Anti-ulcer Activity of Curcumin on Experimental Gastric Ulcer in Rats and Its Effect on Oxidative Stress/Antioxidant, IL-6 and Enzyme Activities

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Objective To investigate the possible mechanism by which curcumin protects stomach during the acute chronic phase of gastric ulcer disease. Methods The rats were divided into four groups and fasted for 2 days with free access to water. On the third day, the animals were fasted for a further 24 h with no access to water followed by surgery. Rats received different doses of curcumin (20, 40, and 80 mg/kg) or vehicle by oral gavage. Nineteen hours after ulcer induction, the rats were killed by decapitation. Stomach was opened along the greater curvature and ulcerative lesions were counted. Total juice acidity, neutrophils activity, mitochondrial activity, total antioxidants, paraoxonase (PON 1)/arylesterase and total peroxides were evaluated. DNA fragmentation (%) and pro-inflammatory cytokine IL-6 level were measured. The level of different gastro-cytoprotective effectors including total antioxidants and paraoxonase (PON 1)/arylesterase activities was measured. Results The anti-ulcer activity of curcumin was displayed by attenuating the different ulcerative effectors including gastric acid hyper-secretion, total peroxides, myeloperoxidase (MPO) activity, IL-6 and apoptotic incidence. Conclusion Curcumin appears to have a propitious protective effect against gastric ulcer development.

Key words: Curcumin; Paraoxonase/Arylesterase activity; Mitochondrial activity and Myeloperoxidase activity

INTRODUCTION

The prevention of gastric ulcer pathogenesis or its recurrence is the main desired goal for many experimental studies in the present era. Ulcer healing requires angiogenesis in granulation tissue at the base of ulcer, together with re-epithelization starting from ulcer margins and subsequent re-establishment of glandular architecture[1]. Although many drugs have been synthesized and established for gastric ulcer treatment, the search for agents having powerful antiulcer activity with possible natural biological occurrence and predictably having no side effects is worthwhile.

Curcumin has been speculated to have promising chemotherapeutic and preventive activities, which could approve avenues for alternative treatment of many diseases. Recently, great attention has been paid to the medical applications of Curcumin in the treatment of human diseases. Since curcumin was shown to exhibit anti-tumor activity and apoptosis in many human cancer cell lines including lung and liver cancer cell lines[2], that is aside its prospective role as a potential immunomodulatory effector in vivo and in vitro studies[3-5]. Currently, curcumin has been used in treatment of pancreatic cancer[6-7], multiple myeloma[8-9], Alzheimer’s disease[10-11], and colorectal cancer[12]. Despite these tremendous strides in investigations of different diseases, the protective role of curcumin in the genesis of gastric ulcer has not been studied well, particularly, its role as a potent antioxidant in vivo.

In the biological system, small amounts of the potentially toxic reactive oxygen species (ROS) could be generated in eukaryotic cells by normal oxidase action or during the course of electron transport in mitochondria or in endoplasmic reticulum. Since electron seeks stability through electron pair ing with various biological macromolecules, e.g. proteins, lipids and DNA, which in turn, leads to protein and DNA damage along with lipid peroxidation. Additionally, a variety of cells, including neutrophils, macrophages, neurons, and endothelial cells, can produce both O2- and NO through the respective actions of various
oxidases and nitric oxide synthases. Superoxide radical ($O_2^-$) reacts extremely rapid with naturally produced nitric oxide (NO$^-$) to give rise of peroxynitrite (ONOO$^-$)/peroxynitrous acid (ONOOH). Therefore, oxidative stress is involved as one of the major pathogenic factors that directly impair cells functions, promotes cellular organelle damage in the cells, including particularly, mitochondria, lysosomes, and nuclei. Since ROS could directly disrupt the mitochondrial membrane that subsequently leads to release of cytochrome C which becomes a part of apoptosis complex, or it leads to membrane rupture of lysosomes in an additive way resulting in release of cathepsins which activate caspase and apoptosis cascade, finally leading to cell death via apoptosis. On the contrary, the cells protect themselves against the destructive effects of ROS by scavenging them through enzyme defense system, or through the antioxidant activities of dietary compounds.

**MATERIALS AND METHODS**

**Animals and Treatment**

Wistar albino male rats weighing 200-250 g were divided into four groups (n=10) and fasted for 2 days with free access to water. On the third day, the animals were deprived of water for a further 24 h and then underwent surgical operation after general anesthesia with Ketamine/diazepam “according to the rules of the Animal Care and Use Committee of the Alexandria University”. Animals in group one served as a standard ulcerative control group with their pylorus ligated and had only vehicle. The pylorus of rats in the other three groups was ligated using the same model. The animals in groups 2-4 were given 20, 40, and 80 mg/kg (dissolved in saline) curcumin, respectively, by oral gavage, and killed after 1 h by decapitation. The gastric juice was collected and the excised stomach was then filled with 15 mL 4% formalin. After 24 h, the fixed stomach was then opened along the greater curvature, gently rinsed in saline, and then pinned open to expose the gastric mucosa. The haemorrhagic and ulcerative lesions were counted and measured under a light microscope by two colleagues who were blinded to the treatment.

**Determination of Gastric Acidity**

Gastric juice samples were back titrated against 0.01 mol/L NaOH using phenolphthalein indicator (1% in absolute ethanol) to the faint pink colored end point. The total acidity was expressed as milliequivalents using the following equation: Total acidity (mEq/L) = mLs NaOH / liter juice $\times$ 50.

**Determination of Mucosal Lesions**

The number of lesion bands of more than 4 mm long was multiplied by the severity factor 2, while lesions of less than 2 mm long were multiplied by severity factor 1. The ulcer index was calculated as the total number of lesions multiplied by the corresponding severity factor.

**Determination of Neutrophil Activity**

Myeloperoxidase (MPO) is the most abundant protein in neutrophils (also found in monocytes). Hypochlorous acid production by myeloperoxidase was determined by measuring accumulation of taurine chloramine$^{[13]}$. Briefly, 10 µL of fresh heparinized blood samples or purified myeloperoxidase (10 nmol/L) was incubated with 10 mmol/L taurine in 10 mmol/L sodium phosphate buffer (pH 7.4) plus 140 mmol/L sodium chloride (PBS) at 21 ℃ for 5 min. The reaction was started by addition of 30 µmol/L hydrogen peroxide and stopped with 20 µg/mL catalase. The amount of taurine chloramine formed was assayed with 5-thio-2-nitrobenzoic acid. The activity of MPO was measured following the decrease of absorbance at 412 nm. One unit is the amount of MPO that can produce 1.0 nmole of taurine chloramine (hypochlorous acid) at pH 6.5 at 25 ℃ during 30 min in the presence of 100 mmol/L chloride and 100 mmol/L of hydrogen peroxide.

**Mitochondrial Viability**

The ability of cell to convert MTT to formazan crystals depends on the mitochondrial dehydrogenase activity. Briefly, 20 µg of fresh tissue homogenates were incubated in 1 mL of culture medium free from phenol red containing MTT (0.5 mg/mL) at 37 ℃ in an incubator for 60 minutes. Extraction of the formazan dye into 1 mL of DMSO was done with vigorous shaking for 1 h at 37 ℃. Then, 100 µL of supernatant was transferred into a 96-well plate and diluted with 100 µL of DMSO per well. The absorbance was measured at 570 nm on a Multiskan plate reader. Cell viability was expressed as a percentage of viable treated cells relative to untreated control cells. The data were normalized to 100%.

**Antioxidant Markers**

Paraoxonase/arylesterase activity was measured using two synthetic paraoxon (diethyl-p-nitrophenyl phosphate) and phenyl acetate. The activity toward paraoxon (PON activity) was estimated by measuring the initial rate of liberation of p-nitrophenol, the absorbance of which was monitored at 412 nm at 25 ℃ in the assay mixture (800 µL) containing 1.0 mmol/L
paraoxon, 1.0 mmol/L CaCl$_2$ and 20 µL of heparinized plasma or gastric juice in 50 mmol/L glycine/NaOH buffer (pH 10.5). The blank sample (incubation mixture without plasma or gastric juice) was run simultaneously to correct spontaneous substrate breakdown. The activity was calculated from $E_{154}$ of $p$-nitrophenol (18 290 M$^{-1}$ cm$^{-1}$) and expressed in U/mL. 1 U of enzyme hydrolyzes 1 nmol of paraoxon/minute. Arylesterase (AR) was assayed according to the initial rate of hydrolysis in the assay mixture (3 mL) containing 1 mmol/L substrate, 1 mmol/L CaCl$_2$ in 20 mmol/L Tris HCl (pH 8.0) and 30 µL of plasma diluted at 1:8. The absorbance was monitored at 270 nm. Blank samples prepared as described above but without plasma, representing nonenzymatic hydrolysis, were subtracted and the activity was calculated assuming $E_{270}$ = 1310 M$^{-1}$ cm$^{-1}$. The results are expressed in U/mL, 1 U hydrolyzes 1 mmol of phenyl acetate/min.$^{[14]}$

Total antioxidant activity and the ability of test samples to scavenge ABTS$^+$ radical cation were compared with trolox standard.$^{[15]}$ A total of 0.384 g of 2, 2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid, ABTS) was dissolved in 100 mL deionized water to make a concentration of 7 mmol/L. ABTS radical cation (ABTS$^+$) was produced by reacting ABTS stock solution with 2.45 mmol/L potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The stock solution was diluted with PBS to an absorbance of 1.6 at 415 nm after 1/50 dilution. Then, 1.0 mL of diluted ABTS$^+$ was added to 10 µL of juice or plasma sample or standard or H$_2$O as a blank, mixed. The decrease in absorbance was recorded 6 min later at 415 nm. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was used as a standard dissolved in water at the concentration of 0.0, 0.500, 1.0, 1.5, 2.0, and 2.5 mmol/L, respectively. Total antioxidant concentration in samples was measured from the standard constructed using Trolox as a standard.

Protein thiol concentrations were quantified spectrophotometrically using 5, 5'-dithionitrobenzoic acid (DTNB)$^{[16]}$ and their serum level was measured.

Total protein in tissue homogenates was estimated as previously described.$^{[17]}$

**Oxidative Stress Markers**

The total peroxide concentration in gastric juice and plasma was determined as previously described.$^{[18]}$ A total of 9.8 mg ammonium ferrous sulphate was dissolved in 10 mL 250 mmol/L H$_2$SO$_4$. This solution was added to 10 mL HPLC-grade absolute methanol containing 79.2 mg butylated hydroxytoluene. Xylenol orange (7.6 mg) was dissolved in the solution by stirring. Blank reagent was prepared similarly with the omission of ammonium ferrous sulphate. Standard H$_2$O$_2$ (10 µL of 30% stock reagent) was diluted in 250 µL of 5 mmol/L PBS (pH 7.4) to get 250 µmol/L that was serially diluted at 1:1 in the buffer to get 125, 62.5, 31.25, 15.625, and 7.812 5 µmol/L. Briefly, 50 µL sample was added to 450 mL reagent and blank reagent in separate Eppendorf's tubes, mixed well and incubated at room temperature for 30 min, centrifuged at 5 000 rpm for 3 min, and 250 mL of the supernatant was transferred into the wells of a microplate, and OD of the formed purple chromophore was recorded at 560 nm against blank reagent-treated samples. Standards were treated similarly without a blank reagent treatment.

**DNA Fragmentation (As An Apoptotic Marker)**

The total and fragmented DNA in the gastric juice of the luminal content was measured colorimetrically as previously described.$^{[19]}$ Hydrolysis of DNA leads to release of free deoxyribose that is colorimetrically measured after reaction with the diphenylamine reagent producing purple chromogen proportional to its concentration. Briefly, the whole juice samples were centrifuged at 5 000 rpm for 10 min to precipitate damaged and intact cells as a pellet from the supernatant containing fragmented DNA in gastric juice. Ice-cold trichloroacetic acid (TCA, 25% in H$_2$O$_2$) was added to supernatant (1, 1, v/v) to precipitate proteins and lipids and centrifuged. The supernatant was recovered for assaying fragmented DNA. The pellet was resuspended in 0.5 mL warm TCA (40 °C), mixed and incubated for 10 min to hydrolyze intact DNA and centrifuged to remove precipitate. The supernatant was used for assay of deoxyribose released from intact DNA. Briefly, 300 µL diphenylamine reagent was added to 150 µL supernatant/standard deoxyribose (0.0-3.2 mg/L)/or redistilled water blank, boiled for 10 min and cooled on ice. The blue color developed was detected at OD 600 nm against reagent blank and the DNA content in samples was measured from the standard curve. Contents in pellet and supernatant of each sample were added to get total DNA content. Fragmented DNA (%) was calculated by dividing supernatant content by the total content and multiplying by 100.

**Total Rate of IL-6 Content (A Pathogenic Marker)**

The IL-6 level in plasma and stomach juice was measured by ELISA according to its manufacturer's recommendations (Quantikine, cat. # PR6000B, R&D Systems, Inc. Minneapolis, MN, USA).

**Data Analysis**

Data were analyzed using SigmaPlot 10 software.
RESULT

Effect of Curcumin Treatment on Gastric Acid Output and Lesion Formation

All doses of curcumin significantly reduced gastric acid output into the gastric lumen. The level of gastric juice acidity was significantly decreased from 38.60±3.516 mEq/L in the control group to 27.50±2.609 mEq/L, 13.10±0.8750 mEq/L and 6.400±0.6000 mEq/L in the treatment groups receiving 20, 40, and 40 mg/kg curcumin, (Fig. 1A). There was also a significant difference between the different treatment groups. The total acidity level was significantly lower in the groups receiving 40 and 80 mg/kg curcumin than in treatment group receiving 20 mg/kg curcumin (P<0.001) and in treatment receiving 80 mg/kg curcumin than in those receiving 20 mg/kg curcumin (P<0.001).

No significant difference was found in mitochondrial activity among all groups. The mitochondrial activity was 85.40%±5.421% in the control group and 88.00%±5.655%, 88.90%±5.658%, and 92.30%±5.047 %, respectively, in treatment groups receiving 20, 40, and 80 mg/kg curcumin (Fig. 3).

The activity of paraoxonase (PON 1)/arylesters was significantly lower (P<0.001) in the control group (88.00±12.96 U/mL/91.20±12.73 U/mL than in treatment groups receiving 20, 40, and 40 mg/kg curcumin (132.0±9.197 U/mL/138.8±18.80 U/mL, 206.1±16.28 U/mL/193.2±15.02 U/mL, and 269.4±26.64 U/mL/187.1±8.580 U/mL) (Fig. 4).

The activity of MPO was significantly higher in the control group (50.10±2.953 U/mL) than in treatment groups receiving 20, 40, and 40 mg/kg of curcumin significantly decreased gastric lesion formation in the gastric wall from 47.80±3.768 lesions/animal in control group to 36.70±1.802 lesions/animal and 16.80±1.604 lesions/animal and 4.400±0.4269 lesions/animal (P<0.001, Fig. 1B) in treatment groups receiving 20, 40, and 40 mg/kg of curcumin. The results also recorded a significant decrease (P<0.001). The number of lesions was significantly smaller in rats receiving 40 and 80 mg/kg curcumin than in those receiving 20 mg/kg curcumin (P<0.001).

Effect of Curcumin Treatment on Enzyme Activities and Total Protein Thiol Group

The total protein thiol level was significantly increased from 4.190±0.3035 nmol thiol/mg protein in the control group to 5.070±0.2712 nmol thiol/mg protein, 6.100±0.2966 nmol thiol/mg protein and 6.104±0.3197 nmol thiol/mg protein in rats receiving 20, 40, and 80 mg/kg curcumin (P<0.001) (Fig. 2).
Curcumin (36.60±2.790 U/mL, 13.20±1.977 U/mL, and 10.80±1.093 U/mL) (P<0.001, Fig. 7), while it was significantly lower in rats receiving 80 and 40 mg/kg than in those receiving 20 mg/kg of curcumin (P<0.001).

**Fig. 3.** Mitochondrial dehydrogenase activity detected by MTT test after pylorus ligation in the control and treatment groups receiving different concentrations of curcumin with the data normalized to 100%.

**Fig. 4.** Activity of plasma paraoxonase 1 and arylesterase detected using synthetic paraoxon (paraoxonase activity) and phenyl acetate respectively after pylorus ligation in the control and treatment groups receiving different concentrations of curcumin.

**Effect of Curcumin Treatment on Oxidative Stress/Antioxidant**

The total antioxidant level was significantly lower in plasma of control group (0.722 ±0.04026 mmol/L) than in treatment groups receiving 20, 40, and 40 mg/kg curcumin (1.145±0.06922 mmol/L, 1.636±0.1449 mmol/L, and 1.950±0.1829 mmol/L) (P<0.001, Fig. 5A), while it was 0.109 ±0.01115 mM/L in gastric juice of the control group and significantly increased in the treatment groups receiving 20, 40, and 80 mg/kg curcumin (0.4974±0.05664 mmol/L, 0.7434±0.04762 mmol/L, and 0.8318±0.04488 mmol/L) (Fig. 5B). In contrast, the total peroxide level was significantly higher in plasma of control group (60.52±5.721 µmol/L) than in plasma of treatment groups receiving 20, 40, and 80 mg/kg curcumin (39.90±4.245, 38.58±4.976, and 20.33±1.642) (P<0.001, Fig. 6A). The total peroxide level was significantly higher in gastric juice of control group (201.8±20.76 µmol/L) than in gastric juice of treatment groups receiving 20, 40, and 40 mg/kg of curcumin (86.78±15.92 µmol/L, 43.87±3.192 µM/L, and 40.75±8.442 µmol/L) (P<0.001, Fig. 6B).

**Fig. 5.** Total antioxidant level in plasma (A) and gastric juice (B) after pylorus ligation in the control and treatment groups receiving different concentrations of curcumin.

**Fig. 6.** Total peroxide level in plasma (A) and gastric juice (B) after pylorus ligation in the control and treatment groups receiving different concentrations of curcumin.
Effect of Curcumin Treatment on Apoptotic Incidence

The fragmented DNA level was significantly higher in control group (46.36%±3.707%) than in treatment groups receiving 20, 40, and 80 mg/kg curcumin (24.51%±2.136%, 10.02%±1.314%, and 6.897%±0.866 2%) (P<0.001, Fig. 8).

Effect of Curcumin Treatment on Level of Proinflammatory Cytokine IL-6

The IL-6 level was significantly higher in plasma of control group (139.9±11.90 pg/mL) than in treatment groups receiving 20, 40, and 80 mg/kg curcumin (86.17±8.739 pg/mL, 52.21±7.166 pg/mL, and 59.77±5.597 pg/mL (P<0.001, Fig. 9A). If we have compared the IL-6 level in stomach with its level in the plasma we will notice that the level of IL-6 is more prominent at the stomach, where IL-6 level was significantly higher in the gastric juice compared with its level at the plasma. The IL-6 level was 317.7±31.10 pg/mL, 166.4±7.956 pg/mL, 153.7±9.315 pg/mL, and 126.9±11.52 pg/mL respectively in gastric juice, while it was 139.9±11.90 pg/mL, 86.17±8.739 pg/mL, 52.21±7.166 pg/mL and 59.77±5.597 pg/mL respectively in plasma of rats receiving 20, 40, and 80 mg/kg curcumin, respectively.

DISCUSSION

Gastric acid hypersecretion is one of the major pathogenic factors for the induction of gastric ulcer disease. The back-diffusion of acid into the mucosa could directly lead to vascular leakage and aggressive damaging effect in the basement membrane of both epithelial and mucosal cells in the gastric wall, which could inhibit the restitution processes in the injured mucosa and induce a progression of apoptosis to deeper layers of the mucosa. In this study, the gastric acidity and the number of lesions in gastric wall of control group were significantly higher than in gastric wall of treatment groups (Fig. 1 A and B). The total thiol level was significantly lower in control group than in treatment groups, due to the increased total peroxide level (Figs. 2 and 6) since, besides their damaging effect, peroxide anions could be reduced into H₂O₂, which has less destructive effect on different organelles and could oxidize the essential thiol (-SH) groups for the activity of some enzymes and inhibit their activities[20]. For instance, protein thiol group plays a crucial role in the detoxification
of xenobiotics. However, the main danger for H$_2$O$_2$ arises from its ability to cross cell membranes rapidly. Once inside the cells, it can probably react with Fe$^{2+}$ and Cu$^{2+}$ ions to form hydroxyl radicals, which may be the origin of many toxic insults. On the other hand, myeloperoxidase, an inflammatory enzyme, has been speculated to have an even more harmful effect than C-reactive protein, due to the production of reactive nitrogen species, such as peroxynitrite and nitrogen dioxide. Furthermore, myeloperoxidase can also use hydrogen peroxide resulting from the reduction of superoxide radicals to oxidize chloride to hypochlorous acid (HPO). In this study, the activity of myeloperoxidase (MPO) in whole blood was significantly higher in control group than in treatment groups (Fig. 8). It is important to notice that the increased basal neutrophil MPO activity is not related to soluble plasma MPO due to intracellular MPO activity$^{[21]}$. Paraoxonase (PON 1) has been emphasized in several studies to have a potential protective effect against oxidation of low-density lipoprotein (LDL) since the oxidized phospholipids and cholesteryl esters are probably the main substrates for its activities$^{[22]}$. Therefore, in this study, both paraoxonase activity and total antioxidant level were significantly lower in control group than in treatment groups (Figs. 4 and 5). In the contrary, the total peroxide level was significantly higher in control group than in treatment groups (Fig. 6).

There is no doubt that the mitochondria are the main source of ATP, which is a source of energy for most cellular functions since the ability of cells to convert MTT to formazan depends on mitochondrial dehydrogenase activity that is lost with cell death and can fix during cellular repair for toxic insults. No significant difference was found in the mitochondrial activity among all groups (Fig. 3), which may be due to the fact that only the healthy and viable cells are attached to the gastric wall, whereas only apoptotic and dead cells are shedding from the gastric wall into the gastric lumen. Therefore, the fragmented DNA level was higher in control group than in treatment groups (Fig. 7). We have shown in a previous study that the presence of fragmented DNA in gastric juice is a stringent morphological criterion for apoptosis$^{[23]}$. However, in necrosis, the membrane is the major site of damage and loses its ability to regulate osmotic pressure among other organelles. Eventually, cell contents are released and eliciting inflammatory reactions accompanied by diffuse inflammatory cells and lymphocytic infiltration. It is noteworthy that the changes in different assayed parameters were more prominent at the stomach level than at the plasma level, which may directly reflect a rather local problem in the stomach region. The last event may be due to the elevated levels of proinflammatory IL-6 (Fig. 9A and B) since the local inflammatory cytokine IL-6 primarily activates neutrophils, lymphocytes and monocytes/macrophages at the inflammatory site (Fig. 4), which in turn initiates different oxidative bursts (Fig. 6), toxic metabolites and lysosomal enzymes responsible for local tissue damage in peptic ulcer disease$^{[24-25]}$. Thus, the proinflammatory IL-6 could predict more precisely the severity and duration of inflammation, particularly in its acute phase, than TNF-α$^{[26]}$.

In conclusion, curcumin exerts its anti-ulcer activity not only by affecting oxidative stress and total antioxidant capacity but also by inhibiting IL-6 secretion and preventing apoptosis in a dose-dependent manner. Further prospective studies are required to investigate the mechanism underlying the inhibitory effect of curcumin on IL-6 signalling and caspase pathways.

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Competing Interests

Potential conflicts do not exist. None of the authors has a commercial interest or other relationship with manufacturers of pharmaceuticals, laboratory supplies, and/or medical devices or with commercial providers of related medical services.

Authors’ Contributions

Muobarak designed the study, performed the biochemical experiments, the statistical analysis and wrote the manuscript as a part of his PhD project; Karolin purchased all chemicals and assays, participated in conceiving the study, the sequence alignment and read the final manuscript.

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