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Role of Nitrites in the Genesis of Adenocarcinoma Associated with Barrett's Esophagus

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Abstract. *Background: Barrett's esophagus (BE) is one of the complications of gastroesophageal reflux disease (GERD) and a premalignant condition. It consists of a process of replacement of the squamous epithelium of the esophagus by intestinal columnar epithelium containing goblet cells, known as specialized intestinal metaplasia with goblet cells, and several factors have been related to its pathogenesis. The objective of this study was to evaluate an experimental model of duodenogastroesophageal reflux and the effect of ingestion of sodium nitrite solution on the genesis of adenocarcinoma associated with Barrett's esophagus. Materials and Methods: Sixty male Wistar rats were divided into four groups. Twenty were not submitted to surgery and served as controls (10 animals ingesting only water and 10 ingesting water plus a solution of sodium nitrite), while the remaining 40 animals were submitted to side-to-side duodenogastroesophageal anastomosis (20 animals ingesting only water and 20 ingesting water plus the sodium nitrite solution). The Vienna classification for dysplasia and adenocarcinoma was used in the analysis of results. Results: After 42 weeks of observation, Barrett's esophagus was found in 26.3% (5/19) of the animals submitted to surgery that had not ingested nitrites compared to 72.3% (13/18) of the animals in the group submitted to surgery and given nitrites. Six cases of adenocarcinoma (33.3%) were also found in this latter group. Barrett's esophagus was not found in any of the animals that were not submitted to surgery. Categories 2, 3 and 5 of the Vienna classification were only found in the animals submitted to surgery that also received sodium nitrite (66.7%). Conclusion: The ingestion of sodium nitrite associated with duodenogastroesophageal reflux plays an important role in the genesis of adenocarcinoma associated with Barrett's esophagus.*

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Adenocarcinoma of the esophagogastric junction is the neoplasia that has most increased in incidence in the past decade and its 5-year prognosis is poor if diagnosis and treatment are not instituted at an early stage (1). Therefore, it is very important to understand the disease process and to institute mechanisms for its prevention. Gastroesophageal reflux, principally the combination of gastric and duodenal contents, is a well-known risk factor for adenocarcinoma of the esophagogastric junction. Individuals with chronic gastroesophageal reflux go on to develop Barrett's esophagus. Lifestyle and habits such as smoking and alcohol consumption are some of the important factors involved (2).

Barrett's esophagus (BE) is one of the complications of gastroesophageal reflux disease (GERD) and consists of a process of replacement of the squamous epithelium of the esophagus by intestinal columnar epithelium containing goblet cells, known as specialized intestinal metaplasia with goblet cells. It is a premalignant condition affecting principally white males over 50 years of age, the incidence of which has been steadily increasing in Western countries. The prevalence in the general population is not precisely known; however, some studies have suggested that it may range from 5 to 9% (3-5). A previous study carried out in this teaching hospital revealed an incidence of 22.4 cases per 100,000 inhabitants or 3.57% of patients with GERD (6).

Intestinal metaplasia is considered a premalignant lesion with an unpredictable progression to adenocarcinoma (7). The sequence of events is well known, metaplasia–dysplasia–adenocarcinoma, during which genetic and molecular changes occur in the cells, induced by diverse factors such as the activation of specific oncogenes, inhibition of tumor suppressing genes and environmental factors (8-10).

Whereas metaplasia is the replacement of squamous epithelial tissue by intestinal columnar epithelium, dysplasia consists of changes in cell architecture. Dysplasia is defined as a truly premalignant condition and the severity of

cytological and architectural abnormalities is used to classify the grade of dysplasia (11).

Differentiation between low-grade dysplasia (LGD) and high-grade dysplasia (HGD) depends on the distribution of the nucleus, which remains confined to the basal half of the cells in so-called LGD, but is found randomly distributed between the basal and apical halves in cases of HGD. The difference between HGD and carcinoma *in situ* is that in the former, the lamina propria is unaffected, whereas in carcinoma *in situ,* the basal membrane is corrupted and the lamina propria invaded (12).

Sodium and potassium nitrites and nitrates are inorganic substances found in nature, in a large variety of food products consumed by man, in drinking water and in fertilizers. They are widely used in the food industry as preservatives for meat, tinned goods and smoked products (13-18).

Since the classic study of Sugimura *et al*. (15) in which adenocarcinoma was successfully induced in the stomach of rats following the use of *N*-methyl-*N*-nitro-*N*-nitrosoguanidine and because of the elevated incidence of gastric carcinoma in countries such as Chile and Colombia where the drinking water and soil are rich in nitrites, and also in Iran where smoked food, particularly fish, is widely consumed, interest in the study of nitrous compounds has increased (16-19).

Experimental models have been used to produce adenocarcinoma with and without the use of carcinogens in the normal esophagogastric junction and in BE (20-22). Chen *et al*. (23) induced carcinomas in an experimental gastroesophageal reflux model in rats in association with the use of iron administered intramuscularly. This procedure is reproducible in the laboratory and is well-tolerated by the animals.

To the best of our knowledge, no studies have been published in which nitrites have been used in association with a gastroesophageal reflux model to study their association with BE and adenocarcinoma in this region of the digestive tract, which was the objective of the present study.

Materials and Methods

The study was carried out in compliance with the Ethical Principles for Animal Experimentation adopted by the Brazilian College for Animal Experimentation (COBEA) and was approved by the Ethics Committee on Animal Experimentation (CEEEA) of the University of Campinas.

Sixty male Wistar rats (*Rattus norvegicus albinus*), considered healthy and specific pathogen-free, of around three months of life and weighing approximately 250 g were used in the study. During the experiment, the animals were housed separately in individual cages, maintained at room temperature under continuous airflow with relative humidity of 50% and day/night light cycles. The animals were fed with standard solid rat chow and given filtered drinking water *ad libitum*. They were observed daily for the evaluation of any possible behavioral or physiological changes.

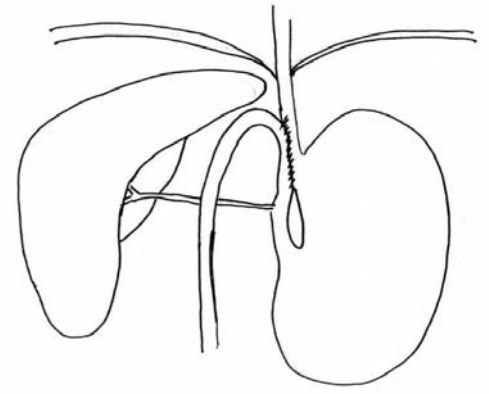

Figure 1. *Schematic representation of side-to-side duodenogastro-esophageal anastomosis.*

Twenty animals were separated into two control groups of 10 animals each. The first group (G1) received the standard diet and filtered water. The second group (G2) received the standard diet and filtered water containing 0.75 mg/ml sodium nitrite in for 42 weeks.

Forty animals (groups G3 and G4) were anesthetized with sodium pentobarbital (30 mg/kg *i.v.*) through the dorsal vein of the tail following 12 hours of fasting. Side-to-side duodenogastro-esophageal anastomosis was performed using 5-0 Prolene sutures, as shown in Figure 1.

Twenty of the animals submitted to surgery (G3) received filtered water from 12 hours after surgery and standard ration from 24 hours after surgery. They were also observed for 42 weeks. One animal in this group died of an unknown cause. The other 20 animals submitted to surgery received filtered water from 12 hours after surgery and standard ration after 24 hours (G4). After the third day following surgery, they were provided with filtered water containing 0.75 mg/ml sodium nitrite. In this group, two animals died of unknown causes. At the end of the observation period, the animals were again submitted to laparotomy followed by euthanasia (G3 and G4), and the esophagogastric junction where anastomosis had previously been performed was removed for histopathological evaluation. The animals of the group G1 and G2 were also submitted to laparotomy and the esophagogastric junction was removed, serving for histopathological studies as controls. The specimens were fixed in 10% buffered formalin, embedded in paraffin blocks, cut into 5 mm slices using a microtome and stained using the hematoxylin-eosin (HE) technique. The criteria used in the histopathological reports are in accordance with the definitions of the Vienna Classification (24) summarized in Table I.

Statistical analysis using Tukey's test for multiple comparisons and Fisher's exact test was carried out. Significance was defined as $p<0.05$.

Results

Statistically significant differences were found between the groups with respect to the occurrence of BE ($p<0.05$), which was found both with and without dysplasia only in the groups of animals submitted to surgery. In G3, BE was found in 26.3% (5/19) of the animals, whereas in G4 it was found in 72.3% (13/18) of the animals. In G4, six animals (33.3%) were found

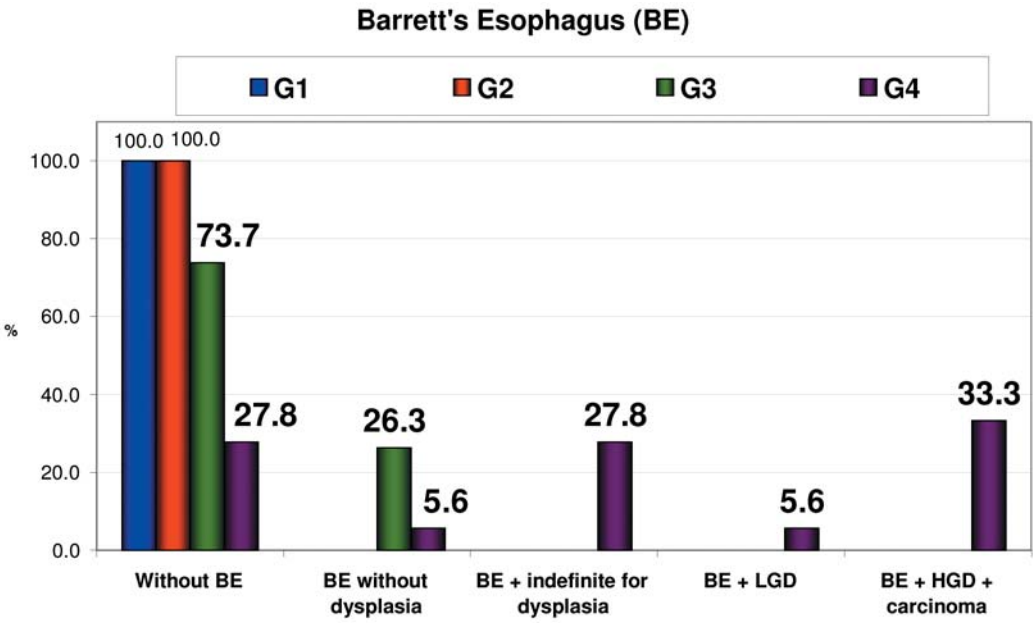

Figure 2. *Distribution of the study groups according to histopathological findings*.

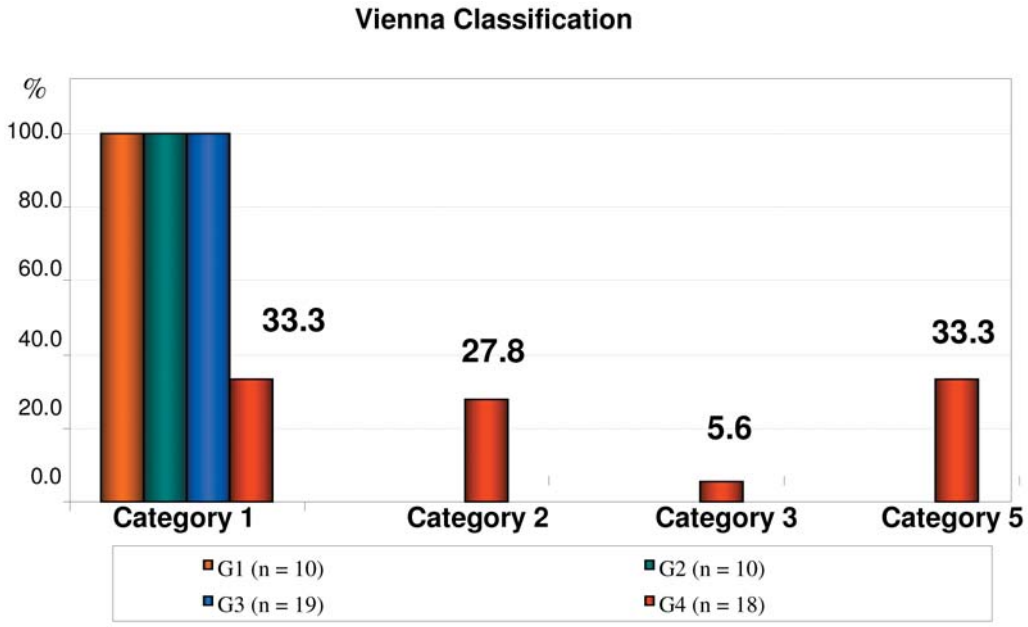

Figure 3. *Distribution of the study groups according to the categories of the Vienna classification*.

to have adenocarcinoma associated with HGD. One case of moderately differentiated adenocarcinoma invading up to the muscular layer, four moderately differentiated adenocarcinomas and one mucinous adenocarcinoma invading the adventitial layer, were found. In G4, five animals with indefinite dysplasia were also found (27.8%). There was no statistically significant difference in the histopathological findings between the animals in G2 and G3 ($p>0.05$) (Figure 2). Categories 2, 3 and 5 of the Vienna classification were only found in the animals submitted to surgery and ingestion of sodium nitrite. Vienna classification category 1 was predominant in groups G1, G2 and G3 (Figure 3).

Table I. *Summarized categories of the Vienna classification.*

Category 1	Negative for dysplasia. Architecture within the limits of normality. Absence of nuclear abnormalities except nuclear stratification. More marked nuclear abnormalities when associated with inflammation or ulceration.
Category 2	Indefinite for dysplasia. Architecture may be moderately distorted. Nuclear abnormalities less marked than those observed in dysplasia.
Category 3	Low-grade dysplasia. Cellular architecture with nucleus confined to the basal half of the cells.
Category 4	High-grade dysplasia. Severe abnormalities, principally if the mucosal surface is involved; however, respecting the lamina propria.
Category 5	Invasive neoplasia. Carcinoma invades the basal membrane of the glands in the lamina propria, but has not invaded the submucosa.

Discussion

The classifications of dysplasia in BE consist of adaptations of those used in other portions of the gastrointestinal tract: mild, moderate or severe (4). Discrepancies between western and eastern pathologists with respect to classification of the grade of dysplasia in BE are common and for this reason the 5-category Vienna classification was proposed in 1998 (4, 7, 24, 25). Later, this classification was reviewed in 2002 by one of its creators (26).

The advantages in the use of this classification system are that it adopts the term dysplasia habitually used by Western pathologists rather than neoplasia, and classifies diagnoses into 5 categories. Moreover, the diagnosis of invasive carcinoma (category 5) requires confirmation that invasion has occurred in at least the lamina propria of the mucosa. Consequently, the Vienna classification is practical and permits greater detail in establishing the grade of dysplasia; nevertheless, it is not yet being used routinely in daily practice and it will be some years yet before its efficacy in the management and treatment of patients is confirmed (27). In the present study, the use of this categorization was extremely helpful in classifying the evolution of cytoarchitectural abnormalities in the animals not submitted to surgery, in those with metaplasia, progressing through LGD and HGD and finally reaching invasive adenocarcinoma.

Experimental models of BE vary, with some investigators performing total gastrectomy (11, 22, 28), and others prefering procedures that facilitate gastric, biliary or biliopancreatic reflux to the esophagus (20, 21, 23, 28, 29, 30). Goldstein *et al.* (22) and Chen *et al.* (23) used experimental models of mixed reflux (gastric and duodenal juices) without the use of carcinogens, administering iron to the animals for 30 and 40 weeks, respectively, and successfully achieved Barrett's esophagus with and without dysplasia and associated adenocarcinomas.

Comparison between the present study and even similar experimental models is difficult in view of the variation in the variables involved, such as the number of animals, race, observation time, type of reflux, the carcinogen used and the route of administration. On the other hand, the use of carcinogens clearly increased the incidence of adenocarcinoma irrespective of the route of administration used. For example, Attwood *et al.* (20) used duodenogastroesophageal anastomosis, similar to the procedure used in the present study, in a group of Sprague-Dawley rats and when carcinogens (2-6-dimethylnitrosomorpholine and methyl-*N*-amyl-nitrosamine) were administered, a high occurrence of both spinocellular carcinoma and adenocarcinoma was recorded, although only one adenocarcinoma was observed in the animals not given carcinogens.

In the present experimental model which facilitated the occurrence of mixed reflux to the esophagus, evidence notable cytoarchitectural abnormalities occurred in the mucosa. The association between the surgical procedure, and the administration of sodium nitrite promoted intense tissue reaction with ulcerative esophagitis, LGD and HGD and invasive adenocarcinoma. These findings once again confirmed without any doubt that this type of reflux is responsible for the etiopathogeny of BE. It must be emphasized that the group of animals not submitted to surgery that also ingested nitrites did not develop BE.

Advanced studies of the molecular and cellular mechanisms of carcinogenesis have concentrated on the *N*-nitrous compounds in an attempt to discover why and how these compounds produce tumors in a large number of animal species with cellular and organic specificity. The *N*-nitrous compounds are currently considered to represent a group of organic substances that result from the interaction between nitrites and amino acids present in food that has been consumed, forming nitrosamines. This nitrosation may occur in the food, in the interior of the digestive tract or *in vitro* (13, 17, 18, 30). The excessive consumption of preserved meat, tinned or smoked products certainly increases the concentration of nitrites in the upper digestive tract, which, in combination with mixed gastroesophageal reflux may perfectly well contribute towards the etiopathogeny of BE and its subsequent progression to dysplasia and adenocarcinoma. Further studies need to be carried out to evaluate these relationships in more detail.

The characteristic morphological changes of the progression of BE to adenocarcinoma in the present animal model were

similar to those recorded in the literature for humans, from and duodenal reflux causing metaplasia, which then progresses dysplasia and adenocarcinoma. It is therefore reasonable to conclude that the mixed reflux of gastric and duodenal juices is the principal factor triggering adenocarcinoma in the esophagogastric junction. A possible means of prevention may lie in implementing changes in dietary habits, avoiding food containing preservatives and opting instead for freshly cooked food, fruit and vegetables.

Finally, it is important that further studies be developed in the future, paying greater attention to patients with BE and to diets in which there is a high consumption of food containing these preservatives.

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