

# The Effect of Tetrahedral Framework Nucleic Acids on the PI3K/AKT Signaling Pathway Regulating VEGF Gene Expression in Oral Submucous Fibrosis

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## Abstract

**Objective:** To analyze the effect of tetrahedral framework nucleic acids (tFNAs) on the PI3K/AKT signaling pathway regulating vascular endothelial growth factor (VEGF) gene expression in oral submucous fibrosis (OSF). **Methods:** Six-week-old SD rats were selected to establish an OSF model. Each group was injected with the corresponding drugs for four weeks before being euthanized. Buccal mucosal tissues were collected for histopathological examination using H&E and Masson staining, as well as immunofluorescence. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was used to detect the expression levels of the PI3K/AKT signaling pathway and its phosphorylation, angiogenesis-related factors (CD34, VEGF), and fibrosis-related factors (type I collagen, COL-I;  $\alpha$ -smooth muscle actin,  $\alpha$ -SMA) in the buccal mucosa. **Results:** tFNAs inhibited the pathological progression of OSF, reduced collagen deposition, and improved the severity of OSF in rats. By upregulating the PI3K/AKT signaling pathway and its phosphorylation levels in OSF mucosal tissues, tFNAs increased the expression of angiogenesis-related factors (CD34 and VEGF) while decreasing fibrosis-related factors (COL-I and  $\alpha$ -SMA). **Conclusion:** tFNAs activate the PI3K/AKT signaling pathway to promote VEGF gene expression, enhance angiogenesis, and ameliorate OSF. This confirms the critical role of angiogenesis in OSF treatment.

## Keywords

Tetrahedral framework nucleic acids; Oral submucous fibrosis; PI3K/AKT signaling pathway; Vascular endothelial growth factor

## 1. Introduction

Oral submucous fibrosis (OSF), also known as oral submucous fibrosis, refers to a pathological condition of the oral mucosa caused by chronic and persistent injury, leading to impaired repair. OSF is a typical chronic disease, and its prevalence has been increasing in recent years. According to reports [1], the global number of OSF patients has exceeded 5 million, making it a significant public health concern worldwide. It is worth noting that the characteristic pathological manifestations of OSF include vascular loss, vascular occlusion, epithelial atrophy, and inflammatory cell infiltration. If left untreated or improperly managed, OSF can lead to structural and functional abnormalities of the oral mucosa. Clinically, OSF presents with symptoms such as oral mucosal ulcers, burning sensations, and rough, pale mucosa. In severe cases, it may cause dysphagia, restricted mouth opening, and even carry a risk of malignant transformation (3%-30%) [2]. Therefore, elucidating the pathogenesis of OSF and developing effective treatments are of great significance.

Vascular endothelial growth factor (VEGF) is a specific growth factor for vascular endothelial cells. Studies have reported [3], that VEGF is highly expressed in OSF tissues and is closely associated with vascular lesions. Research has also emphasized [4], that the PI3K/AKT pathway can regulate VEGF expression in conditions such as myocardial ischemia-reperfusion injury and myocardial infarction, exhibiting a synergistic effect with VEGF expression in OSF development, thereby participating in angiogenesis. However, the use of PI3K/AKT signaling pathway activators, such as insulin-like growth factor (IGF-1), may cause side effects (e.g., pain, hypoglycemia).

Tetrahedral framework nucleic acids (tFNAs) are three-dimensional nanomaterials constructed based on DNA self-assembly technology, offering advantages such as easy synthesis, structural stability, and high bioavailability [5]. They play a prominent role in treating organ fibrosis by activating the PI3K/AKT pathway to enhance cellular autophagy, proliferation, wound healing, and angiogenesis. Therefore, this study investigates the effect of tFNAs on the PI3K/AKT pathway to regulate VEGF, thereby improving the vascular microenvironment in OSF and achieving therapeutic goals. The details are reported below.

## 2. Materials and Methods

### 2.1 Experimental Reagents and Equipment

**Reagents:** SD rats (Changsha Tianqin Co.), chloral hydrate (RWD Life Science), neutral gum (Biosharp), paraformaldehyde (Aladdin), hematoxylin stain (Thermo), eosin stain (Beyotime), primary antibodies (AKT, Proteintech; CD34, Beyotime; VEGF, HUABIO), secondary antibodies (FITC-labeled goat anti-rabbit, Servicebio), ELISA kits (Lianke Co.), absolute ethanol (Servicebio), arecoline (MCE), IGF-1 (MCE), LY294002 (MCE), citrate buffer, PBS buffer, hydrogen peroxide (Servicebio), sodium phosphate dibasic, sodium phosphate monobasic (Fuchen Co.), TUNEL kit (Beyotime).

**Equipment:** Microplate reader (Thermo), confocal microscope (Olympus), chemiluminescence imager (Bio-Rad), rotary microtome (Leica), automatic dehydrator (JunJie Electronics), tissue embedding machine, pathological tissue floating dryer (Zhongwei Electronics), slide scanning imaging system (Shengqiang Technology), real-time PCR system (Thermo Fisher), high-speed low-temperature tissue grinder (Servicebio), low-temperature centrifuge (Xiangyi Instruments), thermal cycler (Thermo Fisher), vortex mixer (Kangjian Medical), UPT ultrapure water system (Chaochun Technology), portable stainless-steel pressure steam sterilizer (Shanghai Shen'an), drying oven (Shanghai Jinghong Laboratory Equipment).

### 2.2 Experimental Methods

#### 2.2.1 Animal Model Establishment and Drug Treatment

(1) OSF Rat Model Establishment: Forty male SD rats (6 weeks old) were acclimatized for 7 days. After adaptation, 8 rats were randomly selected as the blank control group, while the remaining 32 were assigned to the experimental group. The experimental group was further divided into four subgroups (n=8 each): arecoline group, tFNAs treatment group, IGF-1 activator group, and LY294002 inhibitor group. Rats were anesthetized using isoflurane, and the oral cavity was opened. The experimental groups received 50  $\mu$ L of arecoline solution (5 mg/mL in 0.9% saline), while the control group received an equal volume of saline (0.9%). Injections were administered daily for 10 weeks to complete modeling. Body weight was measured every two weeks, and passive mouth opening was assessed under anesthesia every four weeks (three measurements per rat, averaged).

(2) Local Drug Treatment: After 10 weeks, OSF modeling was confirmed. Each group received drug treatment: the control group received saline (0.9%), while the experimental groups received solutions containing arecoline (5 mg/mL) and the respective drugs (IGF-1 at 100 ng/mL, LY294002 at 50  $\mu$ M/mL, or tFNAs at 250 nM/mL). During treatment, changes in buccal mucosa color and texture were observed and recorded, and body weight and mouth opening were measured as before. After four weeks of treatment, rats were fasted for 24 hours, anesthetized with pentobarbital sodium (1%), and euthanized. Bilateral buccal mucosal tissues were harvested, with one side fixed in 4% paraformaldehyde and the other stored at -80°C for further analysis.

#### 2.2.2 Hematoxylin-Eosin (H&E) Staining

(1) Tissue Processing: Dehydration: Gradient ethanol dehydration (75% for 2h at 25°C, 85% and 95% for 1h at 28°C and 30°C, respectively, absolute ethanol I-IV for 20 min each at 35°C). Clearing: Xylene I and II for 25 min and 30 min at 40°C. Paraffin embedding: Paraffin I for 30 min, II/III for 1h at 60°C. Tissues were oriented anatomically, cooled, and sectioned (4-6  $\mu$ m) for staining.

(2) Slice and deparaffinize: Xylene I and II for 30 min each, absolute ethanol I and II for 5 min each, graded ethanol (95%, 85%, 75%) for 5 min each, followed by rinsing.

- (3) Staining: Hematoxylin (0.5% mercuric oxide, 10% ethanol) for 5 min (thin sections) or 8 min (thick sections). Differentiation in 0.5% HCl-ethanol for 5 sec. Bluing in Scott's solution (0.2% NaHCO<sub>3</sub>, 0.3% MgSO<sub>4</sub>, pH 8.5) for 1 min. Eosin staining for 3 min.
- (4) Dehydration and Mounting: Graded ethanol (75%→100%), xylene clearing, and neutral resin mounting.

### 2.2.3 Immunofluorescence (IF) Staining

- (1) Sample Preparation: Fresh tissues were embedded in OCT, frozen, and sectioned (5 μm). Cells were seeded on confocal dishes (2×10<sup>4</sup>/well) and fixed with 4% paraformaldehyde.
- (2) Antigen Retrieval and Permeabilization: Microwave heating in citrate buffer (pH 6.0), followed by PBS washing. Permeabilization with 0.5% PBS-Triton for 15 min.
- (3) Antibody Incubation: Primary antibodies (4°C, 16h), followed by secondary antibodies (Alexa Fluor 488/594, 2h at RT).
- (4) Nuclear Staining and Mounting: DAPI (1 μg/mL) for 5 min. Anti-fade mounting medium and coverslipping.

### 2.2.4 Real-Time Quantitative PCR (RT-qPCR)

- (1) Preparation of working solution in a sterile biosafety cabinet: Add pre-chilled absolute ethanol (-20°C) to buffer BD at a 1:4 ratio. For a 50T reagent kit, add an appropriate volume of ethanol (24 mL), vortex for 30 sec, aliquot, and store at -4°C.
- (2) Tissue homogenization: Take 15-20 mg of fresh tissue sample and place it in a pre-chilled 2 mL grinding tube. Add 350 μL of lysis buffer LB. Homogenize using a tissue grinder under low-temperature, high-speed conditions: 6,500 rpm × 3 cycles (10 sec/cycle, with 30 sec ice bath intervals).
- (3) DNA removal and filtration: Transfer the homogenate to a DNA removal column. Centrifuge at 13,000 × g for 1 min and collect the filtrate in a fresh tube. Add an equal volume of buffer BD, vortex for 10 sec.
- (4) RNA binding: Transfer the mixture to a new RNA binding column. Centrifuge (same parameters as above) and discard the flow-through.
- (5) Protein removal: Add 700 μL of protein removal solution. Incubate at room temperature for 30 sec, then centrifuge and discard the filtrate. Reinsert the DNA removal/RNA binding column into a 2 mL collection tube.
- (6) Wash step: Add 500 μL of wash buffer W. Centrifuge for 30 sec and discard the flow-through.
- (7) RNA elution: Transfer the DNA removal/RNA binding column to a new 1.5 mL centrifuge tube. Add 30–50 μL of RNase-free H<sub>2</sub>O to the center of the membrane. Incubate at room temperature for 1 min, then centrifuge for 1 min. Collect the filtrate, which contains the purified RNA solution.

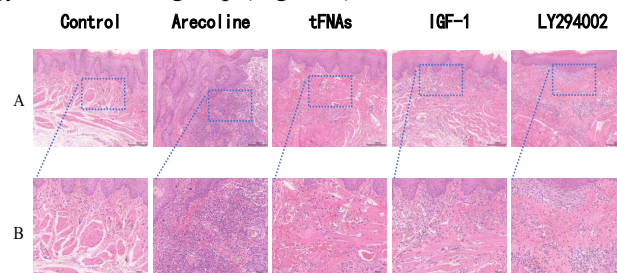
## 2.3 Statistical Analysis

GraphPad Prism 10 was used for statistical analysis. Normally distributed data are expressed as mean ± SD (t-test), while categorical data are presented as percentages (χ<sup>2</sup> test). P < 0.05 was considered statistically significant.

## 3. Results

### 3.1 H&E Staining

Under light microscopy, pathological changes in rat buccal mucosa included epithelial hyperplasia, fibrous tissue proliferation, myofiber degeneration, and inflammatory infiltration. The model and inhibitor groups showed significant epithelial atrophy, thinning, and reduced rete ridges compared to controls. The IGF-1 group exhibited milder lesions, while the tFNAs group showed near-normal epithelial thickness and vascular regeneration. The LY294002 group had similar pathology to the model group (Figure 1).



**Figure 1. Histopathological changes in rat buccal mucosa tissues (H&E staining).**

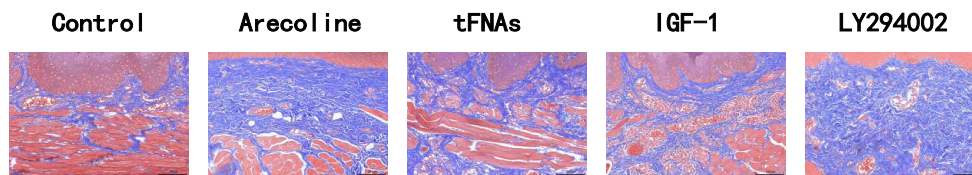
Note: A scale bar: 100 μm; B scale bar: 200 μm.

### 3.2 Masson Staining Experiment

Compared to the normal group, the model group (Arecoline) and the inhibitor group (LY294002) exhibited increased deposition of blue-stained collagen fibers, appearing as patchy aggregates, along with reduced height and number of epithelial rete ridges. In contrast, the activator group (IGF-1) and the treatment group (tFNAs) showed dispersed collagen fibers in a reticular pattern, with significantly improved fibrosis compared to the model group (see Table 1 and Figure 2). Regarding the percentage of fibrous tissue area in buccal mucosal tissues, the activator group (IGF-1) and the treatment group (tFNAs) demonstrated significantly lower values than the model group (Arecoline) and the inhibitor group (LY294002). These findings suggest that tFNAs can markedly alleviate buccal mucosal fibrosis in rats, indicating their potential therapeutic value for OSF.

**Table 1. Percentage of fibrous tissue area in buccal mucosal tissues (mean  $\pm$  SD).**

Group	N (number of rats)	Percentage (%)
Model group (Arecoline)	8	33.83 $\pm$ 9.53
Treatment group (tFNAs)	8	30.98 $\pm$ 6.94
Positive control (IGF-1)	7	34.19 $\pm$ 3.64
Negative control (LY294002)	8	38.14 $\pm$ 8.17



**Figure 2. Masson staining results of rat buccal mucosal tissues (scale bar: 200  $\mu$ m).**

### 3.3 Immunofluorescence Experiment

Compared to the model group (Arecoline), the activator group (IGF-1) and the treatment group (tFNAs) exhibited increased positive expression of PI3K, AKT, and p-AKT in rat buccal mucosal tissues, while the inhibitor group (LY294002) showed decreased expression. This suggests that both tFNAs and the activator IGF-1 can activate the PI3K/AKT pathway.

Additionally, compared to the control group, the model group (Arecoline) demonstrated significantly reduced positive expression of angiogenesis-related factors (CD34 and VEGF) in buccal mucosal tissues, indicating suppressed angiogenesis during fibrosis. In contrast, the activator group (IGF-1) and the treatment group (tFNAs) showed markedly increased expression of CD34 and VEGF, suggesting that tFNAs can activate the PI3K/AKT pathway to enhance angiogenic factors.

Furthermore, compared to the control group, the model group exhibited elevated expression of fibrosis-related factors (COL-I and  $\alpha$ -SMA) in buccal mucosal tissues. However, the activator group (IGF-1) and the treatment group (tFNAs) showed reduced expression of COL-I and  $\alpha$ -SMA compared to the model group, indicating that tFNAs can inhibit fibrosis by activating the PI3K/AKT pathway and promoting angiogenesis.

### 3.4 RT-PCR Detection of mRNA Relative Expression Levels in Tissues

RT-PCR results revealed that the IGF-1 activator group and the tFNAs treatment group had lower mRNA levels of COL-I and  $\alpha$ -SMA but higher mRNA levels of PI3K, AKT, and VEGF compared to the model control group. This suggests that the PI3K/AKT signaling pathway was activated, leading to increased VEGF gene expression and decreased expression of fibrosis-related genes (COL-I and  $\alpha$ -SMA). The upregulation of VEGF gene expression appears to downregulate COL-I and  $\alpha$ -SMA expression. Notably, tFNAs exhibited a more pronounced effect than IGF-1 in elevating VEGF gene expression. In contrast, the LY294002 inhibitor group showed no significant difference in gene expression compared to the model control group. These findings indicate that tFNAs can activate the PI3K/AKT signaling pathway, upregulate VEGF, and suppress fibrosis in OSF rats.

## 4. Discussion

OSF is a pathological process of abnormal repair in oral mucosa caused by chronic injury, characterized by inflammatory reactions, vascular loss, and excessive extracellular matrix deposition. Patients often experience symptoms such as dysphagia, ulcers, and restricted mouth opening. If the condition progresses, it may impair oral function and even carry a risk of malignant transformation, severely affecting patients' physical and mental health [6]. Notably, OSF is prevalent in betel nut-chewing regions (e.g., Hunan, Taiwan, Hainan) and exhibits diverse lesion sites with carcinogenic potential. Studies suggest [7] that OSF-associated malignancies occur in younger patients and display more aggressive biological behavior and poorer prognosis compared to non-OSF cancers.

The pathogenesis of OSF is complex, involving multiple signaling pathways, molecules, and cells, leading to limited treatment options. Surgical interventions are often traumatic and carry a high recurrence risk. Therefore, in-depth research on its molecular mechanisms is essential to provide new insights and therapeutic strategies.

Currently, *in vitro* biocompatibility testing serves as a critical method to evaluate the toxicity or adverse effects of biomaterials and drug carriers in biological systems. These tests allow preliminary assessment of material toxicity at the cellular level, including cell adhesion, proliferation, and morphological changes, enabling rapid screening of potentially toxic materials and reducing development costs and time. Research indicates [8] that tFNAs can promote fibroblast proliferation and migration *in vitro*, regulate cellular functions, and accelerate the repair and healing of damaged mucosa.

Studies have shown [9] that tFNAs activate the PI3K/AKT pathway, enhancing cell autophagy, proliferation, migration, wound healing, angiogenesis, and nerve repair. Another study [10] reported that tFNAs modulate the PI3K/AKT/mTOR pathway to improve avascular retinal regions, restore normal blood flow, and alleviate hypoxia. In this study, using an arecoline-induced OSF rat model, histological observations (H&E and Masson staining) demonstrated that tFNAs increased neovascularization, reduced collagen deposition, and decreased the percentage of fibrous tissue area, suggesting that tFNAs alleviate OSF symptoms. Immunofluorescence and PCR experiments further confirmed that both tFNAs and IGF-1 injections upregulated the PI3K/AKT pathway and its phosphorylation in OSF mucosal tissues, increased angiogenesis-related factors (CD34 and VEGF), and downregulated fibrosis-related factors (COL-I and  $\alpha$ -SMA). These results indicate that tFNAs activate the PI3K/AKT pathway, promote VEGF production, enhance angiogenesis, accelerate collagen degradation, and inhibit fibrosis, thereby influencing OSF progression.

In conclusion, tFNAs activate the PI3K/AKT signaling pathway to promote VEGF gene expression and angiogenesis, highlighting the critical role of angiogenesis in OSF treatment. These findings provide a theoretical basis for developing novel therapeutic strategies for OSF.

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