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Inhibitory Effect of Capsaicin on Interleukin-8 Production by *Helicobacter pylori*-Infected MKN-45 Cells

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Abstract Capsaicin is the active ingredient in chili pepper and has an inhibitory effect on *Helicobacter pylori* growth and NF-κB activation. The present study examined the effect of capsaicin on interleukin (IL)-8 production by *H. pylori* ATCC 43504-infected MKN-45 cells, a gastric epithelial cell line. The viability of the MKN-45 cells treated with capsaicin at 0, 50, 100, 250, and 500 μM was 99, 98, 99, 99, and 85%, respectively. A capsaicin concentration as low as 50 μM significantly inhibited the IL-8 production induced by *H. pylori* ATCC 43504 infection (43.2% of control) during 24 h of incubation. However, low concentrations of capsaicin (50 and 100 μM) did not significantly inhibit the IL-8 production by TNF-α- or PMA-treated MKN-45 cells. Therefore, the overall inhibitory effect of capsaicin on *H. pylori* ATCC 43504 was the sum of *H. pylori* ATCC 43504 growth inhibition, host cell survival, and NF-κB signal cascade inhibition.

Key words: *Helicobacter pylori*, capsaicin, IL-8, NF-κB

Helicobacter pylori is a known causative agent of gastritis and gastric cancer [3], and an increase of interleukin (IL)-8 has been shown in *H. pylori*-positive gastric mucosa and KATO-III cells co-cultured with *H. pylori in vitro* [7]. IL-8 is a small (10 kDa) inducible proinflammatory peptide [28] containing the neutrophil-activating amino acid motif glu-leu-arg, which is a potent neutrophil chemoattractant [16] that stimulates neutrophil migration along a chemotactic gradient and modulates the expression of adhesion molecules [17]. The infiltration of neutrophils into the mucosa is already known to occur in *H. pylori*-induced chronic active gastritis, plus activated neutrophils release proteases and reactive oxygen metabolites that cause gastric mucosal injury [8].

There are several natural compounds that actively inhibit *H. pylori*. For example, the inhibitory effect of different *Bifidobacterium* spp. on the growth of *H. pylori* has been investigated and found to be caused by the bacteriocins from *Bifidobacterium* spp. [2]. In addition, polyfermenticin SCD, named tentatively as the bacteriocin produced by *Bacillus polyfermenticus* SCD, has been shown to exhibit antimicrobial activity against *H. pylori* KCTC 2948 growth [20]. Meanwhile, certain components of Ginseng, polysaccharides, protopanaxadiol, and ponciretin have also been reported to inhibit *H. pylori* growth and infection in Kato-III cells and the Vac A vacuolation of HeLa cells, although a single type of ginsenoside, polyacetylene, or polysaccharide did not exhibit *H. pylori* inhibitory activities [18, 19]. Furthermore, a polysaccharide isolated from the leaves of *Artemisia capillaries* has also shown inhibitory activities against *H. pylori* [31]. Hideki Masuda *et al.* [21] have investigated the inhibitory effects of Gochoonangi roots, stems, and leaves against the growth of *H. pylori* and its urease activity.

Capsaicin is the active ingredient in chili pepper, and several research groups have demonstrated the cytoprotective effect of chili or capsaicin on experimentally induced gastric injury in animal models. Pretreatment with capsaicin also ameliorates aspirin-induced gastric lesions in rats [12]. Similarly, the acute and long-term administration of capsaicin has been found to decrease the gastric injury in rats following the consumption of ethanol [15]. Capsaicin and its analogs are known to have growth inhibition effects on microorganisms

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such as *Zygosaccharomyces rouxii* KFY80 [25]. In addition, capsaicin is already known to inhibit the growth of *H. pylori* at 10 μg/ml *in vitro* with a maximal inhibitory effect at a concentration of 50 μg/ml [14]. However, the mechanism by which capsaicin reduces *H. pylori*-induced gastric injury has not yet been determined.

Accordingly, *in vitro* time- and dose-dependent studies were conducted to examine the production of IL-8, a key player in gastric inflammatory processes, by the stomach cancer cell line MKN-45 in the presence of *H. pylori*. Upon *H. pylori* infection, the MKN-45 cells produced IL-8 in a time- and dose-dependent manner. However, this IL-8 induction was significantly inhibited by the addition of capsaicin in a time- and dose-dependent manner. Since this inhibitory effect of capsaicin on *H. pylori* may be related to the suppression of the NF-κB activation induced by *H. pylori*, as previously shown by Singh *et al.* [29], the suppressive effect of capsaicin on the IL-8 production induced by PMA or TNF-α was examined. The results suggested that capsaicin partially inhibits the NF-κB activation induced by *H. pylori* ATCC 43504, and thus could be used as a potential target for drug development against *H. pylori* ATCC 43504 infection.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Urease-positive, Vac A+, Cag A+ wild-type *H. pylori* ATCC 43504 (Rockville, U.S.A.) was used. The bacteria were grown on blood agar plates supplemented with 5% sheep blood for 48 h at 37°C in a microaerobic system jar (Difco, Sparks, U.S.A.), where the CO_2 and N_2 gas were generated by a Campy pak plus (BBL, Sparks, U.S.A.). The *H. pylori* was inoculated onto a brain heart infusion (BHI) medium containing 5% horse serum (pH 6.8) and incubated for up to 2 days [35]. The optimal stimulation concentration of *H. pylori* ATCC 43504 on the MKN-45 cells was 500 cfu/cell.

Gastric Epithelial Cell Line

The MKN-45 cells [10, 22], a human gastric cancer cell line, were cultured in an RPMI-1640 medium (Gibco, Grand Island, U.S.A.) with 10% heat-inactivated fetal calf serum (Hyclone, Utah, U.S.A.). The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2, then passaged at subconfluence after trypsinization and seeded into 6- or 24-well culture plates (Nunk, Denmark).

Reagents

PMA (Sigma, St. Louis, U.S.A.) and TNF-α (Pharmingen, San Diego, U.S.A.) were used as the stimulators, and capsaicin (Sigma, St. Louis, U.S.A.) as the inhibitor of the IL-8 production by the stimulator-treated MKN-45 cells.

Cytotoxity of Capsaicin Towards *H. pylori* and MKN-45

The cytotoxic effect of capsaicin on the *H. pylori* ATCC 43504 and MKN-45 cells was assessed separately. The *H. pylori* ATCC 43504 was cultured on blood agar plates with capsaicin on paper disks for 4 days, and then the *H. pylori* ATCC 43504 growth inhibition zones were measured. Meanwhile, the MKN-45 cells were cultured in 96-well plates with media containing various concentrations (1,000, 500, 250, 100, 50, 10, and 0 mM) of capsaicin for 48 h, followed by an MTT assay.

IL-8 ELISA

After culturing the MKN-45 cells in 24-well plates overnight, the cells were stimulated by *H. pylori* ATCC 43504 (500 cfu/cell) and PMA (1 μg/ml) or TNF-α (100 unit/ml) in the presence of various concentrations (250, 100, 50, and 0 mM) of capsaicin for 2, 4, 8, 16, and 24 h, respectively. The IL-8 concentrations in the culture supernatants were then determined by an enzyme-linked immunosorbent assay (ELISA). The wells of 96-well microtiter plates (Nunc Inc., Denmark) were pre-coated with goat anti-human IL-8 antibody (R&D Systems 614, McKinley Place, U.S.A., 4 μg/ml in 100 μl of PBS) by incubating overnight at room temperature, washed with PBS containing 0.05% Tween 20 and 0.1% BSA (Sigma, St. Louis, U.S.A.), and blocked with 1% BSA in PBS-Tween 20 for 1 h at room temperature. After a further washing, 100 μl of conditioned media or IL-8 standards (R&D Systems, 614 Mckinley Place, U.S.A.) was added and incubated at room temperature for 2 h. A rabbit anti-human IL-8 (1:1,000, Endogen, Woburn, U.S.A.) primary antibody was then added to the wells at room temperature for 1 h. Alkaline phosphatase conjugated mouse anti-rabbit IgG (Jackson, West Growe, U.S.A.) was used as the secondary antibody. An ELISA Amplification System (Gibco, Grand Island, U.S.A.) was used to develop the enzyme-catalyzed color dye production. The optical density at 494 nm was read using an automated microplate photometer, and the concentrations of IL-8 were determined by comparison with the IL-8 standard curve.

RESULTS

Cytotoxicity of Capsaicin Towards *H. pylori* ATCC 43504 and MKN-45 Cells

The cyotoxic effect of capsaicin on *H. pylori* ATCC 43504 was measured based on the inhibition zone of *H. pylori* ATCC 43504 growth, which was 0, 0, 12, and 32 mm with 0, 250, 500, and 1,000 μM of capsaicin, respectively. The maximal inhibitory effect of capsaicin was at a concentration of 1 mM, whereas the capsaicin concentration of 250 μM had no inhibitory effect on the growth of *H. pylori* ATCC 43504. Incubation with the vehicle (DMSO) alone did not affect the growth of the bacteria. In accordance with the

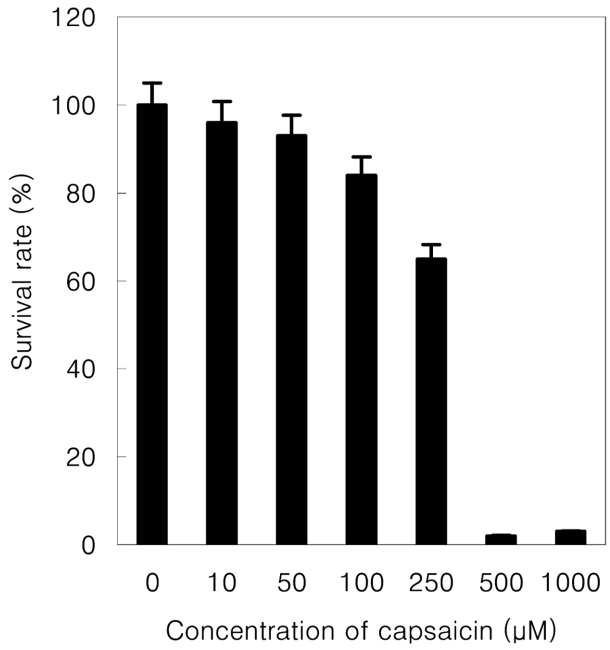

Fig. 1. Viability of MKN-45 cells at various concentrations of capsaicin followed by MTT assay.

findings of Jones *et al.* [14], bactericidal activity against *H. pylori* ATCC 43504 was evident within 4 h of incubation (data not shown). Meanwhile, when examining the cytotoxic effect of capsaicin on the MKN-45 cells, a gastric epithelial cell line, the MKN-45 cells were able to tolerated incubation with up to 500 μM of capsaicin for a period of 24 h (Fig. 1). The viability of the cells treated with 0, 50, 100, 250, and 500 μM of capsaicin was 99, 98, 99, 99, and 85%, respectively. A cytotoxic effect on the MKN-45 cells became evident after 48 h of incubation at capsaicin concentrations of 250 μM (65% survival) and 500 μM (2% survival), whereas a capsaicin concentration of 100 μM or less had a minimal affect on the viability of the MKN-45 cells (data not shown).

Increased IL-8 Production by MKN-45 Cells Infected with *H. pylori* ATCC 43504

The MKN-45 cells infected with *H. pylori* ATCC 43504 for 8 h released an increased amount of IL-8 into the culture media (Fig. 2) and this effect was dose- and time-dependent. The IL-8 production was significantly increased after 2 h, and the highest IL-8 release was observed after 8 h at an infection ratio of 1:500 (host cell: *H. pylori*). No further increase in IL-8 production was evident with bacteria doses above 1:500.

Inhibition of *H. pylori* ATCC 43504-Induced IL-8 Production by MKN-45 Cells Treated with Capsaicin

The effect of capsaicin on the *H. pylori* ATCC 43504-induced IL-8 production by the MKN-45 cells was examined.

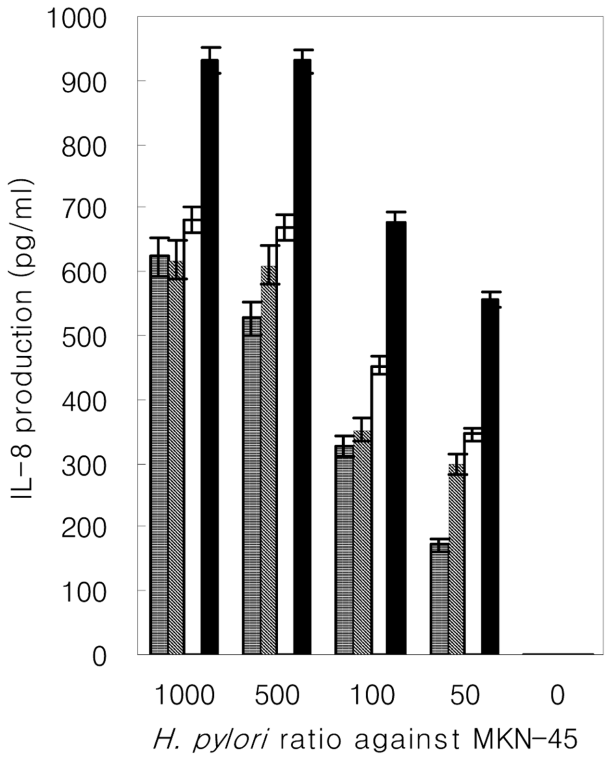

Fig. 2. Effect of MKN-45 cell IL-8 release by *H. pylori* ATCC 43504 infection.
Each measurement is from three replicates. Symbols used: ▤, 1 h; ▧, 2 h; □, 4 h; ■, 8 h.

A capsaicin concentration as low as 50 μM significantly inhibited the IL-8 production induced by the *H. pylori* ATCC 43504 infection (43.2% of control) after 24 h of incubation (Fig. 3). The inhibitory effect of the capsaicin became evident during the first 8 h of incubation and longer exposure to capsaicin returned the IL-8 production to baseline levels.

Partial Inhibition of TNF- and PKC-Dependent IL-8 Production by MKN-45 Cells Treated with Capsaicin

The experimental findings presented here are consistent with previous studies showing that *H. pylori* ATCC 43504 infection induces IL-8 production. The promoter region of the IL-8 gene carries a putative NF-κB binding site, and this transcription factor is believed to be a prime regulatory element for IL-8 expression. Thus, the ability of capsaicin to suppress the IL-8 production by *H. pylori* ATCC 43504-infected cells suggests that capsaicin may inhibit the NF-kB in gastric epithelial cells. Therefore, the effect of capsaicin on other NF-κB activating signals, specifically TNF-α and PMA, was examined. In contrast to the *H. pylori* ATCC 43504-infected MKN-45 cells, low concentrations of capsaicin (50 and 100 μM) did not significantly inhibit the IL-8 production by the TNF-α- or PMA-treated MKN-45 cells (Fig. 4), which is consistent with previous

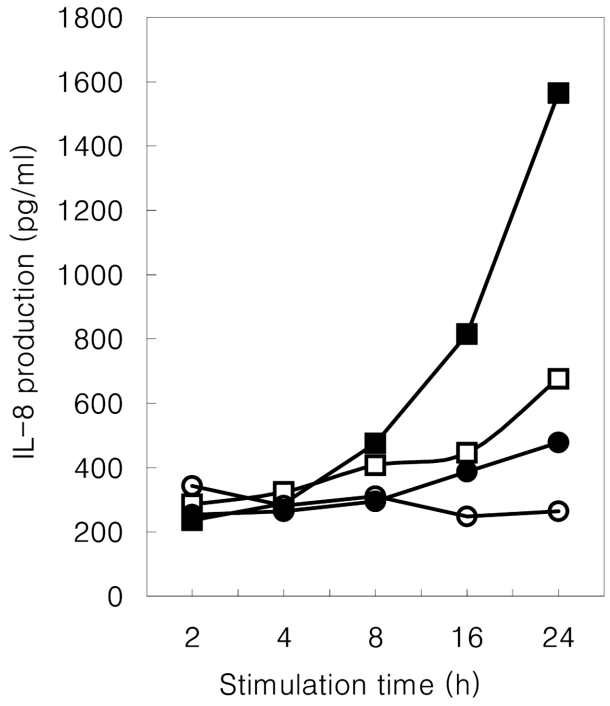

Fig. 3. Time course of IL-8 release by capsaicin-treated MKN-45 cells exposed to *H. pylori* ATCC 43504.
The MKN-45 cell monolayers were infected with *H. pylori* ATCC 43504 (1:500) and treated with various concentrations (0, 50, 100, and 250 μM) of capsaicin. Each measurement is from three replicates. Symbols used: ■, 0 μM; □, 50 μM; ●, 100 μM; ○, 250 μM.

studies [27]. However, a 250 μM concentration of capsaicin significantly suppressed the IL-8 production by the TNF-α- or PMA-treated MKN-45 cells, suggesting that capsaicin interferes with the TNF-α- and PMA-signal cascades leading to IL-8 expression via NF-κB.

DISCUSSION

IL-8 is an important cytokine in the host inflammatory response to *H. pylori* [22, 26], which correlates with its induction in gastric epithelial cells co-cultured with *H. pylori in vitro* [6, 27]. A variety of studies have shown that the gastric epithelium is an important source of chemokines [5, 10], which are released both in response to *H. pylori* [1, 27] and on exposure to endogenous pro-inflammatory mediators [33]. The bacterial induction of epithelial chemokines involves a protein tyrosine kinase (PTK) pathway and NF-κB activation [1]. The upregulation of IL-8 by *H. pylori* may lead to free-radical generation and the release of proteolytic enzymes from activated neutrophils, affecting mucosal integrity [34]. The eradication of *H. pylori* in ulcer patients results in a reduction of antral IL-8 mRNA expression, neutrophil infiltration, and surface epithelial lesions [22], suggesting that inflammatory cytokines

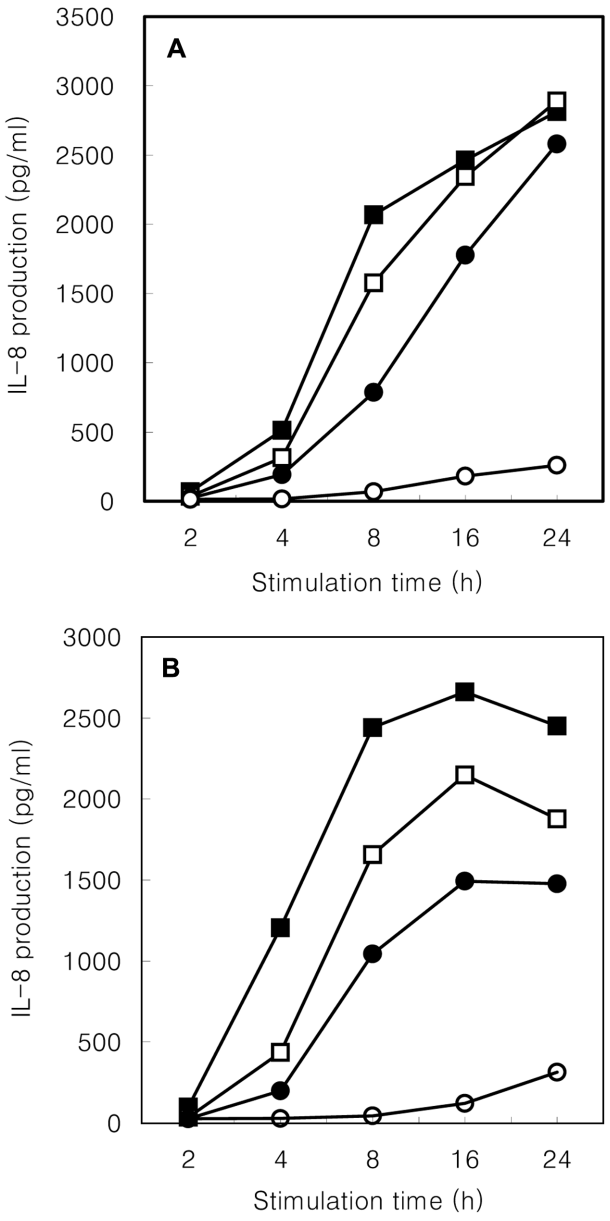

Fig. 4. Time course of IL-8 release by capsaicin-treated MKN-45 cells exposed to TNF-α and PMA.
The MKN-45 cell monolayers were stimulated with 100 units/ml of TNF-α (**A**) and 1 μg/ml of PMA (**B**), and treated with various concentrations (0, 50, 100, and 250 μM) of capsaicin. Each measurement is from three replicates. Symbols used: ■, 0 μM; □, 50 μM; ●, 100 μM; ○, 250 μM.

may play an important role in the mucosal damage seen with *H. pylori* infection. Therefore, this study investigated the hypothesis that the production of IL-8 by gastric MKN-45 cells exposed to *H. pylori*, TNF-α, and PMA is diminished by capsaicin treatment. *H. pylori* significantly enhanced the production of IL-8 from the gastric epithelial cell line MKN-45. Capsaicin then exerted an inhibitory effect on the IL-8 production by the MKN-45 cells when co-cultured with *H. pylori* ATCC 43504, in accordance

with the findings of Kassai *et al.* [16]. The inhibitory effect of capsaicin on the IL-8 production induced by *H. pylori* ATCC 43504 infection became evident after as little as 8 h of incubation, suggesting that the penetration of capsaicin was the limiting factor. Once capsaicin reached its intracellular (cytoplasmic) target(s), the suppressed activation remained stable thereafter.

Since TNF-α and PMA are potent activators of IL-8 expression by epithelial cells via NF-κB, the present study then tested the effect of capsaicin on IL-8 production by MKN-45 cells stimulated with these agents. The data revealed that capsaicin effectively inhibited in a time- and concentration-dependent manner the IL-8 production by MKN-45 cells co-cultured with *H. pylori* ATCC 43504, TNF-α, and PMA *in vitro*, suggesting that capsaicin may interfere with the signal cascades of PMA, TNF-α, and *H. pylori* ATCC 43504 that lead to the expression of IL-8. The primary signal cascade candidate affected by capsaicin is the NF-κB pathway, as PMA, TNF-α, and *H. pylori* are all known to utilize the NF-κB signaling pathway to induce IL-8 expression [13]. In addition, the finding that *H. pylori* is a potent activator of NF-κB also supports this idea, since other NF-κB responsive genes, including TNF-α, IL-1, and IL-6, have been found to be elevated in the gastric mucosa of persons with *H. pylori* [9, 24]. Furthermore, the production of IL-8 in AGS gastric cells by *H. pylori* is regulated via an NF-κB-dependent transcriptional process [13]. In the present study, low concentrations (50 μM and 100 μM) of capsaicin did not significantly inhibit the production of IL-8 by PMA- and TNF-α-stimulated cells, which is in accordance with the previous study of Singh *et al.* [29], where capsaicin (300 μM) failed to suppress NF-κB activation by TNF-α unless the cells were pretreated. However, high doses of capsaicin (250 mM) consistently inhibited the IL-8 production induced by TNF-α and PMA treatment up to 90%. At this point, the reason for this discrepancy is uncertain and under investigation using various cell lines.

Capsaicin is known to block both the degradation of IκBα and the nuclear translocation of the p65 subunit of NF-κB [4, 29]. Similar to capsaicin, 1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphocholine, a synthetic diester phospholipid, and auranofin, an antirheumatic drug, have reported to inhibit PMA-induced NF-κB activation, yet not TNF-α- or IL-1-induced NF-κB activation [32]. Although PMA and capsaicin both activate PKC, only PKC induces NF-κB [30], as shown by the IL-8 production by the MKN-45 cells in the present study. Thus, although capsaicin had no effect on NF-κB activation, it blocked PMA-induced NF-κB activation. Similar to TNF, PMA is also known to generate Reactive Oxygen I, which may play a critical role in the effect of capsaicin on PMA [30]. In addition to inhibiting the growth of *H. pylori in vitro*, capsaicin has also exhibited specific bactericidal activity against a nonpathogenic human commensal *E. coli* strain. In addition, capsaicin continues to exhibit antibacterial activity at reduced pH values, suggesting that the efficacy of capsaicin may be independent of pH [14]. In accordance with these findings, the present data also showed similar effects of capsaicin. However, in contrast to previous findings, the minimum dose affecting *H. pylori* ATCC 43504 growth was 500 μM, which is a lethal dose for the cells tested. Nonetheless, capsaicin inhibited the production of IL-8 by *H. pylori*-stimulated MKN-45 cells at concentrations as low as 250 μM with minimal effect on the cell survival. Thus, the present data revealed that capsaicin exerted an inhibitory effect on the IL-8 production by MKN-45 cells stimulated by *H. pylori* ATCC 43504, confirming that the inhibitory effect was not due to the growth inhibition of the MKN-45 cells, but rather to the inhibition of IL-8 production by the *H. pylori*-stimulated MKN-45 cells and *H. pylori* ATCC 43504 growth.

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