Cigarette Smoke Extracts Delay Wound Healing in the Stomach: Involvement of Polyamine Synthesis

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The association between cigarette smoking and peptic ulcer diseases has been well established. Ornithine decarboxylase (ODC) is crucial for the gastroprotective and mucosal growth promoting effects in gastric ulcer healing. The aim of this study is to elucidate the possible mechanism of how inhibition of ODC activity is involved in the delay of ulcer healing, if any, by cigarette smoke extracts (CSE). CSE were fractionated into chloroform extract (CE) and ethanol extract (EE). In *in vivo* study, rats with acetic acid-induced ulcers were given CE or EE intragastrically (2.5 or 5 mg/kg) once daily for 3 days. Ulcer sizes were significantly larger after CE or EE administration, followed by an increase in myeloperoxidase activity and a reduction in cell proliferation. However, both CSE stimulated the number of microvessels following the increase of basic fibroblast growth factor. In *in vitro* studies, the effect of CE or EE (10, 40, or 100 μg/ml) on cell migration and cell proliferation were measured using an *in vitro* wound model and [^{3}H]-thymidine incorporation assay, respectively. Both CSE delayed cell migration and decreased cell proliferation, which were accompanied with a reduction in ODC activity. Exogenous spermidine (5 or 10 μM) could reverse the inhibitory action on cell proliferation and ODC activity induced by CSE. In conclusion, both CSE significantly delayed ulcer healing as a result of reduction in cell proliferation and cell migration. All these effects are, in part, related to the reduction of polyamine synthesis.

[Exp Biol Med Vol. 227(2):114–124, 2002]

Key words: cigarette smoke extracts; ornithine decarboxylase; myeloperoxidase; gastric ulcer healing

Peptic ulcer disease is a chronic inflammatory disorder that adversely affects the normal integrity of the stomach and duodenum as a result of the noxious action produced by some luminal oxidants from foods, bacteria, and cigarette smoking (1, 2). However, our gastric mucosa is capable of repair after mucosal injury by mucosal restitution or reepithelization by sloughing off the damaged cells. This is achieved by rapid migration of viable cells to the injured site and is finally accomplished by cell proliferation to replace the number of dead cells during ulcer healing (3). Ulcer healing is commenced by the stimulation of number of growth factors in the ulcer margin and ulcer base. It has been shown that inflammation at the ulcer crater by itself is a potent stimulus for the production of different growth factors, including nitric oxide (4), prostaglandin E_2 (5), vascular endothelial growth factor (VEGF) (6), and basic fibroblast growth factor (bFGF) (7). In particular, bFGF induces prostaglandin synthesis (8), which increases blood vessel formation (9) and cell proliferation of endothelial cells (10). Most often, myeloperoxidase (MPO) activity is used as a marker for inflammation because it is an enzyme secreted by leukocytes, with a potent oxidizing capacity (11, 12). It is anticipated that the increase of such an enzyme during the inflammatory process in the stomach would not only damage the tissue, but would also stimulate the production of bFGF to enhance angiogenesis followed by ulcer healing. This intriguing phenomenon has not been examined and is worthwhile for further study.

In addition to bFGF, polyamine is another indispensable factor for normal repair of gastric and duodenal erosions, and it is known to participate in the progression of cell cycle followed by cell proliferation (13). Ornithine decarboxylase (ODC) is a rate-limiting enzyme for the synthesis of polyamines, which are crucial for cell migration and cell proliferation during ulcer healing (14, 15). The increase in ODC activity stimulates cell growth, whereas depletion of ODC with α-difluoromethylornithine (DFMO), a specific inhibitor of ODC, entirely prevents ulcer healing

This research was supported by the University of Hong Kong Committee on Research and Conference Grants (CRCG) and the Hong Kong Research Grant Council (RGC).

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Received May 9, 2001.
Accepted October 13, 2001.

1535-3702/02/2272-0114$15.00

(16, 17). All of these findings suggest that both bFGF and ODC are crucial for ulcer repair in the gastrointestinal tract.

Our preliminary findings have shown that passive cigarette smoking delays gastric ulcer healing (18), which is reported to be due to reduction of epidermal growth factor (EGF) production and ODC activity (19, 20). It is not known which components in cigarette smoke are responsible for these effects, although it has been reported that nicotine in cigarette smoke is not the culprit for ethanol ulceration in rats (21). Since cigarette smoking is an important environmental factor in causing peptic ulcer and its relapse (22–25), the clarification of which components in cigarette smoke and the mechanism of how they affect wound repair in the gastrointestinal tract is pivotal in understanding the pathogenesis of peptic ulcer disease in humans.

In this context, we would like to explore the differential effects of different cigarette smoke extracts (CSE) on gastric ulcer healing in rats and their relationship with the severity of mucosal inflammation and production of bFGF and angiogenesis at the ulcer site. We would also correlate the ulcer healing effect of CSE in rats with cell migration and proliferation in relation with the ODC activity in isolated gastric epithelial cells.

Methods and Materials

Reagents and Drugs. All chemicals and reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Preparation for Cigarette Smoke Extracts. The 'Camel' cigarette (R. J. Reynolds, Winston-Salem, NC) was used in the present study. Smoke from burning cigarettes was bubbled into chloroform and ethanol solvents, respectively, and different fractions of cigarette smoke extract were collected (20). The substances dissolved in ethanol were regarded as ethanol fraction. This fraction was preconcentrated by a rotary evaporator (RE 47, Yamato Scientific Co., Tokyo, Japan) connected to a cooling system (F10, Julabo Laborthechik, Seelbach, Germany) to evaporate the excess ethanol. After evaporation, chloroform was added to the ethanol fraction to extract those substances dissolved in chloroform. The remaining insoluble part was regarded as ethanol extract (EE). The chloroform-soluble fractions were combined and concentrated again following the same procedures, and this was known as chloroform extract (CE). Both CE and EE were finally prepared in 0.1% dimethyl sulfoxide (DMSO) solution before oral administration. A control group followed the same procedure as the CSE groups but without cigarette smoke, and again, this was prepared in 0.1% DMSO and was used as vehicle control. A single batch of both CE and EE was used in the current study.

Both EE and CE were analyzed for their chemical types by the thin-layer chromatography and gas chromatography/mass spectrophotometry methods. The chemicals identified in the EE were mainly alkaloids, including nicotine, whereas terpenoids, phenolic compounds, hydrocarbons, organic acids, fatty acids, and flavonoids were found in the CE, but no alkaloid was observed (21).

Animals and Induction of Gastric Kissing Ulcers. The present study was approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong. Male Sprague-Dawley rats (180–200 g) were reared on a standard laboratory diet (Ralston Purina, Chicago, IL) and were given tap water. They were raised under controlled temperature (22 ± 1°C), humidity (65%-70%), and day-night cycle (12:12-hr light: dark). Rats were starved for 24 hr before ulcer induction, but were allowed free access to tap water. Gastric kissing ulcers were obtained by luminal application of acetic acid as described previously by Tsukimi and Okabe (26), with modifications. In short, the abdomen was opened under ether anesthesia and the stomach was exposed. A clip was used to clamp together the anterior and posterior walls of the stomach. Afterward, 0.12 ml of 60% acetic acid (v/v) was injected using a syringe through the forestomach into the gastric lumen in between the clamped area for 45 sec. Acid was then withdrawn from the lumen and the abdomen was sutured. Afterward, the rats were fed with a standard diet and were given tap water *ad libitum*.

Assessment of Ulcer Size. Altogether, there were six treatment groups with seven rats in each group: normal group without ulcer; the other groups with ulcer: vehicle (0.1% DMSO) treatment as control; 2.5 mg/kg CE; 5 mg/kg CE; 2.5 mg/kg EE; and 5 mg/kg EE. Rats were given 5 ml/kg orally with either the vehicle or the two CSE using an orogastric tube. They were given the treatment once daily for 3 days following the day of ulcer induction, and were sacrificed 3 days after treatment. The stomach was excised, opened along the greater curvature, and rinsed thoroughly with normal saline to remove the attached debris. After the stomach was blotted dry and spread, a transparency was used to trace the outermost lining of the ulcerated tissue and was then copied over a square grid to calculate the ulcer size (in millimeters squared). Both ulcerated and corresponding intact gastric tissues were taken and fixed in 4% formalin at 4°C overnight for histological study. The remaining glandular mucosa around the ulcer (including the ulcer margin and adjacent normal mucosa) was scraped with a glass slide on an ice-cold dish and was immediately frozen in liquid nitrogen. The mucosal samples were stored at −70°C until assay for different parameters.

Measurement of MPO Activity in the Gastric Mucosa. The activity of MPO was described previously (11) with modifications. Tissue samples were homogenized in 50 mM phosphate-buffered saline (PBS, pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) in ice-cold conditions for 30 sec. Homogenates were underwent a freeze-thawed process (liquid nitrogen and a 25°C water bath) and were finally sonicated for 30 sec. This process was repeated three times. The samples were centrifuged (J2-21 centrifuge; Beckman Instruments, Palo Alto,

CA) at 14,000 rpm for 20 min at 4°C. The supernatant was added to a mixture of 2 mM 3,3′,5,5′-tetramethylbenzidine (150 μl), 0.3 M H_2O_2 (50 μl), and 80 mM PBS (pH 5.4, 250 μl) and was incubated for 25 min at 25°C. The reaction was quenched by 0.2 M H_2SO_4 (2.5 ml) and the resulting mixture was subjected to measure the absorbance at 450 nm with a spectrophotometer (DU 650; Beckman Instruments) using horseradish peroxidase as standard.

Immunohistochemistry with Proliferating Cell Nuclear Antigen (PCNA). Mucosal cell proliferation was assessed by immunohistostaining as described by Tarnawaski *et al.* (27) with modifications. The sections were digested with trypsin for 15 min at room temperature and were incubated with a blocking agent (LSAB kit; DAKO, Copenhagen, Denmark) for 1 hr. After blocking, they were incubated with a monoclonal primary antibody against mouse PCNA (1:200; sc-56; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After washing with Tris-buffered saline (TBS), sections were incubated with Link reagent (DAKO) for 1 hr at room temperature. Another washing with TBS, they were incubated with streptavidin (DAKO) for 1 hr. The sections were further incubated with H_20_2-diaminobenzidine (DAB) to visualize the PCNA-positive cells. Finally, the sections were counterstained with Mayer's hematoxylin for 1–2 min, and were cleared by graded ethanol and xylene solutions and mounted. PCNA-positive cells were stained brown and counted under an image analyzer (Q500IW, Leica Imaging Systems, Wetzlar, Germany). The number of PCNA-positive cells was counted with a magnification of ×200.

Determination of Angiogenesis in the Granulation Tissue. The number of microvessels was assessed by immunohistochemical method for von Willebrand factor (28). The procedures were basically similar to the assessment of PCNA-positive cells staining, except the primary antibody used was rabbit anti-human von Willebrand factor (1:200; DAKO). After the sections were mounted, they were counted under an image analyzer (Q500IW, Leica Imaging Systems) at ×200 magnification.

Cell Culture. Normal rat gastric epithelial cell line, RGM-1, was used in this study. RGM-1 (established by Hirofumi Matsui, Institute of Clinical Medicine, University of Tsukuba, Ibaraki, Japan) was obtained from Riken Cell Bank (Tsukuba, Japan). It was passaged into DMEM/F-12 medium (Gibco-BRL, Grand Island, NY) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, and 20% fetal bovine serum (FBS; Gibco-BRL) in an incubator at 37°C, 95% humidity, and 5% carbon dioxide. Cells were treated with different concentrations of CE or EE for 5 hr in duplication. These experiments were repeated three times. Hence, the total sample number was 6 in each treatment group. They were subsequently subjected to different parameter measurements.

[^{3}H]Thymidine Incorporation. This method was described previously (29) with modifications. After treatment, cells were incubated with 0.5 μCi/ml [^{3}H]thymidine (Amersham, Arlington Heights, IL) for 5 hr at 37°C. The solution was aspirated and then washed with 0.5 ml of iced 0.15 M NaCl. Afterward, 10% trichloroacetic acid (TCA) was added into the wells and was incubated for 15 min at room temperature. The well was rinsed with distilled water four times, and this was followed by adding 0.5 ml of 1% sodium dodecyl sulphate (SDS) to each well for incubation for 15 min at 37°C. The content was transferred into a scintillation vial and 0.5 ml of SDS was added to rinse the well; this was all transferred into the same vial. Finally, 9 ml of water-accepting scintillation fluid was added in the vial and it was then vortexed. The amount of [^{3}H]thymidine incorporated was measured using a liquid scintillation spectrometry on a beta counter.

Cell Migration. The wound healing assay was described previously (30) with modifications. Cells were seeded in 24-well culture plates and were cultured in DMEM/F-12 with 20% FBS until confluent. After confluent, monolayers of the cells were achieved, and cells were starved for 24 hr in DMEM/F-12 medium in the absence of FBS. The cells were pretreated with mitomycin C (2 μg/ml) for 2 hr before wound formation. An artificial circular wound of cell-free area 2 mm^2 was made in the center of the monolayer using a plastic blade. The wounded monolayers were then cultured in DMEM/F-12 medium (without FBS supplement) in the presence or absence of different concentrations of CSE (10, 40, or 100 μg/ml). The changes of cell-free area was monitored up to 48 hr using a digital image processor connected to a microscope (Nikon, Tokyo, Japan) and the areas were measured three times with an image analyzing program (Leica, Cambridge, UK).

ODC Activity. Cells were incubated in the absence or presence of spermidine (5 or 10 μM) for 2 hr, and were then incubated with CSE for another 5 hr. Cells were scraped from culture plates and 0.5 ml of ODC buffer (10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 0.05 mM pyridoxal 5′ phosphate, and 5 mM dithiothreitol) was added. Samples were sonicated under an ice-cold conditions for 20 sec and were centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant collected was used for the assay.

ODC activity was determined by the production of $^{14}CO_2$ from DL-[^{14}C] ornithine using a radiometric technique (31). The resulting supernatants (300 μl) were incubated with 2.5 mM L-[1-^{14}C] ornithine at 37°C for 15 min. The liberated $^{14}CO_2$ by the decarboxylation of ornithine was absorbed by a filter paper impregnated with 2 mM NaOH (20 μl), and it was placed inside a plastic well connected to the stopper, which hung over the reaction mixture. The incubation was terminated by the addition of 10% TCA (300 μl) and was incubated for another 10 min. Labeled CO_2 trapped in the filter paper was measured by a liquid scintillation counter.

Western Blotting for bFGF Expression. Cells were harvested in RIPA buffer for Western blot. After sonication and centrifugation, the protein concentration was routinely measured using a protein assay kit (Bio-Rad Labo-

ratories, Hercules, CA). Total cell proteins (50 μg/lane) were separated by 7.5% SDS-polyacrylamide gel electrophoresis overlaid with a 5% acrylamide stacking gel, and were then transferred to Hybond C nitrocellulose membranes (RPN203G, Amersham). The membranes were probed with a polyclonal antibody against bFGF (sc-1360; Santa Cruz Biotechnology) overnight at 4°C. The membranes were developed by the enhanced chemiluminescence system (Amersham) and were exposed to x-ray film (F050099; Fuji, Tokyo). Prestained molecular weight standards (Novex, San Diego, CA) were used as markers. Quantitation was carried out by a video densitometer (Scan Maker III; Microtek International Inc., Taiwan).

Cell Viability. Cell viability was assayed by routine 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method (32, 33). After treatment with CSE at different concentrations, cells were cultured with fresh medium with 2.5% MTT solution (5 mg/ml) for another 3 hr at 37°C. Then, 100 μl of 0.04 N HCl-isopropanol was added and mixed thoroughly. Within 30 min, the color change was recorded spectrophotometrically with the microplate reader (MRX, Dynex Technologies, Chantilly, VA) at 570 nm. The same test was repeated three times and the OD was calculated for statistic analysis.

Administration of Exogenous Spermidine. Cells were pretreated with exogenous spermidine (5 or 10 μM) for 2 hr prior to the incubation with CE or EE for 5 hr. This pretreatment procedure was the same for the assays of [^{3}H]thymidine incorporation and ODC activity.

Statistical Analysis. Results were expressed as the means ± SEM. Statistical analysis was performed with an analysis of variance (ANOVA) followed by a Dunnett *t* test or Turkey's honestly significant difference test, and *P* values less than 0.05 were considered statistically significant.

Results

Effect of CSE on Ulcer Healing in Rats. Ulcer sizes were measured 4 days after ulcer induction. With daily oral administration of either CE or EE (2.5 or 5 mg/kg) for 3 days starting 1 day after ulcer induction, the ulcer size was larger in both CSE-treated groups when compared with the control group (Fig. 1). The highest dose (5 mg/kg) of both CE and EE delayed ulcer healing significantly.

Effect of CSE on MPO Activity in Rats. MPO activity was used as a marker for neutrophil infiltration to signify the degree of inflammation. Gastric mucosal MPO activity was increased 2-fold in the ulcerated gastric mucosa (control) than that in the intact gastric mucosa (normal; Fig. 2). Oral administration (i.g.) of CSE further stimulated MPO activity by 1.5-fold for all doses of treatments when compared with the control group.

Effect of CSE on Angiogenesis in Rats. The growth of new blood vessels was denser at the ulcer margin than the ulcer base in both the control and CSE-treated groups (Fig. 3). The number of microvessels was significantly elevated in all the CSE-treated groups 4 days after ulcer induction when compared with the control group. Also, angiogenesis was significantly increased by 3-fold in the gastric mucosa with ulcer than in intact tissues. This is probably due to the mucosal defensive mechanism in response to inflammation in the stomach.

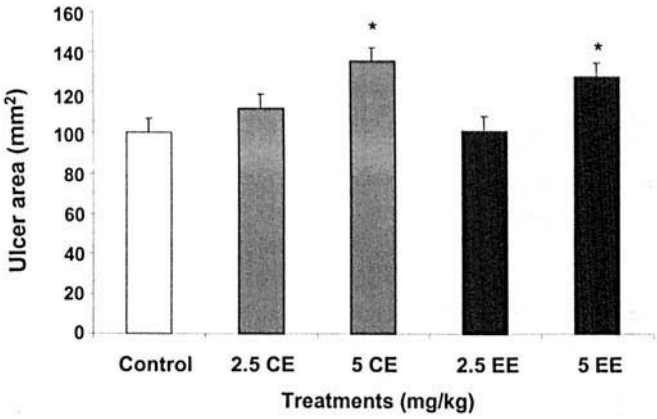

Figure 1. Effect of CE and EE (2.5 or 5 mg/kg) on ulcer sizes 4 days after ulcer induction. Rats were administered with either CE or EE intragastrically once daily for 3 days, and the ulcer size was measured after the last treatment. Values are means ± SEM of seven rats. *$P < 0.05$ vs control group with vehicle treatment.

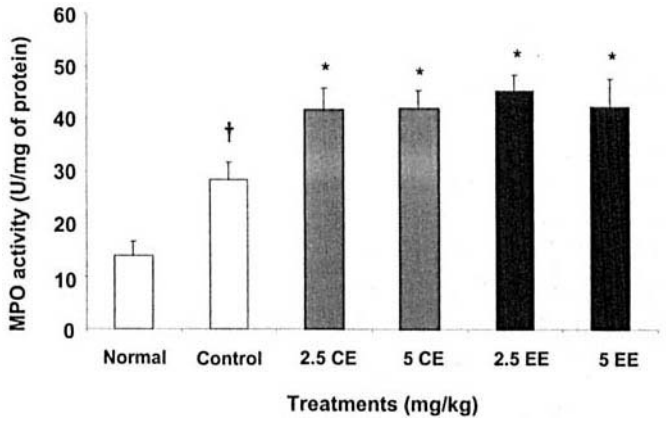

Figure 2. Effect of CE and EE (2.5 or 5 mg/kg) on MPO activity in rat gastric mucosa 4 days after gastric ulceration. Rats were given either CE or EE intragastrically once daily for 3 days, and MPO activity was measured in gastric mucosa. Values are means ± SEM of seven rats. *$P < 0.05$ vs control group with vehicle treatment; †$P < 0.05$ vs normal group without ulcer.

Effect of CSE on PCNA-Positive Cells Staining in Rats. The maximum number of PCNA-positive cells was at the first field away from the edge of ulcer margin in both the control and CSE-treated groups, and decreased markedly with higher concentrations in a dose-dependent manner for both extracts (Fig. 4). The maximum reduction of cell proliferation was found in the 5 mg/kg CE-treated groups, which inhibited 60% of cell proliferation when compared with the control group.

Effect of CSE on bFGF Protein Expression in Rat Gastric Mucosa. Expressions of bFGF were significantly stimulated in a dose-dependent manner in all CSE-treated groups. There was a 2- and 3-fold increase at doses of 2.5 and 5 mg/kg, respectively, for both CE and EE (Fig. 5).

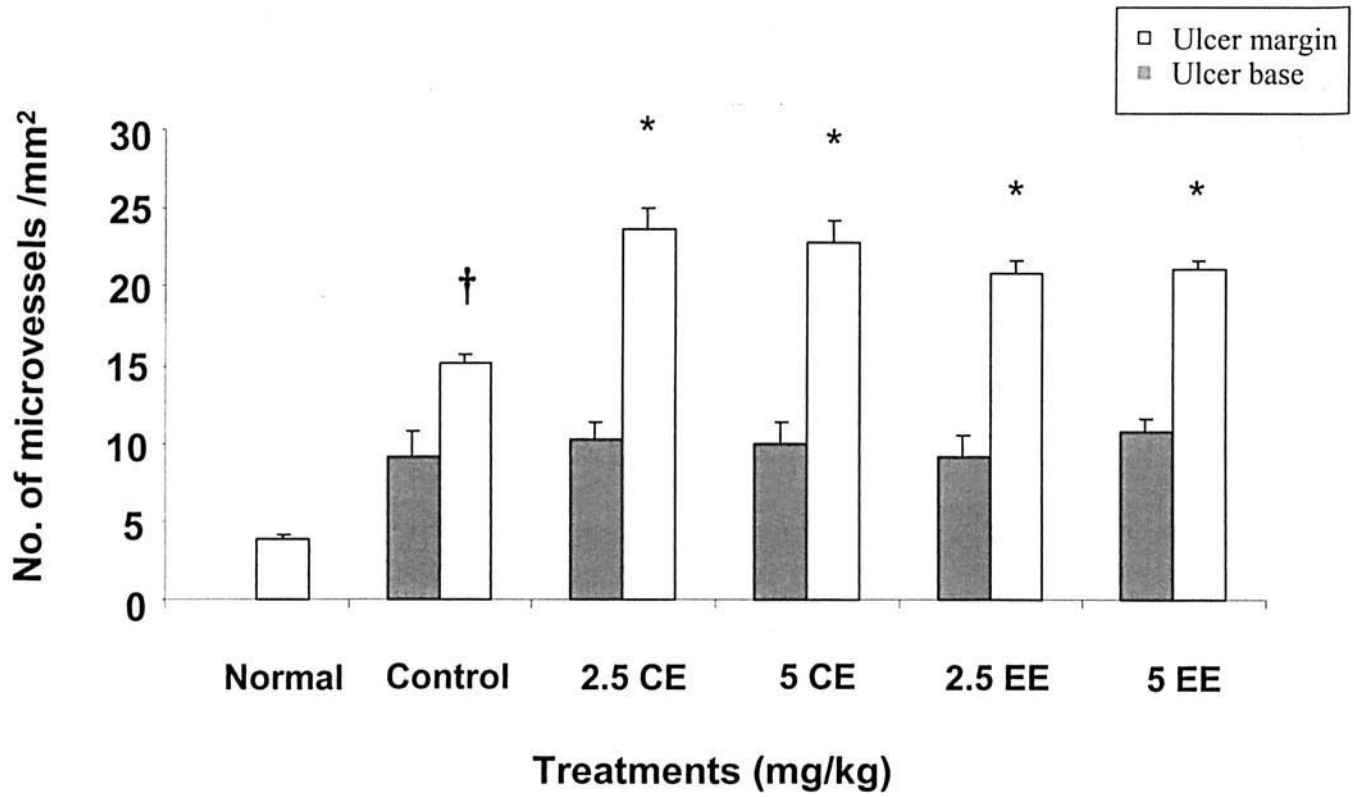

Figure 3. Effect of CE and EE (2.5 or 5 mg/kg) on angiogenesis 4 days after gastric ulceration. Rats were given either CE or EE intragastrically once daily for 3 days, and angiogenesis in gastric ulcerated tissues was visualized by immunohistochemical staining. Values are means ± SEM of seven rats. $^{*}P < 0.05$ vs control group with vehicle treatment; $\dagger P < 0.05$ vs normal group without ulcer.

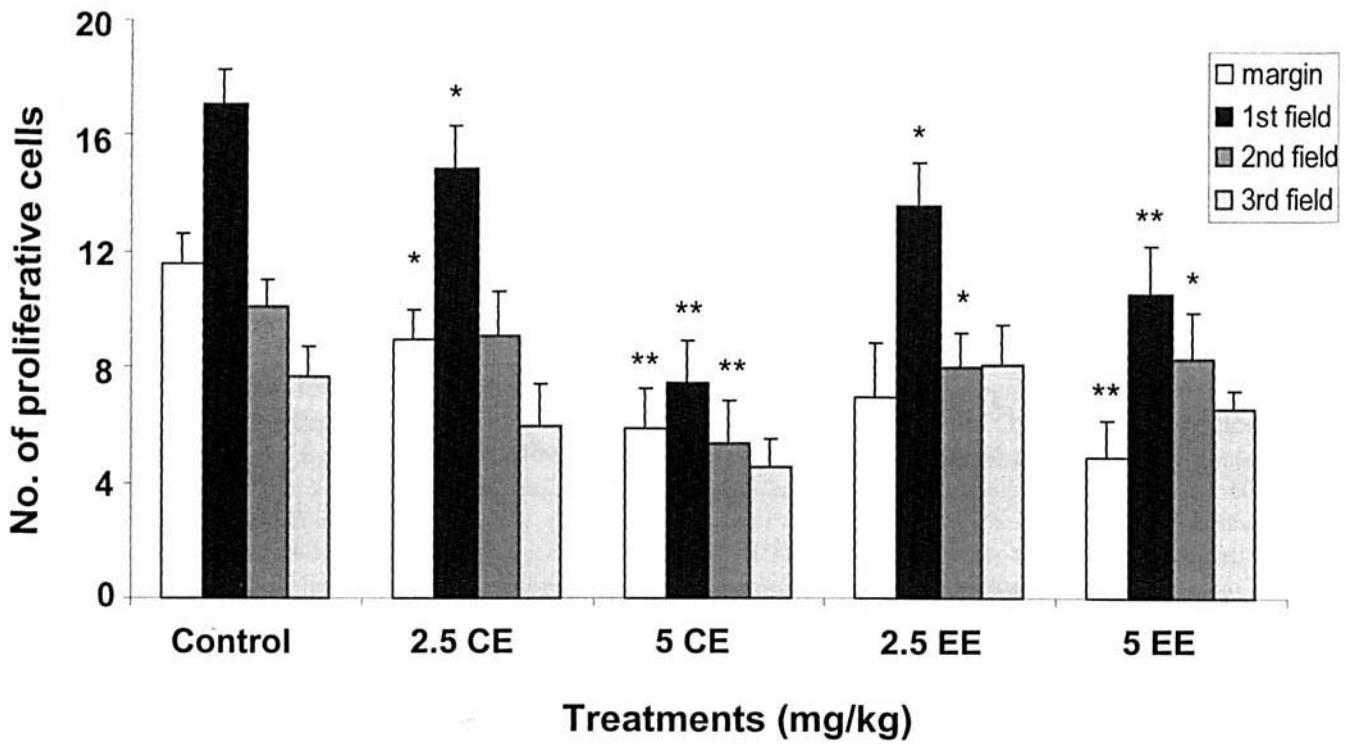

Figure 4. Effect of CE and EE (2.5 or 5 mg/kg) on PCNA-positive cells staining 4 days after gastric ulceration. Rats were given either CE or EE intragastrically once daily for 3 days, and the number of PCNA-positive cells in gastric tissues was counted. Values are means ± SEM of seven rats. $^{*}P < 0.05$, $^{**}P < 0.01$ vs corresponding control group with vehicle treatment.

Effect of CSE on Cell Proliferation in RGM-1 Cells. Cell proliferation was measured using [^{3}H]thymidine incorporation assay. After 5 hr of either CE or EE incubation, cell proliferation was significantly decreased in a dose-dependent manner for both extracts (Fig. 6), yet it exerted no effect on cell viability assessed by MTT test (data not shown). EE at doses of 10, 40, and 100 μg/ml reduced cell proliferation to 60%, 40%, and 30% of the control, respectively. Cell proliferation was inhibited to a greater extent by CE in which 10 μg/ml already suppressed cell proliferation by 60% relative to the control group.

Effect of CSE on Cell Migration in RGM-1 Cells. An artificial wound was made after confluent. The sizes of the wound were all constant at the beginning of cell migration. Cells were incubated with different concentrations of CSE. The rate of migration was different throughout the time course, in which the healing rate was fastest for the first 12 hr, then gradually slowed down thereafter. In the control group, the cell-free area reached 50% after 12 hr and nearly 100% after 48 hr (Fig. 7). Both CSE inhibited restoration in a dose and time-dependent manner at all time points starting from 12 hr. Both CSE (10 μg/ml) markedly suppressed cell migration to about 20% of the cell-free area at 48 hr. In particular, 100 μg/ml CE significantly inhibited restoration, the cell-free area reached a plateau after 12 hr, and the restoration was about 60% at 48 hr when compared with the control group.

Effect of CSE on ODC Activity. Cells treated with CSE significantly reduced the activity of ODC in a dose-dependent fashion (Fig. 8). For EE, the activity was reduced to 88%, 80%, and 62% of the control for 10, 40, and 100 μg/ml respectively. The effect of CE was found to be more potent, as it suppressed the activity to 75%, 69%, and 33% of the control, respectively, for the same concentration as EE. Maximum reduction of ODC activity was noted at 100 μg/ml for both extracts.

Effect of Exogenous Spermidine on Inhibition of ODC Activity and Cell Proliferation Induced by CSE. Addition of exogenous spermidine stimulated cell proliferation in a concentration-dependent manner and reversed the suppression of cell proliferation induced by CSE nearly to the basal level as measured by [^{3}H]thymidine incorporation (Fig. 9, a and b). In the control group, exogenous spermidine (10 μM) could increase the activity of ODC, but not the lower dose (Fig. 10, a and b). Also, exogenous spermidine could significantly reverse the inhibition of ODC activity in all CSE-treated groups, since they all restored to the basal level. Therefore, deficiency of intracellular spermidine may account for the reduced cell proliferation and ODC activity in CSE-treated groups.

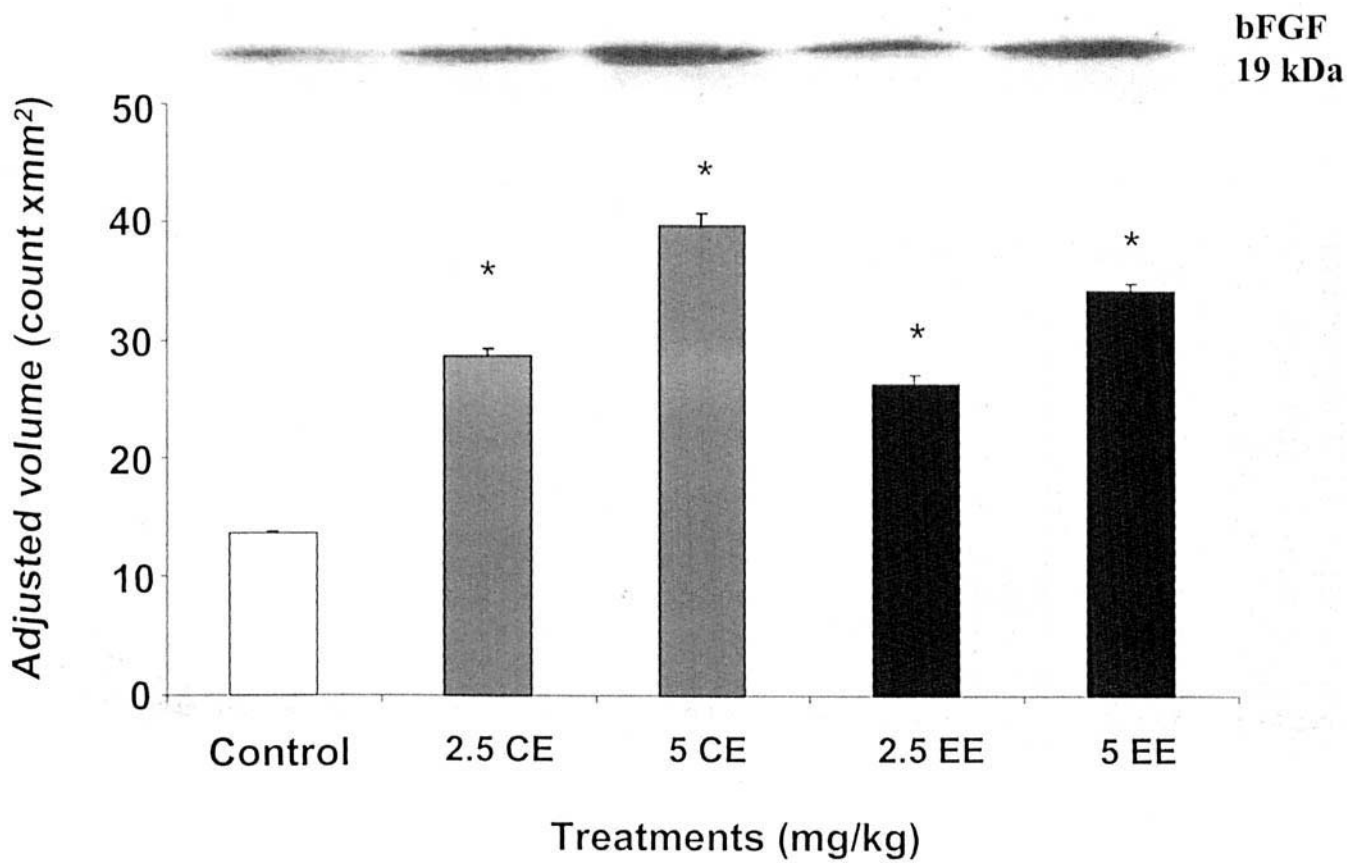

Figure 5. Effect of CE and EE (2.5 or 5 mg/kg) on bFGF protein expression in rats 4 days after ulcer induction. Rats were given either CE or EE intragastrically once daily for 3 days, and bFGF protein expression was measured by Western Blotting. Lane 1, control; Lane 2, CE at 2.5 mg/kg; Lane 3, CE at 5 mg/kg; Lane 4, EE at 2.5 mg/kg; Lane 5, EE at 5 mg/kg. Values are means ± SEM of seven rats. *$P < 0.001$ vs control group with vehicle treatment.

Discussion

Ulcer healing is rather a complex and spontaneous process that involves migration and proliferation of cells to the injured site in order to reconstruct the muscularis mucosae and mucosal architecture (34). Cigarette smoking undoubtedly has an adverse effect on gastric ulcer healing (18, 35). This phenomenon was also observed when using CSE (20). However, the exact mechanism of how CSE delays ulcer healing is not fully elucidated. In order to outline the mechanism, rats were given CSE intragastrically for 3 days after ulcer induction. In the present study, both CE- and EE-treated groups had larger ulcer sizes when compared with the control group (Fig. 1), indicating that CSE could produce some adverse effects on the gastric mucosa that would prolong the healing process in the stomach.

Indeed, CSE further increased the mucosal MPO activity in the ulcerated stomach (Fig. 2). This high gastric mucosal MPO activity suggested that CSE would increase the inflammatory response in the gastric mucosa through neutrophil infiltration (21). This inflammatory response would then trigger the production of various growth factors, especially bFGF (7), to initiate angiogenesis and to promote the healing process in the stomach. Consistent with this phenomenon, the present study also showed that CSE induced more inflammation in the ulcerated mucosa (Fig. 2), followed by more angiogenesis and bFGF protein expression in these groups of animals (Figs. 3 and 5), suggesting that inflammation and angiogenesis are closely related. However, CSE-treated groups still had a bigger ulcer in the early stage of ulceration (Fig. 1), though angiogenesis was significantly increased. Therefore, we believe that both actions play a minor role in the initial part of ulcer healing. It seems that cell proliferation and migration represent the two important processes involved in the early part of ulcer healing. In order to confirm our hypothesis, we further studied whether CSE affects these processes in both gastric mucosa and isolated epithelial cells.

We measured the intensity of cell proliferation using immunostaining with PCNA in gastric mucosa. Our data showed that CSE significantly reduced cell proliferation in the early phase of ulcer healing, that is, 4 days after ulcer induction (Fig. 4) and yet the ulcer size remained large when compared with the control group (Fig. 1), suggesting that cell proliferation is the dominating factor responsible for healing at this stage of ulcer healing. These findings strongly supported the hypothesis that cell proliferation is more predominant than angiogenesis in the early healing process, though angiogenesis was significantly increased in all CSE-treated groups.

In order to further expand the findings in *in vivo*, CSE were applied in a cell culture system to see if the same phenomenon was also observed and to delineate the mechanism of actions involved in wound healing in isolated gastric epithelial cells. In *in vitro* study, both CE and EE at different concentrations (10, 40, and 100 μg/ml) inhibited [^{3}H]thymidine incorporation in a dose-dependent manner (Fig. 6), and this reduction in cell proliferation was not due

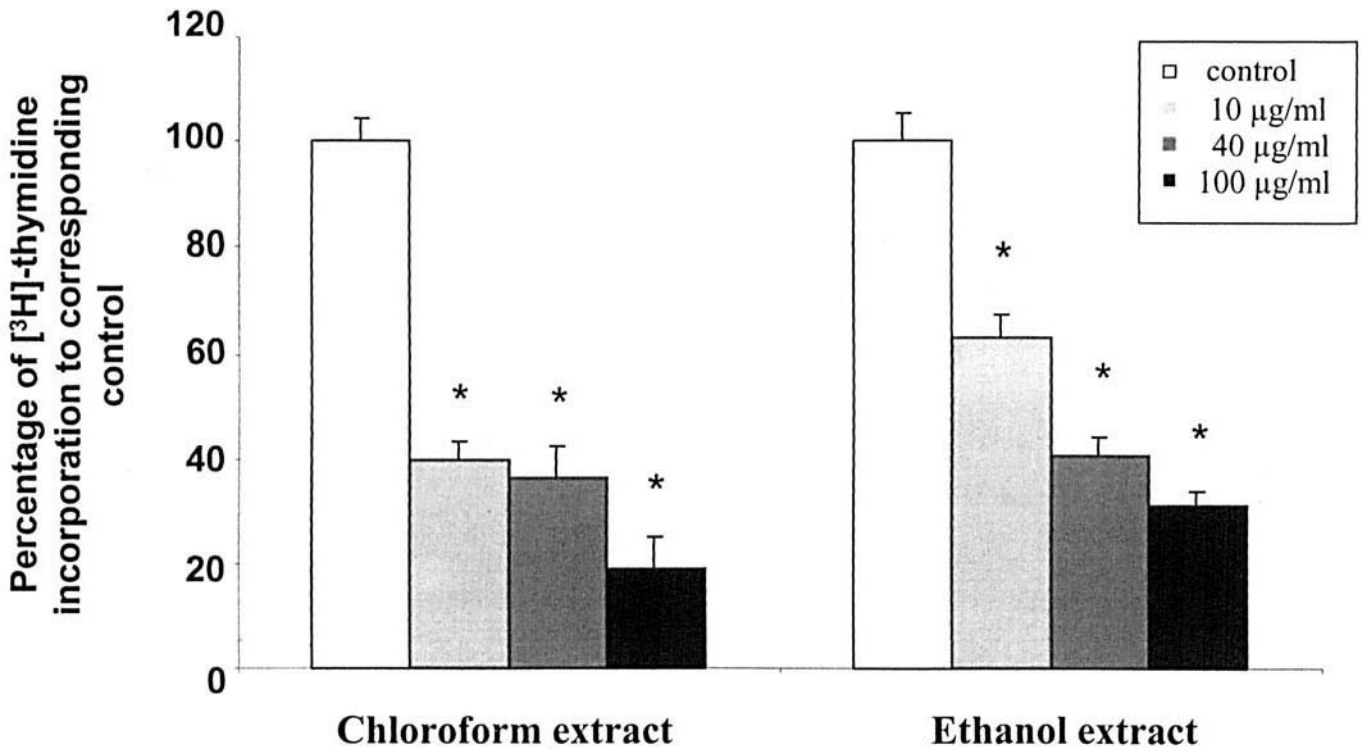

Figure 6. Effect of CE and EE (10–100 μg/ml) on [^{3}H]-thymidine incorporation in gastric epithelial cell line. Cells were incubated with either CE or EE from 10 to 100 μg/ml for 5 hr, and we then measured the [3H]-thymidine incorporation. Values are means ± SEM of six samples. *$P < 0.001$ vs corresponding control group with vehicle treatment.

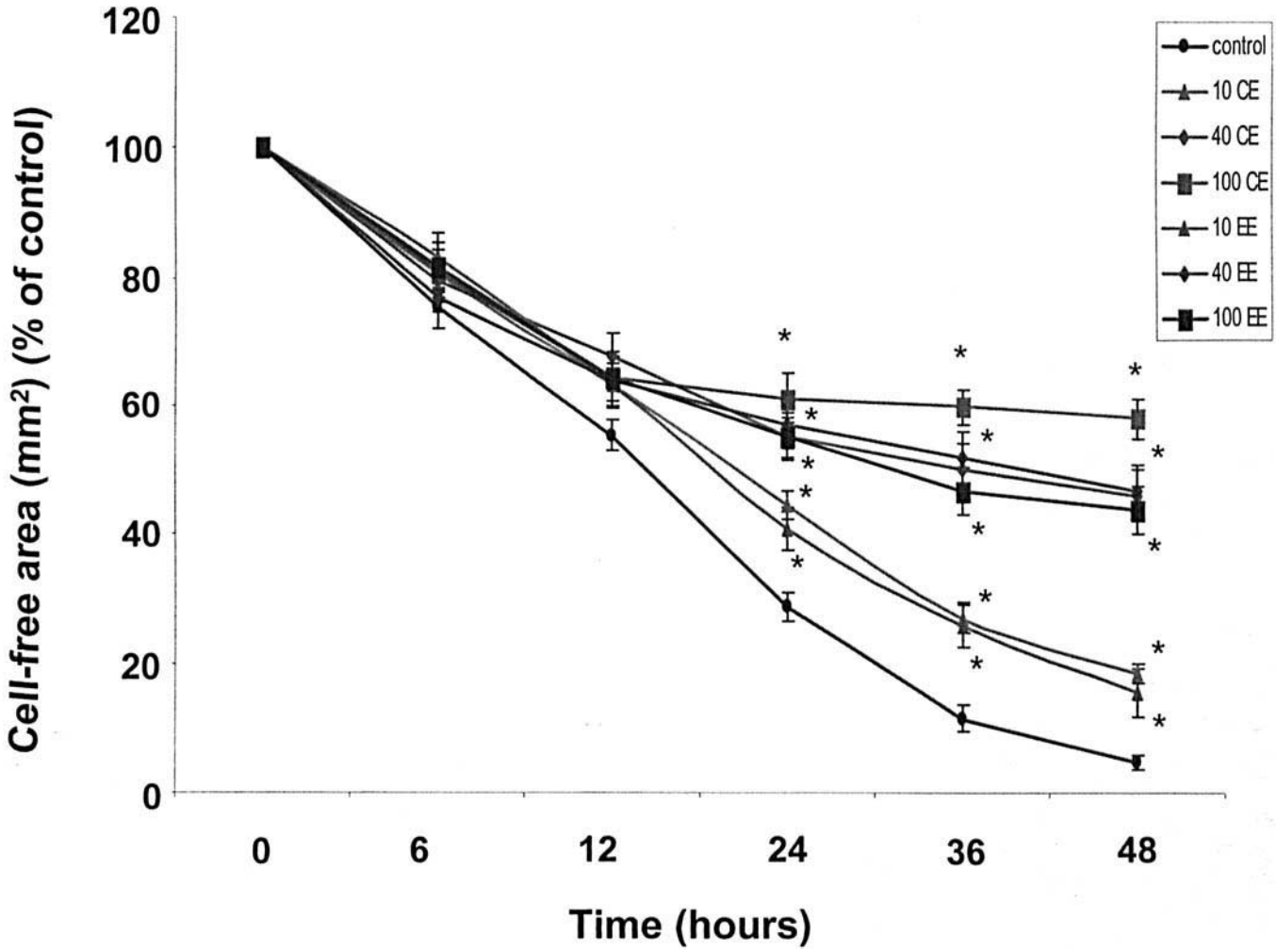

Figure 7. Effect of CE and EE (10–100 μg/ml) on cell migration in gastric epithelial cells. An artificial wound was made until confluent. Cells were incubated with either CE or EE for 48 hr. The cell-free area was measured at 0, 6, 12, 24, 36, and 48 hr. Values are means ± SEM of six samples. $^{*}P < 0.05$ vs control group with vehicle treatment.

to the cytotoxicity, if any, exerted by CSE, which was confirmed by the MTT test (data not shown). This inhibition of cell proliferation leads to an imbalance of cell number, which, as a consequence, could increase ulcer formation and delay ulcer healing. Cell migration is another process involved in the early event in gastric restoration after injury (3). In the present study, it is the first time the effect of CSE on cell migration in gastric epithelial cells has been illustrated. CSE inhibited cell migration in a time- and dose-dependent fashion (Fig. 7), with the highest dose (100 μg/ml) exerting the most potent inhibitory effect. Retardation on cell migration induced by CSE is highly responsible for the slow response of wound healing. This result, together with cell proliferation both in animals and in gastric cell line, put forward the phenomenon that cell migration and cell proliferation are the two important processes responsible for the early phase of gastric ulcer healing, which are adversely affected by CSE.

We further examined the possible mechanism leading to a decrease in cell proliferation and cell migration after CSE treatment. Results from our laboratory have demonstrated that ODC activity is closely related with cell proliferation, which is crucial for wound healing in gastric epithelial cells (36). Consistent with the current study, ODC activity was significantly decreased in both CSE-treated

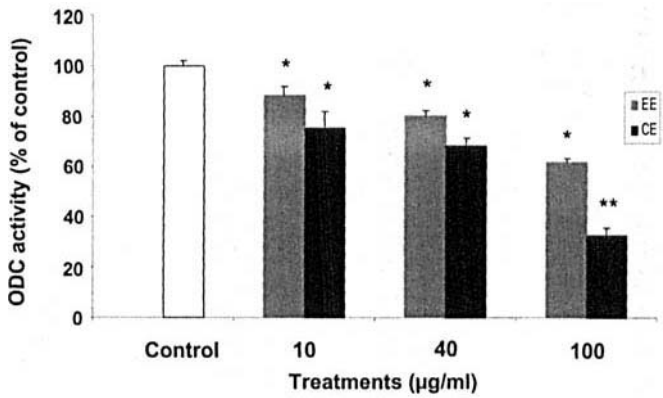

Figure 8. Effect of CE and EE (10–100 μg/ml) on ODC activity in gastric epithelial cell line. Cells were incubated with either CE or EE for 5 hr, and its supernatant was used to test for the activity. Values are means ± SEM of six samples. $^{*}P < 0.05$; $^{**}P < 0.01$ vs control group with vehicle treatment.

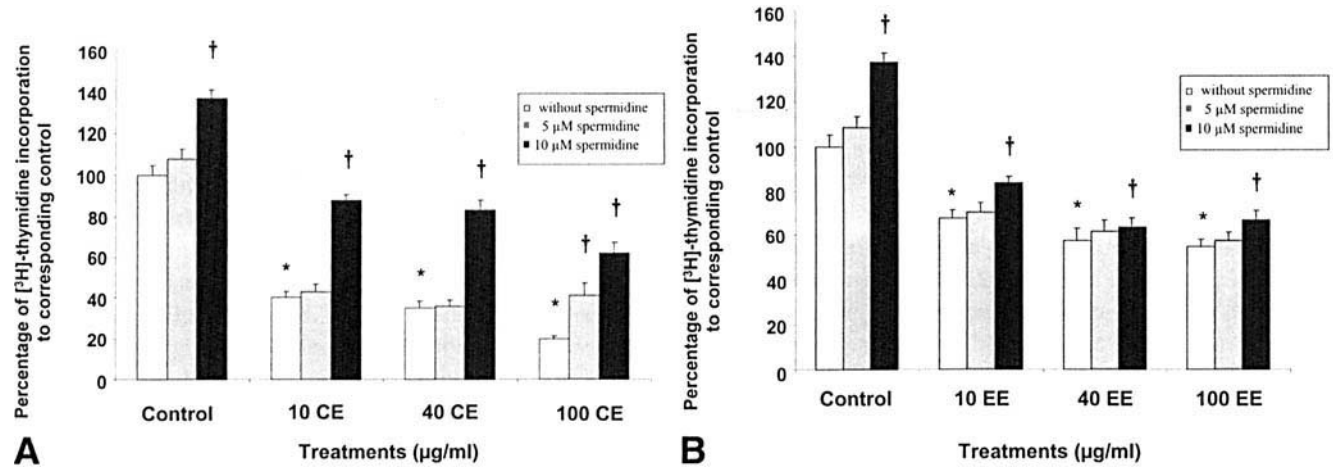

Figure 9. Effect of exogenous spermidine on [^{3}H]-thymidine incorporation in gastric epithelial cell line. Cells were pretreated with exogenous spermidine (5 or 10 μM) for 2 hr and were incubated with CE (a) and EE (b) from 10 to 100 μg/ml for 5 hr, then the amount of DNA synthesis was measured. Values are means ± SEM of six samples. *$P < 0.05$ vs control group (with vehicle treatment) without spermidine; †$P < 0.05$ vs corresponding control group without spermidine.

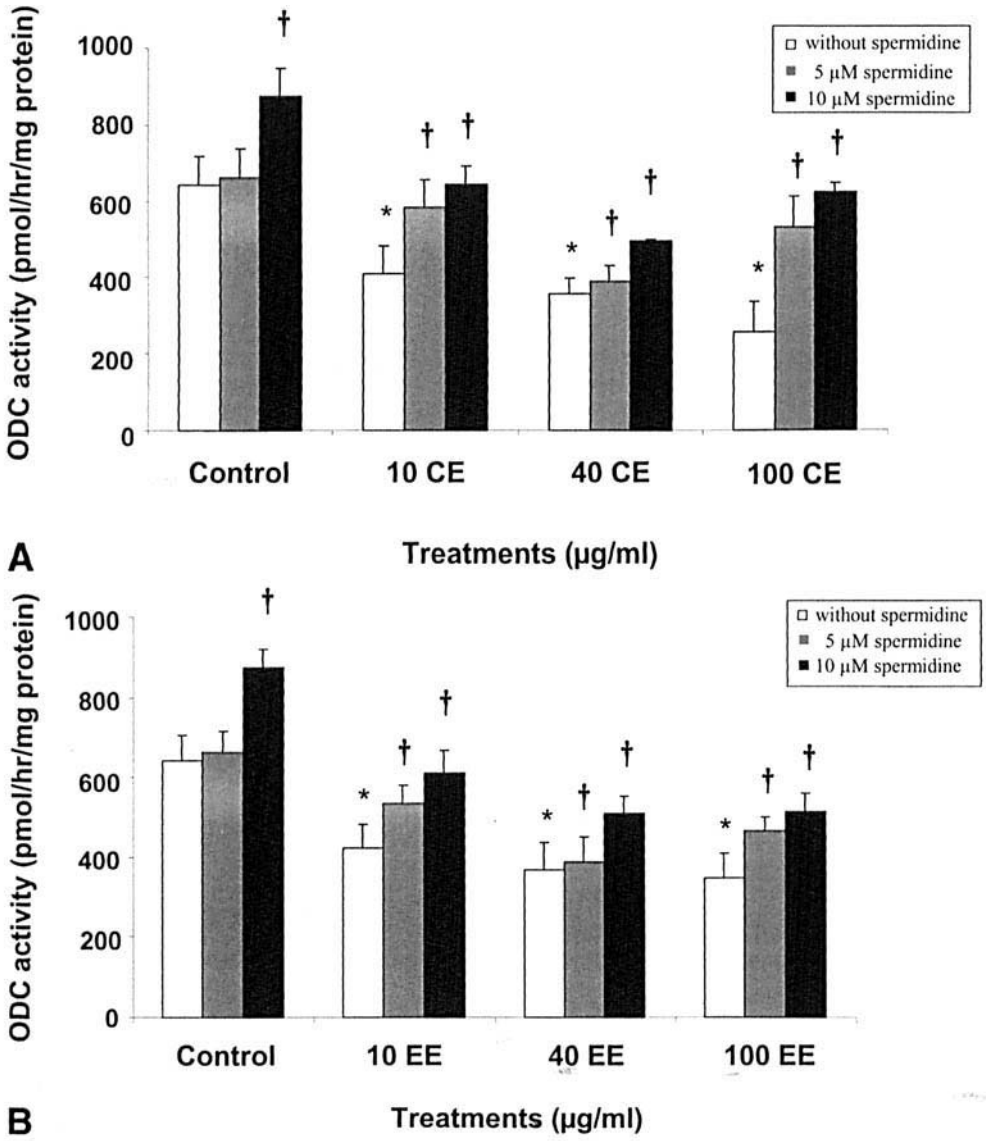

Figure 10. Effect of exogenous spermidine on ODC activity in gastric epithelial cell line. Cells were pretreated with exogenous spermidine (5 or 10 μM) for 2 hr and incubated with CE (a) and EE (b) from 10 to 100 μg/ml for 5 hr, and the supernatant was used to measure the ODC activity. Values are means ± SEM of SIX samples. *$P < 0.05$ vs control group (with vehicle treatment) without spermidine; †$P < 0.05$ vs corresponding control group without spermidine.

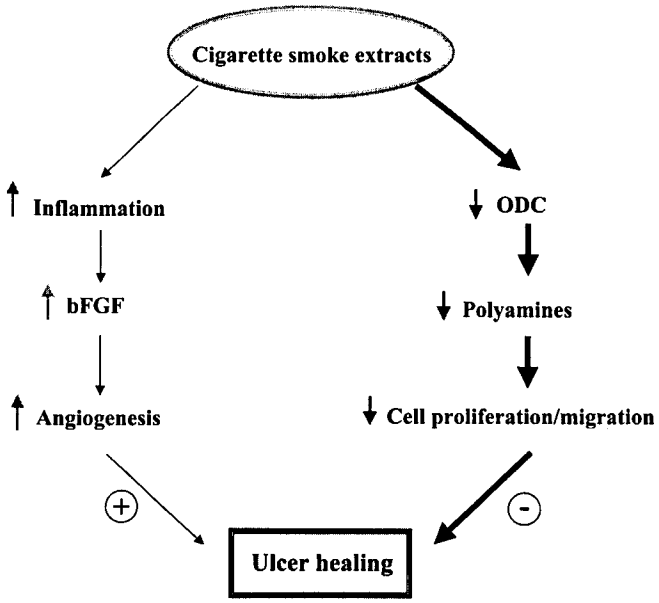

Figure 11. Possible actions of CSE in gastric ulcer healing. + represents stimulation; – represents inhibition.

groups (Fig. 8). However, addition of exogenous spermidine could reverse the inhibitory effect on cell proliferation and ODC activity induced by CSE (Figs. 9 and 10). These findings further support that spermidine deficiency induced by CSE results in a reduction of cell proliferation and migration, which ultimately delays gastric ulcer healing. Figure 11 summarizes the mechanism of actions of CSE on gastric ulcer healing.

Interestingly, the present study demonstrated not only the involvement of the alkaloid, which is largely nicotine in EE, but also the contribution of other components in CE in the delay of ulcer healing. These findings suggest that there is no single compound wholly responsible for the adverse action of cigarette smoking on gastric ulcer healing. This contrasts with the study in ethanol ulceration in which only the components in CE potentiated gastric injury in ethanol-treated animals (21).

In conclusion, CSE greatly inhibited the activity of ODC. The depletion of spermidine subsequently suppressed cell proliferation and perhaps also cell migration in gastric cells. All these findings would explain why cigarette smoking delayed ulcer healing in the stomach. Furthermore, the components found in both extracts are the culprits for these actions, as both CE and EE delayed ulcer healing in rat stomachs and inhibited cell proliferation and migration in gastric epithelial cells.

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