

## Carcinogenesis of the upper gastrointestinal tract induced by N-methyl-N'-nitro-nitrosoguanidine and reflux of duodenal contents in the rat<sup>1</sup>

### Carcinogênese do trato gastrointestinal alto induzida pelo N-methyl-N'-nitro-nitrosoguanidina e pelo refluxo duodenogástrico no rato

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#### ABSTRACT

**Purpose:** To investigate the combined effects of reflux of duodenal contents through the pylorus and treatment with N-methyl-N'-nitro-nitrosoguanidine (MNNG) on the development of lesions in the glandular stomach, at the gastrojejunal anastomosis and in the forestomach of rats. **Methods:** Eighty Male Wistar rats were divided into 4 groups: G1: MNNG + Reflux, G2: Reflux, G3: MNNG and G4: Gastrostomy. MNNG was given in the drinking water (100 mg/ml) for 12 weeks and then two groups (G1 and G2) were submitted to a gastrojejunal anastomosis followed by section of the afferent loop and suture of both stumps to allow reflux of duodenal contents through the pylorus. The animals were sacrificed 18 and 36 weeks after surgery. The lesions obtained in the antral mucosa, at the gastrojejunal anastomosis and in the forestomach were analysed histologically. **Results:** Duodenal reflux induced proliferative lesions at both glandular and squamous mucosa of the stomach. In the antrum, adenomatous hyperplasia (AH) was observed in 20% and 50% of the animals at the 18<sup>th</sup> and 36<sup>th</sup> weeks respectively. Additionally 85% of the animals presented AH at the gastrojejunal anastomosis and 60% developed squamous hyperplasia at the squamous portion of the stomach. MNNG treatment plus duodenal reflux enhanced the development of malignant tumors at both glandular and squamous mucosa, since there were 30% of antral adenocarcinomas and 45% of squamous carcinomas at the 18<sup>th</sup> week and the frequency of these malignant tumors rose to 50% in the antrum and 65% in the squamous mucosa at the 36<sup>th</sup> week. **Conclusion:** The reflux of duodenal contents through the pylorus enhanced the development of proliferative lesions, benign and malignant, in the glandular stomach and in the forestomach of rats.

**Key words:** Upper Gastrointestinal Tract. Duodenogastric Reflux. Animal Experimentation. Rats.

#### RESUMO

**Objetivo:** Investigar os efeitos do refluxo duodenogástrico e sua interação com o cancerígeno químico N-metil-N'-nitro-nitrosoguanidina (MNNG) no desenvolvimento de lesões no estômago glandular, anastomose gastrojejunal e no estômago escamoso do rato. **Métodos:** Foram utilizados 80 ratos Wistar divididos em 4 grupos: G1: MNNG + Refluxo, G2: Refluxo, G3: MNNG e G 4: Gastrostomia. O MNNG foi oferecido na água de beber (100mg/ml) por 12 semanas. A seguir foi feita anastomose gastrojejunal na porção glandular do estômago nos grupos G1 e G2, com secção da alça aferente junto ao estômago e sutura de ambos os cotos para permitir o refluxo do conteúdo duodenal para o estômago pelo piloro. Os animais foram sacrificados 18 e 36 semanas após a cirurgia. As lesões identificadas foram submetidas à exame histopatológico. **Resultados:** O refluxo duodenogástrico levou ao desenvolvimento de lesões proliferativas no estômago glandular e na porção escamosa. No antro, hiperplasia adenomatosa (HA) foi diagnosticada em 20 e 50% dos animais (G2) na 18<sup>a</sup> e 36<sup>a</sup> semanas, respectivamente. Na anastomose gastrojejunal 85% dos animais (G2) apresentaram HA e 60% apresentaram hiperplasia escamosa no estômago escamoso, na 36<sup>a</sup> semana. No grupo MNNG+Refluxo foram identificados na 18<sup>a</sup> semana, 30% adenocarcinomas no antro e 45% carcinomas escamosos. A frequência destas lesões malignas aumentou, respectivamente, para 50% e 65% na 36<sup>a</sup> semana. **Conclusão:** O refluxo duodenogástrico potencializou o desenvolvimento de lesões proliferativas benignas e malignas no estômago glandular e em sua porção escamosa, no rato.

**Descritores:** Trato Gastrointestinal Superior. Refluxo Duodenogástrico. Experimentação Animal. Ratos.

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#### Introduction

Reflux of biliopancreatic secretion has been related with both cancer of the gastric stump following partial gastrectomy<sup>1</sup> and

with antrally located gastric cancer<sup>2</sup>. Similarly, in the esophagus, reflux of gastric and duodenal contents has been involved with development of Barrett's esophagus and adenocarcinoma in the metaplastic glandular epithelium<sup>3-5</sup>.

The low occurrence of spontaneous tumors of the upper gastrointestinal tract in rodents<sup>6</sup>, as well as its low incidence following simulation of clinical conditions such as partial gastrectomy<sup>7</sup> have led to the use of chemical carcinogens in the experimental models of carcinogenesis of the upper gastrointestinal tract. Due to the selectivity of N-methyl-N'-nitro-nitrosoguanidine (MNNG) to the glandular stomach of rodents, it has been the chemical carcinogen most frequently used in experimental studies<sup>8-11</sup>.

We have previously observed the development of polypoid lesions at the gastrojejunal anastomosis in rats submitted to BII gastrectomy without carcinogen treatment<sup>12-14</sup>. Using a surgical procedure to remove reflux of duodenal contents away from the mucosa of the gastric stump, we have found that the lesions reverted after interruption of reflux, thus meaning that the proliferative lesions were not true neoplasms<sup>12</sup>.

The present study was designed to investigate the combined effects of carcinogen treatment with MNNG and reflux of duodenal contents through the pylorus on the development of lesions in the glandular stomach, at the gastrojejunal anastomosis and in the forestomach of rats.

## Methods

Male Wistar rats weighing 180 g were divided into 4 groups considering carcinogen treatment with MNNG and a surgical procedure for induction of reflux of duodenal contents through the pylorus: Group 1: MNNG + Reflux, Group 2 Reflux, Group 3: MNNG and Group 4: Gastrostomy.

In the first 12 weeks, animals from groups 1 and 3 received MNNG (100 mg/ml) dissolved in the drinking water. The solution containing the carcinogen was placed in glass bottles wrapped in aluminum foil, to prevent MNNG decomposition by light. The bottles were changed daily. Control groups (G2, and G4) received only water. At the end of the 12<sup>th</sup> week, when MNNG treatment was finished, the surgical procedures were carried out. They consisted in an anisoperistaltic gastrojejunal anastomosis, with 1.0 cm in length performed in the animal's glandular stomach by using 6-0 polypropylene suture in parallel to the small curvature. Fifteen days after such procedure, Reflux was introduced to the stomach through the pylorus by sectioning the afferent-loop of the gastrojejunal anastomosis and suture of both stumps (Figure 1). In the gastrotomy group (G4) a 10 mm horizontal incision was performed on the anterior wall of the glandular stomach. The animals were sacrificed 18 and 36 weeks after surgery.

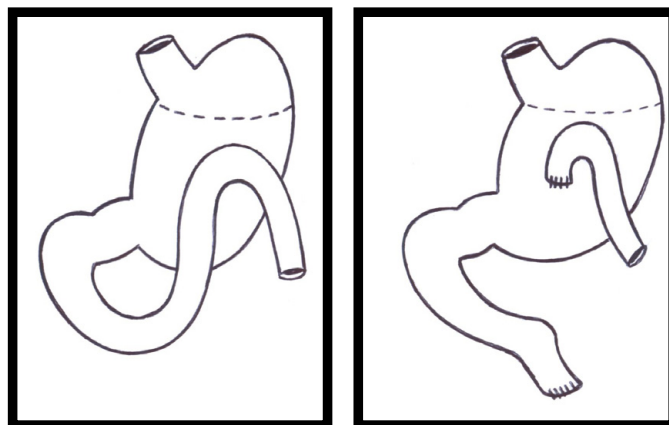


FIGURE 1 – Diagram of surgical procedures

At necropsy, the stomach was removed, opened through the great curvature and the intestinal loop anastomosed to the stomach, whenever present, was opened through the mesenterial border. The specimens were rinsed in saline solution, spread on a cork plate on the serosal surface and fixed by immersion in 4% buffered paraformaldehyde. This fixative solution consisted of 85 ml of distilled water, 6 g of mercury chloride, 5 ml of acetic acid and 10 ml of 40% formaldehyde. After fixation, the samples were rinsed in distilled water and placed in phosphate (PBS) for 60 minutes. Five-micron-thick histological sections were stained by hematoxylin-eosin.

As for the glandular stomach, the diagnosis of adenocarcinoma (Ca) was made when proliferation of glandular structures with cellular atypia, with both endophytic and exophytic growth was found at the histological examination (Figure 2A). Similar proliferative lesions without cellular atypia were diagnosed as adenomatous hyperplasia (Figure 2B).

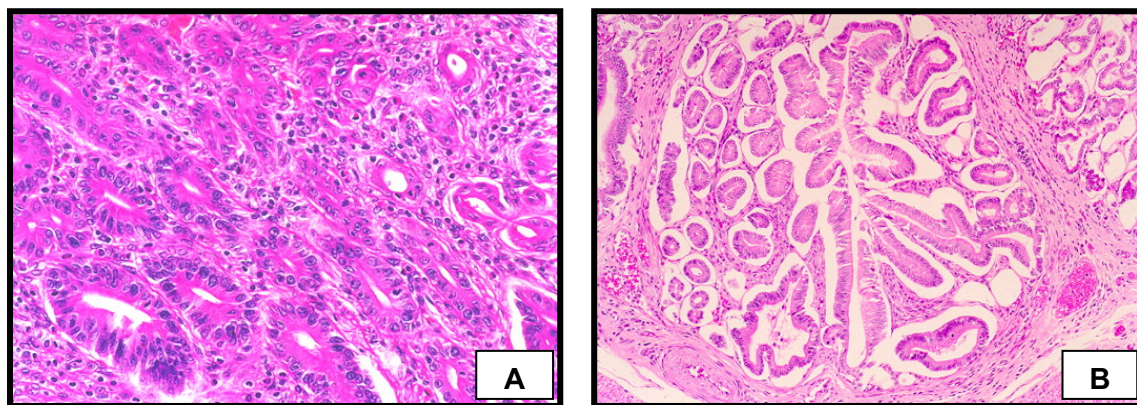
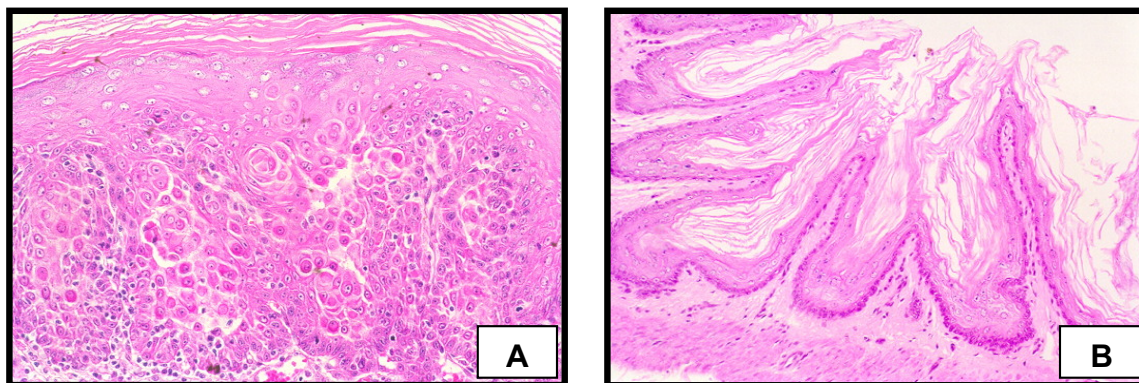


FIGURE 2 – Histology of the lesions in the glandular mucosa. **A)** Adenocarcinoma characterized by proliferation of glandular structures with cellular atypia. **B)** Adenomatous hyperplasia characterized by proliferation of glandular structures without cellular atypia

In the forestomach, distorted proliferation with cellular atypia characterized squamous cell carcinoma (Sca) (Figure 3A), and the epithelium's thickening two to threefold that of normal epithelium and papillomatosis characterized squamous hyperplasia (S.H.) (Figure 3B). The histopathological analysis was performed

without knowledge of the experimental groups. Comparisons between the groups were performed by the Chi-Square test. Differences were considered significant when  $p < 0.05$ . The study was approved by the Ethics Committee on care and use of laboratory animals of the National Research Council.



**FIGURE 3** - Histology of the lesions in the squamous mucosa. **A)** Squamous cell carcinoma characterized by distorted proliferation with cellular atypia. **B)** Squamous hyperplasia characterized by hyperkeratosis and papillomatosis

### Results

The animals submitted to MNNG+ Reflux (Group 1) developed macroscopic lesions in the antral mucosa, at the

gastrojejunal anastomosis and at the squamous portion of the stomach. The frequency of macroscopic lesions observed after 18 and 36 weeks of the surgical procedure is shown on Table 1.

**TABLE 1** - Incidence of macroscopic lesions in the antrum, at the gastrojejunal anastomosis and at the squamous portion of the stomach after 18 and 36 weeks

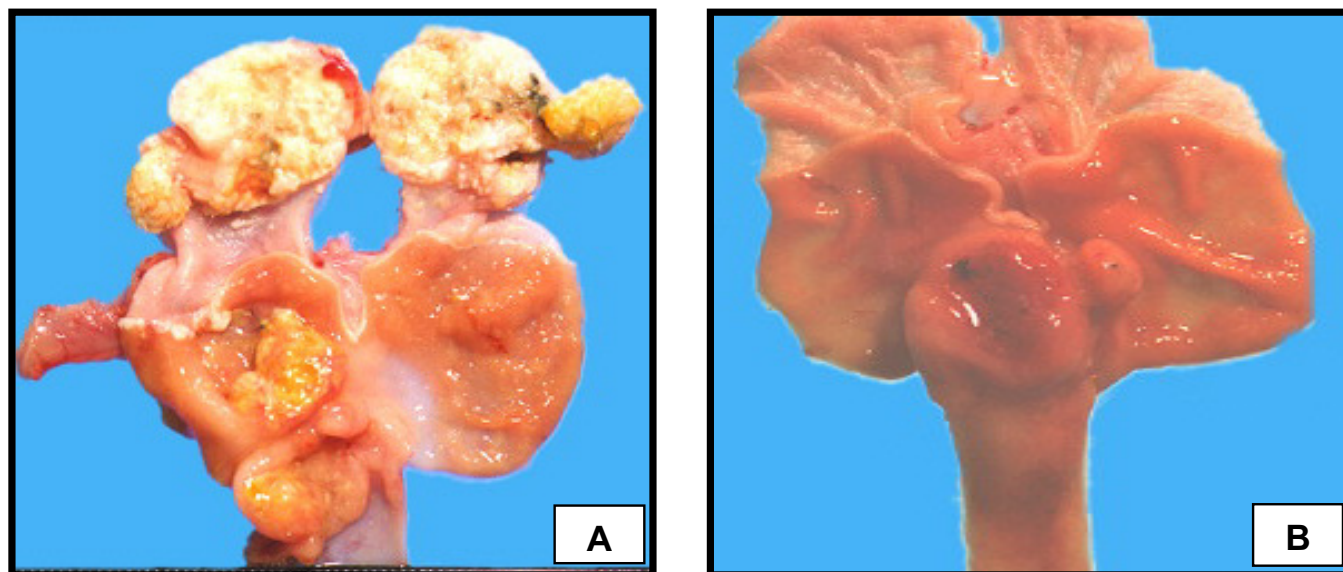
Groups	n	Antrum		Anastomosis		Squamous Stomach	
		18 weeks	36 weeks	18 weeks	36 weeks	18 weeks	36 weeks
1) MNNG + Reflux	20	11	14	3	6	16	16
2) Reflux	20	2	9	4	4	0	4
3) MNNG	20	1	1	-	-	0	0
4) Gastrotomy	20	0	0	-	-	0	0

In the antral region, the lesions were characterized by nodules and ulcers with elevated and indurated edges. At the gastrojejunal anastomosis, elevations with central depression were found on the suture line. In the squamous mucosa, small elevations were detected at the 18<sup>th</sup> week; confluent vegetating lesions with swollen aspect were observed after 36 weeks (Figure 4A).

The animals submitted to Reflux (Group 2) presented macroscopic lesions in the antral mucosa, at the gastrojejunal anastomosis and in the squamous portion of the stomach (Table 1). In the antral region, the lesions were “plaque-like”, indurated, with raised edges and fibrin on the bottom. The development of proliferative lesions were characterized by small elevations, and, on the squamous mucosa, by scattered elevations throughout the epithelium, some of which with central depressions. Such lesions were observed after 18 and 36 weeks. Table 2 shows the results of the histopathologic analysis.

Macroscopic lesions resulting from MNNG treatment (Group 3) were observed only in the antral mucosa (Table 1). They were located in the small curvature and were characterized by reddish nodules and ulcers with raised borders as shown in Figure 4B. The histological diagnosis of the lesions in this group is shown in Table 2.

No macroscopic or histological lesions were found in the gastric mucosa or at the squamous portion of the stomach in the group submitted to gastrotomy (Group 4).



**FIGURE 4** – Macroscopic appearance of the lesions at the 36 week. **A)** Vegetating lesions in the forestomach (G1: MNNG + Reflux). **B)** Ulcer with raised borders in the antral mucosa (G3: MNNG)

Table 2 presents the incidence and percentage of each histopathological diagnosis at 18 and 36 weeks in the 3 analyzed regions in all groups of animals.

**TABLE 2** - Incidence and percentage (%) of macroscopic lesions in the antrum, at the gastrojejunal anastomosis and at the squamous portion of the stomach after 18 and 36 weeks

Groups	n	Antrum				Anastomosis				Squamous Stomach			
		18 weeks		36 weeks		18 weeks		36 weeks		18 weeks		36 weeks	
		AH	Ca	AH	Ca	AH	Ca	AH	Ca	SH	Sca	SH	Sca
1) MNNG + Reflux	20	12 (60)	6 (30)	10 (50)	10 (50)	17 (85)	3 (15)	14 (70)	6 (30)	11 (55)	9 (45)	10 (50)	13 (65)
2) Reflux	20	4 (20)	0 (0)	10 (50)	0 (0)	0 (0)	0 (0)	17 (85)	0 (0)	0 (0)	0 (0)	12 (60)	0 (0)
3) MNNG	20	0 (0)	2 (10)	1 (5)	3 (15)	-	-	-	-	0 (0)	1 (5)	2 (10)	1 (5)
4) Gastrostomy	20	0 (0)	0 (0)	0 (0)	0 (0)	-	-	-	-	0 (0)	0 (0)	0 (0)	0 (0)

AH: Adenomatous Hiperplasia  
 SH: Squamous Hiperplasia  
 Ca: Adenocarcinoma  
 Sca: Squamous Carcinoma

## Discussion

In the present study the development of proliferative lesions in the upper gastrointestinal tract was evaluated using carcinogen treatment with MNNG and reflux of duodenal contents through the pylorus in an experimental model of carcinogenesis.

Our results showed that reflux of duodenal contents through the pylorus induced the development of benign proliferative lesions at both glandular mucosa and the squamous portion of the stomach. In the antrum, adenomatous hyperplasia was observed in 20% and 50% of the animals at the 18<sup>th</sup> and 36<sup>th</sup> weeks respectively. Additionally 85% of the animals presented adenomatous hyperplasia at the gastrojejunal anastomosis and 60% developed squamous hyperplasia at the squamous portion of the stomach. Hence, in this experimental model, the proliferative effects of reflux of duodenal contents could be observed at different sites of the gastric mucosa and it was related to duration of reflux. Additionally, the extent and intensity of reflux is relevant for the development of such benign proliferative lesions<sup>15</sup>. We have observed in a previous study that these benign proliferative lesions were smaller and less frequent when reflux to the stomach was induced through a gastrojejunal anastomosis<sup>15</sup>. It is possible that the gastrojejunal anastomosis may enable the passage of intestinal contents directly from the afferent loop, with little contact with the gastric mucosa.

Although the exact proliferative mechanisms of reflux to the gastric mucosa are not clear, one possibility is that epithelial proliferation may be mediated by peptides with growth-factor activity present in the biliopancreatic secretion<sup>15,16</sup>. The action of these factors may be related to the regulation of gene expression in the epithelial cells, leading to an increase in their proliferative activity.

In the animals receiving only MNNG (G3), we have found 15% of antral adenocarcinomas and 5% of squamous carcinomas at the 36<sup>th</sup> week. This finding highlights that the oncogenic effects of MNNG are not selective for the glandular mucosa of the stomach, as previously described<sup>17-19</sup>.

On the other hand, the combined effects of MNNG treatment with reflux (G1) enhanced the development of malignant tumors at both sites, since we have detected 30% of antral adenocarcinomas and 45% of squamous carcinomas at the 18<sup>th</sup> week and the frequency of these malignant tumors rose to 50% in the antrum and 65% in the squamous mucosa at the 36<sup>th</sup> week (Table 2). Our findings point out the role of reflux of duodenal contents on the development of tumors at the squamous mucosa and are in agreement with previous studies<sup>20,21</sup>.

In rodents, the squamous portion of the stomach is considered to be an anatomic extension of the esophagus<sup>20</sup>, although it is admitted that, in normal conditions, a certain type of anti-reflux barrier exists between the esophagus itself and the squamous portion of the stomach. The surgical procedure used in the present study allowed the prolonged exposure of the squamous mucosa of the stomach to the duodenal contents, thus leading to the development of proliferative lesions, benign and malignant, when combined with carcinogen treatment.

Therefore, the results of the present study disclose a useful protocol to investigate the potential role of reflux of gastroduodenal contents on the carcinogenic process at the squamous mucosa of the upper gastrointestinal tract.

## Conclusion

The reflux of duodenal contents through the pylorus enhanced the development of proliferative lesions, benign and malignant, in the glandular stomach and in the forestomach of rats.

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