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# Effect of celecoxib on protein expression of FAK and Cx43 in DMBA induced rat tongue carcinoma cells

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**Abstract. – OBJECTIVE: The pathogenesis of tongue cancer (TA) has not been fully illustrated. Cyclooxygenase-2 (COX-2) is correlated with the precancerous lesion of oral cavity mucosa and malignant transformation. The focal adhesion kinase (FAK) and gap junction protein connexin 43 (Cx43) are involved in the occurrence and progression of tumors. This study aimed to investigate the effect of celecoxib on the proliferation, malignant transformation, and expression of FAK and Cx43 proteins.**

**MATERIALS AND METHODS: Healthy male Sprague-Dawley (SD) rats (4 months old) were divided into control, model and celecoxib group. 7,12-dimethylbenzanthracene (DMBA) was used to generate tongue mucosal carcinoma, coupled with celecoxib intervention. At 8, 12, 16, and 20 weeks after induction, the rat survival status, the tumor formation rate and the tongue tissue morphology were observed. Meanwhile, the expression of FAK and Cx43 was also evaluated by using immunohistochemistry (IHC).**

**RESULTS: Tumor occurrence rates after induction were 0, 26.67%, 66.67%, and 80% at 8, 12, 16, and 20 weeks, respectively. The celecoxib treatment decreased such rats to 0, 0, 0, and 13.33%, respectively ($p<0.05$ compared to model group). No significant change was observed in control group, whilst model group had mild to severe hyperplasia and squamous carcinoma with elongated time. Celecoxib treatment significantly improved the tissue morphology ($p<0.05$). The model group also had elevated FAK and depressed Cx43 protein expression ($p<0.05$). With elongated time, the FAK expression was further increased whilst Cx43 protein was depressed ($p<0.05$ compared to model group).**

**CONCLUSIONS: The focal application of celecoxib effectively inhibited the DMBA-induced rat TA, possibly via regulating FAK and CX43 protein expression, and inhibiting oral epidermal hyperplasia.**

*Key Words:*
Celecoxib, Tongue cancer, FAK, Cx43 protein.

## Introduction

Oral cavity cancer has a relatively higher incidence. Among those, the most common is squamous cell carcinoma (SCC). Tongue cancer is one common malignant tumor in the oral cavity and has insignificant symptoms during onset. Most patients thus already have infiltration or metastasis at the time of confirmed diagnosis[1,2]. Abundant studies have been performed regarding etiology and diagnosis/treatment of tongue cancer, but still cannot clarify the pathogenesis mechanism. The effective measure for prevention thus is one focus for study[3,4]. The precancerous lesion is one risk factor for SCC. Abnormal hyperplasia is a common pathological manifestation of various precancerous lesions including mucosal erythema, leukoplakia, and human papilloma. Various related factors participate in the progression from the abnormal proliferation of oral epidermal hyperplasia to cancer. Cyclooxygenase-2 (COX-2) is related to the precancerous lesion of oral mucosa and pathogenesis of SCC. COX-2 expression is elevated in human tongue SCC tissues with a more advanced malignancy. COX-2 participates in tumor progression via affecting the expression of the carcinogenic factor, inhibiting cell apoptosis and facilitating invasion/metastasis[5,6]. Selective COX-2 inhibitor celecoxib plays an important role in tumor prevention and treatment[7] and can exert anti-tumor effects via facilitating cell apoptosis, inhibiting cell proliferation or tumor angiogenesis, and modulating immunity. This *in vitro* study showed that celecoxib could inhibit the human tongue SCC Tca8113 cell growth and induce (the) apoptosis, plus the potentiation of the killing effect by chemo-therapy drugs[8]. The focal adhesion kinase (FAK) is correlated with cell proliferation and apoptosis and participates in tumor progres-



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sion and invasion/metastasis via multiple signal pathways such as tyrosine kinase receptor and G protein-coupled receptor[9]. Gap junction protein connexin 43 (Cx43) has decreased the expression level in precancerous lesion and malignant tumor tissues, with decreased expression level as advanced differentiation grade of oral SCC[10]. This study established rat tongue carcinoma model via 7,12-dimethylbenzanthracene (DMBA) induction, observing the effect of intervention by focal application of COX-2 inhibitor celecoxib, in an attempt to observe the effect of celecoxib on FAK and Cx43 protein expression in abnormal hyperplasia and carcinoma tissues in the oral epidermal.

## Materials and Methods

### Experimental Animal Grouping

Healthy male Sprague-Dawley (SD) rats (4 weeks age, body weight 140-160 g) were provided by the Laboratory Animal Center, Chinese Medicine Academy (No. SYXK-2013-0025). Animals were kept in a specific pathogen free (SPF) grade facility with standard food and water. Animals were randomly divided into control group (n=20), model group (n=60) and celecoxib group (n=60). DMBA stimulus combined with trauma was used to induce rat tongue cancer model. The present investigation was approved by the Ethics Committee of Jinan Central Hospital, affiliated to Shandong University (Jinan, China).

### Major Reagent and Equipment

The celecoxib capsule (0.2 g) provided by Pfizer Inc., Public (Brooklyn, NY, USA) was prepared for 6% of paste using matrix, including vaseline, hydroxypropyl methyl cellulose, liquid paraffin, polyethylene glycol 400, and laurocapram (Kemiou, China). DMBA (≥95% purity, Sigma-Aldrich, St. Louis, MO, USA). Acetone (Kemiou, China) was prepared into 1% of solution and was kept in the fridge at a temperature of 4°C. Hydrate chloral, paraformaldehyde (Kemiou, China); mouse anti-rat FAK monoclonal antibody, rabbit anti-rat Cx43 polyclonal antibody (Boster, Wuhan, China); Horseradish peroxidase labeled goat anti-rabbit secondary antibody (Cell Signaling Technology, Danvers, MA, USA). Diaminobenzidine (DAB) staining kit (ZSJQ BioTech., Beijing, China). The Image Pro Plus 6.0 software (Media Cybernetics, Inc., Bethesda, MD, USA) was used to analyze the images. The inverted phase-contrast microscope (Olympus, Tokyo, Japan) was used to observe the images.

### Animal Model Preparation

Rats were anesthetized with 10% of hydrate chloral intraperitoneal injection (until the absence of corneal reflex) and 0-1 grade of limb muscle strength. The left edge mucous of the tongue was scratched using abrasive paper up to the hyperemia, but without bleeding of the tongue mucosa. 1% of DMBA solution was applied on the tongue edge three times per week. Celecoxib group received a focal application of 6% paste daily. The animal survival rate and the tumor formation rate were observed.

### Observation of General Conditions

Before and after induction, rats were observed for mental status, activity, appetite, and fecal. The oral mucosa was checked from reddish, erosion and neoplasia, whose location, size and growth conditions were recorded.

### Hematoxylin-Eosin (HE) Staining for Tongue Tissue Pathology

At 8, 12, 16, and 20 weeks after induction, rats were sacrificed to observe the general conditions of the tongue. Tissues were fixed in paraformaldehyde and sectioned into 5 μm coronal section. HE staining was then performed on paraffin sections, followed by light filed microscopy to analyze the pathology of tongue SCC tissues. The Image-pro plus software was used to analyze tissue thickness of the epidermal layer, keratin, the abnormally proliferated epidermis and the degree of inflammatory infiltration. Average values were measured from 10 points of epidermal samples.

### Immunohistochemistry (IHC) Method for FAK and Cx43 Protein Expression

At 8, 12, 16 and 20 weeks after induction, rats were sacrificed to collect tongue tissues, which were fixed in paraformaldehyde. Paraffin tissue sections (5 μm thickness) were prepared. After de-waxed, tissues slides were washed in phosphate-buffered saline (PBS) pH7.4 for three times (3 min each), followed by 2 min antigen retrieval. 3% of $H_2O_2$ was added on each slide for 10 min at room temperature incubation to block the activity of endogenous peroxidase. Primary antibody (1:100 dilution) was added for 2 h incubation at room temperature, followed by polymer enhancer for 20 min incubation at room temperature. Enzyme-labeled anti-mouse/rabbit polymer

was added for 30 min at room temperature incubation, followed by freshly made DAB substrate. The slide was observed under a microscope for 5 min, followed by hematoxylin counterstaining and 0.1% of HCl differentiation. Tap water was used to rinse tissue sections, which were dehydrated by gradient ethanol (90%, 95% and absolute), and were immersed in xylene for mounting in neutral resin. The image-pro plus software was used to analyze data. The FAK positive expression localizes in the cytoplasm as light yellow to brown color. Cx43 positive expression was on the membrane or cytoplasm as shown by dark yellow granules. Five randomly selected fields under 40X objectives were recorded for positive staining ratio (ImA), average light density of positive staining (ImIn) and IHC index (ImT). ImA = positive staining area in cytoplasm (positive staining area in cytoplasm + negative staining area of cytoplasm) × 100%. ImIn = light density value/staining area of cytoplasm. ImT = ImA X ImIn = light density value (positive area of cytoplasm + negative staining area of cytoplasm). The IHC staining strength was deduced as score 0 (less than 10% positive cells), score 1 (10%-40% positive cells), score 2 (40%-70% positive) and score 3 (more than 70% positive cells). The summation of both scores was divided into weak expression (0-2 scores) and strong expression (3-6 scores).

### Statistical Analysis

The SPSS20.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Measurement data were tested firstly for normal distribution. Those fitted normal distributions were presented as mean ± standard deviation (SD). The comparison among multiple groups was performed by the one-way analysis of variance (ANOVA), which was validated by the Tukey's Post-Hoc test. The LSD test was used in a paired comparison between groups. A statistical significance was defined when $p<0.05$.

## Results

### General Condition and Tumor Formation Rate

The model group had decreased activity and mental drooping. At the late phase of the experiment, due to the food intake difficulty caused by the lesion, rats had decreased body weight. Celecoxib group had improved food, activity and mental status compared to model group. By pathology observation, no tumor occurred in celecoxib group 8 weeks after induction. The cumulative tumor rate at 12, 16, and 20 weeks after induction was 26.67%, 66.67%, and 80%, respectively in model group. With elongated induction time, the tumor formation rate was increased with extended induction time. The tumor formation rates in celecoxib group were 0, 0, and 13.33% at 12, 16 and 20 weeks after induction, respectively, with a significant difference of tumor formation rate between two groups ($p<0.05$). In general conditions, tumor formation rate and tongue were shown in Figures 1, 2, and 3.

### Pathology of Tongue Tissues

Under light field microscopy, after HE staining, no significant pathologic abnormality was observed in control group, with the regular arrangement of keratin epidermal on dorsal tongue, intact morphology, and no inflammatory cell infiltration. Rats in model group had mild, moderate and severe abnormal hyperplasia at 8, 12, and

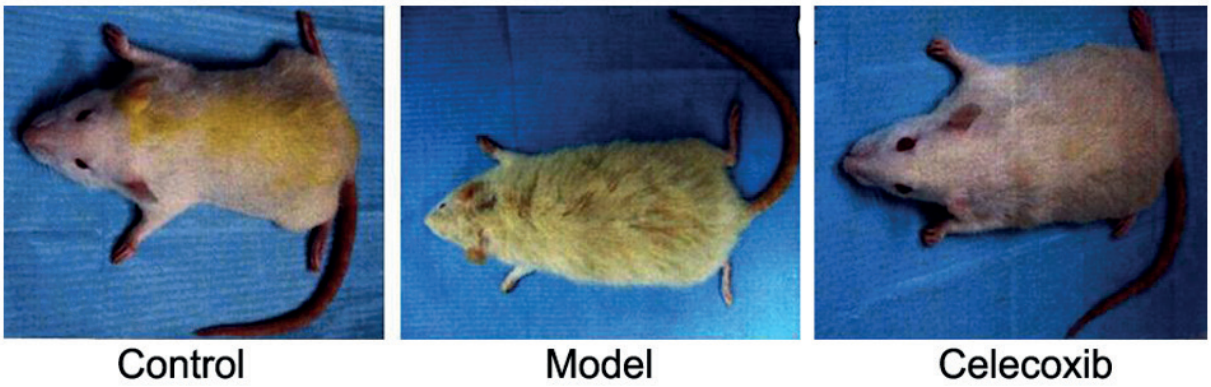


**Figure 1.** General conditions of all rats.

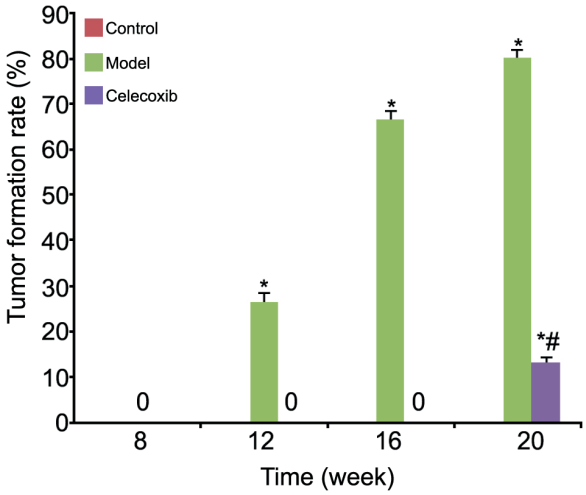


**Figure 2.** Rat tumor formation rate. $^{*}p<0.05$ compared to control group. $^{\#}p<0.05$, compared to model group.

16 weeks after induction with over-keratinization of epidermis in basal cell layers and disappearance of polarity. Epidermal protrusion showed droplet shape, disrupted layering, and dark staining of the nucleus. 20 weeks after induction, pathology developed into highly differentiated SCC. Cancer cells invaded through the basal membrane to reach the lower mucosa and the basal layer. Cancer nest showed concentric circles or a layered arrangement. Celecoxib group had significantly improved pathology injury, as shown in Figure 4.

### *Analysis of Pathology Results*

With elongated DMBA induction time, thickness of tongue epidermal tissues, abnormally proliferated epidermis, keratin, and degree of inflammatory infiltration were all significantly increased ($p<0.05$). Model group had a significant difference compared to celecoxib group ($p<0.05$, Figure 5).

### *FAK and Cx43 Protein Expression in Tongue Tissues*

IHC results showed a weak positive expression of FAK in the oral mucosal epidermis of controlled rats. Model group had a strong positive expression of FAK in basal, spinal and basal layers as shown by light yellow to brown with elongated induction times. The FAK positive expression locates in pe-

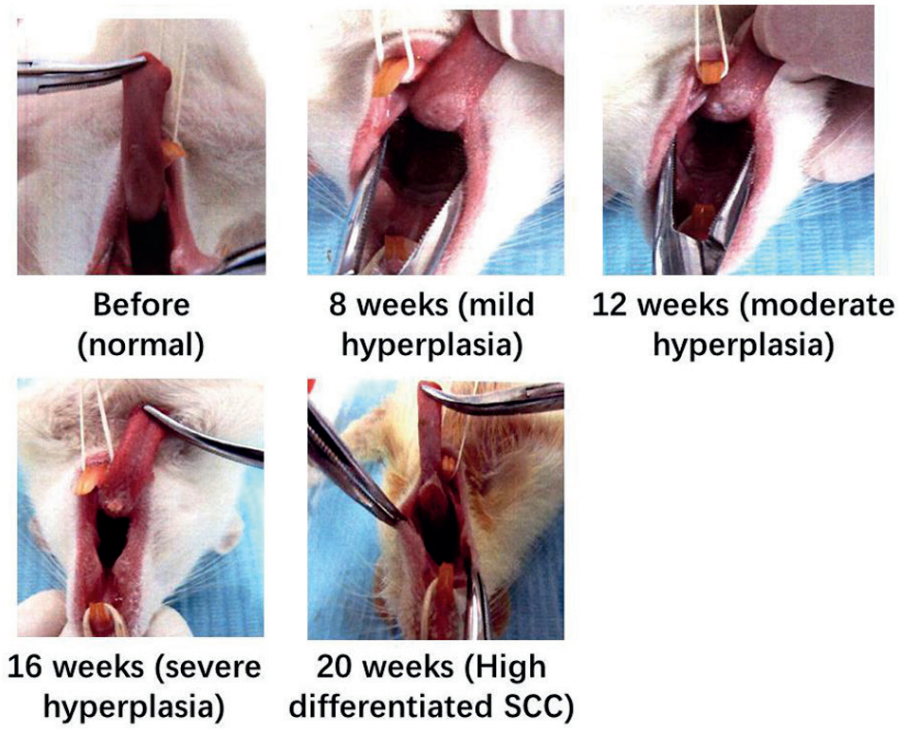


**Figure 3.** Change of rat tongue. Before induction, the dorsal site of the anterior tongue showed pink and wet mucous, and soft tissues with elasticity. With elongated induction time, milk white plaque occurred on the mucosa of the dorsal tongue, which protruded on the surface with harden matter. Mucosal lesion enlarged with elongated time, with partial necrosis. Rats showed limited activity and difficulty of feeding.

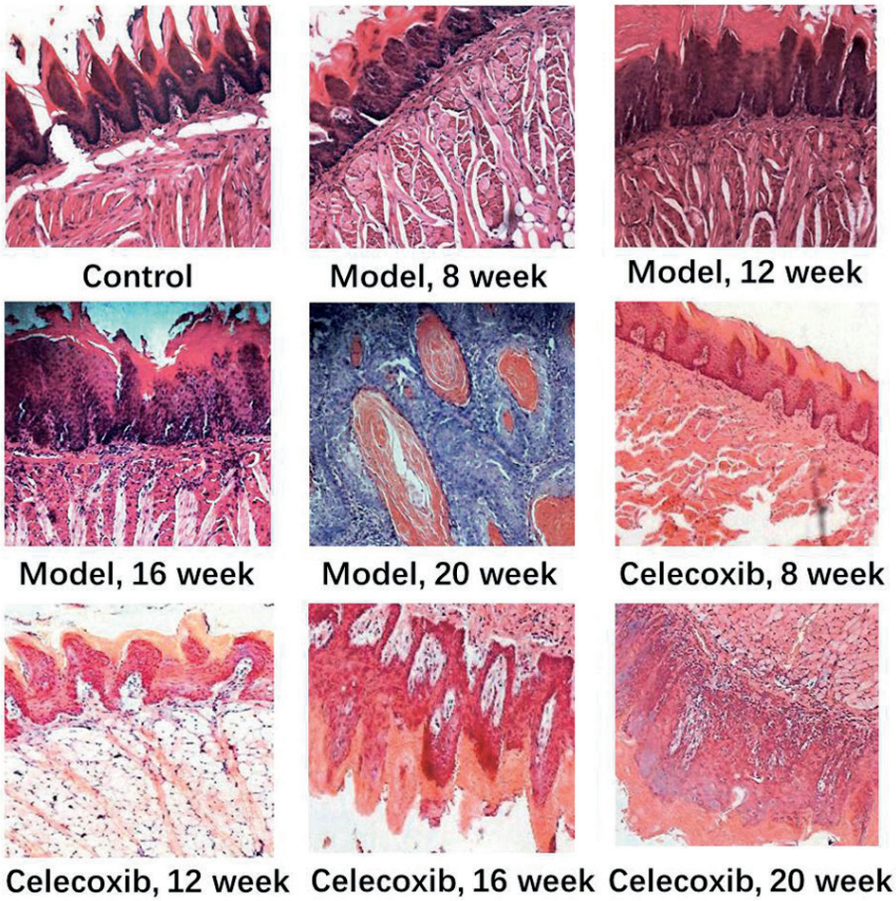


**Figure 4.** Pathology of tongue tissues (HE staining, ×200).

ripheral basal cells around cancer nest of SCC, as shown by dark yellow color and strong positive expression. The Cx43 protein showed weak positive expression in the normal mucosal epidermal basal layer in control group, and strong positive expression in granular and spine layers, whilst in the keratin layer, it had negative expression. With elongated induction time, model group had more abnormal hyperplasia of tongue mucosa and lower Cx43 protein expression. In SCC tissues, the positive expression of Cx43 protein located in the keratinization droplet of SCC tissues and the peripheral cytoplasm of tumor nucleus. Celecoxib group had a weaker positive expression of FAK and Cx43 proteins, compared to model group at the same time. Quantitative analysis revealed a higher FAK positive expression in the model group compared to the Celecoxib group, plus a lower Cx43 protein positive expression ($p<0.05$). With elongated time, FAK positive expression was potentiated whilst Cx43 positive expression was decreased. Compared to model group at the same time-point, celecoxib group had a significant difference of FAK and Cx43 protein expression ($p<0.05$, Figures 6, 7, and 8).

## Discussion

Due to an abundant blood flow and lymphatic tissues in tongue tissues, the tongue SCC is predisposed to invasion or metastasis, causing unfavorable prognosis for late-stage tongue cancer. Currently, the pathogenesis mechanism for tongue cancer has not been fully illustrated. Previous studies showed the involvement of oncogene or tumor suppressor gene such as p53, gene of phosphate and tension homology deleted on chromosome ten (PTEN), cell apoptosis gene (caspase-3, surviving), inflammatory gene [interleukin 8 (IL-8) and tumor necrosis factor α (TNF-α)] in the transformation of oral epidermal

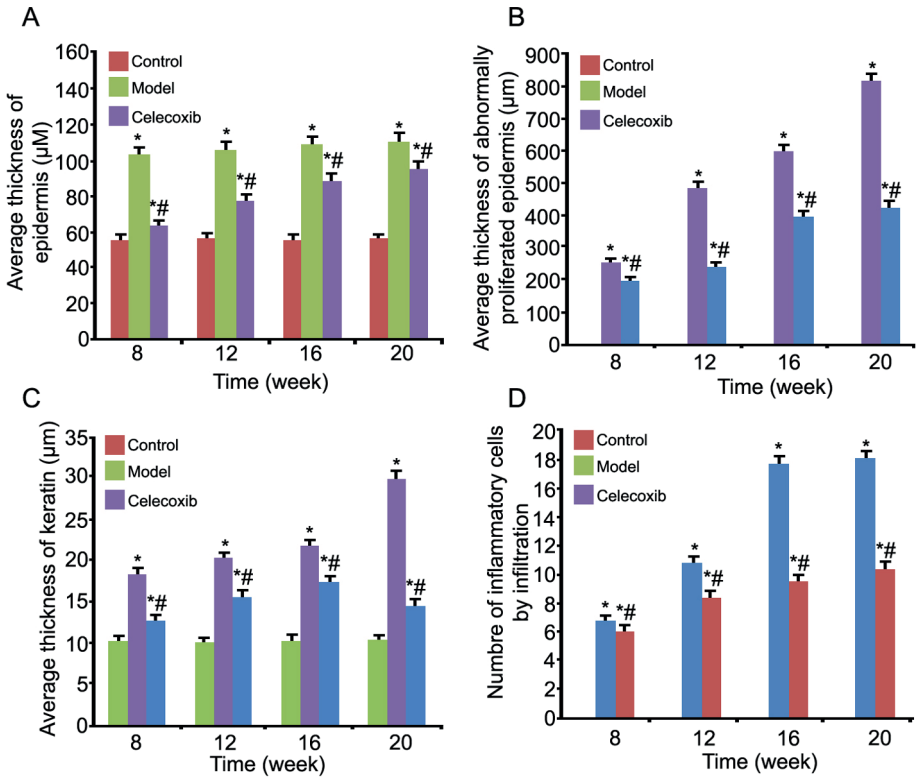


**Figure 5.** Analysis of pathology examination. $^{*}p<0.05$ compared to control group. $^{\#}p<0.05$, compared to model group.

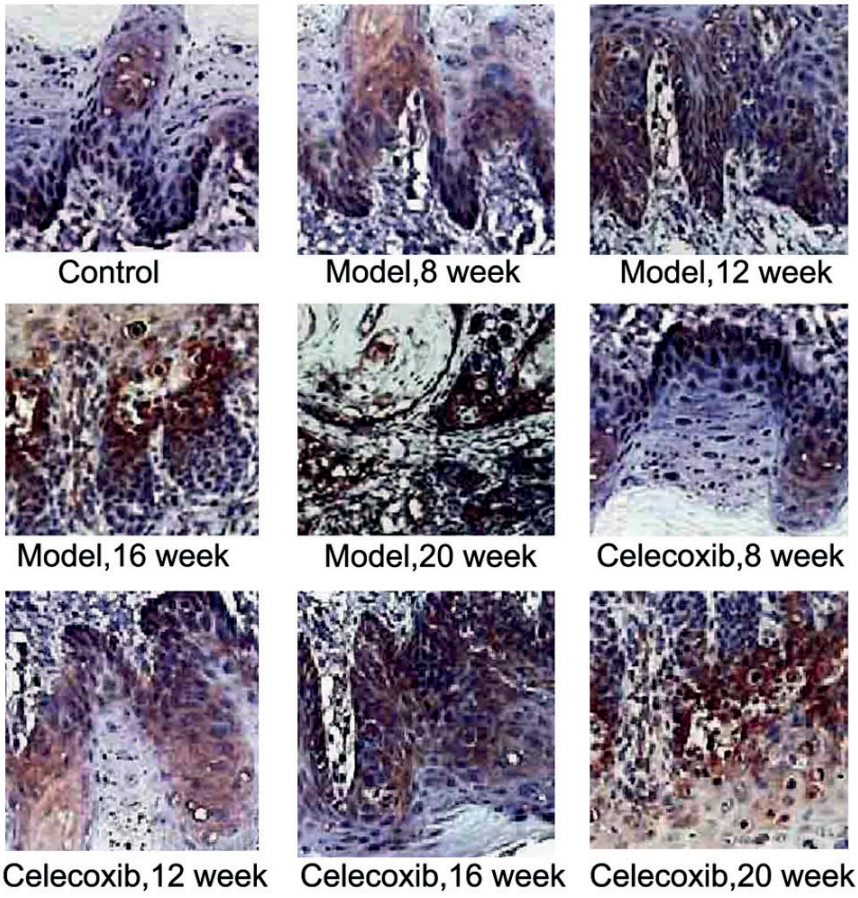


**Figure 6.** FAK protein expression of rat tongue tissues (IHC, ×400).

hyperplasia to cancer. COX-2 expression level was significantly elevated in infiltrative cancer, primary cancer, and abnormally proliferated epidermal tissues. COX-2 over-expression precedes other apoptosis-related biological changes in precancerous lesions[11,12]. As one selective COX-2 inhibitor, celecoxib can inhibit the progression of multiple malignant tumors, including those in the lymphatic hematology system, oral cavity and breast, and can enhance tumor sensitivity to radiotherapy and toxicity of drugs. Celecoxib can induce cell apoptosis via arresting cell cycle progression, the desensitization of cell cycle-dependent enzyme CDK, and the inhibition of protein kinase B[13]. This study observed the effect of the focal application of celecoxib to intervene in the DMBA-induced rat tongue cancer, and its effect on FAK and Cx43 expression in tongue cancer tissues to investigate the functional mechanism. Results showed over 80% of tumor incidence 20 weeks after induction in model group, whilst the tumor incidence was only of 13.3% in celecoxib group, with a statistically significant difference between two groups, suggesting that the focal application of celecoxib could inhibit the occurrence of DMBA-induced tongue cancer in SD rats. Pathology results showed no significant change in control group. Model group showed weak to severe abnormal hyperplasia with elongated time. The celecoxib treatment significantly improved pathology injury of tongue tissues. Through the dynamic observation of the pathology injury condition during the intervention of celecoxib, it is showed that celecoxib effectively inhibited the malignant transformation of the epidermal tissues in DMBA-induced SD rats, and depressed the degree of inflammatory infiltration. FAK is over-expressed in the epidermal tissues derived tumors such as those in pharynx, stomach and colon. Amplification of the FAK

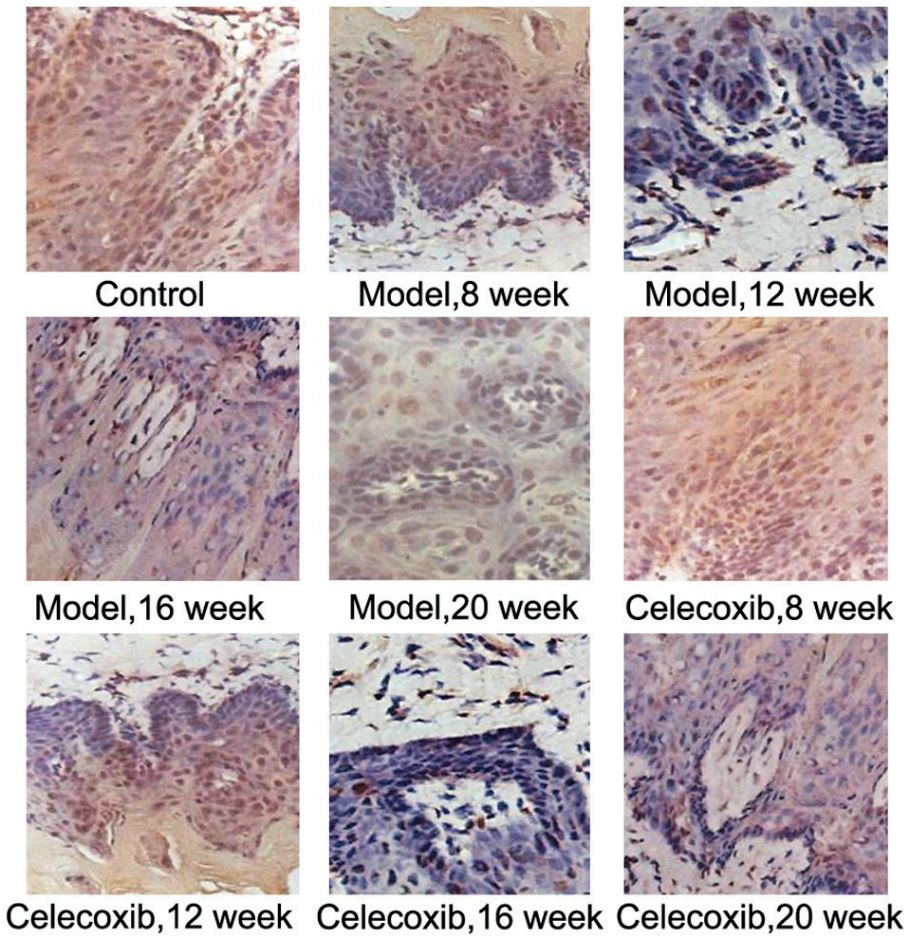


**Figure 7.** Cx43 protein expression in rat tongue tissues (IHC, ×400).

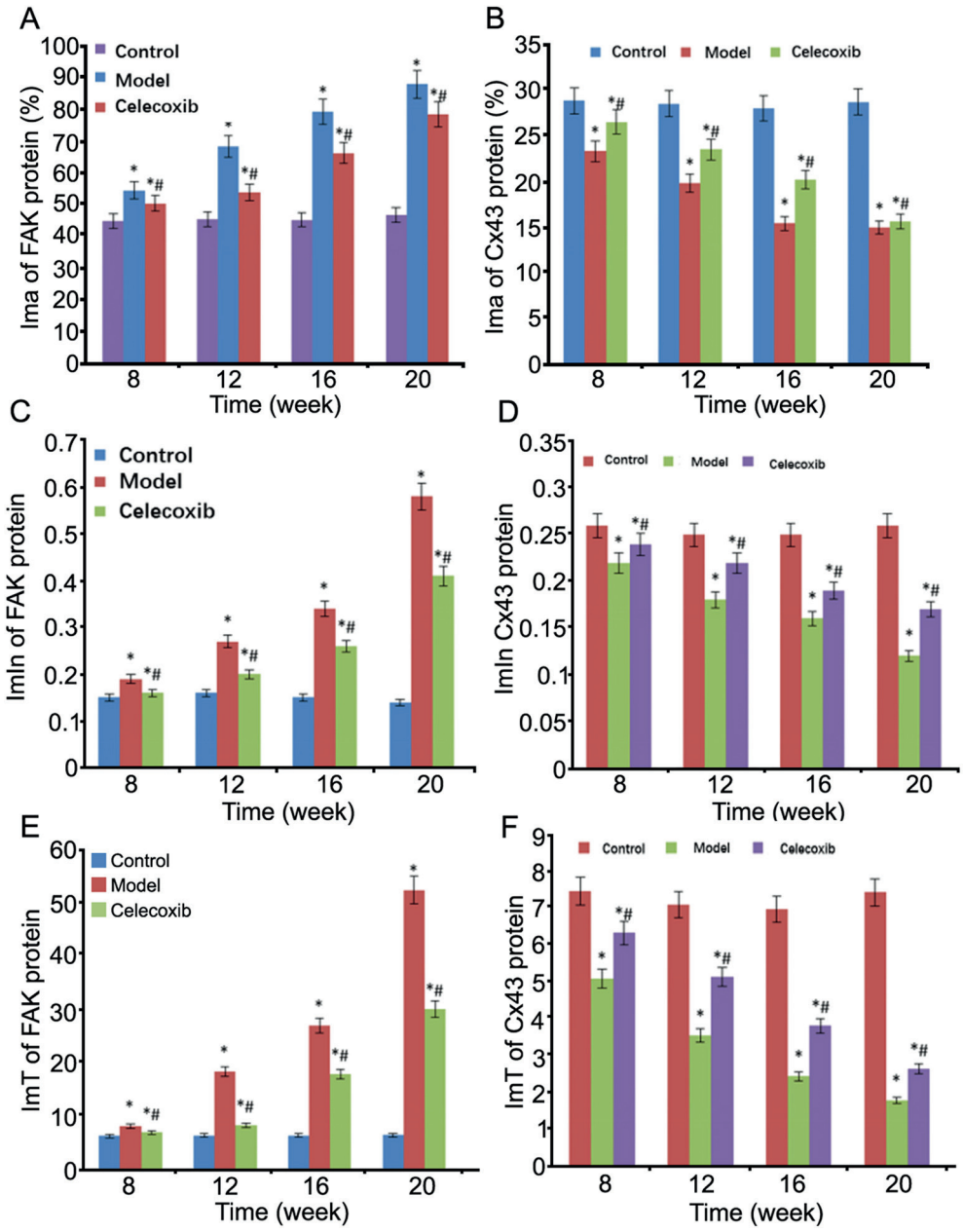


**Figure 8.** Quantitative analysis of IHC staining. ImA, the percentage of positive staining; ImIn, the averaged light density of positive staining; ImT, IHC index. $^{*}p<0.05$ compared to control group, $^{\#}p<0.05$, compared to model group.

gene has been observed in head-neck SCC[14,15]. In this study, model rats had the higher positive expression of FAK protein compared to control group, with further elevated FAK positive expression with elongated time. Compared to those of model group at the same time, celecoxib group had a statistical difference of FAK protein positive expression, indicating the elevation of FAK protein during the dynamic process of rat tongue mucosa tumor transformation. With the advanced malignancy of tongue tissues, FAK protein expression was further up-regulated. FAK locates in the central position of the integrated protein signal pathway. Integrated protein can participate in the malignant transformation of rat tongue mucosa by activating downstream signal pathway and up-regulating FAK expression. Celecoxib may inhibit malignant transformation

of tongue tissues via down-regulating FAK positive expression. In the normal basal layer of human skin, Cx43 protein had a weak expression, with elevated Cx43 protein in spine layer, and negative expression in keratin layer[16,17]. Positive expression of Cx43 protein is probably related to the cell differentiation grade[18,19]. Cx43 had decreased expression in various precancerous lesions and tumor tissues including cervical cancer, papilloma, and SCC tissues. The reverse of Cx43 down-regulation can alleviate malignant phenotype of certain tumors, and down-regulation of Cx43 is probably correlated with the malignant transformation of the epidermis[18,19]. This study observed the dynamic change of Cx43 protein in tumorigenesis of rat mucous. The result showed the lower positive expression of Cx43 protein in model group compared to control group, and lower positive expression with elongated time. Compared to those in model group at the same time, celecoxib group had a statistical difference of Cx43 protein positive expression. In tongue tumorigenesis process of rats, the Cx43 expression gradually decreased, and showed decreased expression in the mild hyperplasia tissues, indicating the correlation between lower Cx43 protein expression and malignant transformation of rat oral cavity mucosal tumorigenesis. It can work as an early event of oral mucosal carcinoma. The IHC staining results showed that celecoxib might inhibit DMBA-induced tongue cancer of rats, decreasing the incidence of oral cavity SCC via regulating FAK and Cx43 protein expression as well as inhibiting abnormal proliferation of the oral epidermis. Some studies showed the participation of FAK gene in angiogenesis during malignant transformation of the rat oral cavity. FAK gene knockout mice had abnormal angiogenesis during the embryonic stage, and FAK gene could facilitate the cancer cell invasion or metastasis[20-22]. This study observed the dynamic change of FAK and Cx43 protein expression in the rat tongue tumorigenesis as well as the intervention effect by celecoxib, leaving its detailed mechanism requiring further studies.

## Conclusions

The focal application of celecoxib effectively inhibited the DMBA-induced tongue tissue tumorigenesis of rats, possibly regulating the FAK and Cx43 protein expression and inhibiting abnormal hyperplasia in oral cavity epidermis.

### Conflict of Interests

The Authors declare that they have no conflict of interests.

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