Research Article

Therapeutic Effect of Selected Natural Products in Gastritis Induced by Helicobacter pylori in Experimental Rats

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ABSTRACT

Gastritis induced marked cellular changes which vary according to histological characters and causative mechanism. We aim to assess the therapeutic role of amoxicillin, black seed oil, curcuminoids either individually or combined on rat model of Helicobacter pylori (H. pylori) gastritis. H. pylori gastritis was induced in rats, followed by administration of Amoxicillin, black seed oil, curcuminoids or their combination for four weeks. Serum gastrin, pepsinogen activity, interleukin-6 (IL-6), and gastric mucosal myeloperoxidase (MPO) activity and prostaglandin E2 (PGE2) were measured. Histopathological examination of gastric mucosa and immunohistochemical reactions for inducible nitric oxide synthase (iNOS), nitrotyrosine (NTR) and DNA fragmentation were also evaluated. Administration of black seed oil and curcuminoids individually for four weeks significantly decreased serum gastrin, IL-6 as well as gastric mucosal MPO and PGE2 and increased total pepsinogen activity compared to untreated rats. Their combination significantly decreased serum gastrin, IL-6, gastric mucosal MPO and PGE2 while total pepsinogen activity demonstrated further improvement. Histological results demonstrated marked improvement in agreements with biochemical markers. Amoxicillin-treated group demonstrated no significant changes regarding these biomarkers with moderate positive reaction for iNOS, NTR and DNA fragmentation. In conclusion, Black seed oil and/or curcuminoids either individually or combined improved H. pylori-associated inflammatory and oxidative injury. However, amoxicillin has failed to induce significant effect.

Keywords: Amoxicillin; black seed oil; curcuminoids; DNA fragmentation; gastrin; IL-6; iNOS; myeloperoxidase; nitrotyrosine; prostaglandin E2

INTRODUCTION

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Heliocobacter pylori (H. pylori) is curved spiraled or S-shaped gram negative bacteria, selectively colonize the gastric epithelium. H. Pylori infection affects about 50% of the world’s population. Clinically, the infection varies from asymptomatic gastritis to peptic ulcer and gastric melanoma. H. pylori changes the microenvironment of gastric mucosa and causes inflammatory and oxidative injury in the gastric mucosa. Oxidative stress is adverse interactions of molecular oxygen (O2) or its reactive derivatives with bio-molecules causing an imbalance between the generation of cell damaging molecules and the cellular capacity for detoxification. Reactive oxygen species and reactive nitrogen species are normally generated by tightly regulated enzymes such as NADPH oxidase isoforms and nitric oxide synthase (NOS), respectively. Prostaglandins (PGs) are lipid mediators of the inflammatory immune response which are derived from oxidative metabolism of arachidonic acid. These lipids are synthesized in large quantities by inflammatory cells in response to both acute and chronic inflammatory stimuli. The primary enzyme responsible for PGs synthesis, cyclo-oxygenase (COX), exists in at least 2 isoforms. COX-1 is constitutively expressed in the gastrointestinal tract and most other tissues, whereas COX-2 is expressed at very low levels in most tissues usually at sites of inflammation. Prostaglandins protect the gastroduodenal mucosa from damage caused by diverse noxious agents. Local inflammation with H. pylori infection is usually characterized by infiltration of polymophonuclear leukocytes (neutrophils) and lymphocytes into the gastric mucosa and increased production of several cytokines. IL-6 is produced by a variety of lymphoid and non-lymphoid cells including activated macrophages, fibroblasts, keratinocytes and endothelial cells. Moreover, myeloperoxidase (MPO), a haemoprotein abundantly expressed in neutrophils, is secreted during neutrophils activation. It plays an important role in defense mechanism of any organism and usually stored in azurophlic granules of neutrophils. Barnett et al. demonstrated that MPO was expressed as a biochemical marker of granulocyte infiltration into various tissues, including the gastrointestinal tract.
Apoptosis, programmed cell death, is a physiological suicide mechanism that occurs during normal tissue turnover and is usually involved in tissue homeostasis. Normally, the rate of cell loss by apoptosis is matched by the rate of new cell production mediated through proliferation. However, apoptosis induced by H. pylori infection without any increase in cell proliferation may lead to loss of mucosal integrity, gastric erosion and ulceration. Loss of gastric glands may lead also to gastric atrophy. Infection with H. pylori leading to increased cell proliferation represents a host response to apoptosis. Furthermore, H. pylori infection raises basal and meal-stimulated serum gastrin concentrations and lowers iron stores, reducing in turn fasting glucose levels. Infection also represents the main cause of non-autoimmune chronic gastritis, which increases gastrin secretion. Hypergastrinemia usually leads to increased proliferation of gastric progenitor cells and a thickened mucosa. Dual therapy with omeprazole plus amoxicillin has gained popularity as an effective, well tolerated treatment for H. pylori. The poor penetration into gastric mucus and inactivation by low pH may be factors that contribute to the limited clinical efficacies of antimicrobial agents that are active in vitro against H. pylori. Using another gastritis model (idoacetamide-induced gastritis), we tested the ability of some natural products including black seed oil and curcuminoids to ameliorate the associated inflammatory and oxidative stress condition. The oil of N. sativa seeds has potential antioxidant properties and can also exert inhibitory effects on the production of the inflammatory mediators as COX and lipoxygenase (LOX) enzymes. Curcuminoids (curcumin I, II, and III) has a proven benefit for the prevention and treatment of a number of inflammatory diseases. They have a wide range of biological and pharmacological activities, including antioxidant, anti-inflammatory and anti-mutagenic in vitro, anti-carcinogenic, hypocholesterolemic in rats and hypoglycemic effects in humans. The current study aims mainly to assess the therapeutic effectiveness of amoxicillin, black seed oil, curcuminoids administration for 4 weeks either individually or in combination form on ameliorating the associated inflammatory and oxidative stress condition in H. pylori infected rats.

**METHODS**

**Animals**

Fifty adult female albino Wistar rats (Rattus Norwegicus), weighing 130 ± 10 g, supplied from the animal farm of the National Research Center, Dokki, Cairo, Egypt, were used in this study. Rats were allowed 7 days for acclimatization at room temperature with a 12 h light/dark cycle before beginning the experimental work. Animals were fed normal rodent chow (El-Nasr Pharmaceuticals, Chemicals Industries, Egypt), allowed free access to drinking water, and observed daily.

**Study drugs**

Amoxicillin capsules were purchased from GlaxoSmithKline, UK. The capsules were grind and prepared as suspension. Black seed oil capsules were purchased from Nigellar® capsules were purchased from Kahira Pharm. and Chem. Ind. Co., Cairo, Egypt. The oil was prepared as a suspension. Curcuminoids were prepared as previously described from dried roots of turmeric (Curcuma longa; Zingiberaceae). Briefly, the roots were extracted with 95% ethanol over night at 28°C, pooled extracts were loaded on silica gel for column chromatography 60-120 mesh, eluted with methanol/chloroform 9:1, evaporated under reduced pressure.

**Isolation of H. pylori**

H. pylori was isolated from an antral biopsy of a 49-years-old male patient, endoscopically diagnosed as having H. pylori infection. Bacteria was identified as small gray gram negative colonies showing positive rapid urease (Christensen’s urea test; Remel Inc., Kansas, USA), cytochrome oxidase (SpotTest Oxidase kit; Difco, USA) and catalase (SpotTest Catalase kit; Difco, USA) tests. The bacterial strain was gently rinsed with sterile physiological saline, individually aliquoted into vials containing 3 ml of 20% glycerol in bruccella broth and maintained at -70°C.

**H. pylori gastritis model**

After acclimatization, an H. pylori gastritis model was induced in 50 rats as previously described. Briefly, each animal received 0.5 ml of H. pylori bruccella broth, containing 2x10^7 CFU/ml, daily in the morning for 1 week. This was followed by 4 weeks treatment period using drug free vehicle (gastritis control group; n=10 rats), 50 mg/kg body weight of amoxicillin suspension orally (Amoxicillin group; n=10 rats), 50 mg/kg body weight of black seed oil suspension orally (black seed oil group; n=10 rats), curcuminoids suspension 50 mg/kg body weight daily (curcuminoids group; n=10 rats) or a combination form of the above mentioned drugs using the same specified doses. The study protocol was approved by scientific committees at Cairo University and the National Research Center, Egypt.

**Sampling**

At the end of each treatment period, rats were fasted overnight. Blood was collected via retro-orbitals bleeding and centrifuged for 15 min at 3,000xg. Serum was collected and diluted into aliquots and stored at -20°C for determination of serum gastrin, total pepsinogen, and IL-6. Rats were then decapitated and stomachs were dissected out, cut along the greater curvature; the mucosa were rinsed with cold normal saline and dried with filter paper. Specimens from the fundus region were cut, kept in 10% formol saline and processed for nitrotyrosine (NTR), inducible nitric oxide synthase (iNOS) and DNA fragmentation immunostaining and histopathological examination (H and E staining). The remaining mucosal parts were quickly scraped, frozen in liquid nitrogen (-170°C) and stored at (-20°C) for determination of PGE2 and MPO activity.

**Biochemical Analyses**

Serum gastrin was determined by competitive immunoassay technique using DRG rat gastrin kit (DRG International, Inc., USA). Serum pepsinogen activity was


Table 1. Serum and gastric mucosal parameters measured in various groups of experimental rats received treatment for 4 weeks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Gastritis control group</th>
<th>Treated groups (4 weeks)</th>
<th>Black seed oil</th>
<th>Curcuminoids</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Gastrin (pg/ml)</td>
<td>209(14)</td>
<td>187(10)</td>
<td>150(23) b</td>
<td>114(5) b</td>
<td>111(7) b</td>
</tr>
<tr>
<td>pepsinogen (U/ml)</td>
<td>92(11)</td>
<td>95(7)</td>
<td>101(11)</td>
<td>120(9) b</td>
<td>112(14) b</td>
</tr>
<tr>
<td>Serum IL-6 (pg/ml)</td>
<td>133(12)</td>
<td>131(11)</td>
<td>117(7) b</td>
<td>105(10) b</td>
<td>115(12) b</td>
</tr>
<tr>
<td>gastric MPO (U/g protein)</td>
<td>236 (9)</td>
<td>205(9)</td>
<td>156(15) b</td>
<td>115(6) b</td>
<td>132(9) b</td>
</tr>
<tr>
<td>gastric PGE2 (pg/mg protein)</td>
<td>406(23)</td>
<td>403(10)</td>
<td>358(13) b</td>
<td>332(13) b</td>
<td>337(8) b</td>
</tr>
</tbody>
</table>

* Standard deviations in parentheses. b Significant differences from gastritis control at P<0.001

Positive immunoreaction was indicated by brown colour. Polyclonal rabbit antibodies for iNOS, NTR and DFF-45 were obtained from Zymed Laboratories, Cairo, Egypt.

**Statistical analyses**

Statistical analysis was performed using the Statistical Package for Social Sciences software (SPSS, Illinois, USA). One-way ANOVA was carried out to test if the means of the measured parameters are different which was followed by LSD post hoc test, taking p<0.05 as statistically significant. All data are presented as mean ± SD.

**RESULTS**

**Biochemical results**

The experimental model of H. pylori gastritis in rats revealed elevated serum gastrin, serum IL-6, gastric mucosal MPO activity and gastric mucosal PGE2 level and depressed serum pepsinogen activity compared to normal rats (P<0.001). Four weeks of administration of black seed oil induced significant decreases in serum gastrin, IL-6, gastric mucosal MPO and PGE2 (P<0.001 each). Similar effects were also observed in curcuminoids-treated group in addition to an increased pepsinogen activity (P<0.001). Amoxicillin-treated group, however, demonstrated non significant changes regarding all the parameters studied. Combined administration of these drugs induced similar results to black seed oil effects with a supplementary improvement in pepsinogen activity (Table 1).

**Histological results**

Histological examination of the stomach fundic region from adult female albino rats of control H. pylori gastritis group showed irregularly arranged atrophic fundic glands, sloughed apical epithelium, irregular gastric pit, vacuolated cytoplasm in some gastric cells, while other have small pyknotic nuclei (Fig 1a). Amoxicillin-treated rats had regularly arranged fundic glands, chief cells, peripherally arranged parietal cells with central rounded nuclei, thin lamina propria and few cells with small darkly stained pyknotic nuclei (Fig 1b). Black seed oil-treated rats had regularly arranged fundic glands, rounded peripherally arranged parietal cells and granular chief cells (Fig 1c). Curcuminoids-treated rats had regularly arranged fundic glands with many cells have vacuolated cytoplasm and lost their nuclei in some cells while few cells appear normal (Fig 1d). Combination-treated rats had regularly arranged fundic glands contain normally...
Immuno-histological examination of gastric mucosa from H. pylori infected rats revealed a strong positive NTR immuno-reactivity, mainly within cells lining the neck region of the gastric gland. Moderately positive and weak reactions were detected in the middle and lower regions of the gland, respectively (Figure2a). NTR staining was observed mainly within the cells of lamina propria. iNOS positive cells were spotted in the lining of the transverse sections of the gastric gland (Figure 3a). This nitrosative stress induced free radical-mediated DNA damage and, as a consequence, apoptosis in the gastric mucosa as indicated by the strong DFF-45 staining in cells lining all parts of the gastric gland (Figure 4a).

Moderate positive reactions to NTR, iNOS and DFF-45 were observed in the gastric mucosa from amoxicillin-treated and black seed oil-treated rats (amoxicillin group: NTR (Figure 2b), iNOS (Figure 3b), DFF-45 (Figure 4b) and black seed oil group: NTR (Figure 2c), iNOS (Figure 3c)).
No reaction was seen in muscularis mucosa to either iNOS or DFF-45 in gastric mucosa from black seed oil-treated rats (Figure 3c). The gastric mucosa from curcuminoids-treated rats also revealed moderate and weak positive reactions to NTR and iNOS in the middle and lower parts of the glands, respectively (Figure 3d). The reaction to DFF-45, however, showed weak positive reaction in the middle and lower parts of the gland with negative reaction in muscularis mucosa (Figure 4d). The combination treatment turned the gastric mucosal reactions to NTR and iNOS to be weakly positive and negative in all layers of the gastric gland, respectively (Figure 2e and Figure 3e). However, the reaction to DFF-45 was weak positive in the upper and middle parts of the gastric gland and negative in the lower parts of the gland (Figure 4e).

**DISCUSSION**

Gastritis, or inflammation of the gastric mucosa, is not a single disease but rather a group of disorders, mostly responsible for observed inflammatory changes in the gastric mucosa [33]. Hypergastrinemia observed in...
subsequent to H. pylori infection was previously reported to be induced by the intra-gastric increase of pH, that cause corpus atrophy, and G cells damage27, 34, and the alkalization of G cell environment caused by bacterial urease35. H. pylori urease represents a potent activator of human mucosal macrophages for IL-6 expression36 which is mainly a heat shock protein-60 (HSP60)-dependent. Its release is likely to occur in vivo and may be capable of reaching mucosal macrophages within the gastric epithelium. This is in consequence to disruption of epithelial tight junctions during H. pylori infection37. Strong positive reactions for iNOS, NTR and DNA fragmentation were observed in H. pylori gastritis rats. Activated neutrophils located in the inflammatory foci and secreting MPO into the extracellular space can convert hydroperoxides into free radicals, indeed lipid peroxidation sequences38. Previous mammalian studies reported that inflammatory cells such as polymorphonuclear cells and macrophages can express iNOS39. Increased expression of iNOS in gastric mucosa of humans, additionally certain tumours may indicate its pathological role of elevated NO production27, 33. The latter is a potent vasodilator synthesized by iNOS which can form potentially genotoxic nitrating species such as peroxynitrite and nitrosating species (nitrosonium ion). NTR, a stable end product of tyrosine nitration residues, can be used as a marker for peroxynitrite and other

Figure 3. A photomicrograph of Immun-peroxidase reaction for iNOS in stomach fundic region of adult female albino rats of (a) control gastritis group showing strong positive reaction in cells lining the transverse sections of the glands. (b) Antimicrobial-treated group showing moderately positive reaction. (c) black seed oil-treated group showing moderate positive reaction in upper, middle and lower parts of the gland. No reaction is seen in muscularis mucosa (MM). (d) curcumin-treated group showing moderate positive reaction in the middle and weak positive in the lower part of the gland. (e) combination-treated group showing negative reaction in the upper, middle and lower parts of the gland. (Arrows, double arrows and short arrow refer to upper, middle and lower parts of the gland respectively; X 200).
nitrating species\textsuperscript{40}. Mucosal inflammation itself provokes apoptosis and atrophy of the epithelial cells due to proinflammatory cytokines and free radicals effects\textsuperscript{41}. Increased gastrin release represents also an inducer for mucosal proliferation\textsuperscript{42}. Gastritis rats received amoxicillin (AMX) demonstrated non significant change in serum gastrin, total pepsinogen activity, IL-6 levels as well as gastric mucosal PGE2. Slight reduction in iNOS, NTR and DNA fragmentation reactions were only observed. This may be due to primary or secondary resistance of H. pylori bacteria to AMX. Previous study reported that most antimicrobial agents can demonstrate excellent activities \textit{in vitro}, but have little or no activity when used \textit{in vivo} as a single therapeutic agents\textsuperscript{43}.

Black seed oil has been extensively studied pharmacologically since it has an antimicrobial activity against a wide range of organisms\textsuperscript{44}. In our H. pylori model, the administration of black seed oil decreased serum gastrin level in agreement with recent study\textsuperscript{21}. Such oil can activate soluble guanylate cyclase enzyme, leading to an intracellular increase of cGMP joined with decreased intracellular Ca\textsuperscript{2+} inhibiting its flux, where Ca\textsuperscript{2+} represents a potent stimulator for gastrin secretion\textsuperscript{21}. It also encouraged a stimulatory release of pepsinogen from chief cells. We previously reported total pepsinogen activity as a biomarker of gastric mucosal status, including atrophic change and inflammation\textsuperscript{27}. Miki and Urita also reported that its gradual decrease may reflect any reduction of the fundic glandular mucosa and

\textbf{Figure 4.} A photomicrograph of Immun-peroxidase reaction for DFF in stomach fundic region of adult female albino rats of (a) control gastritis group showing strong positive reaction in cells lining upper, middle and lower parts of the glands. (b) Antimicrobial treated group showing moderately positive reaction. (c) black seed oil-treated group showing moderate positive reaction in the upper, middle and lower parts of the gland. No reaction is seen in muscularis mucosa (MM). (d) curcumin-treated group showing weak positive reaction in the middle and lower part of the glands. There was negative reaction in muscularis mucosa (MM). (e) combination-treated group showing weak positive reactions in the upper, middle and negative reaction in the lower parts of the gland. (Arrows, double arrows and short arrow refer to upper, middle and lower parts of the gland respectively, X 200).
it can detect also extensive atrophic gastritis regardless of its H. pylori status. The antioxidant properties and the inhibitory influence of black seed oil on IL-6 expression may be responsible for the decrease of serum IL-6. Black seed oil decreased gastric mucosal PGE2 is in agreement with other studies. They demonstrated an inhibitory effect of the thymoquinone (TQ) content of the oil on both COX and LOX enzymes. Furthermore, the moderate positive reactions for iNOS, NTR and DNA fragmentation are an indication of the ability of black seed oil to attenuate the severity of the oxidative stress status. El-Mahmoudy and colleagues highlighted the ability of TQ to suppress the production of NO by macrophages, an effect which may be useful in ameliorating the inflammatory and autoimmune conditions and may be induced through reduction of mRNA and protein expressions of iNOS. This consequently leads to a decline in both NTR and DNA fragmentation.

Rats received curcuminoids for four weeks once daily demonstrated significant decrease in serum gastrin levels joined with increased total pepsinogen activity. Data recorded from preclinical models suggested that curcumin's suppression of the inflammatory response may involve inhibition of COX-2, iNOS and production of cytokines. Accordingly it prevents any damage due to reduced level of inflammatory cytokines. Curcuminoids decreased serum IL-6 significantly in conformity with other studies. Gulcubuck and colleagues indicated that curcumin administration can markedly reduce serum IL-6 levels in the late phase of experimental acute pancreatitis, but failed to prevent tissue injury. In myeloma cells, curcumin has shown to inhibit signal transducer and activator of transcription 3 (STAT3) phosphorylation and hence suppress IL-6 production. Gastric mucosal PGE2 levels demonstrated also significant decrease mostly attributed to suppression of prostaglandin synthesis via regulation of COX-2 expression. Strimpakos and Sharma attributed this also to downregulation of COX-2 protein levels and increased apoptosis for those cells that constitutively express COX-2 protein. Attenuation of PGE2 biosynthesis may be through inhibiting the expression of messenger PGES. This may be mediated through phosphorylation inhibition of cPLA2, expression of COX-2 and the enzyme activity of 5-LOX. Its anti-inflammatory properties were reported to be mediated through inhibition of neutrophils function as reflected by decreased level of gastric mucosal MPO. In response to inhibition of neutrophil infiltration an increase in expression of antioxidant enzymes may occur indeed it exerts protective effect of cell function under different stress conditions.

The decline extent for iNOS, NTR and DNA fragmentation reactions in rats treated with curcuminoids is in agreement with others. As a matter of facts bacterial infection represents an inducer for oxidative stress and curcumin supplementation can attenuate such stress. Similar results was also reported and referred to decreased iNOS levels, NTR indeed decreased NO scavenging and oxidative protein damage. Such changes were mediated by decreased activation of redox-sensitive transcription factors. In particular, Curcumin ability to alter gene transcription and induce apoptosis in preclinical models advocates its potential ability in cancer chemoprevention and chemotherapy. Gastritis rats received combination form (AMX, black seed oil and curcuminoids) demonstrated significant decrease in serum gastrin, IL-6, gastric mucosal MPO and PGE2 joined with increased pepsinogen activity. The eradication of H. pylori can decrease the severity of gastritis and provokes a significant change in serum pepsinogen activity. Immunohistochemistry demonstrated weak positive reaction for iNOS and negative reaction for both NTR and DNA fragmentation after 4 weeks of mixture administration. This may be attributed to certain synergism that has been occurred between these agents in combination form. In conclusions, Curcuminoids and black seed oil were superior to amoxicillin in attenuating H. pylori-associated inflammatory and oxidative injury in rats. A synergistic effect was found upon combining these drugs with amoxicillin. An observation deserves great attention and need to be tested clinically in the future.

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