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***Helicobacter pylori* INFECTION CAN MODULATE THE SUSCEPTIBILITY OF GASTRIC MUCOSA CELLS TO MNNG**

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Abstract: The pathogenesis of stomach cells can be associated with their susceptibility to exogenous dietary irritants, like nitrosamines such as dimethylnitrosamines (DMNA), and to the effects of non-dietary factors, including *Helicobacter pylori* infection. We used *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as a surrogate agent that induces a spectrum of DNA damage similar to DMNA. Using the alkaline comet assay, we showed that antioxidants – vitamins C and E, quercetin, and melatonin – reduced the genotoxic effect of MNNG in *H. pylori*-infected and non-infected human gastric mucosa cells (GMCs). To compare the sensitivity of the stomach and the blood, the experiment was also carried out in peripheral blood. We observed a higher level of DNA damage induced by MNNG in *H. pylori*-infected than in non-infected GMCs. We did not note any difference in the efficacy of the repair of the damage in either type of GMC. *H. pylori* infection may play an important

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Abbreviations used: GMCs – gastric mucosa cells; MNNG – *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, PBL – peripheral blood lymphocytes

role in the pathogenesis of GMCs, as it can modulate their susceptibility to dietary mutagens/carcinogens, thus contributing to gastric cancer.

Key words: *Helicobacter pylori*, DNA damage, DNA repair, MNNG, Melatonin, Vitamin C, Vitamin E, Quercetin

INTRODUCTION

The development of gastroduodenal diseases and gastric cancer is associated with diverse risk factors that can be classified as dietary and non-dietary. The pathogenesis of stomach cells is linked with increased susceptibility to dietary irritants, including *N*-nitroso compounds, and non-dietary factors, mainly *Helicobacter pylori* infection. *N*-nitroso compounds like *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) could be formed endogenously in the gastric lumen. This process can be accelerated by a decrease in the acidity of the stomach environment, as nitrosation can be catalyzed by bacteria at neutral pH [1, 2]. Furthermore, *H. pylori* may promote the formation of endogenous reactive nitrogen intermediates and induce free radicals as part of the host immune response, which may contribute to DNA damage and apoptosis [3]. *H. pylori* is able to colonize the human stomach mainly by the neutralization of its environment, by direct interaction with gastric epithelial cells, and by the induction of several histological changes, including infiltration of the mucosa (chronic gastritis), loss of mucosal mass (ulcer, atrophy), and inflammation through infiltrating lymphocytes with the development of masses (hyperplastic polyps, adenomas, carcinomas and lymphomas) [4-6].

We previously showed that dietary antioxidative substances, including vitamin C and quercetin, can modulate the DNA-damaging effect of MNNG in human peripheral blood lymphocytes (PBLs); it was of interest to establish whether such modulation also occurs in gastric mucosa cells (GMCs) and whether it can be influenced by *H. pylori* infection [7]. In this study, we also used vitamin E and melatonin, antioxidant substances which can easily supplement the diet. It was also interesting to compare the sensitivity of GMCs and PBLs to MNNG in one experiment, so PBLs were used, serving as a positive control, since we had earlier performed some experiments on them using the substances employed in this study [7-9]. MNNG is one of the most powerful mutagens – it methylates oxygen and nitrogen atoms in DNA via the SN_1 mechanism, in which there is a loss of the leaving group and the generation of a carbocation intermediate with the rapid attack of a nucleophile on the electrophilic carbocation to form a new σ bond [10]. We previously showed that reactive oxygen and nitrogen species generated by inflammatory cells in response to *H. pylori* infection may damage the DNA in GMCs, producing oxidized DNA bases and strand breaks [11]. In this study, we evaluated the reaction of the *H. pylori*-infected and non-infected GMCs and PBLs to MNNG in terms of DNA damage and repair. We assessed the potentials of vitamins C and E, quercetin and melatonin to modulate the

DNA-damaging effect of MNNG relative to *H. pylori* infection. We also assessed the ability of infected and non-infected GMCs to repair DNA damage, so in general, the aim of the study was to evaluate the influence of *H. pylori* infection on the reaction of the stomach cells to a mutagenic challenge.

MATERIALS AND METHODS

Subjects

The study population consisted of 40 individuals, including 18 *H. pylori*-infected patients (aged from 30 to 63 years, median 48 years) with a positive ¹³C-urea breath test, and 22 non-infected controls (aged from 28 to 72 years, median 49 years) hospitalized in the Department of Gastroenterology and Internal Diseases, Medical University of Łódź, and in the Outpatient Gastroenterology Department, St. John of God's Hospital, Łódź, Poland. A urea breath test value of over 3‰ was taken to indicate *H. pylori* infection. The study was approved by the Ethics Committee of the Medical University of Łódź, and each person enrolled in the study gave written informed consent.

Cell preparation

Blood was obtained from young, healthy, non-smoking, male donors. Peripheral blood lymphocytes were isolated by centrifugation in a density gradient of Histopaque-1077 (15 min, 280 g). The pellet containing lymphocytes was resuspended in RPMI 1640 medium to give about $1-3 \times 10^5$ cells per ml and further processed.

Human GMCs were isolated from tissue taken during upper digestive endoscopy from the greater curvature of the upper corpus and the antrum of the stomach of all the patients. The extent of *H. pylori* infection was determined by the urea breath test. Biopsy samples were transported to the laboratory in ice-cold Hanks' balanced salt solution within 15 min, and incubated in an aliquot of 1.5 ml digestion mixture consisting of 3 mg proteinase K and 2 mg collagenase in Hanks' balanced salt solution for 1 h at 37°C. The GMCs were centrifuged for 15 min at 280 g and diluted in Hanks' balanced salt solution. The final concentration was adjusted to 10^4 cells per ml in a single cell suspension, and further processed. The isolated GMCs were immediately put in an incubation solution with MNNG and/or antioxidants.

Cell treatment

MNNG was taken from a stock (0.5 mmol/L) solution in dimethyl sulfoxide (DMSO) and added to the cell suspension to give final concentrations of 1 or 5 μmol/L. The GMCs were exposed for 1 h at 37°C to MNNG. To assess the effect of antioxidants on DNA damage, cells were pretreated for 10 min at 37°C with vitamin C at 50 μmol/L, vitamin E at 25 μmol/L, quercetin at 30 μmol/L or melatonin at 10 μmol/L and incubated with MNNG. Vitamin E and quercetin were derived from stock (20 mM) solutions in DMSO. Vitamin C was taken

from a stock (20 mM) solution in water. Melatonin was derived from a stock (10 mM) solution in methanol.

Comet assay

The comet assay was performed under alkaline conditions essentially according to the procedure of Singh *et al.* [9] with some modifications as described previously [11]. The objects were observed at 200× magnification with an Eclipse fluorescence microscope (Nikon, Tokyo, Japan) attached to a COHU 4910 video camera (Cohu, San Diego, CA, USA) equipped with a UV-1 filter block (an excitation filter of 359 nm and a barrier filter of 461 nm) and connected to a personal computer-based Lucia-Comet v. 4.51 image analysis system (Laboratory Imaging, Prague, Czech Republic). 100 images were randomly selected from each sample and the percentage of DNA in the tail of the comet was measured. This quantity is positively correlated with the level of DNA breakage and/or alkali-labile sites and is negatively correlated with the extent of cross-links. All the values in this study were expressed as means \pm SEM. If no significant differences between variations were found, as assessed by the Snedecor-Fisher test, the differences were assessed with the ANOVA test and contrasted with Scheffe's multi-comparison test.

RESULTS

MNNG-induced DNA damage

Fig. 1 shows DNA damage in PBLs, and in non-infected and *H. pylori*-infected GMCs exposed for 1 h to MNNG at 1 and 5 μ M/L. MNNG induced dose-dependent DNA damage in all the types of cells. The extent of the DNA damage was higher in *H. pylori*-infected GMCs than in non-infected GMCs or PBLs. We

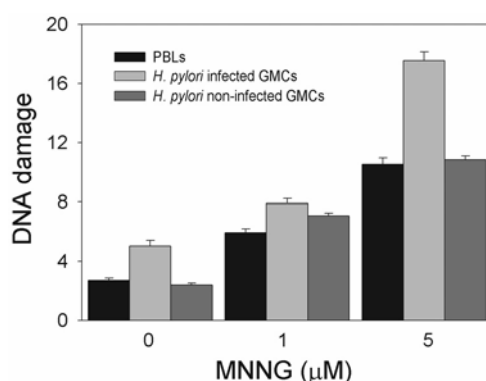


Fig. 1. DNA damage measured as the percentage of DNA in the tail of a comet in the alkaline comet assay performed on human lymphocytes (black column), and on *H. pylori*-infected (grey column) and non-infected (dark grey column) gastric mucosa cells exposed for 1 h at 37°C to N-methyl-N'-nitro N-nitrosoguanidyne (MNNG). The number of cells scored in each treatment was 100. Error bars denote SEM. The results displayed are the means of four independent experiments.

observed an additive effect of *H. pylori* infection and MNNG on DNA damage in gastric mucosa cells. The extent of DNA damage evoked by *H. pylori* (5.02 ± 0.39) and by MNNG at 1 (5.92 ± 0.28) or 5 μM (10.52 ± 0.45) in non-infected GMCs are at an approximately similar level to those induced by MNNG in infected cells (7.90 ± 0.33 for 1 μM and 17.54 ± 0.59 for 5 μM).

The protective action of melatonin, quercetin and vitamins C and E

Fig. 2 shows the extent of DNA damage induced by MNNG after pretreatment with antioxidants: vitamins C and E, quercetin or melatonin. Melatonin at 10 $\mu\text{mol/L}$ decreased the DNA damage caused by MNNG in each type of cell. Quercetin at 30 $\mu\text{mol/L}$ had a lower protective potential than melatonin but a higher one than vitamins C (50 $\mu\text{mol/L}$) and E (25 $\mu\text{mol/L}$) in *H. pylori*-infected and non-infected GMCs. Vitamins C and E more effectively decreased DNA damage caused by MNNG in PBLs than in GMCs. It is worth noting that at 30 $\mu\text{mol/L}$, quercetin itself induced DNA lesions in PBLs.

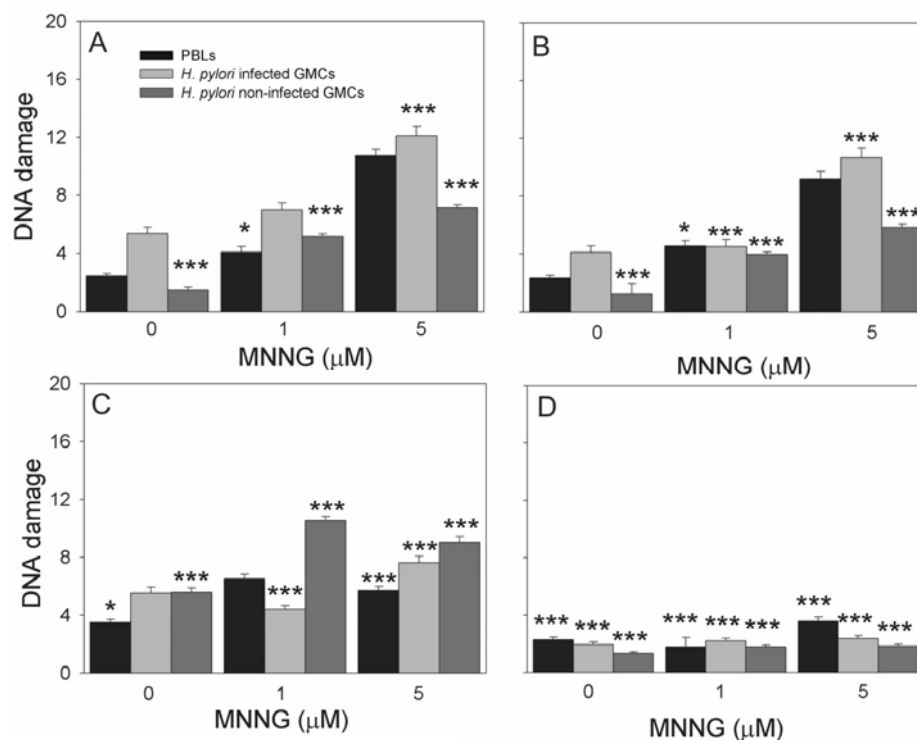


Fig. 2. DNA damage in human lymphocytes (black column), and *H. pylori*-infected (grey column) and non-infected (dark grey column) gastric mucosa cells exposed for 1 h at 37°C to N-methyl-N'-nitro N-nitrosoguanidine (MNNG) in the presence of vitamin C at 50 $\mu\text{mol/L}$ (A), vitamin E at 25 $\mu\text{mol/L}$ (B), quercetin at 30 $\mu\text{mol/L}$ (C), and melatonin at 10 $\mu\text{mol/L}$ (D). DNA damage was evaluated as the percentage of DNA in the tail in the alkaline comet assay. The number of cells analyzed in each treatment was 100. The results displayed are the means of four independent experiments. * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$ compared to the appropriate controls (Fig. 1).

MNNG-mediated DNA damage repair

Fig. 3 shows the extent of DNA damage in *H. pylori*-infected and non-infected GMCs and PBLs exposed to MNNG immediately after exposure and 30, 60 and 120 min thereafter. In all the types of cells, DNA damage was progressively removed over 60 min. We observed a difference in the kinetics of the repair of MNNG-induced DNA damage, which was more efficiently removed in the non-infected than in the *H. pylori*-infected GMCs over the first 30 min of incubation. Similarly, DNA damage induced by hydrogen peroxide at 10 $\mu\text{mol/L}$ for 10 min at 4°C was completely removed after 60 min in GMCs and PBLs.

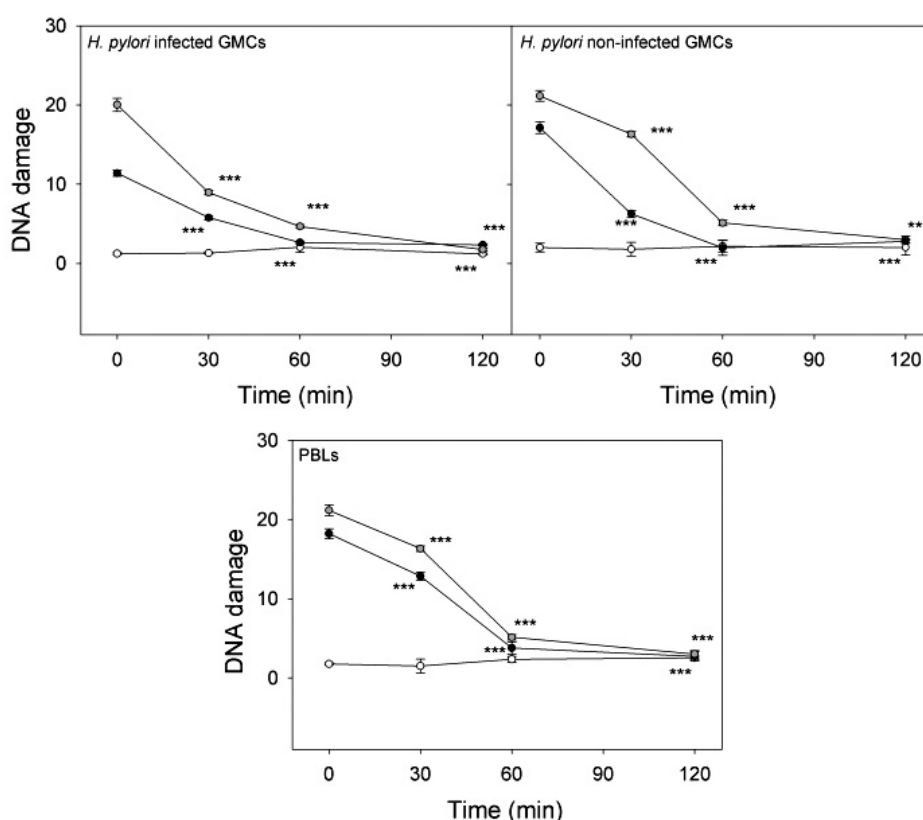


Fig. 3. The time course of the repair of DNA damage in *H. pylori*-infected and non-infected gastric mucosa cells and human peripheral blood lymphocytes (PBLs) treated with N-methyl-N²-nitro N-nitrosoguanidine (MNNG) at 5 $\mu\text{mol/L}$ for 1 h at 37°C (black circles) or with hydrogen peroxide at 10 $\mu\text{mol/L}$ for 10 min on ice (gray circles) compared to the appropriate controls (empty circles). Cells were treated with either chemical, and washed and incubated in a fresh medium for 2 h at 37°C. DNA damage was analyzed as the percentage of DNA in the tail of the comet in the alkaline comet assay. The number of cells analyzed in each treatment was 100. The data presented is the results from four independent experiments. * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$.

DISCUSSION

Gastric mucosa cells are a target for dietary compounds, including harmful environmental pollutants and potentially beneficial antioxidants. The response of these cells to exogenous substances can be modulated by a non-dietary, potentially genotoxic factor, namely *H. pylori* infection, which is a source of neutrophil-generated free radicals, which increase lipid peroxidation in gastric mucosa, resulting in DNA damage [12]. The aim of our investigation was to assess the influence of four antioxidants, melatonin, quercetin and vitamins C and E, on DNA damage and repair induced by surrogate of nitrosamines (MNNG) in association with *H. pylori* infection in GMCs.

Our results show that the higher level of DNA damage induced by MNNG in *H. pylori*-infected GMCs is associated with the additive effect of MNNG and the infection, resulting in a higher susceptibility of the infected GMCs to the DNA-damaging agent. This increased susceptibility may be mediated by an excess of ammonia in a stomach affected by *H. pylori*. Ammonia, a product of *H. pylori* urease, may cause indirect mucosal injury by stimulating neutrophils and phospholipases, and may damage the gastric mucosa by degrading phospholipids and generating precursors of ulcerogenic components. Moreover, the integrity of the gastric epithelium might be destroyed by the products of lipid peroxidation, resulting from the interaction between *H. pylori*-induced free radicals and the GMC membranes [13]. Our results show that DNA lesions induced by MNNG in GMCs may be reduced by antioxidants, especially melatonin and quercetin. Melatonin is a scavenger of a number of reactive oxygen and nitrogen species, and has an ability to reduce lipid peroxidation and may be used as a preventive antioxidant [14].

Quercetin is a dietary flavonoid with numerous biological activities including its antioxidant action against oxidative DNA damage induced by free radicals [7]. *H. pylori* infection is a source of free radicals produced by neutrophils. Additionally, quercetin might exert an anti-inflammatory effect by negatively modulating pro-inflammatory factors, such as interleukine 6, interleukine 8 and TNF-alpha, inhibiting the formation of the inflammatory mediators prostaglandins and leukotrienes, and histamine release. [15, 16]. Our results showed that at high concentrations, quercetin induced DNA damage in PBLs, and this effect might be associated with the absence or a low level of potential prooxidative factors and reactive oxygen species in the reaction environment. This anti-inflammatory action of quercetin and genotoxic effect on PBLs are the reasons why the use of this flavonoid in *H. pylori* infection should be better studied.

The weaker beneficial effect of vitamin C on DNA damage induced by MNNG in GMCs might be correlated with the lower level of ascorbic acid as compared with ascorbyl radicals in *H. pylori*-infected gastric mucosa [17]. DNA damage induced by MNNG was completely removed in *H. pylori*-infected and non-infected GMCs after 1 h, and we observed differences in the kinetics of this

process up to 30 min. This effect might be associated with the accumulation of endogenous modifications of DNA bases in *H. pylori* infection. *H. pylori* can promote the local production of radicals, like *N*-nitroso compounds, which are crucial factors in the early stage of gastric cancerogenesis. Additionally, *H. pylori* induced regenerative hyperproliferation of the antral gastric mucosa, and could disturb their cell cycle mechanisms like DNA repair and apoptosis. An increase in the regenerative proliferation activity of gastric mucosa cells was reported to be correlated with a higher frequency of mutations, reduction of DNA repair efficiency and disturbance of apoptosis [18].

In conclusion, *H. pylori* infection plays an important role in GMC pathogenesis. Dietary antioxidant supplementation might effectively protect against the DNA-damaging effect of MNNG, which is a recognized mutagen and may play a pivotal role in the malignant transformation of gastric epithelium cells.

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