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# Nicotine suppresses gastric wound repair via the inhibition of polyamine and $K^+$ channel expression

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**Abstract**

Nicotine is one of the most representative components in cigarette smoke leading to gastric ulceration. Both ornithine decarboxylase and potassium ion ($K^+$) channels are essential for cell growth and wound repair. The aim of the present study is to elucidate the causative relationship of these two factors during wound healing and the influence of nicotine on this healing process in rat gastric mucosal epithelial cells (RGM-1). Nicotine markedly inhibited cell migration and proliferation in RGM-1 cells. The latter effect was significantly antagonized by a nicotinic receptor blocker, mecamylamine. Nicotine also suppressed ornithine decarboxylase activity significantly. Our data showed that inhibition of cell proliferation and ornithine decarboxylase activity by nicotine was accompanied with a reduction in $K^+$ channel protein expression, all of which were significantly alleviated by spermidine pretreatment. These results suggested that there was a cause/effect link between ornithine decarboxylase and $K^+$ channel on wound repair. Nicotine in cigarette smoke inhibited this healing process and delayed wound repair in gastric epithelial cells. © 2002 Published by Elsevier Science B.V.

*Keywords:* Nicotine; Cell migration; Cell proliferation; Ornithine decarboxylase; $K^+$ channel; Wound healing; Nicotine receptor

## 1. Introduction

Gastric epithelial cells are vulnerable to injury as they are readily exposed to noxious agents. However, gastric mucosa has the ability to repair rapidly after injury and the process of restoration involves cell migration and cell proliferation (Watanabe et al., 1994). Cell migration is an important process for wound healing, inflammatory reactions and metastasis (Lauffenburger and Horwitz, 1996), in which cells migrate from the proliferative zone to restore the constant number of cells. Nevertheless, there are many factors that affect the rate of wound healing.

Ornithine decarboxylase activity is an important element for cell proliferation during ulcer healing (Wang and Johnson, 1990). Levels of intracellular polyamines are tightly regulated by a mechanism that controls biosynthesis, degradation and uptake, and they are highly depended upon the activity of ornithine decarboxylase (Lux et al., 1980). DL-α-Difluoromethylornithine, a specific and irreversible inhibitor of ornithine decarboxylase, strongly suppresses gastric mucosal healing, as well as the expression of c-*fos* and c-*myc* protooncogene (Wang and Johnson, 1994). Furthermore, polyamines are shown to participate in the gastric mucosal healing in rats (Banan et al., 1998; Konturek et al., 1998).

Potassium ion ($K^+$) channel is also involved in the mucosal ulcer healing process. $K_{ATP}$ channel plays a role in gastric mucosal restitution through a polyamine-independent pathway in rats (Rahgozar et al., 2001). However, the voltage-gated $K^+$ ($K_v$) channel was shown to be involved in polyamine-dependent cell migration in intestinal epithelial crypt-6 (IEC-6) cells (Wang et al., 2000). Indeed, cell migration was inhibited by the application of $K^+$ channel blocker in transformed Madin–Darby canine kidney focus cells (Schwab et al., 1994, 1995). In addition, blockade of $K^+$ channel activity was demonstrated to inhibit cell proliferation in human melanoma cells (Nilius and Wohlrab, 1992; Lepple-Wienhues et al., 1996), normal human lymphocytes (Lin et al., 1993) and breast cancer cells (Woodfork et al., 1995). Epidermal growth factor is found to stimulate cell proliferation via the activation of $K^+$ channel activity in fibroblasts (Magni et al., 1991) and breast cancer cells (Wegman et al., 1991). However, the fact that whether inhibition of $K^+$ channel can reduce cell proliferation in gastric epithelial cells remains unexplored.

Experimental studies demonstrated that cigarette smoking significantly delayed gastric ulcer healing in animals (Iwata and Leung, 1995) and in cell lines (Snajdar et al.,

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2001). These data are consistent with the findings in our laboratory that either short-term passive cigarette smoking or cigarette smoke extracts remarkably reduced epidermal growth factor levels and ornithine decarboxylase activity, and resulted in a delay of gastric ulcer healing in rats (Ma et al., 1998, 2000a,b). However, which component(s) in cigarette smoke involved in the delay of ulcer healing is unclear.

Nicotine is a major component in cigarette smoke that exerts detrimental effect to gastric ulceration (Hui et al., 1991; Wong et al., 1986). However, the effect of nicotine on cell proliferation and cell migration is controversial. Preliminary study has showed that nicotine has no direct effect on cell migration in rabbit gastric cell restitution (Sato et al., 1994). Another study demonstrates that the process of migration requires keratinocytes to move from the ulcer margin to cover the defect and nicotine is shown to inhibit the migration of keratinocytes (Zia et al., 2000). Hence, further studies are needed to establish a relationship between nicotine and wound healing in the stomach.

Based on the above studies, we hypothesized that intracellular polyamines may somehow modulate $K_v$ channel which in turn affects cell restoration during wound repair in gastric epithelial cells. Furthermore, it is likely that nicotine, the active ingredient in cigarette smoke could adversely affect this restoring process, and this may partially explain the mechanism how cigarette smoking delays gastric ulcer healing in animals and in humans.

## 2. Methods and materials

### 2.1. Reagents and drugs

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

### 2.2. Cell culture

Rat gastric mucosal epithelial cell line (RGM-1) was obtained from Riken Cell Bank (Tsukuba, Japan) and was kindly donated by H. Matsui (Institute of Clinical Medicine, University of Tsukuba, Ibaraki, Japan). Cells were grown in Dulbecco's Modification of Eagle's Medium/Ham's F-12 medium (GibcoBRL, Grand Island, USA) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin and 20% fetal bovine serum (GibcoBRL) in an incubator with 37 °C, 95% humidity and 5% carbon dioxide.

### 2.3. Cell migration

Cells were seeded in 24-well culture plates and cultured in medium with 20% fetal bovine serum until confluent. After confluent, monolayers of the cells were starved for 24 h in the medium in the absence of serum. Cells were pretreated with mitomycin C (2 μg/ml) for 2 h before a wound was made to inhibit cell proliferation. An artificial circular wound of cell-free area 2 $mm^2$ was made in the center of the monolayer using a plastic blade (Watanabe et al., 1994). The wounded monolayers were then cultured in the medium (without serum supplement) in the presence or absence of nicotine (10, 50 or 200 μg/ml). These concentrations of nicotine were similar to the amounts of nicotine intake in light (10–15 cigarettes per day), moderate (16–30 cigarettes per day), or heavy smokers (>30 cigarettes per day) (Lawson et al., 1998). Sizes of the cell-free area were monitored from time 0 to 48 h using a digital image processor connected to a microscope (Nikon, Tokyo 100, Japan) and the areas were calculated with an image-analyzing program (Leica, Cambridge, England).

### 2.4. [$^3$H]thymidine incorporation

[$^3$H]Thymidine incorporation assay was used to determine the amount of DNA synthesis. The method was described previously (Tones et al., 1988) with modifications. Cells reaching 70–80% confluence were seeded in 24-well culture plates with culture medium. Cells were pretreated in the presence or absence of exogenous spermidine (5 or 10 μM) for 2 h and then incubated in the presence or absence of nicotine bitartrate (10, 50 or 200 μg/ml) for 5 h. In a separate experiment, mecamylamine hydrochloride (20 μM) was co-incubated with nicotine bitartrate (200 μg/ml) for 5 h. After treatment, cells were incubated with 0.5 μCi/ml [$^3$H]Thymidine (Amersham, Arlington Heights, IL, USA) for 5 h at 37 °C. The solution was discarded and then washed with 0.5 ml iced 0.15 M NaCl. Afterwards, 10% trichloroacetic acid was added into the wells and incubated for 15 min at room temperature. The well rinsed with distilled water for four times, and then followed by adding 0.5 ml of 1% sodium dodecyl sulphate into each well to incubate for 15 min at 37 °C. The solution was being transferred into a scintillation vial, an addition of 0.5 ml SDS was added to rinse the well and transferred it into the same vial. Water-accepting scintillation fluid was added into the vial and then vortex. Finally, the amount of DNA synthesis was measured using a liquid scintillation spectrometry on a beta-counter.

### 2.5. Ornithine decarboxylase activity

The method for measuring ornithine decarboxylase activity was described previously (Russell and Snyder, 1968) with modifications. Cells were incubated in the absence or presence of spermidine (5 or 10 μM) for 2 h, and then incubated with nicotine for another 5 h. Cells were scraped from culture plates and 0.5 ml of 10 mM Tris–HCl buffer (containing 1 mM EDTA, 0.05 mM pyridoxal 5′ phosphate, and 5 mM dithiothreitol) was added. Samples were sonicated under an ice-cold condition for 20 s and centrifuged at 14,000 rpm for 10 min at 4 °C. The resulting supernatants (300 μl) were incubated in 2.5 mM L-[1-$^{14}$C]Ornithine at 37 °C for 15 min. The liberated $^{14}CO_2$ was absorbed by a filter

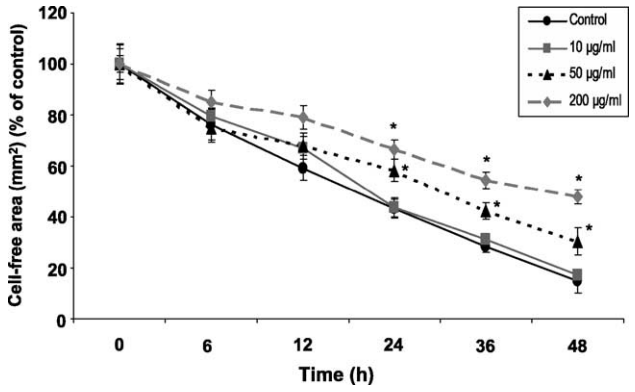


Fig. 1. Effect of nicotine on cell migration in gastric epithelial cells. An artificial wound was made until confluent. Cells were incubated with nicotine (10–200 μg/ml) for 48 h. Cell-free area was measured at time 0, 6, 12, 24, 36 and 48 h. Values are means±S.E.M. of six samples. *$P$<0.05 vs. control group.

paper impregnated with 2 mM NaOH (20 μl), and it was placed inside a plastic well connected to the stopper, which hang over the reaction mixture. The incubation was terminated by the addition of 10% trichloroacetic acid (300 μl) and incubated for another 10 min. Labeled $CO_2$ was trapped in the filter paper and was measured by a liquid scintillation counter.

### 2.6. Western blotting for $K_v1.1\alpha$-subunit expression

Cells were collected in RIPA buffer for Western blot. After sonication and centrifugation, the protein concentration was routinely measured using a protein assay kit (Bio-Rad Laboratories, Hercules, USA). Proteins (50 μg/lane) were separated by 7.5% SDS-polyacrylamide gels electrophoresis overlaid with a 5% acrylamide stacking gel, and then transferred to Hybond C nitrocellulose membranes (Amersham). The membranes were probed with a monoclonal antibody against $K_v1.1\alpha$-subunit (Upstate Biotechnology, Lake Placid, NY, USA) overnight at 4 °C and incubated for 1 h with goat anti-rabbit immunoglobulin G antibody conjugated with peroxidase. The membranes were developed by the enhanced chemiluminescence system (Amersham) and exposed to an X-ray film (FUJI Photo Film, Tokyo, Japan). Quantitation was carried out by a video densitometer (Scan Maker III, Microtek, USA).

### 2.7. Cell viability

Cell viability was measured using 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method (Sato et al., 1995; Hodgson et al., 1994). After incubation with nicotine at different concentrations, cells were incubated with 2.5% MTT solution (5 mg/ml) for another 3 h at 37 °C. Thereafter, 0.04 N HCl-isopropanol was added into the mixture and mixed thoroughly. The color change was recorded using spectrophotometry with the microplate reader (MRX, Dynex Technologies, Sullyfield Circle Chantilly, USA) at 570 nm. The same test was repeated three times and the optical density was calculated for statistic analysis.

### 2.8. Statistical analysis

Results were expressed as the means±S.E.M. Statistical analysis was performed with an analysis of variance (ANOVA) followed by the Turkey's $t$-test. $P$ values less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. Effect of nicotine on cell migration in RGM-1 cells

Mitomycin C (2 μg/ml) was shown to inhibit cell proliferation significantly (data not shown), hence, this concentration was used to pretreat the cells before a wound was made. The sizes of the wound were all the same at the beginning of the assay. In the control group, cell-free area was about 40% at 24 h and almost completely restored after 48 h (Fig. 1). In nicotine-treated groups, wound healing was significantly inhibited in a time- and dose-dependent manner after 48 h. In this experiment, the highest dose (200 μg/ml) exerted an inhibitory effect on wound healing, giving rise to a large cell-free area (about 60%) at 48 h.

### 3.2. Effect of nicotine on cell proliferation in RGM-1 cells

To examine the ability of nicotine to alter gastric epithelial cell proliferation, [$^3$H]Thymidine incorporation was used to determine the amount of DNA synthesis. Exposure to nicotine (10–200 μg/ml) for 5 h significantly inhibited cell proliferation in a dose-dependent manner (Fig. 2). The highest concentration at 200 μg/ml reduced cell proliferation by

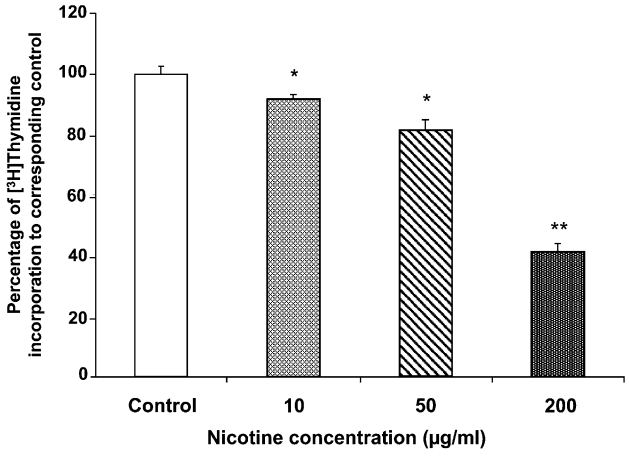


Fig. 2. Effect of nicotine on [$^3$H]Thymidine incorporation in gastric epithelial cell line. Cells were incubated with nicotine (10–200 μg/ml) for 5 h and measured the amount of DNA synthesis. Values are means±S.E.M. of six samples. *$P$<0.05, **$P$<0.001 vs. control group.

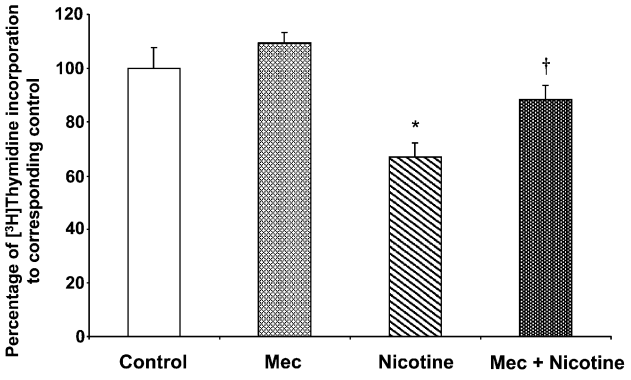


Fig. 3. Effect of mecamylamine (nicotinic receptor blocker) on nicotine-induced inhibition on [$^3$H]Thymidine incorporation in gastric epithelial cell line. Nicotine (200 μg/ml)-treated cells were co-incubated with 20 μM mecamylamine (Mec) for 5 h and measured the amount of DNA synthesis. Values are means±S.E.M. of six samples. *$P$<0.05 vs. control group; †$P$<0.05 vs. nicotine-treated group.

60%. In order to ensure the inhibitory effect of nicotine was not due to the cytotoxicity, MTT test was carried out to test for cell viability. Our results showed that incubation with nicotine for 5 h did not effect cell viability at all concentrations (data not shown). In order to confirm that the effect of nicotine on cell proliferation was nicotine receptor dependent, nicotine-treated cells were co-incubated with 20 μM mecamylamine (a specific nicotinic receptor blocker) for 5 h. Data showed that mecamylamine significantly prevented the inhibition on cell proliferation induced by nicotine (Fig. 3). Addition of mecamylamine could partially reverse the inhibitory action of nicotine back to normal about 90% of the basal level, suggesting that activation of nicotinic receptor inhibited cell proliferation of RGM-1 cells.

### 3.3. Effect of nicotine on ornithine decarboxylase activity

Since ornithine decarboxylase activity is closely related with gastric epithelial cell growth, therefore the inhibition of cell proliferation induced by nicotine may be due to the alteration in ornithine decarboxylase activity. To confirm this hypothesis, cells were incubated with various concentrations of nicotine for 5 h and the ornithine decarboxylase activity was measured. Cells treated with nicotine suppressed the activity of ornithine decarboxylase in a dose-dependent fashion (Fig. 4). A significant inhibitory effect was observed even at lower dose of 10 μg/ml, and the highest concentration 200 μg/ml exerted the most potent effect on the inhibitory action of ornithine decarboxylase activity, which resulted in approximately 50% reduction in the activity.

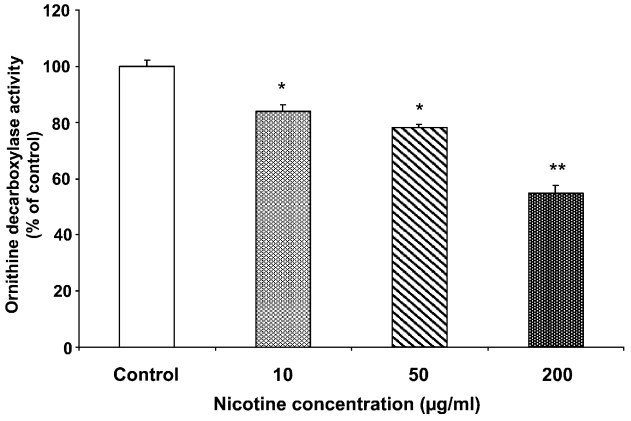


Fig. 4. Effect of nicotine on ornithine decarboxylase activity in gastric epithelial cell line. Cells were incubated with nicotine (10–200 μg/ml) for 5 h, and its supernatant was used to test for the activity. Values are means±S.E.M. of six samples. *$P$<0.01, **$P$<0.001 vs. control group.

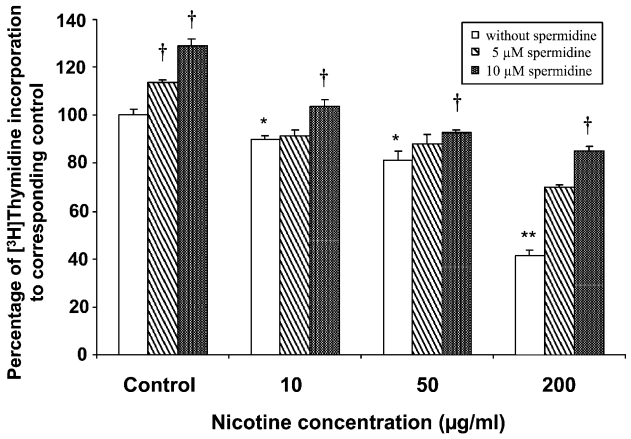


Fig. 5. Effect of nicotine and exogenous spermidine on [$^3$H]Thymidine incorporation in gastric epithelial cell line. Cells were pretreated with or without exogenous spermidine (5 or 10 μM) for 2 h and further incubated with nicotine (10–200 μg/ml) for 5 h, and then measured the amount of DNA synthesis. Values are means±S.E.M. of six samples. *$P$<0.05, **$P$<0.001 vs. corresponding control group without nicotine; †$P$<0.05 vs. corresponding group without spermidine.

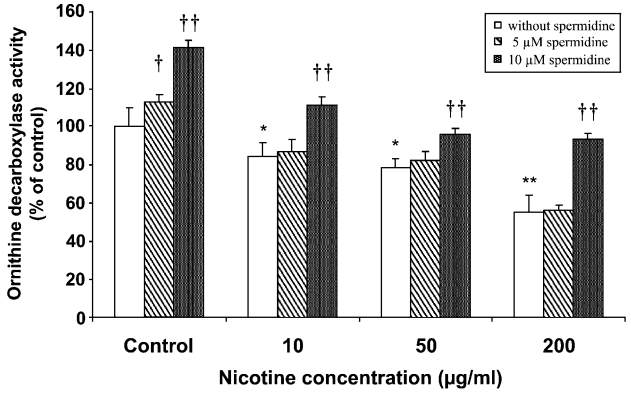


Fig. 6. Effect of nicotine and exogenous spermidine on ornithine decarboxylase activity in gastric epithelial cell line. Cells were pretreated with or without exogenous spermidine (5 or 10 μM) for 2 h and further incubated with nicotine (10–200 μg/ml) for 5 h, and the supernatant was used to measure the ornithine decarboxylase activity. Values are means± S.E.M. of six samples. *$P$<0.01, **$P$<0.01 vs. corresponding control group without nicotine; †$P$<0.05, ††$P$<0.01 vs. corresponding group without spermidine.

### 3.4. Effect of exogenous spermidine on cell proliferation and ornithine decarboxylase activity

In order to ensure the reduction of cell restoration was due to depletion of polyamines, exogenous spermidine was used to see if it could reverse the inhibitory effect of nicotine on cell proliferation and ornithine decarboxylase activity. Cells treated with exogenous spermidine (5 or 10 μM) were able to stimulate cell proliferation in a dose-dependent manner by 13% and 30%, respectively, in the control group (Fig. 5). Our data also showed that exogenous spermidine invalidated the inhibition of cell proliferation by nicotine. Only supplementation with higher dose of spermidine (10 μM) could reverse the inhibitory action of nicotine on the level of [$^3$H]Thymidine incorporation to its basal level in all nicotine-treated groups.

Similar trend was observed on the effect of exogenous spermidine on the activity of ornithine decarboxylase. Briefly, the addition of exogenous spermidine (5 or 10 μM) could effectively increase the activity of ornithine decarboxylase by 12% and 40%, respectively, in the control group (Fig. 6). While in cells treated with nicotine, spermidine significantly reversed the nicotine-induced suppression of ornithine decarboxylase activity. Moreover, 10 μM spermidine was sufficient to attenuate the depressive actions of nicotine on ornithine decarboxylase activity back to its basal level.

### 3.5. Effect of nicotine on $K_v1.1\alpha$-subunit protein expression

Expression of $K_v1.1$ channel was found to be downregulated remarkably by nicotine in a dose-dependent manner (Fig. 7). Maximum reduction was at 200 μg/ml nicotine, which significantly downregulated $K_v1.1$ channel by 50%. Again, exogenous spermidine at the concentration of 10 μM could significantly prevent the inhibitory action induced by nicotine on $K_v1.1$ channel protein expression in RGM-1 cells.

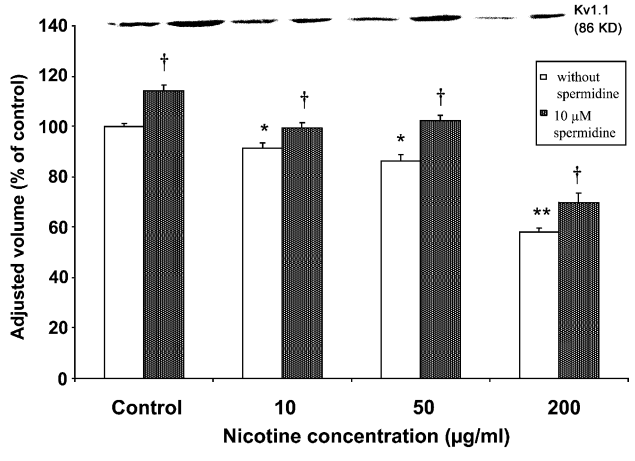


Fig. 7. Effect of nicotine and exogenous spermidine on $K^+$ channel protein expression. Cells were pretreated with or without exogenous spermidine for 2 h and further incubated with nicotine for 5 h. $K^+$ channel protein expression was measured by Western Blotting. Lane 1, control; Lane 2, control+spermidine; Lane 3, 10 μg/ml nicotine; Lane 4, 10 μg/ml nicotine + spermidine; Lane 5, 50 μg/ml nicotine; Lane 6, 50 μg/ml nicotine + spermidine; Lane 7, 200μg/ml nicotine; Lane 8, 200 μg/ml nicotine + spermidine. Values are means ± S.E.M. of six samples. *$P$<0.01, **$P$<0.001 vs. corresponding control group without nicotine; †$P$<0.01 vs. corresponding group without spermidine.

## 4. Discussion

Gastric cell restoration includes cell proliferation and cell migration occurs in the early event after injury (Silen and Ito, 1985). Migration and proliferation of the viable cells from the wounded site could cover the denuded area, therefore, a wound repair model has been established to study the mechanism of restoration. Using this wound repair model, the mechanisms of how nicotine, one of the active ingredients in cigarette smoke, affected wound healing in the stomach were investigated.

Nicotine is shown to delay wound healing by inhibiting epithelialization (Sherwin and Gastwirth, 1990) and cell adhesion (Austin et al., 2001). The adverse effect of nicotine on wound re-epithelialization was suggested via the nicotine receptor-mediated pathway in keratinocytes (Zia et al., 2000). Nicotine was also found to inhibit cell proliferation in a dose- and time-dependent manner in promyelocytic HL-60 leukemia cells (Konno et al., 1986). In contrast, low dose of nicotine can trigger the stimulation of DNA synthesis and cell proliferation in vascular endothelial cells (Villablanca, 1998). In smooth muscle cells, both nicotine and its metabolite cotinine enhance the production of basic fibroblast growth factor, which exerts mitogenic effect, and upregulates the expression of several matrix metalloproteinases that are important in cell migration (Carty et al., 1996). Hence, the effect of nicotine in cell proliferation and migration remains uncertain. Our data showed that treatment with nicotine detrimentally inhibited cell migration and proliferation in gastric epithelial cells (Figs. 1 and 2). This experiment mimics the effects of cigarette smoking, which impinges a negative impact on the recovery of epithelial cells in the stomach during wound healing.

Polyamines have been demonstrated to take part in cell migration in IEC-6 cells, in which inhibition of ornithine decarboxylase activity followed by a reduction of polyamine synthesis in these cells can detrimentally inhibit cell migration (McCormack et al., 1993). In consistent with our results, Wild and his associates reported that exogenous spermidine would increase ornithine decarboxylase activity in rats (Wild et al., 1993). However, other studies demonstrated opposite effect (Mitchell et al., 1996; Yuan et al., 2001). This dichotomy phenomenon may be due to different concentrations of polyamines and systems used in these reports. The current study indicates that the mechanism by which exogenous spermidine can reverse the inhibitory action on cell proliferation by nicotine is either by its direct stimulatory action on cell proliferation or indirectly through

ornithine decarboxylase activation in rats (Konturek et al., 1998) and in isolated gastric epithelial cells (Fig. 6). In any case, suppression of ornithine decarboxylase activity together with the reduction of polyamine synthesis could adversely affect cell migration and proliferation of RGM-1 cells. Previous study also demonstrated the significance of ornithine decarboxylase and polyamines in the same cell line (Ye et al., 2001). From this study, the magnitude of inhibition on ornithine decarboxylase activity and cell proliferation by DL-α-difluoromethylornithine was comparable to the effects of nicotine in the current report (Figs. 2 and 4). In brief, these results point to the importance of ornithine decarboxylase in cell proliferation, which is crucial for wound healing. Our study also demonstrates that the antiproliferative action of nicotine is likely to be mediated through the activation of nicotinic receptors, because the action can be blocked by a specific nicotinic receptor antagonist, mecamylamine (Fig. 3).

There are reports which indicate that $K^+$ and its channel activity are involved in signaling pathway that triggers proliferation and differentiation in various cell types (Dubois and Rouzaire-Dubois, 1993; Boonstra et al., 1981). Voltage-gated $K^+$ ($K_v$) channel blockers, 4-aminopyridine and tetraethylammonium inhibited cell proliferation in T-lymphocytes (DeCoursey et al., 1984), pituitary cells (Vaur et al., 1998) and lung cancer cells (Pancrazio et al., 1993). These studies provide evidence that $K_v$ channel plays a role in mitogenesis. However, little is known about the relationship between polyamines and $K_v$ channels on cell proliferation during wound healing in gastric epithelial cells. We found that nicotine downregulated $K_v1.1$ channel protein expression and addition of spermidine effectively prevented the downregulation of this protein (Fig. 7), suggesting that inhibition of ornithine decarboxylase activity by nicotine would reduce the production of polyamines, which subsequently decrease $K_v1.1$ channel protein expression and delay wound healing in RGM-1 cells. This study is the first time to demonstrate a causal relationship between ornithine decarboxylase and $K_v1.1$ channel activity in gastric epithelial cell repair after wounding. Nicotine could act through the polyamine/$K_v1.1$ channel pathway and thereby delay wound repair. However, the exact mechanisms of how polyamine activates $K_v$ channel protein expression and affects cell restitution, need further investigation.

In summary, nicotine can effectively inhibit cell proliferation and migration in gastric epithelial cells. This suppressive action is the result of the reduction in ornithine decarboxylase activity leading to the depletion of polyamines followed by the downregulation of $K_v1.1$ channel expression in the cell. Taken together, our data reveal for the first time the importance of ornithine decarboxylase activity in the regulation of $K^+$ channel on wound healing in gastric epithelial cells. The current study also validates the hypothesis that nicotine in cigarette smoke is responsible for the delay in gastric ulcer healing.

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