitted three times weekly to the application of a solution of 0.1N (normal) hydrochloric acid directly on the laryngopharyngeal mucosa.

Group II consisted of twelve animals submitted twice weekly to the application of a 0.1N hydrochloric acid solution directly on the laryngopharyngeal mucosa.

Group III consisted of twelve animals submitted three times weekly to the application of a mixture of a 0.1N hydrochloric acid solution with a solution of pepsin diluted to 1mg per 1ml of distilled water, directly on the laryngopharyngeal mucosa.

Group IV consisted of twelve animals submitted twice weekly to the application of a mixture of 0.1N hydrochloric acid solution with a pepsin solution diluted to 1mg per 1ml of distilled water, directly on the laryngopharyngeal mucosa.

Group V consisted of twelve animals treated with a mixture of 400mg of sodium nitrate diluted in 300ml of filtered water and submitted three times weekly to the application of a solution of 0.1N hydrochloric acid directly on the laryngopharyngeal mucosa.

Group VI consisted of twelve animals treated with a mixture of 400mg of sodium nitrate diluted in 300ml of filtered water and submitted twice weekly to the application of a 0.1N hydrochloric acid solution directly on the laryngopharyngeal mucosa

Group VII consisted of ten animals submitted twice weekly to the application of filtered water directly on the laryngopharyngeal mucosa, serving as the control group for this study.

Application was carried out with a common number 1 brush with soft bristles, using non-traumatic separators to provide direct exposure of the laryngopharyngeal mucosa of the rats. Yellow brushes were used exclusively for applying the acid, tan brushes exclusively for the mixture of acid and pepsin, and blue brushes exclusively for the filtered water. The area of application included the risk mucosa in study, namely, the supraglottic laryngeal mucosa, besides the pharyngeal mucosa at the base of the tongue and the cervical esophagus. There was no need for anesthesia because the procedure caused no significant discomfort to the animals.

During the study time they received a diet of standard rations and their weights being monitored monthly. After the period of exposure, the rats were euthanized by prolonged anesthesia with endovenous chloral hydrate in lethal doses of two milliliters (2ml) per kilogram of weight.

The specimens for study were obtained by dissecting the mucosa and the parts were removed *in toto*. That is, similar to the surgical procedure performed in humans and following removal, the specimens were fixed in formalin for 24 hours and then stored in a 70% alcohol solution. Serial sections were prepared on slides based on specimens colored with hematoxylin and eosin and set in paraffin.

Later they were analyzed blindly by two experienced pathologists, who evaluated the presence of inflammatory changes in the laryngeal mucosa, such as ulceration, inflammatory infiltrate and characteristic cellularity, as well as presence of inflammatory exsudate in the laryngeal lumen.

For statistical analysis, the SAS Computor System for Windows (Statistical Analysis System), version 8.02 was used (SAS Institute Inc, 1999-2001, Cary, NC, USA). Histological changes were compared using the Fisher Exact Test. Variance analysis for repeated measurements (ANOVA) was used for variation of weights.

3. RESULTS

Five animals were excluded from the study due to lack of laryngeal mucosa in the material analyzed, two from Group III and one each from Groups I, II and VII.

Among the groups studied no epithelial changes suggestive of dysplasia, intra-epithelial neoplasia or clearly invasive carcinoma were seen. Inflammatory histological changes were observed in varying degrees, such as the presence of lymphocytes, plasmocytes, and

neutrophils. There was also mucosal ulceration, inflammatory infiltrate in the mucosa, and loose inflammatory exsudate in the laryngeal lumen, depending on the substance applied and its frequency.

When the presence of mucosal ulceration was evaluated, a higher frequency and greater intensity in Groups I to IV were obtained, although the difference was not statistically significant ($p = 0.582$) (Table 1).

Evaluating separately the cells that comprised the inflammatory reaction in the histological findings, greater frequency of lymphocytes was seen in Groups I, III and IV ($p = 0.007$) (Table 2). The presence of inflammatory cells was more intense in Groups III and IV ($p = 0.007$) (Fig. 1), as was also the case of plasmocytes ($p<0.001$).

In the evaluation of the intensity of the inflammatory exsudate, divided into degrees of 0 to 3, greater frequency of degrees 2 and 3 in Groups I, III and IV ($p<0.001$) were seen. Studying the amount of loose inflammatory material in the laryngeal lumen, more intense inflammatory reaction could be seen in Group III ($p<0.001$).

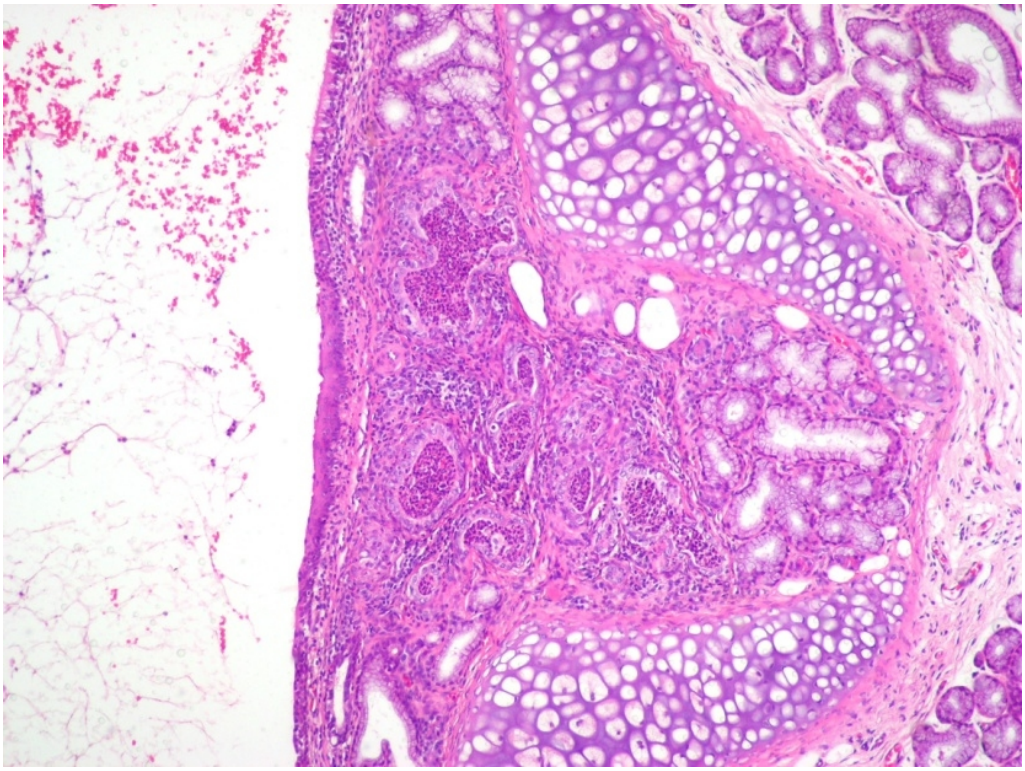

Fig. 1. Severe lymphocyte infiltrate in the larynx. (HE 400X).

Table 1. Analysis of the comparative degree of ulceration. fisher test: p = 0.582

Ulceration	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII	Total
0	8 (72.73%)	8 (72.73%)	8 (80.00%)	10 (83.33%)	1 (91.67%)	1 (91.67%)	9 100.00%)	65
1-3	3 (27.27%)	3 (27.27%)	2 (20.00%)	2 (16.67%)	(8.33%)	(8.33%)	0 (0.00%)	12
Total	11	11	10	12	2	2	9	77

Table 2. Comparative analysis of the presence of lymphocytes. fisher test: p = 0.007

Lymphocytes	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII	Total
0	1 (9.09%)	1 (9.09%)	0 (0.00%)	0(0.00%)	2 16.7%)	7 (58.33%)	1 (11.11%)	12
1	4 (36.3%)	6 (54.5%)	5 (50.0%)	8 (66.67%)	9 75.0%)	4 (33.33%)	4 (44.44%)	40
2	2 (18.2%)	4 (36.3%)	4 (40.0%)	2 (16.7%)	1 8.33%)	1(8.33%)	4 (44.44%)	18
3-4	4 (36.3%)	0 (0.00%)	1(10.0%)	2(16.7%)	0(0.00%)	0(0.00%)	0(0.00%)	7
Total	11	11	10	12	12	12	9	77

4. DISCUSSION

Approximately 5% of patients with head and neck squamous cell carcinoma (SCC) have no history of chronic use of tobacco or alcohol [31]. This fact would seem to reinforce the existence of other factors involved in the genesis of such tumors, and one of these co-factors may be the reflux.

The GERD is directly implicated in various pathologies of the upper airway. One of the first publications on the changes in the larynx epithelium due to reflux was that published by Cherry and Margulies, who reported contact ulcers in the posterior third of the vocal folds of three patients not explained by vocal abuse [32]. All complained clinically of hoarseness associated with dyspeptic symptoms, although no findings of reflux were confirmed with digestive endoscopy or monitoring of esophageal pH.

Koufman showed that pharingolaryngeal and gastroesophageal refluxes are different entities, each with its specific symptomatology, with infrequent pyrosis and regurgitation in the former. He also observed that laryngeal epithelium is more susceptible to tissue injury than is esophageal epithelium [33].

Other authors relate PRL with inflammatory changes in the larynx such as chronic laryngitis [34], persistent dysphonia [35], contact ulcers [36], throat clearing, laryngeal granuloma, globus pharyngeus, cervical dysphagia and subglottic stenosis [37]. Pharyngolaryngeal reflux has also been associated with respiratory diseases such as chronic coughs, asthma, apnea in sleep and chronic obstructive lung disease [38].

by Gabriel and Jones [39], who evaluated 101 cases of laryngitis that progressed to laryngeal SCC, although there was no mention of heavy smoking or alcoholism among the patients analyzed. Glanz and Kleinsasser evaluated 35 cases of laryngeal carcinoma among 841 patients with chronic laryngitis and persistent dysphonia for over two years and found multicentric foci of SCC permeating the inflammatory infiltrate in the histological analysis. Once again, there was no reference to smoking or alcoholism in the series studied [40].

In a clinical study, Kaufman analyzed the most prevalent symptoms and laboratory findings in 225 patients with DRGE following examination of esophageal pH-metria. Among the patients studied, symptomatology similar to that described previously was found and, in 31 cases, laryngeal SCC was also associated. In this same study, the injury mechanisms of hydrochloric acid associated with pepsin in the subglottic mucosa of 20 dogs were experimentally studied. These animals were divided into three groups, with applications of saline solution, pepsin alone, neutralized acid and sodium hydroxide (control group); 0.1N hydrochloric acid and mixture of 0.1N hydrochloric acid with pepsin diluted to 0.3mg/ml. It was seen that, in the group submitted to the application of hydrochloric acid, there was greater latency in healing the inflammatory process than in the control group, and that there was no healing in the group submitted to the mixture of acid and pepsin [41].

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Mercante et al. analyzed 274 patients with CEC in the oral cavity, pharynx and larynx and found 29.3% of GERD confirmed with digestive endoscopy. Only 92 of these patients were not smokers, but a higher percentage of GERD was found in those with laryngeal CEC (21.7%) than in the general population (5%), $p = 0.0001$, indicating that the reflux might be considered a co-promoter of carcinogenesis [42].

Rubin et al. [43], Nurgalieva et al. [44] and Akbayr et al. [45] studied the possible action of *Helicobacter pylorii* in larynx carcinogenesis, although these authors presented conflicting results.

Recent study of Langevin et al. [46] analyzed 631 patients with laryngopharyngeal squamous cell carcinoma and compared with 1234 control subjects. After adjusting for age, gender, smoking, alcohol consumption, HPV16 seropositivity, and other parameters, non smokers and non drinkers with reflux disease patients, the authors concluded that gastric reflux is an independent risk factor for squamous cancers of the pharynx and larynx.

Experimentally, the tumorigenic action of hydrochloric acid in high concentrations in the oral mucous of hamsters was well established in the work by Adams et al [20]. The authors used five different groups of animals, applying solutions of dimethylbenzathracene (DMBA) associated or not with hydrochloric acid, with and without the addition of porcine pepsin. It was ascertained that the combined action of the hydrochloric acid with the pepsin fostered the emergence of neoplastic lesions of greater dimensions than those in the control group, although less latency time for their appearance was not seen. Other subsequent experimental studies confirmed these hypotheses [21-25].

Studies by Adhami et al. [47] in a dog model employed pepsin, conjugated bile acid, unconjugated bile acids and trypsin at pH 1-2, 4-5, and 6-7 applied bilaterally to laryngeal sites three times per week for a total of 9-12 applications. They found that pepsin alone or combined with other agents resulted in significant. The authors concluded that acid refluxate, pepsin and conjugated bile acid are the most injurious agents affecting laryngeal tissue, causing severe histological inflammation.

In the present study, only substances involved in GERD were used, namely, hydrochloric acid in the concentrations found in the organism (0.1N), pepsin, and nitrate. The animals were submitted to applications in series for a period of six months, verifying how much exposition would be necessary to cause any inflammatory response.

We not found any published studies correlating sodium nitrate with reflux and malignant neoplasms of the upper aerodigestive tract. Sodium nitrate and its derivatives are considered co-promoters of carcinogenesis, especially the nitrosamine group. The nitrate is absorbed in the small bowels or generated endogenously by nitrous oxide and secreted by the salivary glands. The bacterial flora of the tongue reduces the nitrate into nitrite, and the nitrite in contact with the acid pH reacting with secondary amines can be converted into nitrosamines, which are known to be carcinogenic [28-29].

Although pre-neoplastic changes were not found, greater intensity of inflammatory reaction was seen in the groups where the mixture of hydrochloric acid and pepsin ($p<0.05$) was applied, with greater tendency in the group with three weekly applications, confirming its injurious action. The presence of mucosal ulceration was seen more often and with greater intensity in the two groups with the highest exposure to acid and pepsin, without statistical difference ($p = 0.582$). As in the study by Adams and others, the synergic effect between acid and pepsin was responsible for the higher injury to tissue. And the histological changes found are in agreement with those described by Lewis [48], who characterized the inflammatory injuries in the larynx of rats submitted to experimentation.

Corroborating the data found in this study, the work by Goldberg et al. [22], although performed on cats, found more injury in the esophageal mucosa submitted to the application of a mixture

with higher concentrations of acid and pepsin, although no pre-neoplastic changes were seen. Lillemoe et al. [23], in an experiment with New Zealand rabbits, ascertained that the pepsin in pH acid has a more irritating effect on the esophageal mucosa than do trypsin, bile or acid, analyzed separately, also without dysplastic changes having been noted.

Finally, new studies in the future employing the usual markers of carcinogenesis such matrix metalloproteinases (MMPs), interleukins (Ils), chemokine receptors (CXC), etc, that could help us to understand the actual epithelial response to the aggression and to elucidate the etiology of these tumors may contribute.

5. CONCLUSION

The primary etiology of malignant neoplasias of the upper aerodigestive tract is the synergic action of smoking and ingestion of alcohol. Other factors have secondary action in the development of these neoplasms. Although pre-neoplastic lesions were not observed, only mucosal inflammatory changes and ulceration, it is possible that GERD and PLR are co-carcinogenic factors due to the inflammatory action of hydrochloric acid potential zed by pepsin, but data of the current study could not corroborate this hypothesis. Further studies are needed to clarify that possible association.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

The authors have declared that no competing interest exists.

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