Apoptosis and proliferation in gastric epithelium due to *Helicobacter pylori* : An immunohistochemical and ultrastructural study

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Abstract

The effect of H. pylori infection on gastric epithelial cell apoptosis and proliferation is contradictory. Using immunohistochemistry and electron microscopy, this study sought to demonstrate gastric epithelial changes (ie, apoptosis and proliferation) due to chronic H. pylori infection.

Methods : **Eighteen female 6- to 8-week old Swiss Albino mice were inoculated intragastrically with 3 doses of 10^9 CFU/mL H. pylori prepared in a Brucella Broth in 5 days. Nine others served as a control group. At the end of 28 weeks, tissue specimens from the gastric antrum were excised and examined immunohistochemically (epithelial growth factor for regeneration and Caspase-3 for apoptosis) and electron microscopically. Immunohistochemical assessment was performed using the indirect peroxidase-antiperoxidase method.**

Results : **In the H. pylori-infected group, EGF staining in gastric epithelium was found to be decreased significantly compared to that in control group ($P < 0.001$). Caspase-3 reactivity was commonly observed in surface epithelial cells and glandular epithelial cells in H. pylori-infected group and totally it was statistically significant compared to Caspase-3 staining in control group ($P < 0.001$). Electron micrograph images demonstrated numerous apoptotic cells with condensed chromatin.**

Conclusion : **Chronic H. pylori infection of 28 weeks' duration increases apoptosis in gastric epithelium ; however, increased apoptosis does not induce proliferation.** (Acta gastroenterol. belg., **2006**, 69, **191-196**).

Key words : Helicobacter pylori, gastric epithelium, immunohistochemistry, electron microscopy.

Introduction

Since the first report (1) of its isolation in 1983, Helicobacter pylori (H.*pylori*) has been accepted as an important human pathogen for the development of gastritis, gastroduodenal ulcer, and gastric cancer (2). How this organism interacts with the gastric epithelium to cause these diseases remains unclear (3).

In 1994, a working group of the WHO International Agency for Research on Cancer concluded that H. *pylori* is a group I carcinogen in humans and plays a causal role in the development of gastric cancer. Gastric mucosal integrity is maintained by a balance between the rate of epithelial cell regeneration and the rate of epithelial cell loss. H. pylori infects the gastric mucosal layer in close proximity to gastric epithelial cells, but does not penetrate the epithelium (4). Bacterial adherence to the gastric epithelium has been proposed as an important colonization/virulence factor, but whether H. pylori affects the balance between epithelial cell proliferation and cell loss is unclear. Increased epithelial proliferation in H. *pylori*-induced gastritis has been reported (5,6). Also, there are some studies that propose that H. *pylori* infection occurs with increased apoptosis (7,8). Cell proliferation and apoptosis are essential events in the cellular turnover of gastric tissue (9). Programmed cell death, or apoptosis, is a distinct form of cell death that can be distinguished morphologically by the condensation and margination of nuclear chromatin (10). There are some studies that describe the effects of H. *pylori* on gastric epithelium ; however, there are contradictory results about whether H. *pylori* increases apoptosis and proliferation in gastric epithelium.

The aim of this study was to show apoptotic and proliferative changes in gastric epithelium in experimental H. *pylori* infection via immunohistochemistry and electron microscopy.

Material and methods

Animals

Six- to 8-month-old female Swiss albino mice weighing 20-25 g were obtained and housed in Başkent University, Medical and Surgical Experimental Research Center (temperature 20 ± 2°C, humidity 50% ± 10%, using a 12-h light-dark cycle). Mice were supplied with standard laboratory diet and tap water *ad libitum*. All experimental procedures involving animals were approved by the ethical animal committee of Başkent University, Faculty of Medicine, Ankara, Turkey.

Preparation of animals for assays

Mice were separated into two groups : the first group (n = 18) was composed of mice with chronic H. *pylori* infection ; the second group (n = 9) served as a control. The first group was inoculated with H. *pylori* as described below. Saline was administered to the control group in the same manner. Mice were observed for 28 weeks before removal of the gastric antrum for

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evaluation of bacterial colonization, and immunohistochemistry and electron microscopy. At the completion of the experimental protocol, all mice were killed.

Infection of mice

The H. *pylori* strain NTCC 11637 was cultured in a microaerophilic environment (CampyPack Plus, Becton-Dickinson, Cockesville, Md, USA) on Brucella agar supplemented with 5% sheep blood for 3 days at 37°C. H. pylori were suspended in Brucella Broth supplemented with 2.5% fetal calf serum. The final concentration was adjusted to 10^9 bacteria/mL. Mice were inoculated intragastrically for a 3-day period with 0.1 mL bacterial suspension via a polyethylene stomach catheter. For the control group, only saline solution was administered.

Detection of H. pylori colonization

Tissue specimens were homogenized in 0.5 mL Brucella Broth. Ten-fold dilutions were prepared, and 100 mL of each dilution was placed on Brucella agar with 5% sheep blood supplemented with H. *pylori*-selective supplement (Oxoid, Hampshire, England). Results were obtained after 3-7 days of incubation under microaerophilic conditions. Also Gram and Hematoxylin-Eosin stain preparations were examined under light microscopy.

Immunohistochemistry

Each animal's gastric antrum was fixed in buffered formalin and embedded in paraffin. Sections 4-5 µm thick were cut and placed onto Polysine slides and stained using the peroxidase-antiperoxidase immunohistochemical method. EGF for regeneration and Caspase-3 for apoptosis were used as primary antibodies. Phosphate Buffered Saline (PBS) (pH = 7.4) was used as buffer. Endogen peroxidase was quenched with 3% hydrogen peroxide (Lab Vision Corp/Neomarkers, Fremont, Calif, USA), washed in PBS, and incubated with normal goat Ig (Lab Vision). Sections were incubated with primary antibodies (EGF-R (NeoMarker, anti-rabbit Ig) and Caspase-3 (NeoMarker, rabbit Ig)) for 60 minutes, and then treated with a biotinylated antibody for 20 minutes followed by streptavidin horseradish peroxidase (Lab Vision, USA). The peroxidase complexes were visualized using DAB (Lab Vision, USA) for EGF-R and AEC (Lab Vision, USA) for Caspase-3. Mayer's hematoxylin was used as the background stain.

Surface, neck, and basal regions were separated as Jiang *et al.* (11) described. Surface epithelium, gland neck region and basal region were scored according to staining intensity.

Staining intensity was ranked using (-) for negative, (-/+) for negative to weak, positive staining, (+) for weak positive staining, (+/++) for weak to moderate positive staining, (++) for moderate positive staining, (++/+++) for moderate to strong positive staining and (+++) for strong positive staining. Points from 0 to 6 were given to increasing densities, respectively. Scores of three regions in each group were summed and a total staining score for each group was calculated.

First comparison were made between total staining scores of the control group EGF and Helicobacter pylori-infected group EGF, second between those of control group Caspase-3 and Helicobacter pylori-infected group caspase. The results of those two comparisons were evaluated statistically.

Electron microscopy

All tissues were fixed in 0.1 M phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde for 2 hours. Samples were then postfixed in 1% osmium tetroxide for 1 hour and dehydrated in a series of graded alcohols. Propylene-oxide–treated specimens were embedded in Araldyte CY 212 according to the manufacturer's protocol. Semithin sections, which had been stained with toluidine blue, were analyzed under a light microscope. Uranyl acetate-lead citrate-stained ultrathin sections were prepared, and transmission electron microscopy was performed using a LEO 906E electron microscope (Leo Company, Germany).

Statistical analysis

Data were analyzed using Mann-Whitney U test. Results were shown in graphical displays and expressed as median and quartiles (M, 25^{th}-75^{th}). *P* values less than 0.05 were considered statistically significant. Statistical analyses were performed using SPSS 13.0 software (Statistical Package for the Social Sciences, version 13.0, SSPS Inc, Chicago, Ill, USA).

Results

Immunohistochemical detection of Caspase-3 and EGF-R

In animals in the control group, a partial EGF reaction of surface epithelium was seen, and there was some weak reactivity. Dense EGF staining was observed in glandular epithelium. Staining was dense, especially in the neck regions. Immunoreactivity was mild to strong in the basal regions (Fig. 1 a-b).

In mice in the chronic H. pylori-infected group, EGF reactivity was generally poor and partially negative. Staining was poor to mild in the gastric glands. There was a mild EGF-R reaction in the neck glands and poor reaction in the basal regions (Fig. 1 c-d).

Reaction to Caspase-3 was generally poor in the control group. In surface epithelium, immunoreactivity rates changed from negative to poor. In the neck and basal regions of the gastric glands, reactivity was poor to mild (Fig. 2 a-b).

In the infected group, reactivity to Caspase-3 was poor to mild in surface epithelium ; however, it was strong in the neck and deep parts of glands (Fig. 2 c-d).

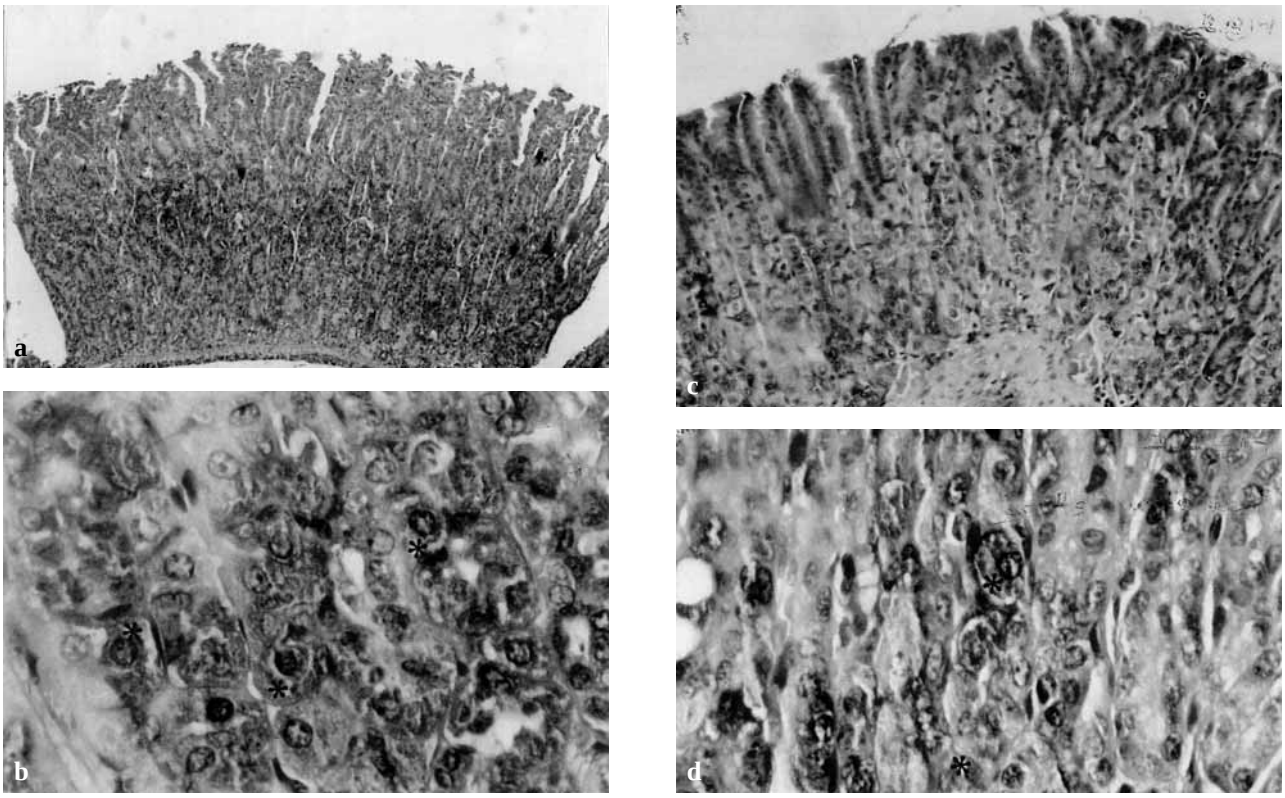

Fig. 1. — EGF-R immunohistochemical staining of animals in control (a-b) and chronic H. pylori-infected (c-d) groups. (a) EGF-R immunoreactivity in the control group is especially apparent in the glandular neck and basal regions. (b) At higher magnifications, strong reactivity (✱) is seen (c). In the chronic H. *pylori*-infected group, EGF-R immunoreactivity is generally poor and is seen at few sites. (d) At higher magnification, reactivity strength ranges from mild to poor (✱) (Immunoperoxidase – Hematoxylin, a-40X, b-400X, c-100X, d-400X).

EM findings

Apoptotic changes were seen in the gastric epithelium of mice in the H. pylori-infected group. The nuclei of the apoptotic cells showed electron-dense chromatin along the inner surface of the nuclear membrane, with deeply stained patches of chromatin condensation (Fig. 3).

Statistical findings

Total Caspase-3 immunreactivity scores of the control and H. *pylori* groups were compared in Figure 4A. Significant differences were found between the total scores of the groups ($P < 0.01$).

Total EGF immunreactivity scores of the control and H. *pylori* groups were compared in Figure 4B. Significant differences were found between the total scores of the groups ($P < 0.01$).

Statistical results for group comparisons were shown in Table 1.

Discussion

The present study demonstrated the effects of H. *Pylori* infeciton on apoptosis and proliferation in gastric epithelium. The most significant findings were the strong EGF reactivity in neck and basal segments of the glands of the control group ; strong Caspase-3 reactivity in neck and basal segments of the glands of the H. *Pylori* group.

Gastric epithelial turnover is a dynamic process. It is characterized by continous cell proliferation, which is counterbalanced by continous cell loss. The biological principle that mediates the homeostasis of epithelium is programmed cell death, or apoptosis (12). Short-term upregulation of apoptosis that is not accompanied by a matched increase in cell proliferation could result in cell loss and thereby might cause mucosal damage. On the other hand, long-term increases in the apoptotic rate could be a stimulus for a lasting increase in cell proliferation ; this hyperproliferation could promote the development of neoplasia (13). So any effect disturbing this balance will cause serious health problems.

H. *pylori* infection of the gastric mucosa is primarily responsible for gastritis, and has been reported to be implicated in various gastrointestinal diseases, such as gastric ulcer, adenocarcinoma and lymphoproliferative disorders (14-17).

H. *pylori* infection increases both apoptosis and proliferation, implying increased epithelial cell turnover

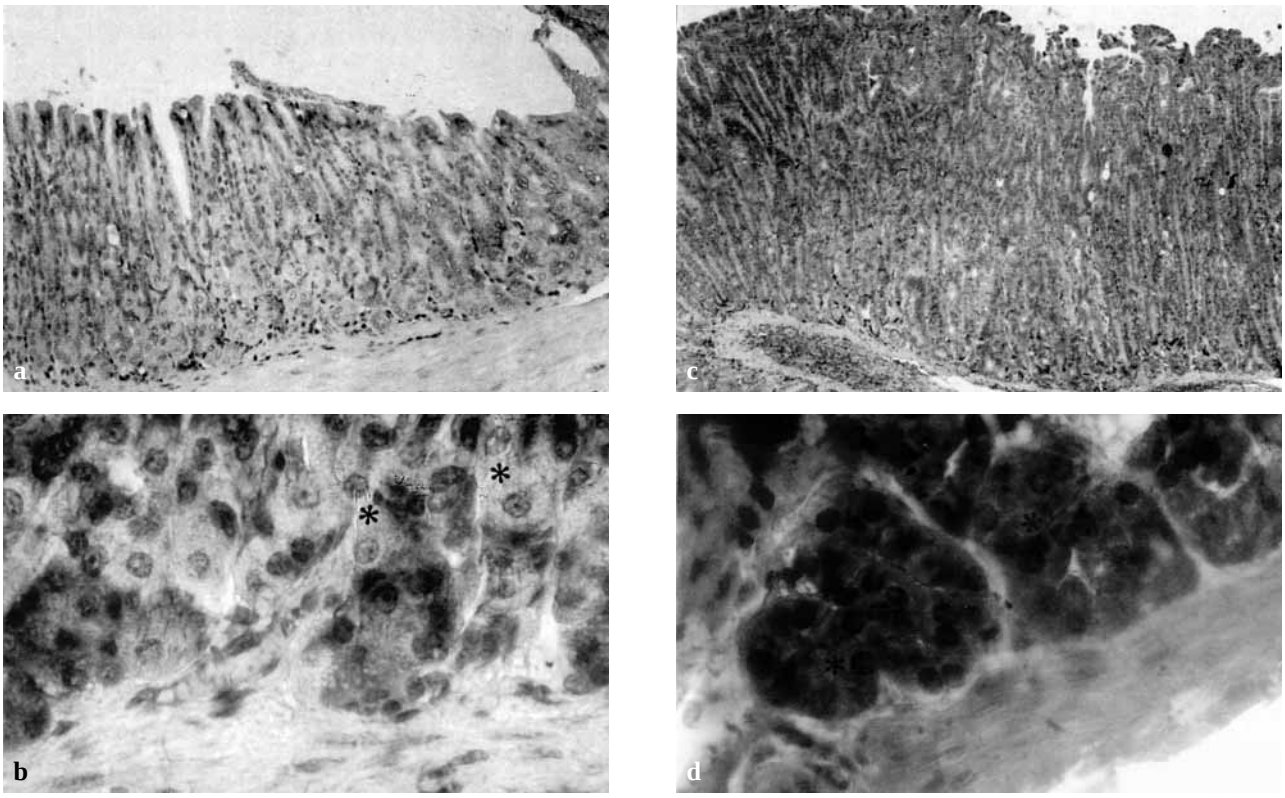

Fig. 2. — Caspase-3 immunohistochemical staining of control (a-b) and chronic H. pylori-infected (c-d) groups. (a) Immunoreactivity in gastric glandular epithelium of the control group is poor to mild. (b) At higher magnification, poor reactivity in glandular epithelium is seen (✱). (c) In the chronic H. *pylori*-infected group, strong Caspase-3 immunoreactivity is apparent in glandular neck and basal regions. (d) At higher magnification, glandular cells showing strong immunoreactivity are seen (✱). (Immunoperoxidase – Hematoxylin, a-40X, b-400X, c-40X, d-400X).

(19,20). In the present study, apoptosis was observed significantly in the antral glandular epithelium in H. *pylori* infected group. But there was weak EGF reactivity in glandular epithelum in antrum in H. *pylori* infected group. Lynch et al reported mild increase of gastric epithelial proliferation as a common feature in H. *pylori*-induced gastritis (21). Also several studies demonstrated increases in epithelial cell proliferation and apoptosis in H. *pylori*-induced gastritis in human and animal models (22-25). The weakness of the EGF reactivity in H. *pylori*-induced gastritis demonstrated in this study may arise from the animals we used. We have performed our experiments on mice and the gastritis induced in mice appears less intense than in humans (26,27) or is restricted to the use of highly selected mouse-adapted strains (28). However studies with gerbils were found to give more identical results to those with humans consistent with the results of some studies. (22,29-31) Recent studies indicated that short-term inection in Mongolian gerbils is a suitable model for evaluating the effects of H.*pylori* virulence factors. In those studies, apoptosis in the glandular epithelial cells and epithelial cell proliferation of H.*pylori* infected gerbils was significantly higher than those of uninfected controls (32).

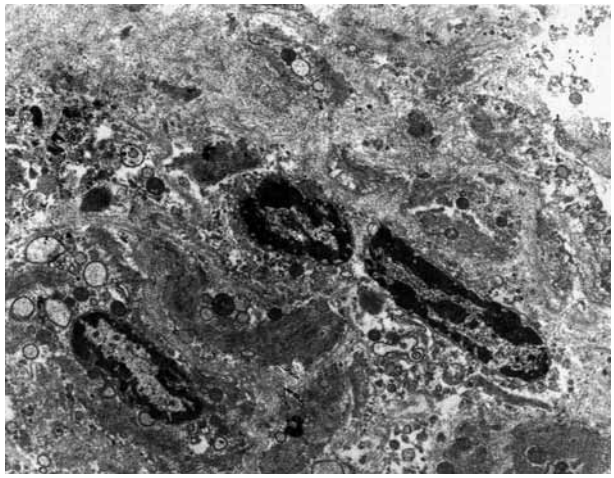

Fig. 3. — Transmission electron microscopy demonstrating apoptotic cells in gastric epithelium in the chronic H. *pylori*-infected group (Uranyl acetate-lead citrate, 4646X).

No increase in epithelial cell proliferation in uninfected mice were reported by Court *et al.* (22) and Suzuki *et al.* (25). In contrast, the present study describes weak EGF reaction in surface epithelial cells

Table 1. — **Statistical results for group comparisons**

	n	M, 25th-75th	Z	p
Control Caspase-3	9	4.00, 2.50-4.50	-4.255	0.0001
H. pylori Caspase-3	18	10.50, 8.00-11.00		
Control EGF	9	7.00, 6.50-8.50	-4.328	0.0001
H. pylori EGF	18	3.00, 1.75-3.00		

and strong reactivity in neck and basal segments of gastric glands. This can possibly be explained by our 28 weeks of experimental period compared to 8 weeks of Court's and 18 monts of Suzuki's study.

Regulation of apoptosis is complex and occurs from both outside and inside the cell (33). Extracellular signals can either supress or activate apoptosis. Apoptosis suppressing molecules are survival factors like growth factors. Apoptosis activating factors are death-inducing molecules like TGF-α or related peptides, CD95 ligand, or tumor necrosis factor alpha (TNF α) and related molecules. These signals act via binding to specific receptors on cell surface. When apoptosis is induced by either intracellular or extracellular pathways, specific intracellular proteases, termed caspases become activated. They are capable of systematic destruction of the cell. Till now, the caspase family were found to be consisted of 10 different members (34).

A major class of intracellular regulators of apoptosis is the Bcl-2 protein family. The Bcl-2 protein family encompasses several proteins. Some activates apoptosis while some suppress via caspase activation or inhibition (35).

A biochemical hallmark of apoptotic cell death is nonrandom degradation of DNA. It is also the key to morphological studies of apoptosis. In the present study, the nuclei of the apoptotic cells showed electron-dense chromatin along the inner surface of the nuclear membrane, with deeply stained patches of chromatin condensation as previously described in a study by Yagmurdur *et al.* (36). Specific labeling of nuclear fragments let us visualize the apoptotic cells (37).

In any subsite of the stomach, the proliferating stem cells are located in the neck of gastric glands, and epithelial migration occurs bidirectionally. By means of bromodeoxyuridine (BrdU) labelling, 4,8% of epithelial cells in the antrum were found to be in the DNA synthesis phase of the cell cycle (38). Gastric epithelial renewal is influenced by various physiologic stimuli. In the antrum epithelial proliferation is hardly influenced by gastrin. So inconsistent results can based on any unidentified physiologic conditions.

In conclusion, according to the data presented in this study, H.*pylori* infection is likely to cause apoptosis more than epithelial proliferation. But detailed studies with more sensitive methods are needed.

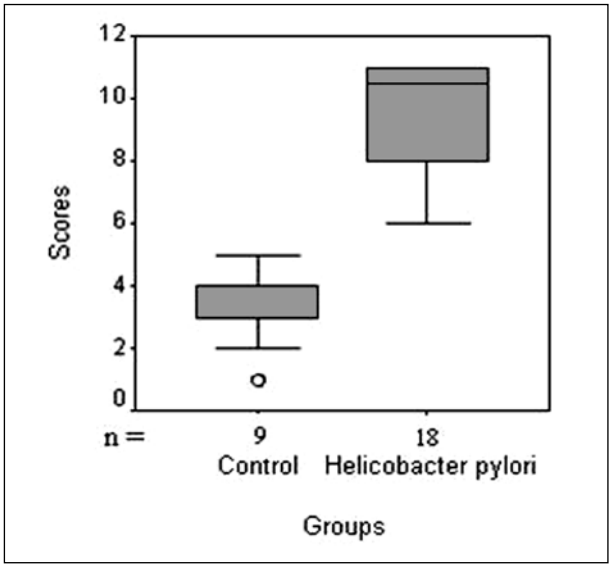

Fig. 4a. — Comparison of the total Caspase-3 immunreactivity scores of the control and *Helicobacter pylori* group.

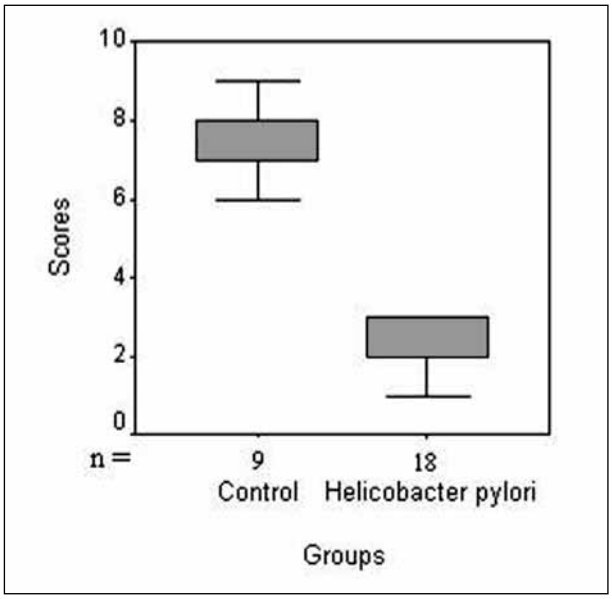

Fig. 4b. — Comparison of the total EGF immunreactivity scores of the control and *Helicobacter pylori* group.

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