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# Mechanism of the antiulcerogenic effect of *Ganoderma lucidum* polysaccharides on indomethacin-induced lesions in the rat

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**Abstract**

Many cytokines, in particular tumor necrosis factor (TNF)-α have been known to play an important role in the pathogenesis of gastric mucosal lesions caused by various factors such as drugs and *Helicobacter pylori* infection. Our previous studies have shown that the polysaccharide fractions isolated from the fruiting bodies of *Ganoderma lucidum* (GLPS) prevented indomethacin- and acetic acid-induced gastric mucosal lesions in the rat. However, the mechanisms remain unclear. This study aimed to investigate whether GLPS had a direct mucosal healing effect in the indomethacin-treated rat, and to explore the possible mechanisms by determining the gastric mucosal mRNA and protein levels of TNF-α and ornithine decarboxylase (ODC) activity. In addition, the effects of GLPS on the cellular proliferation, ODC and c-Myc protein expression and mucus synthesis in the rat gastric cell culture (RGM-1) were examined. The present study demonstrated that GLPS at 250 and 500 mg/kg by intragastric input caused ulcer-healing effect in the rat; this was accompanied with a significant suppression of *TNF-α* gene expression, but with an increased ODC activity. In RGM-1 cells, GLPS at 0.05, 0.25 and 1.0 mg/ml significantly enhanced [$^3$H]thymidine incorporation and ODC activity in a concentration-dependent manner. However, these effects were abrogated by the addition of the ODC inhibitor, DL-α-difluoromethyl-ornithine (DFMO). GLPS at 0.25–1.0 mg/ml also increased mucus synthesis, as indicated by the increased D-[6-$^3$H]glucosamine incorporation in RGM-1

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cells. Furthermore, GLPS at 0.05–1.0 mg/ml increased the c-Myc protein expression. These findings indicated that GLPS produced a mucosal healing effect in the rat model, perhaps due partly to the suppression of *TNF*-α and induction of *c-myc* and *ODC* gene.


*Keywords:* Polysaccharide; Ulcer; c-Myc; tumor necrosis factor-α

## Introduction

Studies in *in vitro*, animals and patients have indicated that various cytokines (in particular TNF-α and interleukins (IL)) play important roles in the pathogenesis of acute and chronic gastric mucosal lesions caused by various factors such as nonsteroidal anti-inflammatory drugs (NSAIDs) and *Helicobacter pylori* infection [1–10]. This has been indicated by the increased gastric TNF-α levels in animal models with drug (e.g. NSAID)-, ethanol-, acetic acid-induced gastric ulcers and gastric mucosal cell cultures treated with NSAIDs, increased gastric lesions by direct exposure of TNF-α and other cytokines, and the ulcer-healing effect of anti-TNF-α agents such as pentoxifylline [4]. In the rat, treatment of lipopolysaccharides from *Helicobacter pylori* markedly impaired the ulcer healing, due partly to increased expression and release of proinflammatory cytokines such as IL-1β and TNF-α [11]. However, in patients with gastric ulcers, the mucosal production of cytokines such as interleukin-8 and TNF-α showed a significant decrease after eradication of *Helicobacter pylori* [12].

The proliferative response of gastric mucosal cells may be of importance in maintaining gastric mucosal integrity and accelerating ulcer healing. Gastric epithelia line the gastrointestinal mucosa to form an important protective barrier to a number of toxic xenobiotics. Superficial damages to the mucosa will lead to increased mucosal epithelial cell proliferation and migration, which is considered to be mediated by certain growth factors, cytokines and polyamines [13,14]. The latter are important intracellular mediators of cell proliferation in normal and cancer cells. Polyamines have been shown to play important roles in the ulcer healing process [15]. Depletion of polyamines or inhibition of polyamine synthesis results in inhibition and/or apoptosis of mucosal cell proliferation [15]. The synthesis of polyamines is mainly controlled by the rat-limiting enzyme ODC, which has been shown to be regulated by a number of proteins such as c-Myc and c-Fos protein [16,17].

We have previously reported that the *Ganoderma lucidum* polysaccharide (GLPS) fractions produced a healing effect on indomethacin-induced gastric mucosal damage in the rat [18]. However, the mechanisms for the ulcer-healing effects of GLPS remain unknown. The present study aimed to investigate whether GLPS had a direct stimulatory effect on the healing of indomethacin-induced gastric mucosal lesions in the rat, and the role of TNF-α in this process. In addition, the effect of GLPS on the proliferation of RGM-1 cells, a cell line derived from the gastric mucosa of adult Wistar rats [19], was examined. Furthermore, as ODC is the initial rate-determining enzyme in polyamine biosynthesis [14], and the growth-related cellular oncogenes such as c-*myc* have been reported to be transcriptional factor for ODC [16,17], the levels of ODC activity and c-Myc proteins were investigated.

## Materials and methods

### *Chemicals and reagents*

Indomethacin, bicinchoninic acid and dimethyl sulfoxide were obtained from Sigma–Aldrich Chemical Co. (Sydney, Australia). Dulbecco's modified Eagle's medium (DMEM), F-12 nutrient mixture, heat-inactivated fetal bovine serum, penicillin, trypsin and streptomycin were all purchased from Life Technologies Inc. (Auckland, New Zealand). A commercial rat TNF-a ELISA kit was obtained from Alexon–Trend, Inc. (Minneapolis, MN). The polyclonal antiserum raised in rabbit recognising rat c-Myc was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-rabbit immunoglobulin G raised in donkey was purchased from ICN Biomedicals (Auckland, New Zealand). Electrophoresis products were obtained from Bio–Rad (Auckland, New Zealand). All other reagents were of analytical or HPLC grade as appropriate.

### *Extraction of polysaccharides from Ganoderma lucidum*

The polysaccharide fractions were prepared from the fruiting bodies of *G. lucidum*, which were collected from southern China as described previously [20,21]. All extracts were finally pooled, and the polysaccharide-enriched fractions were precipitated by the addition of 75% (v/v) ethanol. The polysaccharide-enriched fraction was further purified by high-performance anion-exchange and gel filtration chromatography. The molecular size of polysaccharide was determined by gel filtration chromatography and the phenol-sulfuric acid method [22,23], and the concentrations of uronic acids and proteins were also determined [24,25].

### *Gastric ulcer induction in the rat*

Experiments were performed on male Wistar Kyoto rats (185 – 220 g). Rats were housed under constant temperature (22 ± 1 °C), lighting (12-h cycle) and humidity (55%) according to institutional guidelines. Sterile food and water was available *ad libitum*. The Auckland University Animals Ethics Committee approved all animal procedures. All of the rats were fasted for 24 hours before experiment. Rats (n = 6 for each group) were treated with indomethacin (50 mg/kg, subcutaneous injection) alone, or in combination with intragastric GLPS (250 or 500 mg/kg, p.o). Control rats received a vehicle. Indomethacin was dissolved in sterile saline solution with a drop of Tween 80. Rats were killed by ether anesthesia 1, 3 or 6 days after drug treatment to assess ulcer formation and ulcer healing. The stomach was removed rapidly, opened along the greater curvature, and thoroughly rinsed with normal saline. The size of the lesions was measured by a single-blind method. Gastric mucosa (including the ulcer base and both sides of the ulcer margin in ulcerated stomach) was taken from the stomach by scraping the tissue with a glass slide on a glass dish on ice. They were wrapped in a piece of aluminum foil and immediately frozen in liquid nitrogen, and then stored under − 70 °C until assay.

### *Determination of TNF-α in rat gastric mucosa*

The TNF-α level in gastric mucosa was assayed by ELISA as described previously. Briefly, gastric mucosal samples taken from the rat stomach were homogenized in 100 mg sample/0.9 ml PBS (pH 7.4)

containing 0.75 μg/ml PMSF, 1 μg/ml leupeptin and 5 μg/ml aprotonin for 30 sec. The homogenates were then centrifuged at 20000 *g* for 20 min. The supernatant (100 μl) was added to the 96-well microtiter plate precoated with monoclonal anti-rat TNF-α antibody and incubated for 2 h at 37 °C. After thorough washing, the substrate solution was added. Color development was allowed for 15 min and the reaction was stopped by application of stop solution. Color absorbance was read at 450 nm. The amount of protein in the sample was determined using the bicinchoninic acid (BCA) method [25], and the mucosal TNF-α level was expressed as picogram per milligram protein.

*Northern blot analysis of TNF-α*

Rat gastric mucosal tissue was minced and the total cellular RNA extracted using Trizol (Life-Technologies, Inc., Auckland, New Zealand). Total RNAs (10 μg) were separated by electrophoresis on a formaldehyde-denaturing 1% agarose gel and transferred overnight to a nylon Hybond-$N^+$ membrane (Amersham). The membrane was hybridized with a 391-bp cDNA fragment (open reading frame base 259–649) of rat TNF-α, which was labeled with [α-$^{32}$P]dCTP using a random priming kit (RTS Radprime DNA labeling system; LifeTechnologies, Inc.) by the reverse transcriptase polymerase chain reaction method. Its nucleotide sequence was confirmed to be identical to the reported sequences by an automatic ABI Prism 7700 DNA sequencing system (Perkin–Elmer). Hybridization was performed at 42 °C for 16 hours, and membranes were washed twice for 20 minutes each at 53° C in 0.1 × standard saline citrate/0.1% sodium dodecyl sulfate. Membranes were then stripped and rehybridized with a $^{32}$P-labelled cDNA probe for human β-actin to determine loading of the lanes. The signal intensity was quantified by laser densitometric scanning.

*Cell culture*

RGM-1 cells were obtained from Riken Cell Bank, Koyadai, Japan. The cells were maintained in a 1:1 DMEM/F-12 medium containing 20% (v/v) fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in a water-saturated atmosphere of 5% $CO_2$ at 37 °C. Trypan blue exclusion assay assured the viability of the cells after 24–48 hr periods of GLPS exposure. Passages 8–16 were used in the experiments. There were no significant changes in biochemical characteristics from passages 8–16.

*Cell viability estimation*

Cell viability was estimated using sulforhodamine B assay [26]. RGM-1 cells ($5 \times 10^3$/100 μl) were seeded into a 96-well plate and incubated for 24 hr. They were then incubated with GLPS (0.05, 0.25, and 1.0 mg/ml) at 37 °C for 24 hr. At the end of incubation, the medium was aspirated, and the cells were fixed by incubation for 60 min with 0.2 ml ice-cold 10% (w/v) trichloroacetic acid (TCA). The remaining cells were further incubated with 0.1 ml 0.4% (w/v) SRB in 1% acetic acid for 15 min on a shaker. The unbound dye was removed by rinsing with 1% acetic acid, and cell-bound dye was extracted with 0.1 ml 10 mM unbuffered Tris base at pH 10.5, and the color change in the extract was measured at 596 nm.

The choice of GLPS concentrations (50–1000 μg/ml) in all *in vitro* experiments was based on the possible blood concentration of GLPS achieved *in vivo*. If it were assumed that the bioavailability of GLPS was approximately 75%, the drug concentration in the rat blood (a 200-g rat has about 20 ml blood) would be about 375 μg/ml for a 500-mg/kg dose of GLPS extract which has been administered

for *in vivo* studies. Indeed, the bioavailability and actual concentration of GLPS in gastric mucosa were unknown; we selected concentrations of GLPS from 50 to 1000 μg/ml which were in the range of the predicted blood concentrations *in vivo*.

### *[$^3$H]Thymidine incorporation assay*

To evaluate the effect of GLPS on DNA synthesis in RGM-1 cells, the incorporation of [$^3$H]thymidine (Amersham) into DNA was determined. Briefly, RGM-1 cells were seeded into a 24-well plate and cultured for 24 hr in the presence of 20% (v/v) fetal bovine serum. They were then washed twice with Hanks' balanced salt solution, followed by incubation with 1 ml/well of the medium containing GLPS (0.05, 0.25 and 1.0 mg/ml) for 24 hr. [$^3$H]Thymidine (0.5 μCi) was then added to each well, and the cells were further incubated for 5 hr. The incorporation of [$^3$H]thymidine into cells was measured with a microbeta liquid scintillation counter (Perkin–Elmer).

### *Immunoblot analysis*

The RGM-1 cells were harvested at 4 °C with RIPA buffer (50 mM Tris–HCl at pH 7.5, 150 mM NaCl, 0.1% [w/v] SDS, 0.5% [w/v] α-cholate, 2 mM EDTA, 1% [v/v] Triton X-100, 10% [w/v] glycerol) containing 1 mM of phenylmethylsulfonyl fluoride and 10 μg/ml of aprotinin. The cell suspension was sonicated on ice for 20 sec and centrifuged at 20 000 *g* at 4 °C for 20 min using a Beckman centrifuge (Beckman Coulter, Inc., Fullerton, CA). The supernatant (30 μg of total protein) was denatured and separated by electrophoresis on a 10% [w/v] SDS-polyacrylamide gel according to Laemmli (1970) [27] using a Mini-Protein II system (Bio–Rad). Prestained protein standard with full range colour mark (Bio–Rad) was also loaded and separated. After electrophoresis, the separated cellular proteins were transferred onto Hybond-P membrane (Amersham). Detection of c-Myc was carried out using a primary antibody recognizing rat c-Myc raised in rabbit. Biotinylated donkey against rabbit IgG was used as secondary antibody. Streptavidin–horseradish peroxidase was finally bound to the secondary antibody and the light signal was developed using the ECL system (Amersham). The intensity of blots was quantified using the MD30 image analysis system (Leading Edge, Adelaide, Australia) and a video camera mounted on a Leitz Diaplan microscope.

### *Determination of ODC activity in the gastric mucosa and RGM-1 cells*

The ODC activity in gastric mucosa and RGM-1 cells was determined by measuring the amount of $^{14}CO_2$ liberated from L-[1-$^{14}$C]ornithine [28]. Gastric mocosal tissues were collected as previously described and homogenized in 0.1 M sodium potassium phosphate buffer (pH 7.4) using an automatic homogeniser at 300 rpm (IKA Labortechnik, Staufen, Germany). The homogenates were centrifuged at 20 000 *g* for 20 min at 4 °C. The resulting supernatants served as enzyme source for the assay of ODC activity.

RGM-1 cells ($3 \times 10^5$/ml) were seeded into a 6-well plate and incubated for 24 hr. They were then incubated with GLPS (0.05, 0.25, and 1.0 mg/ml) at 37 °C for 6 hr. The polyamine spermidine was included as control. At the end of incubation, medium was aspirated and discarded. The remaining cells were washed twice with PBS. The cells were then scraped from the plates and placed in 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, 0.05 mM pyridoxal phosphate, and 5 mM dithiothreitol.

The cells were then sonicated on ice for 15 min, and then centrifuged at 20 000 *g* for 20 min at 4 °C. The supernatants were again used as enzyme source. The protein contents of the supernatants arising from gastric mocosa and RGM-1 cells were determined by BCA method as described previously [25]. Typical incubation (total volume = 300 μl) for the ODC activity assay containing 1.0 mg/ml supernatant protein and 2.5 mM L-[1-$^{14}$C]ornithine was performed at 37° for 20 min in a shaking water bath. The $^{14}CO_2$ liberated from the decarboxylation of ornithine was trapped by a piece of filter paper soaked with 20 μl of NaOH (2.0 M). The paper was placed inside a well connected to the stopper and suspended above the reaction mixture. The reaction was stopped by the addition of 0.3 ml of 10% (w/v) TCA. The radioactivity of $^{14}CO_2$ trapped in the filter paper was determined using a microbeta liquid scintillation counter (Perkin–Elmer). The enzyme activity was expressed as pmol $^{14}CO_2$ liberated per mg of protein per hr.

*Determination of mucus synthesis in RGM-1 cells*

The effect of GLPS on the mucus synthesis in RGM-1 cells was determined by measuring incorporation of D-[6-$^{3}$H]glucosamine into gastric mucosal glycoprotein as described [29]. Cells ($1 \times 10^5$) were incubated in 24 well culture plates for 16 hr, then washed twice with $Ca^{++}/Mg^{++}$-free PBS, followed by incubation with 0.5 ml of the medium containing [′ H]glucosamine HCl (Amersham) in the presence of GLPS (0.05, 0.25 and 1.0 mg/ml) for 8 hr at 37 °C in 5% $CO_2$. At the end of incubation, the medium was aspirated and discarded, and the remaining cells were washed twice with $Ca^{++}/Mg^{++}$-free PBS, solubilised with 0.4 ml NaOH (0.3 M), and neutralised with 0.4 ml HCI (0.3 M). The resulting cellular proteins were precipitated by the addition of 0.5 ml of 50% (w/v) TCA. The pellet was washed twice with 10% (w/v) TCA, once with chloroformmethanol solution (1:1, v/v), and finally dried in the air. The dried pellet was then dissolved in 25 μl of dimethyl sulfoxide and the fractions were subjected to Sepharose CL-4B column chromatography for isolation of mucin, as described by Takahashi and Okabe [29]. Radioactivity in the void fractions was measured as the amount of mucus synthesized by the cells. Mucus synthesis was expressed a [$^{3}$H] glucosamine incorporation (cpm) per $1 \times 10^5$ cells.

*Data analysis*

Data were expressed as means ± SD. Statistical analysis was performed with two-way analysis of variance (ANOVA) with a Tukey–Kramer test. Student's *t* test for paired data was used when appropriate. *P* values less than 0.05 were considered statistically significant.

## Results

*Effect of GLPS on gastric ulcer healing in the rat*

As shown in Table 1, treatment of rats with indomethacin at 50 mg/kg by subcutaneous injection produced typical time-dependent acute lesions in the gastric mucosa, with a mean value of 5.6 ± 0.7, 7.8 ± 1.1 and 8.6 ± 0.9 at day 1, 3 and 6 respectively. The vehicle-injected group showed no visible mucosal lesions (data no shown). Co-administration of GLPS at 250 and 500 mg/kg by intragastric input caused a dose-dependent decrease in the number of gastric lesions, with the mean number of lesion

Table 1
Effects of GLPS on the number of indomethacin-induced gastric lesions in rats

| Treatment | Dose | Day 1 | Day 3 | Day 6 |
|---|---|---|---|---|
| Indomethacin alone | 50 mg/kg, s.c | 5.6 ± 0.7 | 7.8 ± 1.1 | 8.6 ± 0.9 |
| + GLPS | 250 mg/kg, p.o | 4.1 ± 0.4[a] | 5.5 ± 0.5[a] | 6.5 ± 0.6[a] |
| + GLPS | 500 mg/kg, p.o | 3.1 ± 0.5[a] | 3.7 ± 0.3[a] | 4.3 ± 0.7[a] |

Data were the mean ± SD (n = 6).
[a] $P < 0.05$, indomethacin + GLPS vs indomethacin alone.

formation at day 1–6 reduced by 24.4–29.5% and 44.6–52.6% at 250 and 500 mg/kg GLPS respectively ($P < 0.05$), compared to the control rats receiving indomethacin only.

*Effect of GLPS on gastric mucosal ODC activity in the rat*

In domethacin treatment caused a sustained increase in gastric mucosal ODC activity compared to control rats receiving vehicle only (Table 2). Co-administration of GLPS at 250 and 500 mg/kg resulted in a significant increase in a dose-dependent manner, with the mean values of $^{12}CO2$ formation at day 1–6 increased by 9.0–12.2% and 19.1–23.3% at 250 and 500 mg/kg GLPS respectively ($P < 0.05$), compared to the control rats receiving indomethacin only.

*Effect of GLPS on indomethacin-induced gene expression of TNF-α in the rat gastric mucosa*

There was no detectable TNF-α expression in the gastric mucosa of control rats treated with vehicle. However, treatment of indomethacin at 50 mg/kg induced marked expression of TNF-α mRNA, with maximal expression 24 hr after administration and declining thereafter (Fig. 1A). Coadministration of GLPS at 250 and 500 mg/kg caused a significant suppressed gastric mucosal transcription of TNF-α mRNA in dose-dependent manner (Fig. 1 A), with the mean values of TNF-α mRNA at day 1–6 decreased by 34.2–95.3% and 76.8–100% at 250 and 500 mg/kg GLPS respectively ($P < 0.01$), compared to the control rats receiving indomethacin only. At day 6, the transcription of TNF-α was nearly or totally inhibited by GLPS at 250 and 500 mg/kg.

Consistently, treatment of indomethacin induced TNF-α proteins in the gastric mucosa, as measured by ELISA (Fig. 1B), with peak level 24 hr after administration and declining thereafter. It is likely that some of the measured TNF-α proteins derived from contaminating blood as the stomach was not

Table 2
Effects of GLPS on gastric mucosal ODC activity in the rat

| Treatment | Dose | ODC activity ($^{14}CO_2$ formation, cpm) | | |
|---|---|---|---|---|
| | | Day 1 | Day 3 | Day 6 |
| Vehicle only | | 21467 ± 547 | 22126 ± 521 | 22512 ± 279 |
| Indomethacin alone | 50 mg/kg, s.c | 23124 ± 324 | 24342 ± 732 | 27165 ± 823 |
| + GLPS | 250 mg/kg, p.o | 25223 ± 412[a] | 27321 ± 291[a] | 29615 ± 526[a] |
| + GLPS | 500 mg/kg, p.o | 27532 ± 425[a] | 30021 ± 612[a] | 32015 ± 517[a] |

Data were the mean ± SD (n = 6).
[a] $P < 0.05$, indomethacin + GLPS vs indomethacin alone.

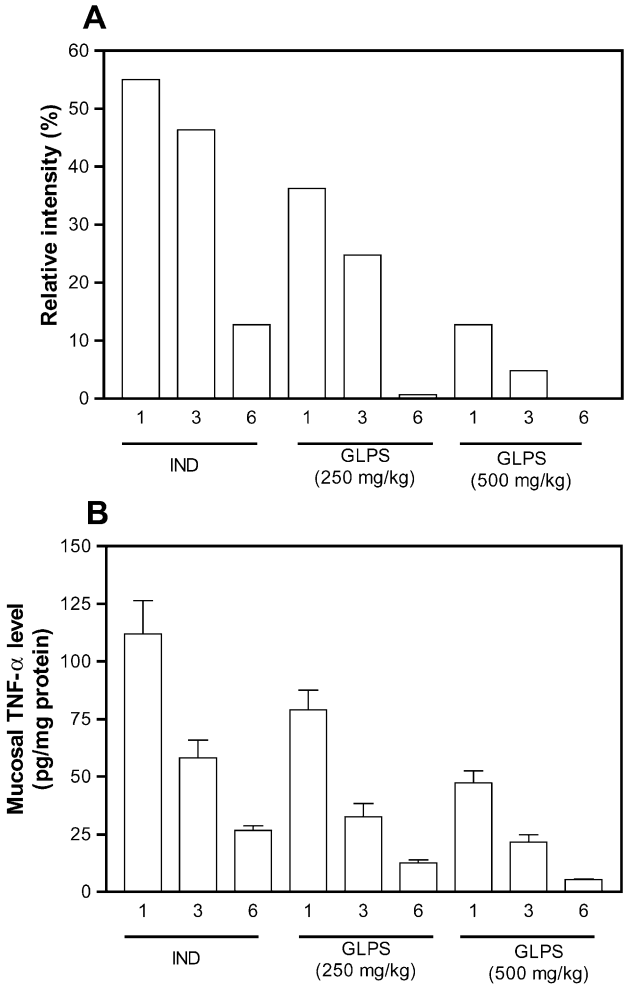


Fig. 1. Effect of *Ganoderma lucidum* polysaccharides (GLPS) on the mRNA (A) and protein (B) expression of TNF-α in the gastric mucosa of rats, as determined by Northern blot analysis and ELISA assay. Rats (n = 6) were injected subcutaneously with indomethacin (IND, 50 mg/kg) with or without coadministered intragastric GLPS at 250 and 500 mg/kg. Data are the mean ± SD from 6 determinations.

perfused. Coadministration of GLPS at 250 and 500 mg/kg caused a significant decrease in gastric mucosal levels of TNF-α proteins as determined by ELISA in dose-dependent manner (Fig. 1 B), with the mean values of TNF-α protein at day 1–6 decreased by 29.3–52.4% and 57.8–79.8% at 250 and 500 mg/kg GLPS respectively ($P < 0.01$), compared to the control rats receiving indomethacin only. The inhibitory effects of GLPS on TNF-α protein production were less pronounced than those observed for TNF-α mRNA, in particular at 500 mg/kg GLPS.

Interestingly, we found that although indomethacin treatment led to an increased formation of gastric mucosal lesions over time up to 6 days, the TNF-α mRNA and protein levels reduced 3 days after indomethacin administration. The reason for this is unclear. However, it may be due to the aggregation of macrophages which were the primary producer of TNF-α in gastric mucosal tissue declined at early

stages of ulcer formation process, but other factors that damage mucosal epithelia were still overwhelming over healing factors over longer time, and thus formation of lesions continued.

*Effect of GLPS on the proliferation of RGM-1 cells*

Our SRB assay showed that GLPS at 0.05 and 0.25 mg/ml caused marginal increase ($P > 0.05$) in the growth of RGM-1 cells, but higher concentration (1.0 mg/ml) of GLPS significantly ($P < 0.05$) increased the growth of RGM-1 cells (Fig. 2A). GLPS concentration-dependently increased the

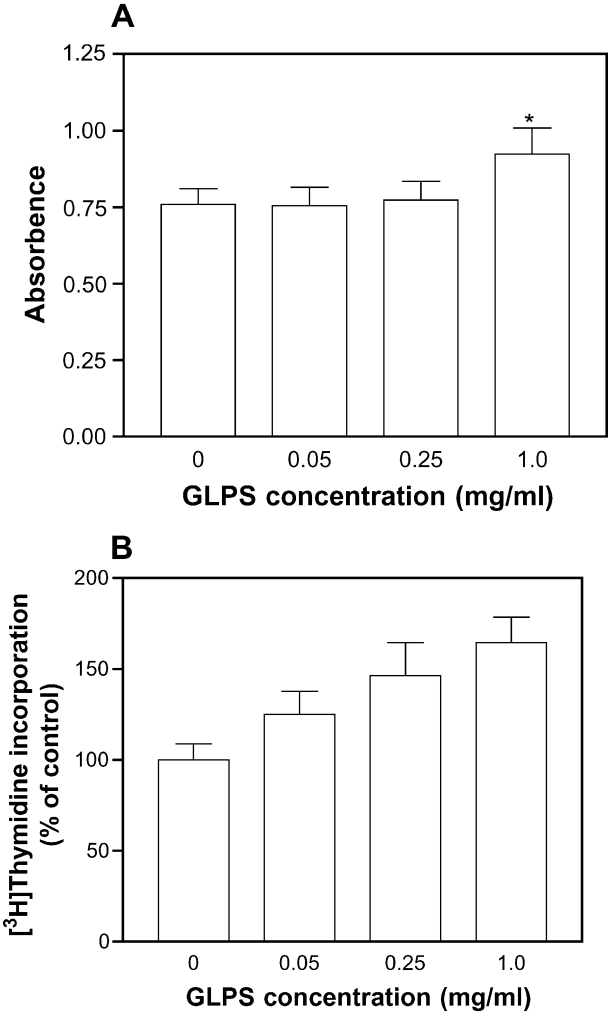


Fig. 2. Effect of *Ganoderma lucidum* polysaccharides (GLPS) at 0.05, 0.25 and 1.0 mg/ml on the cell viability (A) and [$^3$H]thymidine incorporation (B) in RGM-1 cells. To assess cell viability, cells were incubated with GLPS for 24 hr, and cellular proteins were stained with sulforhodamine B and absorbance was read at 596. The [$^3$H]thymidine incorporation assay was performed by incubating cells with GLPS for 24 hr, [$^3$H]Thymidine (0.5 μCi) was then added to each well, and the cells were further incubated for 5 hr. Radioactivity was read by a β-counter. Data are the mean ± SD from at least 5 determinations. * $P < 0.05$.

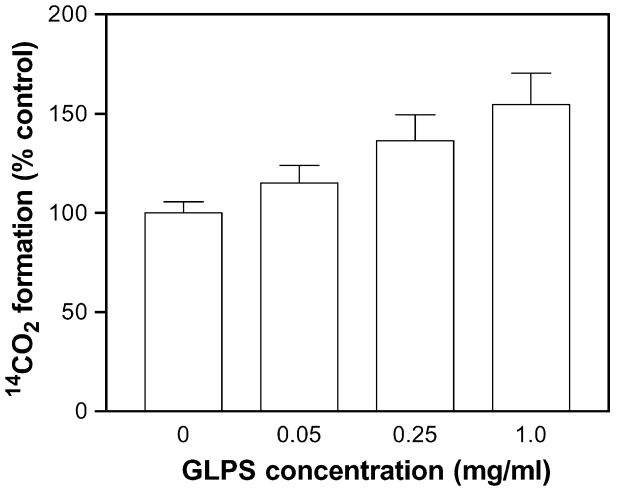


Fig. 3. Effect of *Ganoderma lucidum* polysaccharides (GLPS) on ODC activity in RGM-1 cells. The cells were incubated with GLPS (0.05, 0.25 and 1.0 mg/ml) for 6 hr, and the ODC activity was assessed by the amount of $^{14}CO_2$ liberation. Data are the mean ± SD from 6 determinations.

[$^3$H]thymidine incorporation by RGM-1 cells, with a 25% ($P < 0.05$), 47% and 65% ($P < 0.01$) increase in the DNA synthesis at 0.05, 0.25 and 1.0 mg/ml of GLPS respectively (Fig. 2B). DMFO significantly ($P < 0.05$) suppressed the [$^3$H]thymidine incorporation induced by GLPS.

*Effect of GLPS on ODC activity, c-Myc protein expression and mucus synthesis in RGM-1 cells*

As shown in Fig. 3, the addition of GLPS at 0.05, 0.25 and 1.0 mg/ml significantly ($P < 0.05$) increased the ODC activity in RGM-1 cells by 15.0%, 36.3% and 54.6% respectively, as indicated by the

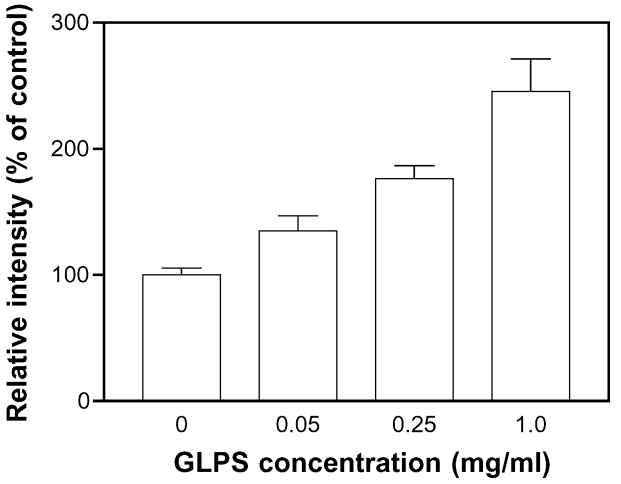


Fig. 4. Effect of *Ganoderma lucidum* polysaccharides (GLPS) on the expression of c-Myc protein in RGM-1 cells. The cells were incubated with GLPS for 6 hr, and cellular proteins were extracted and subjected to SDS-PAGE. Detection of c-Myc was carried out using a primary antibody recognizing rat c-Myc raised in rabbit. Signals were analyzed by an image analysis system.

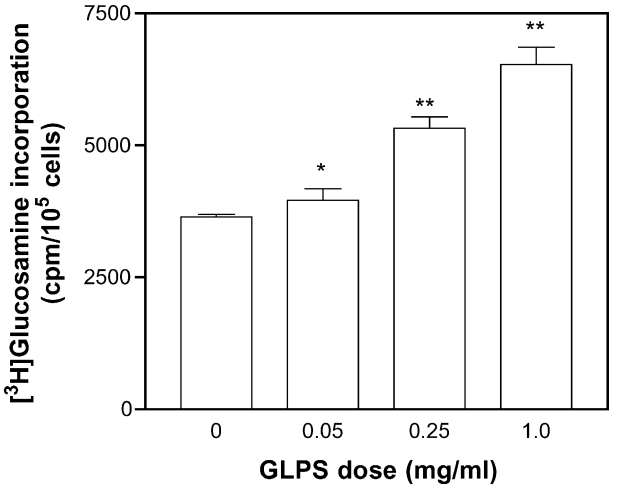


Fig. 5. Effect of *Ganoderma lucidum* polysaccharides (GLPS) on the incorporation of D-[6-$^3$H]glucosamine by RGM-1 cells. Cells were incubated with [′H]glucosamine HCl (Amersham) in the presence of GLPS (0.05, 0.25 and 1 mg/ml) at 37 °C in 5% $CO_2$ for 8 hr. Cellular mucin was isolated and purified by strong base and acid, followed by Sepharose CL-4B column chromatography. * $P < 0.05$; ** $P < 0.01$.

increase of $^{14}CO_2$ formation. Addition of spermidine at 10 mg/ml caused a 51.2% increase in the formation of $^{14}CO_2$, which is similar to the effect induced by GLPS at 1.0 mg/ml. However, addition of DFMO at 10 mM totally suppressed the enhancing effect of spermidine on ODC activity of GLPS.

Immunoblot analysis showed that c-Myc protein expression as determined by Western blotting analysis in RGM-1 cells was significantly ($P < 0.05$) enhanced by the treatment of GLPS in a concentration-dependent mode, with an increase by 35.0%, 76.3% and 145.6% of c-Myc proteins at 0.05, 0.25 and 1.0 mg/ml GLPS respectively (Fig. 4).

In addition, GLPS concentration-dependently increased the [$^3$H]glucosamine incorporation by RGM-1 cells, with an increase by 8.6% ($P > 0.05$), 46.0% ($P < 0.05$) and 79.3% ($P < 0.01$) at 0.05, 0.25 and 1.0 mg/ml repsectively (Fig. 5).

## Discussion

Peptic ulcer disease is generally considered as the consequence of an imbalance between aggressive factors and the maintenance of mucosal integrity through endogenous defence mechanisms [30]. Some active constituents, in particular polysaccharides from several medicinal herbs have been shown to have ulcer-healing effects, and they have traditionally been used for the prevention and treatment of peptic ulcer disease [31–33]. The present study demonstrated that the extracted polysaccharide fractions from the medicinal mushroom *G. lucidum* had potent healing effect on imdomethacin-induced gastric lesions in the rat. This was accompanied with a marked suppression of gene expression of TNF-α. Cytokines such as TNF-α play a central role in the formation of gastric ulcers by initiating the early inflammatory process through stimulating the migration of leukocytes (in particular neutrophils) into the inflammatory sites and secretion of other cytokines such as IL-8 and intercellular adhesion molecules [34,35]. TNF-α

decreased gastric blood flow and upregulated gastric mucosal gene expression for gastrin, cyclo-oxygenases and vascular endothelial growth factor [11,36]. Therefore, the suppression of TNF-α production would greatly facilitate ulcer healing by enhancing various healing mechanisms such as increased epithelial cell proliferation and gastric blood flow and decreased epithelial apoptosis. The results suggested that polysaccharides from natural sources might represent a useful approach for the treatment of peptic ulcer disease wherein cytokines may be considered as the potential therapeutic targets [37]. This study also indicated that the gastric mucosal level of ODC activity was significantly increased by GLPS treatment. Increased ODC activity will enhance the production of polyamines, which have been reported to stimulate ulcer healing [38,39].

The mechanisms for the modulating effects of GLPS on TNF-α and ODC activity remain unknown. We suggest that local direct effects of GLPS on gastric mucosa may play an important role. GLPS may directly bind to the infiltrated immune effector cells (e.g. macrophages), subsequently altering their activities. Studies have indicated that polysaccharides from *G. lucidum* (in particular active β-D-glucans) can bind to leucocyte surfaces through specific receptors or serum-specific proteins, leading to alteration of the activities of macrophage, T-helper, NK cells, and other effector cells [40,41]. In addition, intragastric administration of GLPS will form a layer of artificial mucus, which may provide a temporary protection for gastric mucosa against damaging factors. However, the small molecular weight polysaccharides (e.g. β-D-glucan) in GLPS might be absorbed into blood, and subsequently modulate the activities of immune effector cells. β-D-glucans are potent immuno-modulating agents *in vitro* and *in vivo* [42]. It is notable that GLPS always induces the production cytokines such as TNF-α in *in vitro* and in animal studies, contributing to its anti-cancer activity [43], which is in contrast to its inhibiting effect on TNF-α production in the stomach. It appears that the cytokine-modulating effect of GLPS would be tissue-specific.

Our *in vitro* studies in RGM-1 cells showed that GLPS promoted the proliferation of rat gastric epithelial cells, as indicated by the SRB assay, and [$^3$H]thymidine incorporation. This was accompanied by an increased ODC activity and c-Myc protein. DFMO suppressed the [$^3$H]thymidine incorporation and ODC activity induced by GLPS, suggesting that the increased ODC activity might play a central role in the activities of GLPS. Ye et al. [39] have reported that both the ODC activity and ODC protein are increased by a crude polysaccharide isolated from *Angelica sinesis* in RGM-1 cells. As ODC is also important for the growth of cancer cells, it would be interesting to investigate the effect of GLPS on the proliferation of tumor cells.

c-Myc as a transcription factor is able to regulate a number of target genes including *ODC* [44]. c-Myc has been reported to upregulate ODC gene expression in rat hepatocytes following insulin stimulation [45]. Heterodimers of c-Myc/Max bind cooperatively to the two adjacent, canonical E-boxes (CACGTG) located in the rat *ODC* gene and induce ODC mRNA expression [46]. Myc-Max heterodimers stimulate transcription, whereas Max homodimers, or heterodimers between Max and members of the Mad family of basic/helix-loop-helix/leucine zipper proteins, suppress gene transcription [47]. c-Myc has a key role in the signaling network controlling the balance between cellular proliferation and apoptosis [44]. It is rapidly induced and remains elevated in quiescent cells following mitogenic treatment [48]. Although the exact intracellular molecular events following GLPS treatment remain unclear, c-Myc could be upstream of ODC activation in the signal transduction pathway. c-Myc usually has a short (about 10 min) half life, but mitogenic stimulation can extend its half life to about 60 min [49]. The increased c-Myc in RGM-1 cells by GLPS may be due to enhanced expression of *c-myc* gene at mRNA and protein level and/or prolonged half life of c-Myc protein. The present study suggested that there might be an interplay between c-*myc* and *ODC* gene in RGM-1 cells following GLPS treatment,

resulting in an increase in cell proliferation. However, as c-Myc protein couples cellular proliferation with the induction of apoptosis under specific growth conditions, the possibility of apoptosis of mucosal epithelia following GLPS treatment could not be excluded. It would be interesting to investigate whether GLPS could cause apoptosis in gastric cancer cells via c-Myc protein.

In summary, this study indicates that GLPS accelerates ulcer healing by suppressing damaging factors such as toxic cytokine production and simultaneously enhancing mucosal repair by stimulation of cell proliferation in gastric epithelial cells. The latter was considered to be through the upregulation of c-Myc and c-Fos, which subsequently activated ODC expression.

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