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## ASCORBIC ACID ATTENUATES ASPIRIN-INDUCED GASTRIC DAMAGE: ROLE OF INDUCIBLE NITRIC OXIDE SYNTHASE

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Aspirin (ASA) represents an important risk factor for gastric mucosal injury. Recently, vitamin C releasing aspirin (ASA-VitC) has been shown to reduce gastric toxicity of ASA in animal model of gastric injury. The aim of the present study was to compare the effect of ASA and ASA-VitC on the gastric mucosal damage before and after *Helicobacter pylori* (Hp) eradication in 10 young healthy Hp-positive volunteers. All subjects underwent endoscopy at day 0 (before ASA or ASA-VitC treatment) and at day 3 following treatment (1.6 g ASA/day or 1.6 g ASA + 0.96 g Vit C/day). In addition, *in vitro* experiments were performed in which gastric mucosal cell line (MKN-45 cells) was incubated with ASA or ASA-VitC alone or in combination with *H. pylori*. Expression of constitutive and inducible NO synthase (cNOS, iNOS) was analyzed by Western blot. Moreover, COX-2 expression was analyzed in gastric biopsies at mRNA and protein level by RT-PCR and Western blot, respectively. In humans, treatment with ASA-VitC induced significantly less gastric mucosal lesions than plain ASA. Furthermore, in comparison to plain ASA, ASA-VitC caused stronger inhibition of cNOS and increase in iNOS expression in the gastric mucosa. *In vitro* studies demonstrated a significant increase in iNOS expression in MKN-45 cells incubated with Hp. This effect was aggravated by the addition of ASA, but not ASA-VitC, to MKN-45 cells incubated with *H. pylori*. Both ASA and ASA-VitC stimulated the COX-2 expression in the gastric mucosa. We conclude that ASA-VitC in comparison with ASA induces less gastric mucosal damage and this protective effect may be due to its inhibitory effect on iNOS expression.

Key words: *Acetylsalicylic acid; vitamin C, cyclooxygenase-2, inducible nitric oxide synthase*

## INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin are widely used as anti-inflammatory, analgesic drugs and in the prevention of cardiovascular events (1). However, the major limitations of their clinical application are serious gastrointestinal side-effects, especially peptic ulcerations and gastrointestinal bleeding (2). Ulcers are found at endoscopy in 15-30% of patients using NSAIDs regularly. The annual incidence of upper gastrointestinal bleeding is approximately 1-1.5% in patients taking regularly NSAIDs. Important risk factors for these gastrointestinal events include old age, prior history of upper gastrointestinal events, use of corticosteroids or anticoagulants, and high-dose or multiple NSAIDs (including NSAID plus low-dose aspirin) (3).

Multiple gastroprotective strategies have been applied in order to limit the development of adverse gastrointestinal events including NO-releasing NSAIDs (CINODs), COX-2 inhibitors or use of medical cotherapy (eg. proton pump inhibitor, misoprostol) (4, 5). One of the new strategies to limit the gastric mucosal injury induced by aspirin was adding an ascorbic acid to the native aspirin (aspirin-vitamin C). Our previous animal studies demonstrated that vitamin C attenuates the deleterious effect of aspirin on ulcer healing due to its antioxidant activity by mechanism involving preservation of gastric microcirculation and attenuation of lipid peroxidation and release of proinflammatory cytokines (6). In addition, aspirin-vitamin C in comparison with native aspirin significantly attenuated the expression of proinflammatory cytokines due to its anti-oxidant effect and inhibition of NF $\kappa$ B activity (7). Furthermore, Becker *et al.* demonstrated that aspirin-vitamin C induces less gastric mucosal damage due to the increase in expression and activity of heme oxygenase-1 (HO-1) (8). HO-1 plays an important role in gastroprotection against NSAID by making cells more resistant to apoptotic death (9).

The purpose of the present study was: 1) to compare the effect of aspirin (ASA) with that of aspirin combined with ascorbic acid (ASA-VitC) on gastric mucosal damage in *H. pylori* infected subjects before and after eradication therapy; 2) to assess the effects of ASA and ASA-VitC on the gastric mucosal gene and protein expression of constitutive and inducible NO-synthases (cNOS, iNOS) and cyclooxygenase-2 (COX-2) and 3) to analyze the effects of ASA and ASA-VitC in the presence or absence of *H. pylori* on iNOS mRNA expression in gastric cell line (*in vitro* experiments)

## MATERIAL AND METHODS

*Subjects and study design*

Ten *H. pylori* positive healthy volunteers of both sexes (5 males, 5 females) between 18 and 28 years of age, weighing 65-80 kg entered this study. All subjects were examined for the presence of

*H. pylori* infection during the endoscopic examination using rapid urease test (HUT-test, ASTRA, Wedel, Germany). Pre-study screening ensured that all volunteers had a normal physical examination, full blood count, standard blood chemistry including coagulation tests, and that women had a negative pregnancy test. The study was approved by the appropriate Institutional Review Ethical Committee at Erlangen-Nuremberg University and all subjects gave written informed consent to participate prior to inclusion.

All subjects underwent routine endoscopy at day 0 (before ASA without or with vitamin C (ASA-C) or placebo treatment) during which multiple biopsies (Mannick *et al.*, 1996) were taken from the oxyntic mucosa. On the day following endoscopy, patients were randomized to following treatment groups; ASA or ASA-C (Aspirin Plus C from Bayer, Leverkusen, Germany). The study medication was taken twice daily (0.8 g unbuffered ASA or 2 tablets of ASA (1.6 g) Plus C) after breakfast at 08:00 h and before bed time summing up to a daily total dose of 1.6 g ASA or 1.6 g ASA plus 0.96 g vitamin C. The medication was given for three days. The controls were taking placebo tablets (containing only hydroxypropyl methylcellulose and starch without ASA). For evaluation of gastric mucosal damage, in all subjects the gastric microbleeding was determined and the gastroscopy was performed on day 0 and 3 after start of treatment. The rate of gastric microbleeding was determined as follows: each subject swallowed 16 French gauge orogastric tube. The stomach was rinsed of debris with 100 ml of distilled water, then 100 ml of test solution was instilled into the stomach for 10 min washing period. Mean gastric microbleeding for three 10 min washing periods was calculated and expressed as mean bleeding rate in ml/day. The standard, unsedated, upper gastrointestinal endoscopy was performed by one investigator using an Olympus GIF 100 endoscope and recorded on video tape that was evaluated for mucosal damage using the Lanza score system by the second investigator, being unaware of the treatment given and the *H. pylori* status. Grading score ranged from 0 = normal to 4 = large area of submucosal hemorrhage with active bleeding or widespread involvement of the stomach. During both gastroscopies, gastric mucosal samples were taken from corpus for the molecular analyzes (polymerase chain reaction, Western blot, nuclear factor kappa B activity). After the termination of both treatment regimens (ASA or ASA-C) or placebo, all subjects underwent eradication using triple therapy including amoxicillin (2x1 g per day) plus clarithromycin (2x 500 mg per day) plus pantoprazole (40 mg twice per day) given for 10 days. The *H. pylori* status was assessed using antigen stool-test, confirmed during endoscopy by rapid urease test as stated above. Four weeks after successful eradication of *H. pylori*, all subjects underwent in random order the same treatments and all above endoscopic procedures were repeated in the same manner as before eradication of *H. pylori*.

### *Reverse-transcriptase polymerase chain reaction (RT-PCR)*

For reverse transcription-polymerase chain reaction (RT-PCR) analysis, biopsy specimens were homogenized in 1 ml of TRIZOL (Total RNA Isolation Reagent, Gibco BRL, Karlsruhe, Germany) and RNA was recovered according to the manufacturer's instructions before resuspension in 10-20  $\mu$ l of diethyl pyrocarbonate-treated water and quantification.

Human complementary DNA (cDNA) was generated by reverse transcription of total RNA extracted from mucosal biopsy specimens using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Stratagene, Heidelberg, Germany) and oligi-(dT)-primers (Stratagene, Heidelberg, Germany). The cDNA (2  $\mu$ l) was amplified in a 50  $\mu$ l reaction volume containing 2 U Taq polymerase, dNTP (200  $\mu$ M each) (Pharmacia, Freiburg, Germany), 1.5 mM MgCl<sub>2</sub>, 5  $\mu$ l 10x polymerase chain reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH=8.3) and primers used at final concentration of 1 mM (all reagents from Takara, Shiga, Japan). The mixture was overlaid with 25  $\mu$ l of mineral oil to prevent evaporation. The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk). The nucleotide sequence of the primers for

inducible NO synthase and COX-2 were based on the sequences of the published cDNAs (iNOS sense: CGG TGC TGT ATT TCC TTA CGA GGC GAA GAA GG; iNOS antisense: GGT GCT GCT TGT TAG GAG GTC AAG TAA AGG GC, COX-2 primers purchased by Ambion USA, GAPDH sense: GTC TTC ACC ACC ATG GAG AAG GCT; GAPDH antisense: CAT GCC AGT GAG CTT CCC GTT CA). All primers except for COX-2 were synthesized by GIBCO BRL/Life Technologies (Eggenstein, Germany). Polymerase chain reaction products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Location of predicted products was confirmed by using 100-bp ladder (Takara, Japan) as a standard size marker. The intensity of bands was quantified using densitometry unit from Kodak Digital Science.

### *Western blot analysis*

Using TRIZOL reagent, proteins were extracted from the same biopsy samples as mentioned above. Approximately 10 µg of total protein extracts were loaded on SDS-polyacrylamide gels and run 40 mA, followed by transfer on nitrocellulose membrane (Protran, Schleicher&Schuell, Germany) by electroblotting. 3% bovine serum albumin (BSA) (Sigma Aldrich, Germany) in TBS/Tween-20 buffer (137 mmol NaCl, 20 mmol Tris-HCl, pH 7.4, 0.1 % Tween-20) was used to block filters for at least 1 h at room temperature. Specific primary antibody against iNOS (rabbit polyclonal, dilution 1:200; Santa Cruz, USA), cNOS (rabbit polyclonal, dilution 1: 500, Santa Cruz, Santa-Cruz, CA, USA), COX-2 (mouse monoclonal, dilution 1: 1000, Santa Cruz, USA) or β-actin (mouse monoclonal, dilution 1:5000; Sigma Aldrich, Germany) was added to the membrane, followed by an anti-rabbit-IgG or anti-mouse-IgG horseradish peroxidase (HRP conjugated secondary antibody (dilution 1:40 000 or 1:20 000) dissolved in 1% non-fat milk in TBS-Tween-20 buffer. Incubation of primary antibody was followed by 3 washes with TBS-Tween-20 buffer for 5 min. incubation of the secondary antibody was followed by 6 washes for 5 min. Immunocomplexes were detected by the SuperSignal West Pico Chemiluminescent Kit (Pierce, USA). Thereafter, the developed membrane was exposed to an X-ray film (Kodak, Wiesbaden, Germany).

### *Cell culture*

Human gastric *MKN-45* cells were cultured at 37°C in 75 cm<sup>2</sup> flasks containing RPMI supplemented with 10% FBS and antibiotics under a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. For isolation total RNA and proteins, the *MKN-45* cells were cultured in 60mm Petri dishes. The medium was changed 24 h before addition of the following factors: *H. pylori* MOI 1:25 or 1:50, ASA 1mM and/or vitamin C 0,6mM. After indicated time cells were washing twice with 1xPBS and the RNA or protein isolation was performed.

### *Statistical analysis*

Results are expressed as means ± standard error of the mean (S.E.M.). The significance of the difference between means was evaluated using analysis of variance followed by Duncan's test or, when appropriate, by Wilcoxon's rank sum test with a level of confidence at P<0.05.

## RESULTS

### *Gastric mucosal injury*

All subjects completed the study and no major side effects were recorded. In the control group treated with placebo at day 0 and 3, the mucosal damage was

negligible and these results are not included for the sake of clarity. Plain ASA caused significant ( $P < 0.05$ ) increase in gastric mucosal damage according to Lanza score both in *H. pylori* positive and negative subjects as compared to that in these subjects treated with ASA-C (Fig. 1). After successful eradication (the eradication rate was 100%), ASA induced significantly more lesions (about two-folds) than before eradication therapy, but after therapy with ASA-C of the *H. pylori*-eradicated and non-eradicated patients, the gastric lesion index was significantly lower than that in those treated with plain ASA.

*Gastric mucosal protein expression of inducible and constitutive NO synthase (iNOS, cNOS)*

In all *H. pylori* positive subjects, constitutive NO synthase (cNOS) was detected as a strong signal. The treatment with plain ASA resulted in a significant ( $P < 0.05$ ) down-regulation of the protein expression for constitutive NO synthase. In Hp-positive subjects taking ASA-VitC the reduction of cNOS was not observed. After eradication therapy, no significant change in cNOS expression was found as compared to initial value in *H. pylori* positive patients before this therapy. In subjects successfully eradicated, therapy with plain ASA led to a significant ( $P < 0.05$ ) downregulation of the protein expression for cNOS. In contrast, in these *H. pylori* eradicated subjects receiving ASA-C, this down-regulation of the expression for cNOS was not observed and this expression was significantly higher than in plain ASA treated *H. pylori* negative subjects. (Fig. 2).

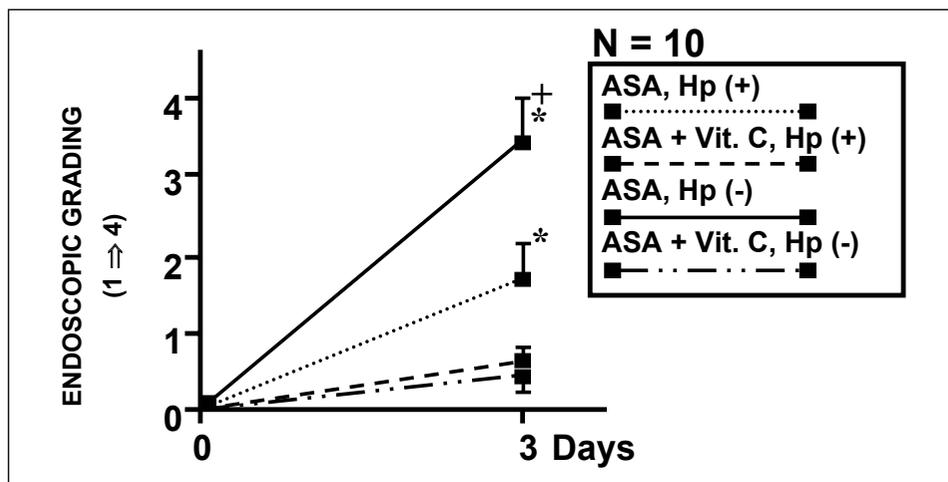


Fig. 1. Endoscopic grading of gastric injury according to the Lanza score in patients treated with ASA or ASA-C before (Hp+) and after successful eradication (Hp-). Asterisk indicates significant ( $P < 0.05$ ) increase above the value obtained with ASA-C treatment. Cross indicates significant increase as compared to the value obtained in (Hp+) subjects treated with plain ASA.

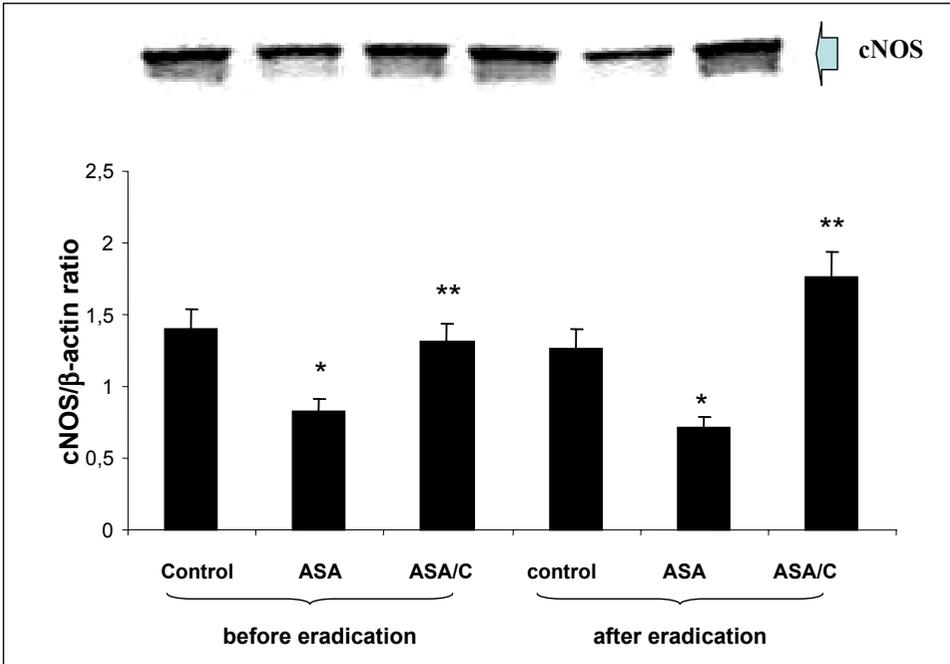


Fig. 2. Representative Western blot and densitometric analysis of the protein expression of cNOS in the gastric mucosa of human Hp+ve volunteers treated with ASA or ASA-vitamin C before and after successful eradication. Asterisk indicates significant ( $P < 0.05$ ) decrease below the value obtained in Hp+ve or Hp-ve controls. Double asterisks indicate significant increase as compared to the values obtained in Hp+ve or Hp-ve subjects treated with plain ASA.

The iNOS expression (Fig. 3) was detected in the gastric mucosa of *H. pylori* positive controls. Following administration of plain ASA, the ratio of iNOS over  $\beta$ -actin increased significantly indicating that the protein expression of iNOS was significantly upregulated by ASA. In subjects taking ASA-C, the iNOS expression was significantly reduced as compared to that observed in ASA-treated group. The eradication therapy led to a small but significant down-regulation of iNOS expression in the gastric mucosa of control subjects. In contrast, in subjects successfully eradicated and taking ASA, a strong upregulation of iNOS expression was detected that reached several times higher level than before eradication therapy. In subjects treated with ASA-C, this increase in iNOS protein expression was significantly attenuated, but was still higher than before eradication therapy.

#### *COX-2 expression in the gastric mucosa*

In all *H. pylori* positive subjects, the mRNA and protein expression for COX-2 was detectable. In *H. pylori* positive subjects receiving plain ASA, a strong and significant increase in the expression of COX-2 at mRNA and protein level was

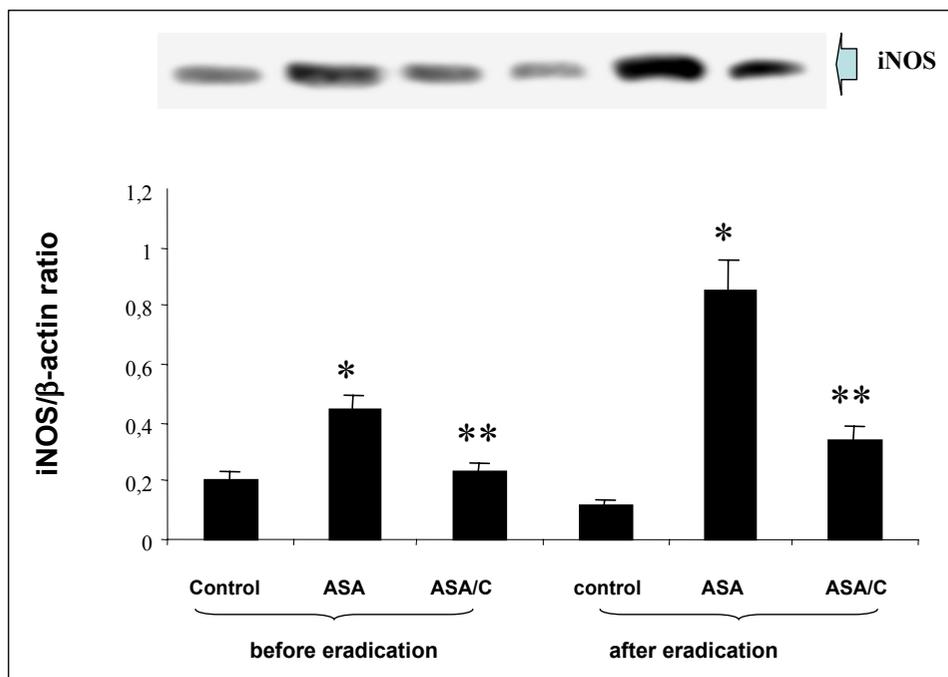


Fig. 3. Representative Western blot and densitometric analysis of the protein expression of iNOS in the gastric mucosa of human Hp+ve volunteers treated with ASA or ASA-vitamin C before and after successful eradication. Asterisk indicates significant ( $P < 0.05$ ) increase above the value obtained in Hp+ve or Hp-ve controls. Double asterisks indicate significant decrease as compared to the values obtained in Hp+ve or Hp-ve subjects treated with plain ASA. Cross indicates significant decrease as compared to Hp+ve control.

observed. Similarly, after ASA-C therapy a significant increase of the COX-2 expression was found. After successful eradication therapy, in control subjects treated with placebo, the expression of COX-2 decreased significantly as compared to that observed in these subjects before eradication therapy. Treatment with plain ASA of eradicated subjects resulted in a strong and significant upregulation of COX-2. In eradicated subjects receiving ASA-C treatment, the expression of COX-2 was also upregulated, however, this increase was weaker as compared to that in plain ASA treated subjects (Fig. 4, 5).

*Effect of ASA and ASA-VitC on gene expression of inducible and constitutive NO synthase (iNOS, cNOS) in gastric cell line (MKN-45) in the presence or absence of H. pylori*

Incubation of MKN-45 cell line with *H. pylori* was accompanied by a significant increase in iNOS expression. In contrast, treatment of cells with ASA or ASA-VitC had no significant effect on iNOS mRNA expression.

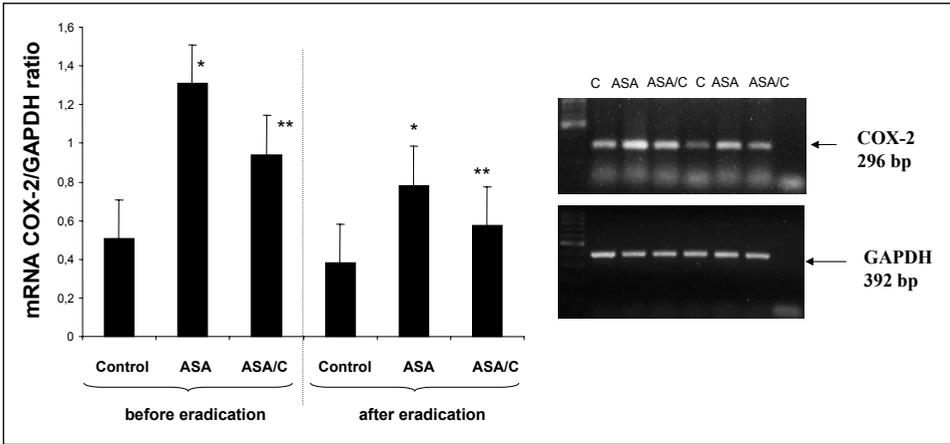


Fig. 4. Representative RT-PCR and densitometric analysis of the mRNA expression of cyclooxygenase-2 (COX-2) in the gastric mucosa of human Hp+ve volunteers treated with ASA or ASA-vitamin C before and after successful eradication. Asterisk indicates significant ( $P < 0.05$ ) increase above the value obtained in Hp+ve or Hp-ve controls. Double asterisks indicate significant decrease as compared to the values obtained in Hp+ve or Hp-ve subjects treated with plain ASA. Cross indicates significant decrease as compared to Hp+ve control.

In MKN-45 cells incubated with both ASA and *H. pylori*, a strong up-regulation of iNOS was found. In contrast, in the same gastric cell line incubated with ASA-Vit.C in the presence of *H. pylori* no increase, but rather decrease in iNOS expression was observed (Fig. 6).

#### DISCUSSION

The present study demonstrates that one of the important mechanisms by which aspirin damages gastric mucosa is increased production of NO due to overexpression of inducible NO synthase. Excessive nitric oxide release has been reported to exert detrimental effects attributed to reactive nitrogen species, such as oxides of nitrogen and peroxynitrate, which are formed by the reaction of nitric oxide with oxygen and superoxide, respectively (10). The important source of NO production induced by aspirin seems to be gastric epithelial cells because our *in vitro* experiments showed an iNOS expression in these cells. Interestingly, the presence of *H. pylori* alone induced the iNOS expression and was a *condition sine qua non* for the stimulatory effect of aspirin on iNOS expression. Incubation of aspirin alone with MKN-45 cells did not cause any significant change in iNOS expression. This observation is discrepant to that found in humans treated with aspirin. After successful eradication, the upregulation of iNOS expression in gastric mucosa induced by aspirin was significantly higher than before *H. pylori* treatment.

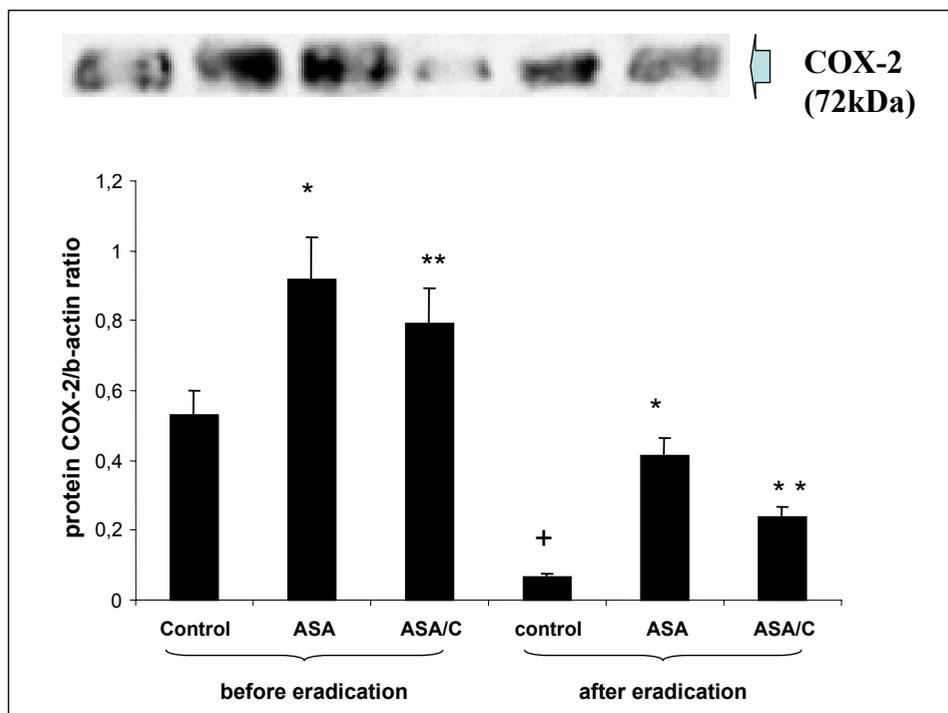


Fig. 5. Representative Western blot and densitometric analysis of the protein expression of COX-2 in the gastric mucosa of human Hp+ve volunteers treated with ASA or ASA-vitamin C before and after successful eradication. Asterisk indicates significant ( $P < 0.05$ ) increase above the value obtained in Hp+ve or Hp-ve controls. Double asterisks indicate significant decrease as compared to the values obtained in Hp+ve or Hp-ve subjects treated with plain ASA. Cross indicates significant decrease as compared to Hp+ve control.

The addition of ascorbic acid to aspirin attenuates its deleterious effect on the gastric mucosa. The lesion score according to endoscopic Lanza classification in subjects treated with ASA-VitC was significantly lower than that observed in subjects treated with plain aspirin. Interestingly, the lesion score remained at low level after successful eradication in subjects treated with ASA-VitC. It is of importance that ASA-VitC significantly attenuated the expression of iNOS and prevented the decrease in cNOS expression observed in subjects treated with aspirin. ASA-VitC attenuated also the increase in iNOS mRNA expression observed in MKN 45 cells incubated with live *H. pylori*.

The mechanism by which ascorbic acid attenuates the iNOS expression remains to be clarified in further studies. However, previous studies postulated that the down-regulation of NF $\kappa$ B may result in the decrease in iNOS expression.

Finally, the present study analyzed the changes in expression of COX-2, the rate limiting enzyme involved in arachidonic acid metabolism, thereby generating

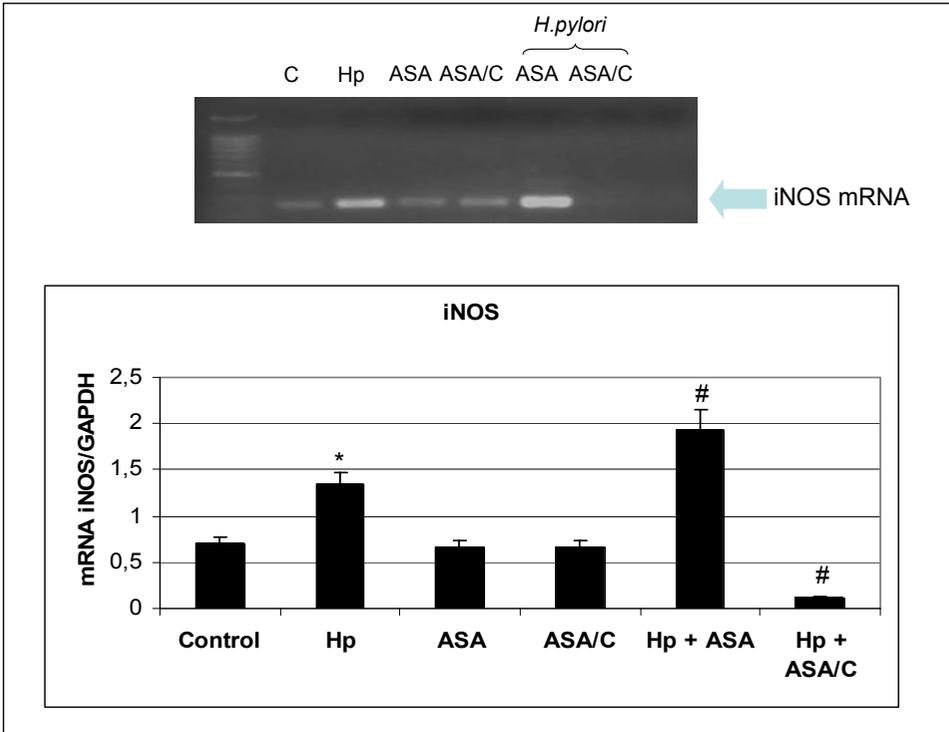


Fig. 6. Effect of *H. pylori*, ASA, ASA-VitC and combination of ASA or ASA-VitC with *H. pylori* on the mRNA expression of iNOS in MKN 45 cell line. Asterisk indicates significant ( $P < 0.05$ ) increase above the value obtained in control cells. Cross indicates significant change as compared to cells incubated only with *H. pylori*.

prostaglandins which play a crucial role in supporting and sustaining the inflammatory response (12). The present study demonstrated a significant upregulation of COX-2 expression at mRNA and protein level in subjects taking aspirin or aspirin-VitC. This observation is keeping with previous results (12). However, the combination of ascorbic acid with aspirin was associated with a significant attenuation in COX-2 overexpression in gastric mucosa. The possible explanation for this phenomenon could be the antioxidant effect of ascorbic acid and decrease of reactive oxygen species which are known strong stimulator of COX-2 expression. In other words, the augmentation in the oxidative stress by ascorbic acid could have implication on COX-2 expression in gastric mucosa. In terms of COX-2 activity, both forms of aspirin are known as strong inhibitors of COX-2 activity and the overexpression observed after the treatment with both substances may reflect the reaction to inhibition of COX-2 activity (Fig. 7).

In conclusion: (1) ASA-VitC in comparison with plain aspirin induces smaller gastric mucosal damage and this protective effect results probably from decreased

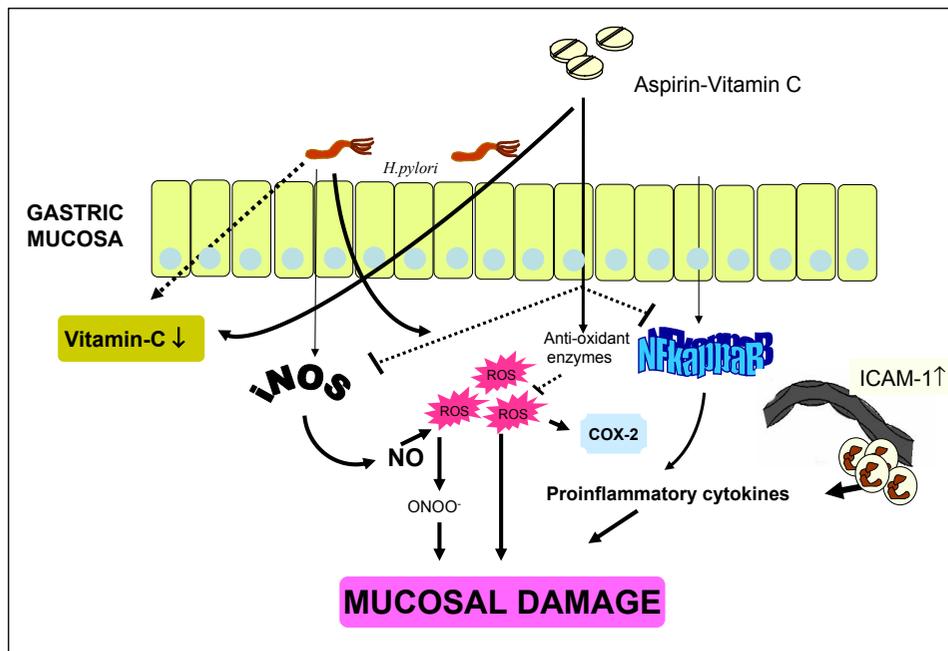


Fig. 7. Schematic diagram showing the attenuation of gastric mucosal damage by ASA-VitC.

NO production due to the attenuation of iNOS expression and (2) eradication of *H. pylori* increases susceptibility of gastric mucosa to damaging effect of aspirin possibly due to reduction in COX-2 expression and COX-2 activity induced by Hp infection and this is accompanied by enhanced expression of inducible NO synthase and downregulation of constitutive NO synthase.

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