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RESEARCH ARTICLE

HIGH DEGREE OF GASTRIC MUCOSA COLONIZATION WITH COCCOID FORMS OF *HELICOBACTER* REDUCE THE EFFICIENCY OF ITS ERADICATION

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ABSTRACT

Helicobacter pylori (HP) eradication therapy do not always achieve eradication. One of the probable reasons for the ineffectiveness of eradication treatment is bacillary-coccoid transformation of HP. We present that in cases when HP eradication was not achieved after the first line eradication therapy, patients usually had had a high degree of gastric mucosa colonization with coccoid forms of HP before the start of *H. pylori* eradication therapy. So, we confirmed the hypothesis of helicobacter coccoid forms can avoid eradication.

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INTRODUCTION

It is reasonable to suppose thatstandard courses of HP eradication therapy approved by Maastricht Consensus do not always achieve Helicobacter pylori eradication (Kim, 2014) The widespread use of anti-*Helicobacter pylori* eradication therapy has led to remarkable advances in the treatment of acid-related diseases. However, there is still no evidence that patients with high gastric mucosa colonization with coccoid forms of HP are significantly less amenable to H. pylori eradication therapy. This report presents our own data demonstrating that, in patients for whom HP eradication was not achieved after standardized therapy there was often a high degree of pre-therapy gastric mucosa colonization with coccoid forms of HP.

MATERIALS AND METHODS

Patients and eradication

Our study was performed in a group comprising of 54 patients (32 men and 22 women aged 35 to 65) with newly diagnosed chronic HP-associated gastritis (ICD-10K29.3) after having completed one course of prior eradication therapy for H. pylori. Also, all these patients were tested for HP coccoids before eradication therapy. All 54 patients were treated with standard 7-day course of clarithromycin 500 mg *b.i.d.*, amoxicillin 500 mg *f.i.d.* and omeprazole 20 mg *b.i.d.*, according to Maastricht 2-2000 recommendations.

Inclusion criteria

- Men and women older than 18 years;
- Histopathological diagnosis of chronic HP-associated gastritis with low grade inflammation
- Prior treatment with first line HP eradication therapy 2-3 months previously (Maastricht 2-2000)
- Helicobacter detection including coccoid HP concentration estimation using immunocyto chemistry

Exclusion criteria

- PPI and antibiotic therapy completed over 6 months ago (?)
- active infection
- erosive and ulcerative changes in the gastric mucosa
- All patients provided written informed consent.

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Immunocytochemical method for detecting coccoid forms of HP

Helicobacter in the gastric mucosa was examined in stamp-smears of the antral biopsy specimens obtained during fiber optic upper GI endoscopy. Cytologic smears were fixated with a mixture of 1:1 alcohol-acetone for 10 minutes, air dried, and then endogenous peroxidase was inactivated in a 1% sodium azide solution (Merck) for 30 min. The samples were washed twice with double-distilled water and immersed for 5 minutes in Tris-NaCl buffer (pH 7,6). Before the application of the pre-immune swine serum (Novocastra), the area for immunocytochemistry was localized with hydrophobic barrier pen (DakoCytomation). After incubation with a preimmune serum (30 min at room temperature), rabbit polyclonal antibodies (NCL-HPp, Novocastra) against cell wall antigens of Helicobacter pylori (1:100) were applied, and these preparations were then incubated for one hour at +370C. Upon the completion of the first antibody labeling, the preparations were put into two changes of buffer for 5 min each time, and then anti-rabbit swine biotinylated antibodies (DakoCytomation) were applied.

After the second antibody was applied, the preparations were incubated for 30 minutes at room temperature. The next step in the immunocytochemical procedure, preceded by washing preparations in two changes of buffer, was the application of an imaging system made of a soluble complex of avidin and biotinylated horseradish peroxidase (DakoCytomation) for 30 minutes at room temperature. The substrate used for the immunocytochemical reaction was 3.3'-diaminobenzidine (DAB) (Novocastra). The samples were tinted with hematoxylin for contrast. It should be noted that the results of H. pylori infection immunocytochemical verification obtained with the use of polyclonal rabbit antibodies from DakoCytomation as the first antibody in the process completely matched the results in cases when the first antibodies used were rabbit polyclonal antibodies from Novocastra. As a positive control we used histological sections of biopsies infected with HP, as recommended by the manufacturer of antibodies. Microscopic examination of the stained cytological slides was effected with the use of immersion objective (x100) on a Leica DM 4000 B microscope.

The degree of colonization of the gastric mucosa with coccoid forms of HP was assessed against a semi-quantitative scale that we developed (KravtsovVIu, 2006)

- **(-)** coccoid forms of HP are not present (coccoid forms of HP are not present in either of tested fields)
- **(+)** single coccoid forms are present (A coccoid bacterial cell is detected in at least one of the fields tested)
- **(++)** HP coccoid cells are present (HP coccoid cells are detected in each field tested but their quantity does not exceed 10% of all HP bacterial cells (spiral and coccoid forms)).
- **(+++)** coccoid forms of HP are present in significant quantities (coccoid forms are detected in each field tested and their part exceeds 10% of all HP bacterial forms (spiral and coccoid forms)).

Statistical processing was performed with STATISTICA.5.1 software. The protocol for the research project was approved by the appropriate ethics committee at a meeting The Nikiforov Russian Center of Emergency and Radiation Medicine EMERCOM of Russia as appropriate ethical standards.

RESULTS

We will start our presentation of the results with a description of the H. pylori cells identified by the immunocytochemical method in the biopsies from the gastric antrum - As a result of the immunocytochemical reaction in the cytological smears, HP cells (spiral and coccoid forms) were stained light to dark brown with diaminobenzidine Spiral forms of H. pylori varied from 3-5 mm in length and were about 0.5 mcm in diameter (Fig.1).

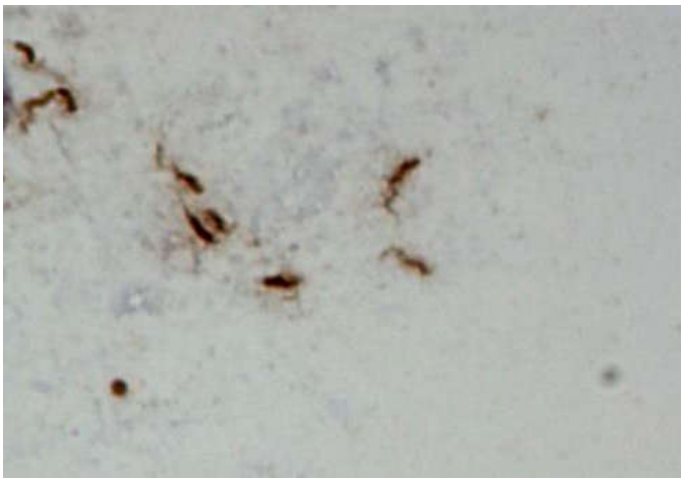

Figure 1. Bacterial cells of spiral forms and coccoid form of Helicobacter pylori, identified by immunocytochemistry in the biopsy of the gastric mucosa

Below we list the criteria according to which the bacterial cells, observed in the cytological smears of gastric mucosa stained using theimmunocytochemical method, should be attributed to the HP coccoid forms: - the presence of species-specific antigens, i.e. brown staining with DAB; - homogeneous uniformity of staining;

- The presence of species-specific antigens, i.e. brown staining with DAB;
- Homogeneous uniformity of staining;
- Geometrically regular round shape;
- Mono and diplococcal forms;
- A diameter of 0.5-1.0 mcm.

We followed these criteria strictly in our study. Before their first course of eradication treatment, the 54 patients in the study group had differing degreesofcoccal gastric mucosa colonization. This enabled us to raise and solve the question of whether eradication therapy has the same efficiency in patients who had a considerable quantity coccoid forms of HP observed in the gastric mucosa biopsies (classified as " +++ ") (Figure 2) as for patients who were found to have no coccoid forms (Figure 3) or only sporadic cases of coccoids, i.e., classified as "-", "+" and "++". In other words, the question was whether the

degree of gastric mucosa colonization with coccoid forms had any impact on the efficiency of eradication therapy.

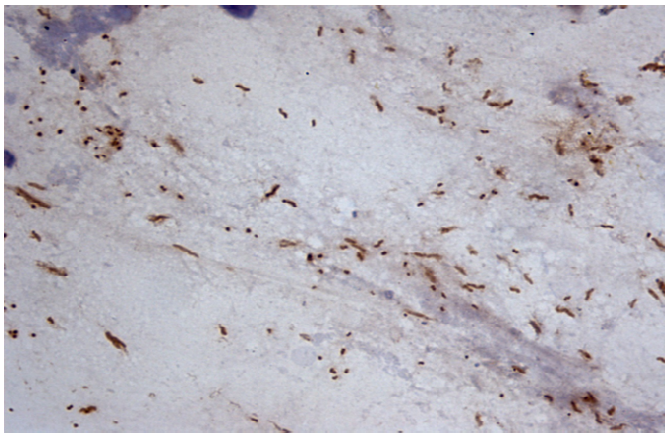

Figure 2. Field of view in immunocytochemically stained cytological smears (prints) from biopsies of gastric mucosa with a high ("+++") degree of colonization with coccoid forms of HP

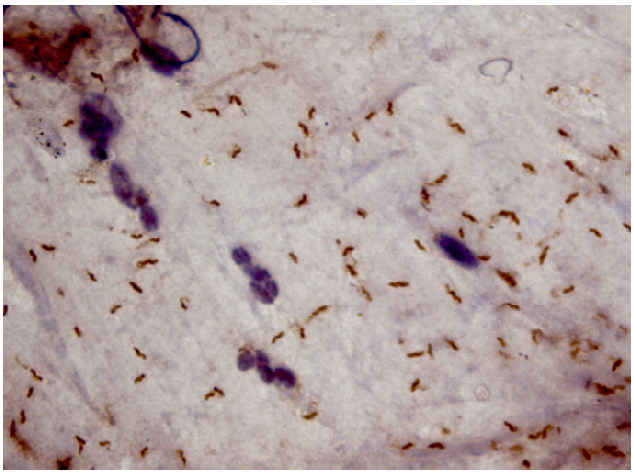

Figure 3. Field of view in immunocytochemically stained cytological smears (prints) from biopsies of gastric mucosa with a low ("+") degree of colonization with coccoid forms of HP

2 or 3 months after their first course of eradication therapy all 54 patients underwent a new test for the presence of H. pylori infection, which was conducted using the same immunocytochemical method. The post-treatment test for HP revealed that 40 of 54 patients had had acheived eradication however HP colonization persisted in 14 of 54 patients. We focused our research on these 14 patients, and in each case we considered the degree of gastric mucosa colonization with coccoid forms of HP that the patient had before treatment. It that 11 of the 14 patients who were not cured had the highest degree of HP coccoid colonization, classified as "+++", beforetreatment. Among the patients who had achieved eradication as a result of treatment, 36 of 40 had only sporadic or no coccoid forms of HP before treatment (Fig. 4). We can also say that in our group of 54 patients undergoing eradication treatment, 15 people were classified as "+++" in terms of coccoid colonization before treatment. The results of the post-treatment test for HP showed that only 4 of the 15 patients with high level of HP coccoids colonization were cured. 11 of these patients had not achieved eradication. A chi-squared test provided statistical evidence for inefficiency of eradication therapy in the group of patients with a high ("+++") degree of gastric musoca colonization with coccoid forms of HP ($p < 0.001$). Thus, the findings of our study suggest that a high degree of colonization of the gastric mucosa with coccoid forms of HP significantly reduce the efficiency of its eradication.

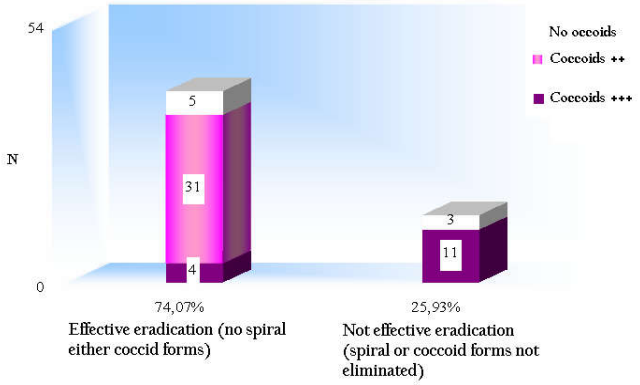

Figure 4. Distribution of patients with low and high levels of gastric mucosa colonization with coccoid forms of HP prior to eradication treatment, in the groups with efficient and inefficient eradication

Note to Figure 4. The group of patients (54 people) tested for HP by immunocytochemical method before eradication therapy, which we classified into three subgroups: with no cocci found ("-"), with the presence of some quantity of cocci ("+" and "++") and with the presence of a significant quantity of cocci (i.e., according to the scale we proposed for semi-quantitative evaluation of cocci; however, patients who were classified as "+" and "++" in respect of cocci colonization are shown as the same group here.

DISCUSSION

Coccoid forms of HP were discovered during attempts to cultivate helical forms of HP obtained from patients with HP colonization. In the context ofin vitro culture mediums, the transition of helical forms of HP to coccoid forms is associated with increased pH and accumulation of toxic metabolic by-products (Catrenich, 1991). For a long time, it was thought that the coccoid forms of HP were only degenerate bacterial cells of H. pylori. There is now no doubt that HP in coccoid form can also be considered a viable and infectious agent. A study using electronic microscopy by Saito *et al* (2003), Showed that HP cocci have two forms — they are either large and loose (degenerative) or small and dense (true) cocci (Saito, 2003) The viability of coccoid forms of HP was proven experimentally (Sisto, 2000 and Poursina, 2013). The fact that HP bacterial cells survive exposure to antibiotics by keeping their coccoid form is supported by the findings of a study by Brenciaglia M.F. *et al* (2000). They showed that the 4-week culture of coccoid forms of HP were viable and were passaged after treatment with amoxicillin, erythromycin, gentamycin and metronidazole (Brenciaglia, 2000). We assume that in our study, coccoid forms of HP survived antibiotic therapy and were responsible for the subsequent generation of new spiral forms of HP.

Antibiotics also induce transformation of bacterial cells of HP from helical forms to cocci (Brenciaglia, 2000 and Jamshid Faghri, 2014). We can add that this transformation can occur not only in vitro but also in vivo, in gastric mucosa, as our study demonstrated cases where patients only had helical forms of HP in gastric mucosa prior to eradication but after eradication coccoid forms were also found. Apparently, in these particular cases, HP bacterial cells transformed from helical to coccoid forms in response to antibiotic therapy. Coccoid forms of HP leave the intestine with the feces (Kravtsov, 2012) and can survive in the environment, which creates a fecal-oral pathway for the spread of HP infection (Delport, 2007). HP coccoids have evolutionarily adapted to survive in hostile environments, and, unlike spiral forms of HP, have increased resistance to external influences (Cellini, 2014) Studies show that coccoid forms of HP have a reduced level of DNA and RNA biosynthesis (Sisto, 2000) and a large reserve of ribosomal RNA (Trebesius, 2000) therefore antibiotics used in standard eradication first line will not have the required impact on the HP coccoids. A high degree of colonization of the gastric mucosa with coccoid forms of HP significantly reduces the efficiency of its eradication. We emphasize that this observation applies so far only to chronic gastritis. As for gastric ulcers, we do not have enough data to analyze a group of patients in our clinic. We should note that even the selection of chronic gastritis patients we studied comprised of only 54 people. This was due to a small number of those wishing to complete the costly immunocytochemical test on HP. However, we can still demonstarte that in gastric ulcers, coccoid forms of H. pylori are detected more frequently. In gastric ulcers, a high intensity of HP colonization with coccoids (over 40%) was shown by Perry *et al* (2004). It is possible that the recurrence of peptic ulcer associated with bacillary-coccoid and cocci-bacillary transformations of HP. Thus, we have shown that in cases when HP eradication was not achieved after treatment, patients usually had had a high degree of gastric mucosa colonization with coccoid forms of HP before the start of the course of H. pylori eradication therapy. Presented phenomenon was established for first line eradication according to Maastricht 2-2000 recommendations. We hope that the data presented in this report will contribute to the further optimization of eradication therapy of H. pylori.

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