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# Effects of Erythromycin on the Proliferation and Apoptosis of Cultured Nasal Polyp-Derived Cells and the Extracellular Signal-Regulated Kinase (ERK)/Mitogen-Activated Protein Kinase (MAPK) Signaling Pathway

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Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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**Background:** Erythromycin and its derivatives have been used to treat nasal polyposis and reduce inflammation, but the mechanism of action remains unclear. The extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) pathway proteins are expressed in nasal polyps. The aim of this study was to investigate the effects of erythromycin on cell proliferation, apoptosis, and the expression of p-MEK1 and p-ERK1 on cultured nasal polyp-derived cells.

**Material/Methods:** Nasal polyp-derived cells (n=32) and control cells from normal inferior turbinate tissue (n=32) were divided into four groups: the control group; the erythromycin-treated (100 μM) group; the selumetinib-treated (2 nM) group; and the erythromycin + selumetinib-treated group. Western blot was used to detect p-MEK1 and p-ERK1 proteins. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect mRNA expression of *BCL-2* and *BAX*. Flow cytometry detected expression of Ki-67 and cell apoptosis. Cell apoptosis was evaluated by terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL). Spectrophotometry assessed caspase-3 activity.

**Results:** The expression of Ki-67 was significantly increased, and cell apoptosis was significantly reduced in untreated nasal polyp-derived cells compared with controls. Erythromycin treatment significantly decreased cell proliferation and the expression of p-MEK1 and p-ERK1, and increased apoptosis in nasal polyp-derived cells compared with control cells. Selumetinib treatment had a synergistic effect with erythromycin to reduce the expression of p-MEK1 and p-ERK1, reduce cell proliferation, and increase cell apoptosis.

**Conclusions:** In cultured cells derived from nasal polyps, erythromycin treatment reduced cell proliferation and increased apoptosis by inhibiting the activation of the ERK/MAPK signaling pathway.

**MeSH Keywords:** Apoptosis • Erythromycin • Nasal Polyps

**Full-text PDF:** https://www.medscimonit.com/abstract/index/idArt/910934







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## Background

Nasal polyps are a common upper respiratory chronic inflammatory disease characterized by edema of the nasal mucosa, inflammatory cell infiltration, and gland hypertrophy. Currently, nasal polyps are treated by endoscopic sinus surgery, but the postoperative recurrence rate remains between 20–30% [1,2]. The mucociliary function of the sinonasal mucosa is an innate defense mechanism of the human nasal airway. Cell proliferation results in cell renewal and repair of the sinonasal mucosa. It has been shown that increased cell proliferation in nasal polyps may play an important role in the repair of the epithelial and sub-mucosal defects associated with nasal polyps [3]. A previously published study has shown that non-eosinophilic nasal polyps showed an increased change in the surface epithelium and more local maxillary involvement [4]. Changes in the dynamics in growth and proliferation in human nasal epithelial stem/progenitor cells (hNESPCs) from nasal polyps have been shown to be an important phenomenon in the pathogenesis of nasal polyps [5]. Also, the nasal epithelium plays a crucial role in innate and adaptive immune responses, and alterations in epithelial barrier function and host defense responses may contribute to the pathogenesis of chronic rhinosinusitis and nasal polyps [6]. Therefore, increased understanding of the changes of nasal polyp-derived cells may provide a molecular basis for the future diagnosis treatment of nasal polyps.

The extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) signaling pathway can activate a variety of nuclear transcription factors and participate in multiple intracellular protein kinase reactions to regulate the transcription and expression of related target genes, which affect cell biology, proliferation, apoptosis, inflammatory response, and other biological processes [7,8]. It has been shown that the expression or functional activity of key proteins in the ERK/MAPK pathway were increased in nasal polyps, suggesting that overexpression of components of the ERK/MAPK signaling pathway has a role in the pathogenesis of nasal polyps [9].

Erythromycin, a macrolide antibiotic, is widely used in clinical antibacterial therapy [10,11]. Hashiba et al. [12] observed that long-term, low-dose macrolide antibiotics have a therapeutic role in chronic sinusitis with nasal polyps. Ichimura et al. [13] found that the use of erythromycin in the treatment of chronic sinusitis and nasal polyps exerted a therapeutic effect by shrinking the polyps. Several studies have now shown that erythromycin can affect cell proliferation, apoptosis, and the release of inflammatory cytokines release through regulation of the MAPK signaling pathway [14–16]. However, the biological mechanisms for the effects of erythromycin on nasopharyngeal polyps remain unclear.

Therefore, the aim of this study was to investigate the effects of erythromycin on cell proliferation, apoptosis, and the expression of p-MEK1 and p-ERK1 and the activation of the ERK/MAPK signaling pathway on cultured nasal polyp-derived cells.

## Material and Methods

### Reagents and instruments

Dulbecco's Modified Eagle's Medium (DMEM)/F12, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Rabbit anti-ERK1 monoclonal antibody and p-ERK1 polyclonal antibody were purchased from Abcam (Cambridge, MA, USA). Mouse anti-β-actin monoclonal antibody was purchased from Cell Signaling Technology (CST) (Beverly, MA, USA). TRNzol Universal total RNA isolation reagent was purchased from Beijing Tiangen (Beijing, China). Quantitative real-time polymerase chain reaction (qRT-PCR) reagent TransScript Green One-Step qRT-PCR SuperMix was purchased from Transgene (Beijing, China). The 5-ethynyl-2'-deoxyuridine (EdU) thymidine analog cell proliferation detection kit was purchased from Molecular Probes Inc. (Eugene, OR, USA). Radioimmunoprecipitation assay (RIPA) cell lysis buffer and the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay kit were purchased from Beyotime (Jiangsu, China). Alexa Fluor® 488 (modified fluorescein) anti-human Ki-67 and Alexa Fluor® 488 mouse IgG1 kappa isotype control antibodies were purchased from BioLegend (San Diego, CA, USA). The MEK1 inhibitor, selumetinib, was purchased from Selleckchem (Houston, TX, USA). Erythromycin was purchased from Sigma-Aldrich (St Louis, MO, USA). Biological safety cabinets and cell incubators were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Polyacrylamide gel electrophoresis (PAGE) and real-time PCR instruments were purchased from Bio-Rad (Hercules, CA, USA). The flow cytometer used in this study was purchased from Beckman Coulter (Brea, CA, USA). The inverted fluorescence microscope was purchased from Nikon (Tokyo, Japan).

### Patients

Thirty-two patients with nasal polyps included 21 men and 11 women, aged between 24–52 years, who underwent their first endoscopic sinus surgery in our inpatient department between January 2016 and May 2017, were enrolled in the study. Exclusion criteria included a history of previous nasal sinus surgery, glucocorticoid or antihistamine treatment, a history of allergic rhinitis, chronic bronchitis, bronchial asthma, poor tolerance to aspirin, or other serious systemic conditions. All patients underwent unilateral or bilateral nasal computed tomography (CT) imaging with a diagnosis of nasal polyposis confirmed by histopathology, which also excluded the presence of fungal and other infectious organisms.





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A further 32 patients were included as controls, who underwent endoscopic nasal sinus surgery at the Nangang Branch of Heilongjiang Provincial Hospital for deviation of the nasal septum. The control group included 22 men and 10 women, aged between 22–49 years. The inferior turbinate mucosal tissue was sampled as control tissue.

In all patients included in the study, tissue samples were collected with informed consent of the patients. The study was reviewed by the Nangang Branch of Heilongjiang Provincial Hospital Ethics Committee. The specimens were collected and stored at -80°C overnight, then transferred to liquid nitrogen for long-term storage.

### TUNEL assay

The nasal polyp tissue and normal inferior turbinate mucosa tissue were sectioned for cell extraction and used to evaluate cell apoptosis according to the instructions on the TUNEL apoptosis kit. Briefly, the tissue samples were fixed in 4% paraformaldehyde for 30 min and washed twice in phosphate buffered saline (PBS). Next, the sample was incubated at room temperature for 5 min with the TUNEL assay solution containing 10% terminal deoxynucleotidyl transferase (TdT) enzyme and 90% fluorescent labeling solution. The sample was washed with PBS in triplicate, sealed with fluorescence quenching liquid, and observed under a fluorescence microscope.

### Nasal polyp cell isolation and culture

There were 32 patients with nasal polyps included in the study. The nasal polyp tissue was placed in a Petri dish and washed two or three times with PBS containing 1% streptomycin. The tissue was cut into pieces and digested in DMEM/F12 medium containing 0.1% type IV collagenase at 37°C for 2h. After filtering using a 100 μm cell strainer, the solution was transferred to a 50 mL centrifuge tube and centrifuged at 300×g for 10 min. The cell pellet was resuspended in DMEM/F12 medium containing 10% FBS and placed into a 10 cm cell culture dish at 37°C and incubated in an incubator containing 5% $CO_2$. The cells in the logarithmic growth phase were used for further experiments.

### Detection of Ki-67 expression

The cells from the digested tissue were fixed with 80% methanol for 5 min and permeabilized using 0.1% PBS-Tween for 20 min. Then, the cells were incubated in the primary anti-human Ki-67 antibody, in the dark, at room temperature for 30 min and washed twice with PBS. The cells were analyzed using a Coulter FC500 MCL flow cytometer.

### Nasal polyp-derived cell grouping and treatment

The isolated nasal polyp cells in the logarithmic phase were divided into four groups: the control group; the erythromycin-treated (100 μM) group; the selumetinib-treated (2 nM) group; and the erythromycin + selumetinib-treated group. The cells were collected after 72 h of treatment. Cell proliferation was measured by the EdU assay, cell apoptosis was evaluated by flow cytometry, caspase-3 activity was assessed by spectrophotometry, and protein expression was detected by Western blot. Quantitative real-time polymerase chain reaction (qRT-PCR) was used for the detection of expression of mRNA for the anti-apoptotic factor *BCL-2* and the pro-apoptotic factor *BAX*.

### Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using the TRNzol Universal kit followed by one-step qRT-PCR through TransScript Green One-Step qRT-PCR SuperMix to detect the relative expression of anti-apoptotic factor *BCL-2* mRNA and pro-apoptotic factor *BAX* mRNA. The 20 μL reaction system included 1 μg RNA template, 0.3 μM primer, 10 μL 2×TransStart Tip Green qPCR SuperMix, 0.4 μL RT Enzyme Mix, and deionized water. The reaction conditions included reverse transcription at 45°C for 5min, followed by 40 cycles of 94°C for 5 s and 60°C for 30 s. The reaction was performed using a Bio-Rad CFX96 real-time PCR amplifier to collect the data.

### Western blot

The tissue and cells were lysed in RIPA buffer at 4°C for 30 min. After centrifuging at 10000×g for 10 min, the supernatant was transferred to a new Eppendorf tube. A total of 50 μg of protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (40 V, 300 min) and transferred to polyvinylidene difluoride (PVDF) membranes (250 mA, 120 min). After blocking with 5% dried skimmed milk powder at room temperature for 60 min, the membrane was incubated with primary antibody overnight at 4°C (p-ERK1, 1: 1000) (p-MEK1, 1: 2000) (β-actin, 1: 10000). The membranes were washed three times with PBST and then incubated with horseradish peroxidase (HRP)-labeled secondary antibody (1: 20000) for 60 min at room temperature. The membrane was washed three times with PBST, and the enhanced chemiluminescence (ECL) substrate was added at room temperature for between 2–3min. Finally, the membrane bands were scanned.

### Detection of cell apoptosis

The cells were enzymatically digested and resuspended in binding buffer. Then, 5 μL Annexin-V conjugated with fluorescein isothiocyanate (FITC) and 5 μl propidium iodide (PI) were





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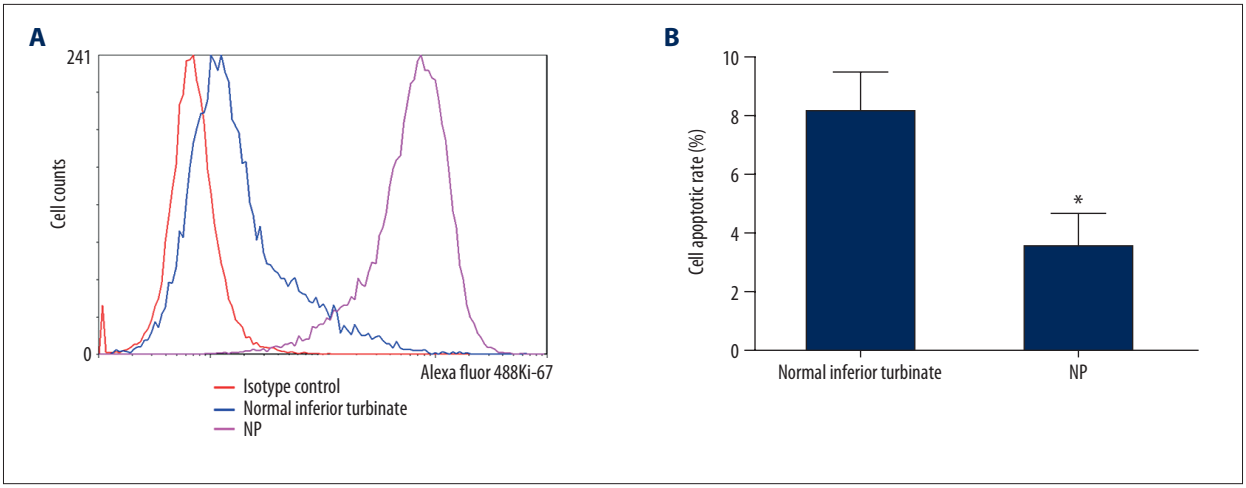


**Figure 1.** Cell proliferation and apoptosis in nasal polyp-derived cells. (**A**) Ki-67 expression detected by flow cytometry. (**B**) Cell apoptosis detected by the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay. * P<0.05, compared with the control.

added to the cells, and then analyzed using a Coulter FC500 MCL flow cytometer.

### Cell proliferation assay

A Click-iT EdU Alexa Fluor® 488 Flow Cytometry Assay Kit was used to test cell proliferation. The cells were incubated in 10 μM EdU for 2 h and then divided into the four treatment groups: the control group; the erythromycin-treated (100 μM) group; the selumetinib-treated (2 nM) group; and the erythromycin + selumetinib group. After 48 h incubation, the cells were digested with a reaction solution containing Alexa Fluor® 488 at was added at room temperature, in the dark, for 30 min. The cells were examined using a Coulter FC500 MCL flow cytometer.

### Detection of caspase-3 activity using a colorimetric spectrophotometry assay

Caspase-3 activity was evaluated according to the manufacturer's instructions. A pNA calibration curve was used to evaluate the 96-well plate Spectrophotometry assay and to calibrate the A405 value. The cells were seeded in 96-well plate and incubated with the Ac-DEVD-pNA spectrophotometry substrate for caspase-3 (CPP32) at 37°C for 2 h. Finally, the activity of caspase-3 was evaluated at A405 using a microplate reader.

### Statistical analysis

All data analysis was performed using SPSS version 18.0 software. The measurement data were shown as the mean ± standard deviation (SD) and comparisons were made using a t-test. A P-value <0.05 was considered to be statistically significant.

## Results

### Cell proliferation and apoptosis in nasal polyp-derived cells

Flow cytometry showed that Ki-67 expression in nasal polyp-derived cells was significantly increased compared with the cells from normal inferior turbinate tissues (Figure 1A), which suggested that increased cell proliferation might be involved in the pathogenesis of nasal polyps. The terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay showed that the apoptotic rate in nasal polyp-derived cells was 3.5%, which was significantly lower than that of normal inferior turbinate-derived cells, at 8.2% (Figure 1B).

### Extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) signaling pathway activation and anti-apoptotic factor expression in nasal polyp-derived cells

Quantitative real-time polymerase chain reaction (qRT-PCR) showed that the expression of anti-apoptotic factor *BCL-2* mRNA was increased, while the expression of pro-apoptotic factor *BAX* mRNA was decreased in nasal polyp cells compared with normal inferior turbinate cells (Figure 2A). Western blot showed that the expressions of p-MEK1 and p-ERK1 in cells from nasal polyps were increased compared with cells from normal inferior turbinate tissue, indicating that the activity of ERK/MAPK pathway was enhanced in nasal polyps (Figure 2B).





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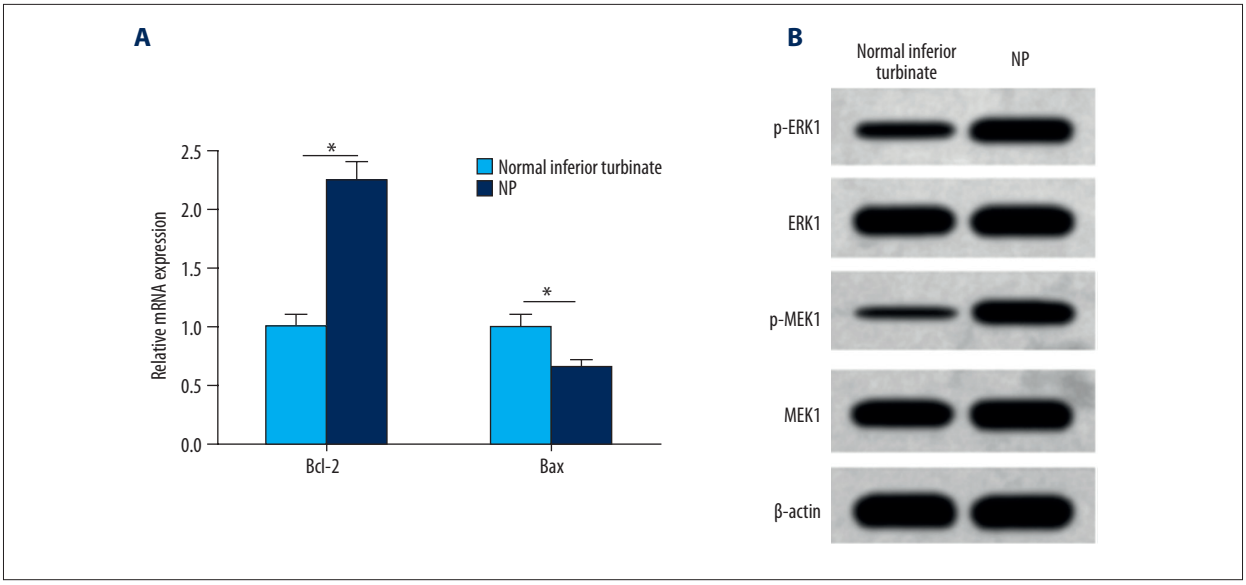


**Figure 2.** ERK/MAPK signaling pathway activation and expression of anti-apoptotic factors were increased in nasal polyp-derived cells. (**A**) Bcl-2 and Bax expression detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). (**B**) Protein expression detected by Western blot. * P <0.05, compared with the control.

### Erythromycin significantly inhibited the proliferation of cells derived from nasal polyps and induced cell apoptosis *in vitro*

The 5-ethynyl-2'-deoxyuridine (EdU) staining showed that the EdU positive rate of nasal polyp cells in the erythromycin-treated group was significantly lower when compared with the untreated group and was dose-dependent (Figure 3A). Flow cytometry showed that erythromycin treatment was significantly associated with apoptosis of nasal polyp cells and was dose-dependent (Figure 3B).

### Erythromycin reduced nasal polyp-derived cell proliferation and induced cell apoptosis through inhibition of the ERK/MAPK signaling pathway

Western blot showed that 100 μM erythromycin significantly downregulated the expression of p-MEK1 and p-ERK1 in nasal polyp cells. Also, the MEK1 inhibitor selumetinib significantly inhibited the expression of p-MEK1 and p-ERK1 and showed a synergistic effect with erythromycin in reducing the activity of the ERK/MAPK signaling pathway (Figure 4A). EdU staining showed that selumetinib further enhanced the inhibitory effect of erythromycin on the proliferation of nasal polyp cells (Figure 4B). Flow cytometry demonstrated that the combination of selumetinib and erythromycin promoted the rate of apoptosis of nasal polyp cells compared with selumetinib or erythromycin treatment alone (Figure 4C). Spectrophotometry showed that the activity of caspase-3 in nasal polyp cells treated with selumetinib and erythromycin was significantly increased when compared with treatment with selumetinib or erythromycin alone (Figure 4D).

## Discussion

Nasal polyps are a common upper respiratory tract chronic inflammatory condition characterized by a high degree of nasal mucosal edema. Nasal polyps mainly affect adults and can be located in the maxillary sinus, ethmoid, middle nasal meatus, and middle turbinate. The prevalence rate of nasal polyposis in adults has been reported to be between 1–2% [17,18]. Nasal polyps are also associated with the occurrence of other respiratory diseases, including asthma, pneumonia, and bronchiectasis.

Cell proliferation and apoptosis are regulated by multiple molecules and mechanisms that regulate the physiological or pathological growth of tissues and organs. The aberrant dynamic balance of cell proliferation and apoptosis leads to the occurrence of many diseases, such as polyps [19,20], precancerous lesions [21], and tumors [22]. In the present study, the finding that apoptosis and cell proliferation of cells derived from untreated nasal polyp tissue was increased has been supported by a previous study [3].

The mitogen-activated protein kinase (MAPK) signaling pathway is present in many eukaryotic cells. MAPK can be activated by a variety of extracellular stimuli, including cytokines, growth factors, neurotransmitters and G protein-coupled receptor ligands, through intracellular receptor tyrosine kinases, G-protein coupled receptors, and cytokine receptors [7,8]. The extracellular signal-regulated kinase (ERK)-mediated MAPK signaling pathway is a classical and major pathway, and Ras/Raf/MEK/ERK is the main transduction mode for ERK/MAPK signaling [23,24]. Recent studies have shown that interleukin





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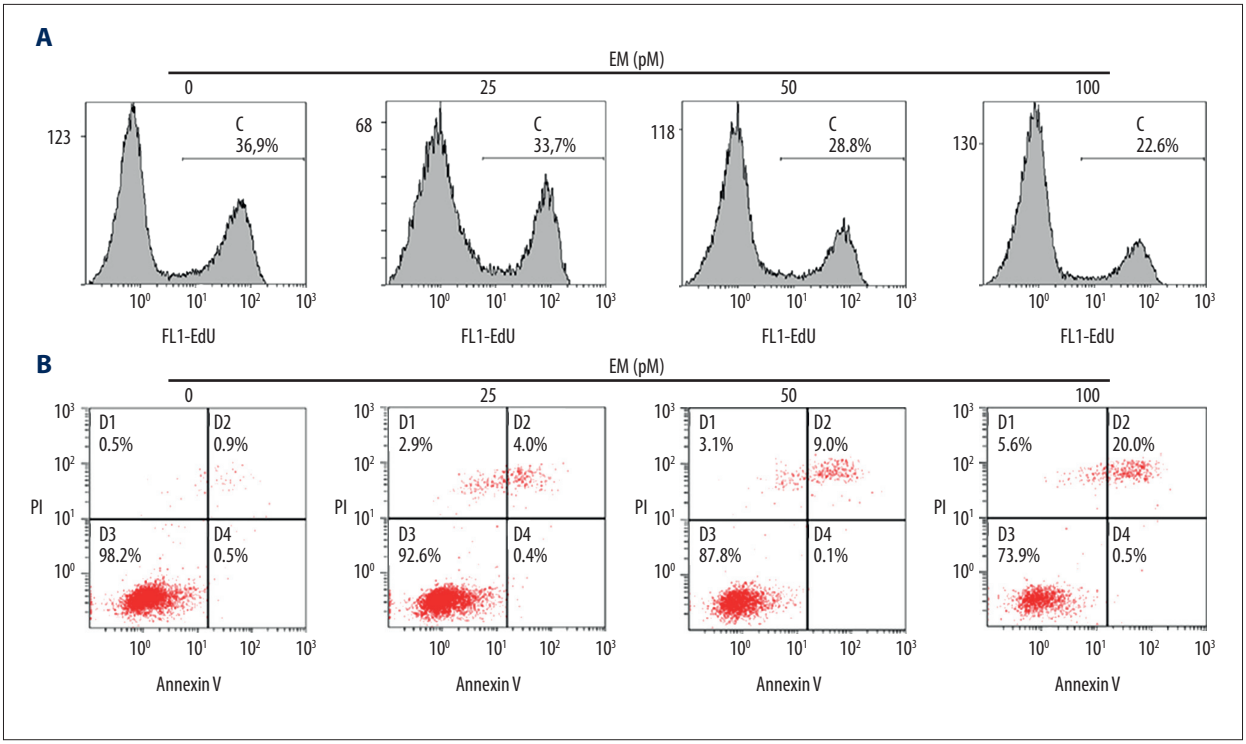


**Figure 3.** Erythromycin significantly inhibited nasal polyp-derived cell proliferation and induced cell apoptosis *in vitro*. (**A**) Cell proliferation detected by 5-ethynyl-2'-deoxyuridine (EdU) thymidine analog staining. (**B**) Cell apoptosis detected by flow cytometry. * P<0.05, compared with the control.

(IL)-1β regulates the expression of glucocorticoid receptor isoforms in nasal polyps *in vitro* via p38 MAPK and JNK signal transduction pathways, supporting the role of MAPK in the development of nasal polyps [25]. Another study has shown that overexpression of the ERK/MAPK pathway is closely related to the occurrence and development of nasal polyps [9]. The findings of the present study showed that the levels of p-ERK and p-MEK in nasal polyp tissue-derived cells were elevated, indicating activation od the ERK/MAPK signaling pathway.

Erythromycin is an antibiotic that is commonly used to treat a wide range of bacterial infections, including respiratory tract infections, skin infections, chlamydia infections, pelvic inflammatory disease, and syphilis. Erythromycin, combined with spiramycin, has been shown to inhibit the proliferation of human mononuclear cells [26]. Also, erythromycin, and its derivatives, have been shown to inhibit the proliferation of T lymphocytes by inhibiting NF-kappaβ mRNA and protein expression [27]. However, the biological and molecular mechanisms by which erythromycin can affect nasal polyp-derived cells remain unclear. In this study, nasal polyp cells were isolated and cultured *in vitro*. The results of this preliminary study showed that proliferation of nasal polyp-derived cells was inhibited by erythromycin in a dose-dependent manner, which was consistent with the findings from previously published studies [26,27].

Previous studies have also shown that erythromycin downregulated MAPK activity and inhibited HIV-1 replication, indicating that erythromycin has a role in regulating the MAPK signaling pathway and cell proliferation [14–16]. In the present study, erythromycin significantly downregulated the expression of p-MEK1 and p-ERK1 in nasal polyp-derived cells, whereas the ERK/MAPK pathway inhibitor selumetinib showed a synergistic effect with erythromycin to reduce cell proliferation and promote cell apoptosis; erythromycin inhibited the proliferation of nasal polyp-derived cells and induced apoptosis by suppressing the activation of the ERK/MAPK signaling pathway.

Pace et al. [28] found that the expression of the inflammatory cytokine IL-19 in nasal polyps was significantly increased compared with normal controls and that increased IL-19 expression promoted cell proliferation. The findings from this previously published study indicated that inflammation may be involved in the pathogenesis of nasal polyps [28]. Kim et al. [29] showed that Ki-67 expression and the proliferation index of epithelial cells in non-eosinophilic nasal polyps with lower airway inflammation were markedly increased compared with eosinophilic nasal polyps, indicating that in addition to inflammation, abnormal proliferation of epithelial cells is also a major factor in the pathogenesis of nasal polyps. In a previously published study that used human nasal epithelial cells in an *in vitro* model, lactoferrin was shown to interact with SPLUNC1 to reduce





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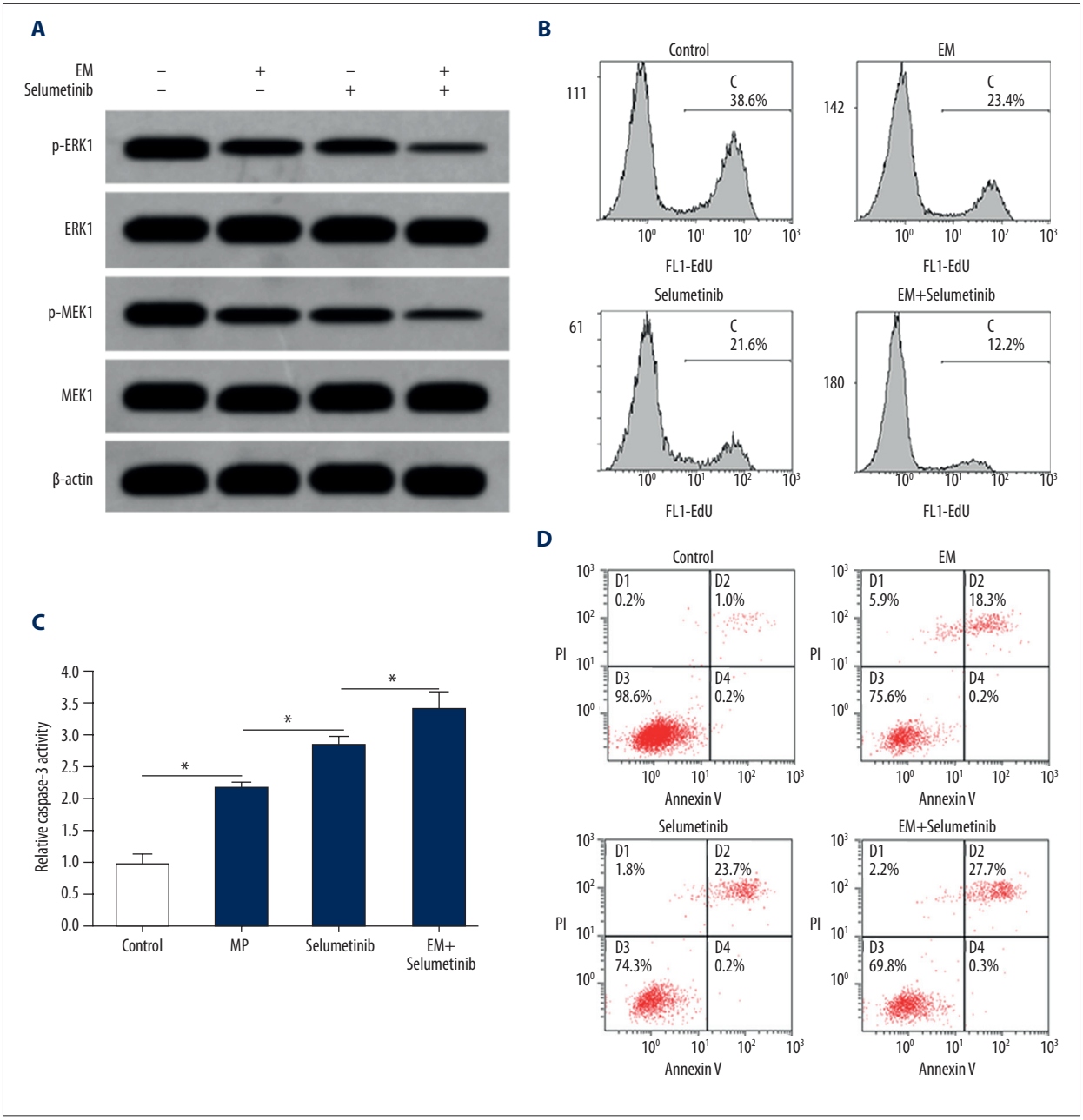


**Figure 4.** Erythromycin reduced nasal polyp-derived cell proliferation and increased cell apoptosis *in vitro* via inhibition of the ERK/MAPK signaling pathway. (**A**) Protein expression detected by Western blot. (**B**) Cell proliferation detected by 5-ethynyl-2'-deoxyuridine (EdU) thymidine analog staining. (**C**) Caspase-3 activity detected by spectrophotometry. (**D**) Cell apoptosis detected by flow cytometry. * P<0.05, compared with the control.

lipopolysaccharide-induced inflammation of human nasal epithelial cells by downregulating the MEK1/2-MAPK signaling pathway [30]. Oda et al. [16] demonstrated that erythromycin inhibited toxin-induced TNF-α synthesis and secretion by inhibiting the activity of the ERK/MAPK pathway in neutrophils, in a similar manner to the ERK1/2 inhibitor, PD98059.

The findings of the present study, together with the findings of previously reported studies, support the potential role of erythromycin in the regulation of inflammation associated with nasal polyps and inhibit cell proliferation by inactivating ERK/MAPK signaling. Future studies are needed to further investigate the role of erythromycin, and its derivatives, on inflammation, as well as to determine the role of alternative pathways, such as NF-kappaβ, involved in the regulatory effects of erythromycin.





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## Conclusions

Increased activation of extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) signaling has been previously shown to be associated with nasal polyposis. In this *in vitro* study, cells were derived from nasal polyp tissue and were compared with cells derived from normal nasal tissue, following treatment with erythromycin. Erythromycin treatment reduced cell proliferation and increased apoptosis by inhibiting the activation of the ERK/MAPK signaling pathway.

### Conflict of interest

None.

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