Eupatilin alleviates inflammation and epithelial-to-mesenchymal transition in chronic rhinosinusitis with nasal polyps by upregulating TFF1 and inhibiting the Wnt/β-catenin signaling pathway

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Eupatilin alleviates inflammation and epithelial-to-mesenchymal transition in chronic rhinosinusitis with nasal polyps by upregulating TFF1 and inhibiting the Wnt/β-catenin signaling pathway

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Abstract
Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is a multifactorial inflammatory disease characterized by high prevalence and morbidity. However, its pathogenesis is still obscure. This work focuses on the effects of Eupatilin (EUP) on inflammation reaction and the epithelial-to-mesenchymal transition (EMT) process in CRSwNP.

Methods: In vivo and in vitro CRSwNP models were established based on BALB/c mice and human nasal epithelial cells (hNECs) to investigate the effects of EUP on EMT and inflammation in CRSwNP. Protein levels of TFF1, EMT-related factors (E-cadherin, N-cadherin, and Vimentin), and Wnt/β-catenin signaling-related proteins (Wnt3α and β-catenin) were assayed via western blotting. Pro-inflammatory factors (TNF-α, IL-6, and IL-8) were assessed via ELISA assay.

Results: EUP treatment significantly reduced the number of polyps, epithelial thickness, and mucosal thickness in CRSwNP mice. Besides, EUP treatment also suppressed inflammation reaction and EMT events in CRSwNP mice and SEB-challenged hNECs in a dose-dependent manner. Also, EUP treatment dose-dependently upregulated TFF1 expression and inhibited Wnt/β-catenin activation in CRSwNP mice and SEB-challenged hNECs. In addition, TFF1 inhibition or Wnt/β-catenin activation partially abated EUP-mediated protection against SEB-induced inflammation reaction and EMT events in hNECs.

Conclusions: Taken together, our findings highlighted the inhibitory role of EUP on the inflammation and EMT processes in CRSwNP in vivo and in vitro via upregulating TFF1 and inhibiting the Wnt/β-catenin signaling, suggesting EUP could be a promising therapeutic agent for CRSwNP.

Keywords: Chronic rhinosinusitis with nasal polyps, TFF1, Wnt/β-catenin, Eupatilin
Introduction

Chronic rhinosinusitis (CRS) is a highly prevalent inflammatory disease of the nasal cavity and paranasal sinuses (Zou et al., 2016). Based on the absence or presence of nasal polyps (NPs), CRS can be typically divided into CRS without NPs (CRSsNP) and CRS with NPs (CRSwNP) (Soyka et al., 2012). Even though CRSwNP only accounts for about 30% of CRS cases (Kim et al., 2019), it often causes nasal stuffiness and olfactory dysfunction, thereby consequently leading to a decline in the quality of life (Yilmaz et al., 2020). CRSwNP is characterized by persistent inflammation and tissue remodeling in the nasal mucosa lesions (Tomassen et al., 2016). As a critical process of tissue remodeling, epithelial-mesenchymal transition (EMT) is also deeply implicated in CRSwNP development (Zhou et al., 2020). Furthermore, aberrant EMT is correlated to recurrent polyposis and severity of CRSwNP (Takahashi and Schleimer, 2021). To identify novel therapeutic targets and develop effective therapeutic drugs for CRSwNP treatment, it is necessary to elucidate the underlying molecular mechanisms of EMT and inflammation in CRSwNP.

Trefoil factors (TFFs), a group of highly conserved peptides, are expressed and secreted by epithelial cells in mucous membranes (Thim and May, 2005). It has long been recognized that TFF1, a member of the TFF family, plays a critical role in epithelial surface protection and repair (Kjellev, 2009). So far, TFF1 has been identified as an essential regulator of inflammation reaction in gastric neoplasia (Soutto et al., 2011), gastric mucosal injury (Zhang et al., 2020), diabetic retinopathy (Zhang et al., 2022), and pterygium (Buron et al., 2008). Besides, TFF1 also exerts inhibitory effects on EMT events in pancreatic intraepithelial neoplasm (Yamaguchi et al., 2018). In addition, studies have proven that TFF1 is downregulated in CRSwNP (Mihalj et al., 2019; Yao et al., 2019). However, its role in CRSwNP has not been well elucidated.

Eupatilin (EUP), an ingredient found in Artemisia Asiatica (Nageen et al., 2020), is of diverse biological activities, including anti-inflammation (Song et al., 2018), anti-oxidation (Liu et al., 2019), anti-apoptosis (Lou et al., 2019), and neuroprotective efficacies (Sapkota et al., 2017). It has been reported that EUP suppresses collagen-induced inflammation in a murine model of arthritis (Kim et al., 2015), ELS-induced production of pro-inflammatory cytokines in BV2 cells (Fei et al., 2019b), and NF-κB-mediated inflammatory reaction in intestinal epithelial cells (Kim et al., 2009). Besides, EUP also inhibits the EMT process in TGF-β2-stimulated retinal pigment epithelial cells (Cinar et al., 2021) and glioma cells (Fei et al., 2019a). Despite the known biological effects of EUP, there have been no reports on its role in CRSwNP.

In this study, we postulated that EUP might ameliorate inflammation and EMT processes in CRSwNP by upregulating TFF1 and inhibiting the Wnt/β-catenin signaling pathway. Herein, the murine and cellular models of CRSwNP were established to validate this hypothesis in vivo and in vitro through experimental methods.
Materials and Methods

CRSwNP modeling and drug treatment
This study was permitted by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University. Briefly, 4-week-old male BALB/c mice acquired from Shanghai Laboratory Animal Center (Shanghai, China) were randomly categorized into Control, CRSwNP, CRSwNP+EUP (5 mg/kg), CRSwNP+EUP (10 mg/kg), and CRSwNP+EUP (15 mg/kg) groups. To establish the CRSwNP model, BALB/c mice were challenged with ovalbumin (OVA) and staphylococcus aureus enterotoxin B (SEB) according to a previous study (Kim et al., 2013). As for EUP treatment, mice received intraperitoneal injection of EUP at different concentrations (5, 10, and 15 mg/kg). 24 hours after all treatment, all mice were sacrificed to collect specimens for the following experiments.

H&E staining
H&E staining was applied for histological analyses. The number of polyp-like lesions (mucosal elevations with inflammatory cell infiltration and microcavity formation) was counted in high-power fields (×400). The epithelium thickness of nasal mucosa (distance between the apices of epithelial cells and the upper border of the subepithelial glands zone) and mucosal thickness were measured via NIS-Elements BR 3.0 system (Nikon Eclipse, Japan).

ELISA
ELISA assay was applied to detect levels of pro-inflammatory cytokines and chemokines (TNF-α, IL-6, and IL-8) in nasal mucosa lysate and cell supernatant from each group with commercial ELISA kits (MyBioSource) as per the manufacturer’s instructions.

Western blotting
RIPA lysis buffer was used to extract the total proteins from nasal mucosa and hNECs. Then, the proteins were respectively separated by SDS-PAGE and transferred onto PVDF membranes (Beyotime). Next, PVDF membranes were incubated in the primary antibodies overnight, rinsed with TBST, and cultured with secondary antibody for another 2 h. Finally, ECL Western Blotting Substrate Kit (Abcam) was adopted to react with the HRP on the blots. Relative levels of protein bands were analyzed via ImageJ2X software (NCBI).

Cell culture and treatment
Human nasal epithelial cells (hNECs) were acquired from BeNa Culture Collection (Beijing, China) and cultured in airway epithelial cell growth media in a humidified atmosphere (37°C; 5% CO₂). Briefly, hNECs were divided into Control, SEB, SEB+EUP (10 µM), SEB+EUP (25 µM), SEB+EUP (50 µM), SEB+EUP (50 µM)+si-TFF1, and SEB+EUP (50 µM)+BML-284 (0.1 µM) (Wei et al., 2022). To simulate CRSwNP in vitro, hNECs were exposed to SEB (5 µg/mL) for 48 hours (Yoon et al., 2020). For EUP treatment, hNECs were pretreated with at different concentrations (10, 25, 50, 100 µM) (Choi et al., 2011). For BML-284 treatment, treated hNECs were finally treated with BML-284 at 0.1 µM.
Statistical analysis
All experiments were repeated 3 times. The acquired data were expressed as means±SD. All statistical analyses were performed on GraphPad 6.0 software, with comparison evaluated via one-way ANOVA or students’ t-test. P-value < 0.05 indicates statistical significance.

Results

EUP inhibits polyp formation in CRSwNP mice
To investigate the functional role of EUP in CRSwNP, an in vivo model of CRSwNP was established based on BALB/c mice via OVA and SEB challenges. Then, CRSwNP mice were treated with EUP at different concentrations (5, 10, and 15 mg/kg). The histopathological changes of the mice were evaluated via H&E staining (Fig. 1A). It was found that EUP treatment dramatically reduced the number of polyps in CRSwNP mice (Fig. 1B). CRSwNP mice exhibited greater epithelium thickness of nasal mucosa than Control group, while EUP treatment markedly reduced epithelium thickness of nasal mucosa in a concentration-dependent manner (Fig. 1C). In addition, EUP treatment also abated the increase in mucosal thickness of CRSwNP mice (Fig. 1D). These results suggested EUP treatment suppressed NP formation in CRSwNP mice.

EUP inhibits mucosal inflammation and EMT process in CRSwNP mice
To further explore the effects of EUP on mucosal inflammation and EMT events in CRSwNP mice, pro-inflammatory cytokines and chemokines (TNF-α, IL-6, and IL-8) and EMT-related proteins (N-cadherin, Vimentin, and E-cadherin) were detected in nasal mucosa of mice from each group by means of ELISA and Western blotting assays. It was shown that TNF-α, IL-6, and IL-8 levels were upregulated in CRSwNP mice compared with mice in Control group, which was abated by EUP in a dose-dependent manner (Fig. 2A-C). In addition, CRSwNP mice exhibited a significant increase in N-cadherin and Vimentin levels but an obvious decline in E-cadherin level; however, EUP treatment concentration-dependently reduced N-cadherin and Vimentin levels and increased E-cadherin level (Fig. 2D). Therefore, EUP might mitigate inflammation and EMT process in CRSwNP mice.

EUP inhibits inflammatory reaction and EMT process in SEB-treated hNECs
To explore the underlying molecular mechanism of EUP in treating CRSwNP in vitro, a cellular model of CRSwNP was established by exposing hNECs to SEB. Then, SEB-treated hNECs were subject to EUP at different concentrations (10, 25, and 50 µM). As illustrated by ELISA results, EUP treatment substantially abrogated SEB-induced increase of TNF-α, IL-6, and IL-8 levels in hNECs in a dose-dependent manner (Fig. 3A-C). Besides, SEB-triggered changes in N-cadherin, Vimentin, and E-cadherin protein levels in hNECs were also abrogated by EUP (Fig. 3D). Altogether, EUP alleviated SEB-induced inflammation response and EMT event in hNECs.

EUP inhibits inflammation and EMT by upregulating TFF1
Studies have proven that TFF1 is lowly expressed in CRSwNP (Mihalj et al., 2019; Yao et al., 2019). Besides, EUP can also upregulate TFF1 expression (Choi et al., 2009). Therefore, EUP might upregulate TFF1 expression in CRSwNP. To confirm the above hypotheses, Western blotting was employed to determine protein levels of TFF1 in nasal mucosa and hNECs. As shown in Fig. 4A and B, TFF1 expression was downregulated in CRSwNP mice and SEB-treated hNECs, which was partly abated
by EUP treatment in a concentration-dependent manner. To further assess the effects of TFF1 on EUP-mediated inhibition of inflammation and EMT in vitro, TFF1 was knocked down in hNECs with si-TFF1. The silencing efficiency was confirmed by Western blotting analysis (Fig. 4C). Then, hNECs were divided into Control, SEB, SEB+EUP (50 µM), and SEB+EUP (50 µM)+si-TFF1 groups. It was shown that TFF1 inhibition partly eliminated the inhibitory effects of EUP on TNF-α, IL-6, and IL-8 levels in SEB-challenged hNECs (Fig. 4D-F). In addition, TFF1 knockdown also reversed the impact of EUP on N-cadherin, Vimentin, and E-cadherin protein levels in SEB-challenged hNECs (Fig. 4G). Thus, EUP might impair CRSwNP-related inflammation and EMT processes via upregulation of TFF1 expression.

**Activation of Wnt/β-catenin signaling partly reverses the suppressive effects of EUP on inflammation and EMT events**

Wnt signaling is upregulated in CRSwNP and is positively related to inflammation and EMT events (Boscke et al., 2017). A former report has demonstrated that TFF1 can deactivate the Wnt/β-catenin signaling (Hasebe et al., 2022). From all the above, it was speculated that TFF1-dependent Wnt/β-catenin may be also involved in EUP-mediated protection against CRSwNP. Consistently, EUP dose-dependently reversed the upregulation of Wnt3α and β-catenin levels in both CRSwNP mice and SEB-treated hNECs (Fig. 5A and B). However, TFF1 silencing evidently abrogated the EUP-caused inhibition on Wnt3α and β-catenin levels in hNECs (Fig. 5C).

To further confirm the above hypothesis, a Wnt agonist, BML-284, was applied (Song et al., 2020). Then, hNECs were divided into Control, SEB, SEB+EUP (50 µM), and SEB+EUP (50 µM)+BML-284 groups. As shown in Fig. 5E and F, BML-284 substantially abolished EUP-triggered inhibition on Wnt/β-catenin activation. In addition, BML-284 remarkably eliminated EUP-induced suppression of inflammation and EMT in SEB-challenged hNECs (Fig. 5D and E). Collectively, Wnt/β-catenin activation might abate the inhibitory effects of EUP on inflammation and EMT processes in CRSwNP.

**Discussion**

As a refractory disease of the upper airway (Fernandez-Bertolin et al., 2015), CRSwNP is mainly featured with inflammation and dysregulated EMT (Bae et al., 2020). Clinically, CRSwNP often causes symptoms such as nasal discharge, stuffiness, and olfactory dysfunction, which remarkably affects the quality of life of CRSwNP patients (Yılmaz et al., 2020). Therefore, it is extremely important to discover novel therapeutic agents for CRSwNP treatment.

The anti-inflammatory and anti-EMT properties of EUP have been identified in previous studies. It was reported that EUP inhibited inflammatory responses to hepatic ischemia-reperfusion injury (Lee et al., 2016), DSS-induced colitis (Zhou et al., 2018), and bronchial epithelial inflammation (Jung et al., 2012). In addition, EUP also suppressed EMT events in prostate cancer cells (Serttas et al., 2021), glioma cells (Fei et al., 2019a) and TGF-β2-challenged retinal pigment epithelial cells (Cinar et al., 2021). However, the exact effects of EUP in CRSwNP remain to be elucidated. In this study, EUP significantly attenuated the number of nasal polyps, epithelium thickness of nasal mucosa, and mucosal thickness in CRSwNP mice in a concentration-dependent manner. Consistent with previous studies, EUP dose-dependently ameliorated inflammation and EMT process in CRSwNP mice and SEB-treated hNECs. Taken together, EUP alleviated CRSwNP severity, inflammatory response and EMT in vitro and in vivo in a dose-dependent manner.
TFF1, a member of the TFFs, has been found to protect mucosa and promote epithelial healing (Liang et al., 2022). Besides, TFF1 also exerts suppressive effects on inflammation and/or EMT in gastric mucosal injury (Zhang et al., 2020), pterygium (Buron et al., 2008), diabetic retinopathy (Zhang et al., 2022), and pancreatic intraepithelial neoplasm (Yamaguchi et al., 2018). Additionally, it has been demonstrated that TFF1 is downregulated in CRSwNP (Mihalj et al., 2019; Yao et al., 2019). Besides, TFF1 expression can be upregulated by EUP (Choi et al., 2009). Similarly, we observed significant TFF1 downregulation in CRSwNP mice and SEB-treated hNECs. It was also shown that TFF1 expression in CRSwNP mice and SEB-treated hNECs was upregulated with the increase of EUP concentration. In addition, TFF1 inhibition substantially abrogated the inhibitory effects of EUP on SEB-induced inflammation and EMT in hNECs. Our results implied that EUP diminished inflammation and EMT in CRSwNP by upregulating TFF1.

A previous study has reported that TFF1 can inhibit the activation of the Wnt/β-catenin signaling pathway (Hasebe et al., 2022). The Wnt signaling pathway is abnormally activated and positively associated with inflammation and EMT events in CRSwNP (Boscke et al., 2017). Therefore, it was conjectured that TFF1 upregulation by EUP might ameliorate inflammation and EMT in CRSwNP via inactivating the Wnt/β-catenin signaling. Our results showed that EUP treatment dose-dependently abated the increase of Wnt3α and β-catenin levels in CRSwNP mice and SEB-treated hNECs; however, TFF1 inhibition considerably reversed EUP-induced deactivation of Wnt/β-catenin signaling in hNECs. Furthermore, it was also illustrated that BML-284, a Wnt activator, partially abolished EUP-induced suppression on the inflammation and EMT of SEB-challenged hNECs.

**Conclusion**

To sum up, our study is the first to elucidate the inhibitory effects of EUP on the inflammation and EMT processes in CRSwNP. Based on murine and cellular CRSwNP models, our findings demonstrated that EUP ameliorated inflammation and EMT processes in CRSwNP by upregulating TFF1 and inhibiting the Wnt/β-catenin signaling pathway in vivo and in vitro (see graphical abstract in Fig. 6), suggesting EUP may be a potential therapeutic agent for CRSwNP patients.

**Declarations**

**Ethical Approval**

This study was permitted by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

HS and QW designed this study, performed all the experiments and prepared the figures. YZ and QW drafted the initial manuscript and analyzed the data. QW reviewed and revised the manuscript. All authors read and approved the final manuscript.

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Not applicable.
Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

References


**Figure Legends**

**Figure 1. EUP inhibits polyp formation in CRSwNP mice.**
Mice were induced with CRSwNP and treated with EUP at different concentrations (5, 10, and 15 mg/kg). (A) H&E staining of nasal mucosa of mice. Scale bar=150 µm. (B) The number of polyps in each group. (C) Epithelium thickness of nasal mucosa in each group. (D) Mucosal thickness in each group. *P < 0.05; ** P < 0.001; ***P < 0.005.

**Figure 2. EUP inhibits mucosal inflammation and EMT process in CRSwNP mice.**
Mice were induced with CRSwNP and treated with EUP at different concentrations (5, 10, and 15 mg/kg). (A-C) TNF-α, IL-6, and IL-8 levels in nasal mucosa from each group were assessed by ELISA. (D) N-cadherin, Vimentin, and E-cadherin protein levels in nasal mucosa from each group were assessed by Western blotting. *P < 0.05; ** P < 0.001; ***P < 0.005.

**Figure 3. EUP inhibits inflammatory reaction and EMT process in SEB-treated hNECs.**
hNECs were stimulated with SEB and treated with EUP at different concentrations (10, 25, and 50 µM). (A-C) TNF-α, IL-6, and IL-8 levels in hNECs from each group. (D) N-cadherin, Vimentin, and E-cadherin protein levels in hNECs from each group. *P < 0.05; ** P < 0.001.

**Figure 4. EUP inhibits inflammation and EMT by upregulating TFF1.**
(A and B) TFF1 protein level in nasal mucosa and hNECs from each group. (C) TFF1 protein level in hNECs transfected with si-NC or si-TFF1. Then, transfected hNECs were stimulated with SEB and treated with EUP. Briefly, hNECs were divided into Control, SEB, SEB+EUP (50 µM), and SEB+EUP (50 µM)+si-TFF1 groups. (D-F) TNF-α, IL-6, and IL-8 levels in hNECs from each group. (G) N-cadherin, Vimentin, and E-cadherin protein levels in hNECs from each group. *P < 0.05; ** P < 0.001; ***P < 0.005.
Figure 5. Activation of Wnt/β-catenin signaling partly reverses the suppressive effects of EUP on inflammation and EMT events. (A) Wnt3α and β-catenin protein levels in nasal mucosa and hNECs from each group. (B) Wnt3α and β-catenin protein levels in hNECs from each group. Then, hNECs were divided into Control, SEB, SEB+EUP (50 µM), and SEB+EUP (50 µM)+BML-284 groups. (C) Wnt3α and β-catenin protein levels in hNECs from each group. (D) TNF-α, IL-6, and IL-8 levels in hNECs from each group. (E) N-cadherin, Vimentin, and E-cadherin protein levels in hNECs from each group. *P < 0.05; ** P < 0.001.

Figure 6. Graphical abstract.
Relative protein expression of N-cadherin

Relative protein expression of Vimentin

Relative protein expression of E-cadherin

TNF-α concentration (pg/mL)

IL-6 concentration (pg/mL)

IL-8 concentration (pg/mL)
HISTOLOGY AND HISTOPATHOLOGY

In vivo

In vitro

Inflammation

Epithelial-to-mesenchymal transition

OVA+SEB

TFF1

Wnt/β-catenin

Eupatilin