Chemoprevention by celecoxib in reflux-induced gastric adenocarcinoma in Wistar rats that underwent gastrojejunostomy[1]

Quimioprevenção pelo celecoxibe no adenocarcinoma gástrico induzido por refluxo em ratos Wistar submetidos à gastrojejunostomia

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ABSTRACT

Purpose: To evaluate chemoprevention by celecoxib in cases of reflux-induced gastric adenocarcinoma, in Wistar rats that underwent gastrojejunostomy. **Methods**: Sixty male Wistar rats of average age three months underwent surgery and were distributed into three groups: group 1, exploratory laparotomy; group 2, gastrojejunostomy; and group 3, gastrojejunostomy and daily celecoxib administration. After 53 weeks, the animals were sacrificed. Changes in the mucosa of the gastric body of group 1 and in the gastrojejunal anastomosis of groups 2 and 3, observed in histopathological and immunohistochemical examinations, were compared. All statistical analyses were performed using Epi-Info®, version 3.4.3. **Results**: Comparison between groups 2 and 3 relative to the presence of adenocarcinoma showed a statistically significant difference (p=0.0023). Analysis of the association between groups 2 and 3 relative to COX-2 expression also showed a statistically significant difference (p=0.0018). **Conclusion**: Celecoxib had an inhibiting effect on gastric carcinogenesis induced by enterogastric reflux in an animal model.
Key words: Adenocarcinoma. Stomach. Chemoprevention. Cyclooxygenase inhibitors. Rats.

RESUMO

Objetivo: Avaliar a quimioprevenção pelo celecoxibe no adenocarcinoma gástrico induzido por refluxo, em ratos Wistar, submetidos a gastrojejunostomia. **Métodos**: Sessenta ratos machos Wistar, com média de idade de três meses foram operados e distribuídos em 03 grupos: Grupo 1 - Os animais foram submetidos a laparotomia exploradora. Grupo 2 - Os animais foram submetidos a gastrojejunostomia. Grupo 3 - Os animais foram submetidos a gastrojejunostomia e tomaram celecoxib, diariamente. Após um período de 53 semanas, os animais foram sacrificados. As alterações da mucosa do corpo gástrico dos animais do grupo 1 e da anastomose gastrojejunal dos animais dos grupos 2 e 3 foram analisadas no exame histopatológico e imuno-histoquímica e foram comparadas. Todas as análises estatísticas foram realizadas pelo programa Epi Info®, versão 3.4.3. **Resultados**: No cotejo entre os animais dos grupos 2 e 3 com relação à presença de adenocarcinoma observou-se uma diferença estatística significante (p=0,0023). A análise de associação entre os grupos 2 e 3 com relação à expressão da COX-2, também evidenciou uma diferença estatística significante (p=0,0018). **Conclusão**: O celecoxib teve efeito inibidor da carcinogênese gástrica, induzida pelo refluxo em ratos.
Descritores: Adenocarcinoma. Estômago. Quimioprevenção. Inibidores de ciclooxigenase. Ratos.

Introduction

Cyclooxygenase, also known as prostaglandin endoperoxide synthase, is the key enzyme needed for prostaglandin synthesis from arachidonic acid. Two types of cyclooxygenase have been identified: COX-1 and COX-2. In many situations, COX-1 enzyme is produced constitutively in the gastric mucosa, while there is high production of COX-2 at inflammation sites and in neoplastic tissue[1,2,3].

Over the last few years, celecoxib (a specific COX-2 inhibitor) has been used to reduce the number of polyps in a rat model for adenomatous polyposis and in cases of duodenal adenomatous polyposis in humans[4,5]. This drug is believed to act primarily by inhibiting the cyclooxygenase enzyme, with the advantages of having fewer gastric and renal side effects and not inhibiting platelet function.

Chemoprevention is taken to mean the use of drugs or natural agents with the purpose of preventing, inhibiting or to reversing the processes of carcinogenesis[6].

Some unanswered questions motivated us to evaluate chemoprevention by celecoxib in cases of reflux-induced gastric adenocarcinoma, in Wistar rats that were subjected to the carcinogenic model of gastrojejunostomy[7,8,9,10,11,12].

Methods

This study was approved by the Research Ethics Committees of the Postgraduate Surgery Programs of UNIFESP and UNCISAL.

Sixty male Wistar rats, of average age three months and average weight 300g and acclimatized to the conditions of the vivarium of the Foundation and University of Health Sciences of Alagoas (UNCISAL), underwent surgery with intra-abdominal anesthesia of ketamine hydrochloride (50 mg/ml), at a dosage of 0.2 ml/100g of the animal's weight. The animals were randomly divided into three groups: group 1, consisting of 10 animals that underwent explorative laparotomy; group 2, consisting of 25 animals that underwent gastrojejunostomy; and group 3, consisting of 25 animals that underwent gastrojejunostomy and received celecoxib orally (10 mg/kg/day). After a 53-week observation period, the animals were sacrificed and their stomachs were removed for macro and microscopic analysis.

Anatomopathological examination

1 – First stage (hematoxylin-eosin staining). All the surgical specimens were fixed in buffered formalin and the routine used in the pathology laboratory of Santa Casa de Misericórdia of Maceió, Alagoas, was followed. The mucosa of the gastric body of the group 1 animals and mucosa of the gastrojejunal anastomoses of the group 2 and 3 animals were evaluated and compared. The microscopic mucosal abnormalities found were chronic gastritis, foveolar hyperplasia, intestinal metaplasia, dysplasia and adenocarcinoma. Chronic gastritis was defined by the presence of mononuclear cells in the submucosa and lamina propria of the stomach. Foveolar hyperplasia was defined as stretching and twisting of the gastric foveolae. Intestinal metaplasia was recognized morphologically by the presence of goblet cells[13]. The definition and classification of dysplasia obeyed the criteria proposed by Ming *et al.*[14]. Adenocarcinoma was defined by the presence of atypical gastric glandules that invaded the submucosa, muscularis propria or serosa[15].

For the purposes of analyzing the occurrences of chronic gastritis, the foveolar hyperplasia and intestinal metaplasia were grouped and named inflammatory-metaplastic abnormalities.

2 – Second stage (immunohistochemistry). Representative blocks were selected from each case for immunohistochemical examination by means of the two-stage polymer technique, using the polyclonal antibody anti-COX-2/H62 (Santa Cruz Biotechnology Inc, USA), in accordance with Shi *et al.*[16]. The negative control was processed without the primary antibody and the positive control was obtained from cases of gastric adenocarcinoma that were already known to express COX-2.

Immunohistochemical evaluation

A Leitz optical microscope was used, with magnifications of 40x and 400x. The analysis was performed by two pathologists without previous knowledge of the study groups. COX-2 expression was deemed to be present when fine granulation of brown color was observed in the cytoplasm of the tumor cells[17].

Statistical methods

All the statistical analyses (frequencies of adenocarcinoma, dysplasia, inflammatory-metaplastic lesions and analyses of associations between groups 2 and 3) were performed using the Epi-Info® software, version 3.4.3. For the nullity hypothesis, p was set to be less than 5% or 0.05. An asterisk (*) was placed on the cases with statistical significance.

Results

Inflammatory-metaplastic lesions were observed in 12 cases (48%) among the group 2 animals and in 24 cases (96%) among the group 3 animals. Chronic gastritis, foveolar hyperplasia and intestinal metaplasia were included. No microscopic abnormalities of the gastric mucosa were observed in the group 1 animals.

A slight degree of dysplasia was observed in three cases (12%) among the group 2 animals. There was no dysplasia among the group 1 and 3 animals.

Adenocarcinoma was observed in 10 cases (40%) among the group 2 animals and in one case (4%) in group 3. All the tumors presented vegetative growth, of well-differentiated type, and had developed at the level of the gastrojejunal anastomosis (Figures 1 and 2).
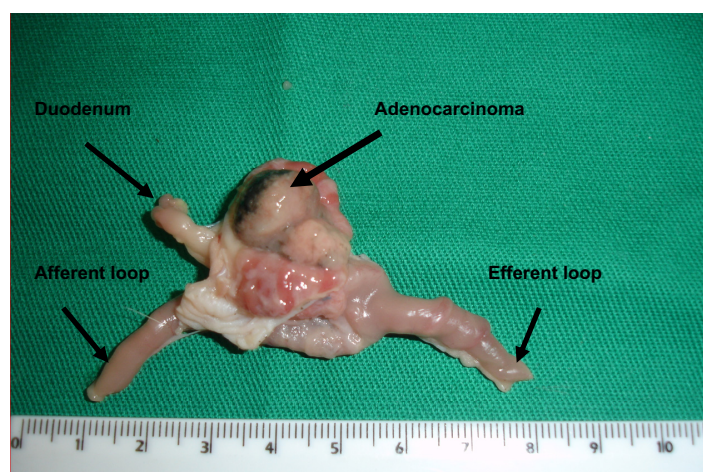

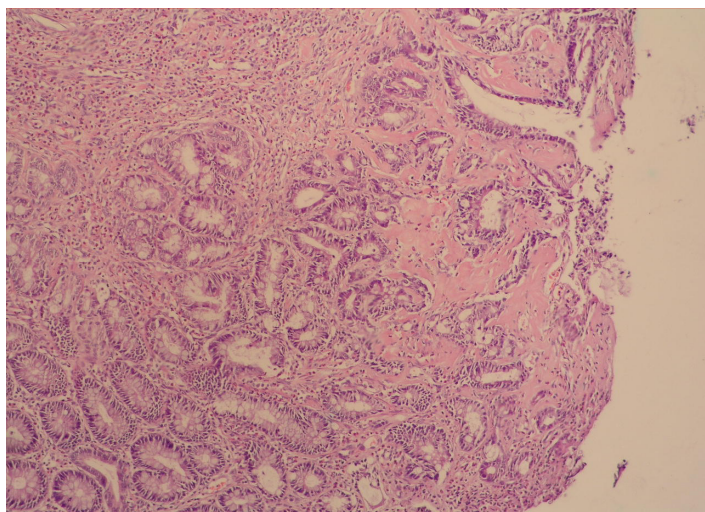**FIGURE 1** – Photograph of a stomach with adenocarcinoma and anatomical references

FIGURE 2 – Photomicrograph of a well-differentiated adenocarcinoma. Hematoxylin-eosin (40x)
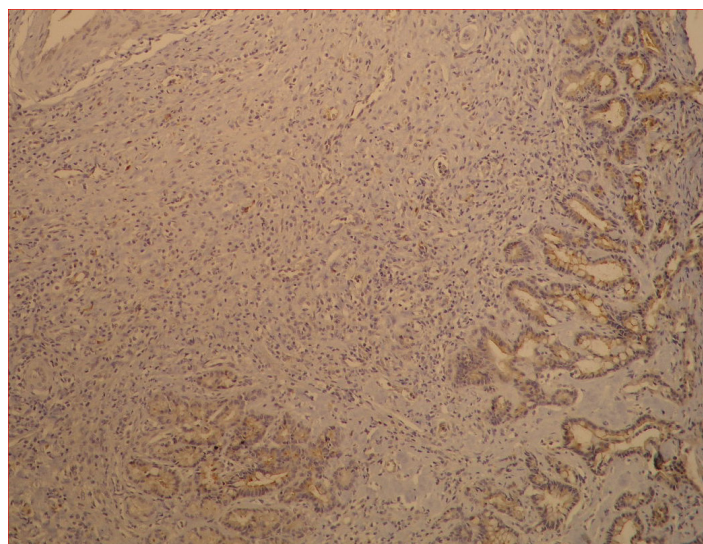

FIGURE 3 – Photomicrograph of a well differentiated adenocarcinoma. Fine brown-stained cytoplasmic pigmentation. Positive for COX-2 (two-stage polymer technique, 40x)

In this experiment, COX-2 expression was observed in four out of the 36 cases (11.1%) of inflammatory-metaplastic lesions, in one out of the three cases (33.3%) of dysplasia and in nine out of the eleven cases (81.8%) of adenocarcinoma (Figures 3 and 4).

Comparison between groups 2 and 3 in relation to the presence of adenocarcinoma showed a statistically significant difference in adenocarcinoma development: Fisher's exact test, p=0.0023*; Odds Ratio: 16 (1.85-137.97); RR: 10 (1.38-72.39) (Table 1).
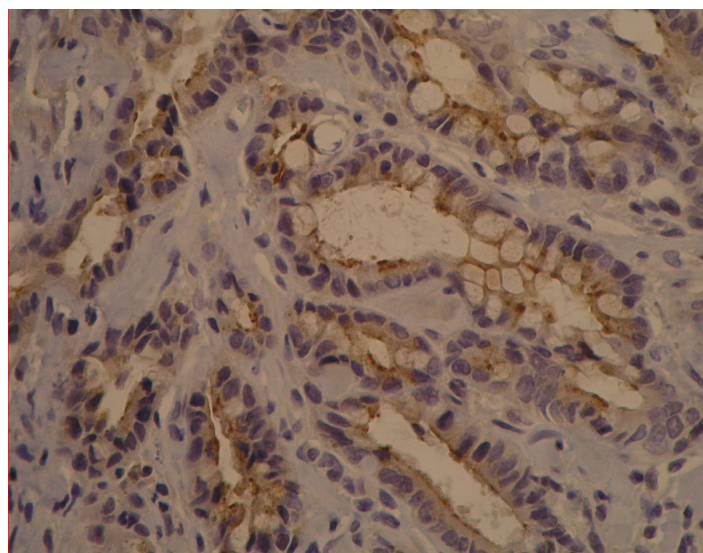

FIGURE 4 – Photomicrograph of a well-differentiated adenocarcinoma. Fine brown-stained cytoplasmic pigmentation. Positive for COX-2 (two-staged polymer technique, 400x)

TABLE 1 – Analysis of the association between groups 2 and 3 in relation to the presence of gastric adenocarcinoma

| GROUP | Cancer | | | | TOTAL |
| | Yes | | No | | |
	Frequency	%	Frequency	%	
Group 2	10	40	15	60	25
Group 3	1	4	24	96	25
TOTAL	11	22	39	78	50

Fisher's exact test, p= 0.0023*; Odds Ratio: 16 (1.85 – 137.97); RR: 10 (1.38 – 72.39)

Analysis of the association between groups 2 and 3 in relation to COX-2 expression showed a statistically significant difference: Fisher's exact test, p=0.0018*; Odds Ratio: 10.10 (2.05 – 54.95); RR: 6.00 (1.49 – 24.10) (Table 2).

TABLE 2 – Analysis of the association between groups 2 and 3 in relation to COX-2 expression

| GROUP | COX-2 | | | | TOTAL |
| | Yes | | No | | |
	Frequency	%	Frequency	%	
Group 2	12	48	13	52	25
Group 3	2	8	23	92	25
TOTAL	14	28	36	72	50

Fisher's exact test, p=0.0018*; Odds Ratio: 10.10 (2.05 - 54.95); RR: 6.00 (1.49 – 24.10)

Analysis of the association between COX-2 expression and gastric adenocarcinoma in groups 2 and 3 showed a statistically significant difference between the adenocarcinoma that expressed COX-2 and the adenocarcinoma that did not express it: Fisher's exact test, p=0.000034*; Odds Ratio: 27.29 (4.99 – 230.94); RR: 11.57 (2.84 – 47.03) (Table 3).

TABLE 3 – Analysis of the association between COX-2 expression and gastric adenocarcinoma in groups 2 and 3

| COX-2 | Cancer | | | | TOTAL |
| | Yes | | No | | |
	Frequency	%	Frequency	%	
Yes	9	64.3	5	35.7	14
No	2	5.6	34	94.4	36
TOTAL	11	22	39	78	50

Fisher's exact test, p=0.000034*; Odds Ratio: 27.29 (4.99 – 230.94); RR: 11.57 (2.84 – 47.03)

Discussion

In studies on gastric carcinogenesis, rats have often been used to evaluate lesions induced by carcinogens[8,9], enterogastric reflux[10,11], *Helicobacter pylori* infection[12] or associations between these agents[8,9,12]. An enterogastric reflux model was used in this experiment, without gastric resection, since this is less traumatic and easy to reproduce. After a 53-week period, the animals were sacrificed and their stomachs were analyzed. The macroscopic and microscopic abnormalities observed were most evident at the level of the gastrojejunal anastomosis, and the histological analyses were therefore focused on that region.

Adenocarcinoma was observed in 10 cases (40%) among the group 2 animals and in one case (4%) among the group 3 animals. There were no cases among the group 1 animals. All the tumors presented vegetative growth, of well-differentiated type, and they developed at the level of the gastrojejunal mucosa, close to the afferent jejunal loop. No cases of metastatic dissemination to the liver or abdominal cavity were observed. The incidence of adenocarcinoma in models for reflux-induced gastric carcinogenesis has ranged from 7 to 70.8% (mean of 31%) among the various authors[8,9,10,11,18]. The reasons for this diversity of results may be due to differences in the intensity of the reflux provided by the techniques used, in exposure duration and in the susceptibility of the rat lineages used. By choosing gastrojejunostomy as the model for enterogastric reflux in cases of gastric carcinogenesis, the multiple stages of this chain of events from chronic gastritis to intestinal metaplasia, dysplasia and, finally, adenocarcinoma were demonstrated.

COX-2 expression was not detected in the normal gastric mucosa in this study, using optical microscopy. However, using electron microscopy, another study has already reported detection of immunoreactivity of mucosal cell cytoplasm in the fundus and pylorus regions of rats[19]. Other researchers, investigating COX-2 expression in gastrointestinal tract tissues of rats, dogs, monkeys and humans, by means of immunoblot and biochemical techniques, have found variations in its expression between these segments, along with differences between the species. COX-2 expression was

shown in only three macrophages out of 22 rats examined[20]. In the present study, it was observed in one out of the three cases of dysplasia (33.3%) among the group 2 animals. With regard to adenocarcinoma, expression was observed in nine out of eleven cases (81.8%), in the tumor cell cytoplasm. These results, relating to COX-2 expression in gastric adenocarcinoma in rats, have never been published previously in the literature. In clinical studies, COX-2 was expressed in 51 to 76% (mean of 73%) of gastric tumors according to Northern blot or RT-PCR, in 67 to 83% (mean of 73%) according to Western blot and in 43 to 100% (mean of 62%) according to immunohistochemistry[21]. The great variability in the expression of this protein in literature can be explained by the use of many types of antibodies, the differences in the methods used for quantifying reaction positivity and the way in which the specimens were fixed. Because this is an immunological reaction, antigen recovery depends on the state of conservation of the paraffin blocks and the adequacy of specimen fixing in buffered formalin. Use of inadequate solutions of formalin may block the protein binding sites on the antibody, thereby not allowing antigen recovery.

Comparing groups 2 and 3 in relation to the presence of adenocarcinoma, a statistically significant difference between the animals in these groups was observed with regard to cancer development, such that the group 3 animals were protected from having cancer through the use of celecoxib (p=0.0023). It was also observed, in analyzing the association between groups 2 and 3 in relation to COX-2 expression, that the group 2 animals had statistically significant greater COX-2 expression than seen in the group 3 animals (p=0.0018). Analysis of the association between COX-2 expression and adenocarcinoma in groups 2 and 3, a statistically significant difference was observed between the adenocarcinomas that expressed COX-2 and those that did not express it (p=0.000034). Some other studies have proved that the specific COX-2 inhibitor (celecoxib) and sulindac have a chemopreventive effect in animal models that have been inoculated with human gastric cancer cells (xenografts)[22,23]. In another study, celecoxib and indomethacin were also effective in inhibiting the growth of lineages of human gastric cancer cells (AGS and MKN), through inducing apoptosis and stopping the cell cycle and not by suppressing COX-2 and prostaglandin E_2[24]. In a model for gastric carcinogenesis using N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), celecoxib and indomethacin were observed to provide chemoprevention by a mechanism independent of COX-2[25]. A complementary study comparing the kinetic cell abnormalities in the gastric mucosa of rats treated with celecoxib at the dosages of 5, 10 and 20 mg/kg/day and indomethacin found that treatment with both drugs inhibited the proliferation of the gastric tumor in over 65% of the cases (p<0.02). Celecoxib induced increased apoptosis at a dependent dosage (p<0.05), and was most evident at the dosage of 10 mg/kg/day26.

In our experiment, celecoxib was observed to be effective for chemoprevention against carcinogenesis induced by enterogastric reflux. The mechanisms responsible for this chemopreventive action are not well known. However, it is known that the main mechanism for the action of this drug as an anti-inflammatory and analgesic agent would be through inhibition of cyclooxygenase-2, thereby blocking the synthesis of prostaglandins from arachidonic acid. There is evidence that chemopreventive mechanisms may function through inhibition of angiogenesis either via a COX-dependent route or not. Other mechanisms could arise through induction of tumor cell apoptosis or inhibition of cell proliferation. We believe that the chemoprevention mechanism of this drug may result from the sum of the effects already mentioned.

Conclusion

Celecoxib had an inhibiting effect on reflux-induced gastric carcinogenesis in rats.

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