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Ketotifen fumarate attenuates feline gingivitis related with gingival microenvironment modulation

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ABSTRACT

Gingivitis is evidenced by inflammation of the free gingiva, and still reversible. If left untreated, it may then progress to periodontitis. In the present study, the therapeutical effect of ketotifen fumarate on gingivitis was explored. Domestic cats with varying degrees of gingivitis naturally were enrolled in this study. Subgroups of animals were treated twice daily for one week with or without ketotifen fumarate (5 mg/kg). Effects of ketotifen fumarate were measured on gingival index, cells accumulation, mediators release, receptor-ligand interaction, oxidative stress, MAPK and NF-κB pathways, epithelial barrier and apoptosis. Ketotifen fumarate attenuated the initiation and progression of gingivitis, inhibited the infiltrations of mast cells, B lymphocytes, T lymphocytes, macrophages, neutrophils and eosinophils as well as the release of IgE, β-hexosaminidase, tryptase, chymase, TNF-α, IL-4, and IL-13, influenced endothelial cells, fibroblasts and epithelial cells proliferation and apoptosis, and induced Th2 cells polarization, where ketotifen fumarate also might affect their interactions. Ketotifen fumarate reduced the oxidative stress, and inhibited NF-κB and p38 MAPK related with mast cells and macrophages accumulation. Ketotifen fumarate improved the aberrant expression of ZO-1 and inhibits the following apoptosis. On the other hand, these cells and mediators augmented functional attributes of them involving SCF/c-Kit, α4β7/VCAM-1 and IL-8/IL-8RB interactions, thus creating a positive feedback loop to perpetuate gingivitis, where an inflammation microenvironment was modeled. Our results showed a previously unexplored therapeutic potential of ketotifen fumarate for gingivitis and further suggest that, in addition to biofilms, targeting inflammation microenvironment could be new strategy for the treatment of gingivitis/periodontitis.

1. Introduction

Ketotifen is a benzocycloheptathiophene derivative with anti-allergic, antihistaminic, antimicrobial and antitumor properties [1,2]. Ketotifen is well absorbed orally, with peak plasma drug concentrations within 2 to 4 h and 75% protein bound, which is metabolized to the inactive form, ketotifen-*N*-glucuronide, and active norketotifen [1]. Although stimulation of appetite, weight gain and drowsiness, ketotifen is appreciably better tolerated. At present, ketotifen has been used clinically or experimentally for conditions ranging from inflammation-related diseases [3–11] to infectious diseases [1,12–18]. Ketotifen has different modes of action that could explain its pharmacological effects. First, ketotifen regulates cyclic adenosyl 3′,5′-monophosphate (cAMP) phosphodiesterase and methyltransferase in cell membrane and histamine receptor, antagonizes calcium entry, and balances β2-adrenoceptor [14] functioning in immunomodulation and homeostasis of mast cell (MC), eosinophil, macrophage, neutrophil, basophil, and T lymphocyte. Second, ketotifen inhibits lipoxygenase, myeloperoxidase, NADPH oxidase and Na-K-ATPase to prevent the release of inflammatory mediators and mucosal damage [8,19]. Third, ketotifen suppresses the expressions of CDC42, Rac, and Rho to block inflammatory cells migration [2]. Fourth, ketotifen improves metabolic profiles, and ameliorates the status of inflammation and oxidative stress functioning in systemic homeostasis [7]. These cells and agents act

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synergistically with other local inflammatory mediators and modify the inflammatory microenvironment. Thus, it is reasonable to suppose that ketotifen exhibits these pharmacological activities possibly through modulating inflammation microenvironment, in addition to target bacterial biofilms.

Gingivitis is evidenced by the free gingiva inflammation, characterized by edema, hyperemia, and bleeding, either local or diffuse [20]. The etiology of gingivitis is the accumulation of dental plaque and is related with several adjunct or risk factors that may increase the susceptibility to the disease. Gingivitis progression could be influenced by the gingival microenvironment, where bacterial biofilms and the inflammatory cells play important roles during gingivitis development and progression. Among those immune cells, mast cells (MCs) were frequently found in the gingiva [21], and their densities were also found to significantly increase in chronic periodontitis/gingivitis [22], which contribute to the immunopathogenesis of periodontitis and may offer therapeutic target [23]. In addition to MCs, epithelial cells, leukocytes, endothelial cells, etc., may also contribute to the gingivitis. Understanding their role and interactions within inflammatory responses provides important insights into the pathogenesis of gingivitis, which also helps us to understand the course of this disease, the regulation and function of inflammatory cells, and develop new therapeutic approaches.

With therapy by eliminating dental plaques, gingivitis is reversible [24]. If left untreated, it may then progress to periodontitis, where inflammatory effects decompose alveolar bone, cementum, and periodontal ligament. Furthermore, the loss of alveolar bone, periodontal membrane can hardly be repaired. Periodontitis may be associated to various systemic diseases, leading to diabetes [25], arteriosclerosis [26], cardiovascular disease [27], stroke and adverse pregnancy outcomes [28–30]. Thus, it should be better to prevent and treat periodontal disease in its initial stage, namely gingivitis.

Therefore, ketotifen would be a new option for treating gingivitis, and its potential use deserves clinical trials. In this study, the effects of ketotifen fumarate on gingivitis in feline model as well as its cellular, molecular mechanisms under gingival microenvironment were examined, and the etiopathogenesis of gingivitis was further investigated.

2. Materials and methods

2.1. Animal & ketotifen fumarate

Domestic shorthair cats (female) with varying degrees of gingivitis naturally, which were of medium size (average weight, 4.56 ± 0.65 kg) and ranged in age from 3 to 10 years, were identified for participation in the study. All these animals were clinically evaluated for severity of gingivitis by visual observation, palpation and probing at Veterinary Teaching & Collaboration Hospitals (Beijing, Luoyang and Zhengzhou), respectively. The gingival index (GI) was recorded using the criteria for the GI system [31,32]. Healthy, age-matched control cats were obtained from commercial providers and evaluated to be free of gingivitis according to the same diagnostic criteria.

Cats were divided into four groups with the ascending GI. Groups 1–4 with different gingivitis were further divided into two subgroups, a ketotifen fumarate subgroup of 6 cats and a control subgroup of the other 6 cats. Ketotifen fumarate (5 mg/kg, i.g.) was orally given twice daily for one week. Under deep anesthesia with propofol (8 mg/kg body weight, i.v.), GI for each cat was checked, gingival samples and peripheral blood were collected. For examination and sampling smoothly, all animals were periodically checked and did not show a strong pinch of the hind paw and an eye blink reflex to tactile stimulation of the cornea during anesthesia.

All cats were housed in stainless steel cages at the animal facilities of Veterinary Teaching & Collaboration Hospitals and allowed to acclimate to the environment for 7 days before being studied. All animals received a complete premium dry food with water ad libitum, and were clinically healthy, as determined by a full clinical physical examination. All laboratory personnel and animal caretakers involved in the study were blinded to the treatment design. This study was approved by the Ethical Committee on Laboratory Animals Care and Use of the Beijing Institute of Animal Husbandry and Veterinary Medicine, China Academy of Agricultural Sciences, Beijing.

2.2. Blood & tissues collection

Fresh EDTA-anticoagulated blood samples were collected from feline saphenous vein or jugular vein. All samples were analyzed within 6 h after blood collection.

All molars and premolars gingival specimens were split in half, one for snap-frozen and stored at −80 °C, and the other for fixation in 10% formalin. Formalin-fixed tissues were paraffin wax-embedded through graded alcohols. The sections were cut at 5 μm, stained with hematoxylin and eosin (HE), or toluidine blue (TB), and the other sections were used for immunohistochemical labeling.

2.3. Hematological analysis

Quantitative determinations of white blood cells, hemoglobin, hematocrit, platelets, and other biochemical factors were performed by a Celltac E MEK-7222 hematology analyzer (Nihon Kohden Corporation, Tokyo, Japan) using standard commercial reagent kits.

2.4. β-Hexosaminidase assay

Gingiva were obtained and frozen as described above. Gingiva (100 mg wet weight) were minced and homogenized in 1% Triton X-100 in Hepes-Tyrode buffer with IKA® Ultra Turrax homogenizer T10 basic on ice. After centrifugation (2000 ×*g*, 4 °C), the supernatants were stored at −80 °C until analysis. 50 μl each sample was incubated with equal volume 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M citrate buffer, pH 5, in a 96-well microtiter plate at 37 °C for 1 h, and then 200 μl 0.2 M glycine (pH 10.7) was added to each well. The plate was measured at 405 nm using a microplate reader (Thermo Scientific, USA), and the results were expressed as percentage β-hexosaminidase activity relative to control.

2.5. Enzyme-linked immunosorbent assay (ELISA)

1 ml ice-cold PBS was added to 0.1 g fresh gingival tissue, which was additionally treated with a protease inhibitor cocktail (Complete Roche, Mannheim, Germany). All the tissues were minced, and homogenized using the homogenizer at 1500 ×g for 3 min on ice. The precipitate was removed by centrifugation at 3000 ×*g* for 15 min at 4 °C and the supernatants were stored at −80 °C until analysis. The supernatants were freeze-dried and resuspended in enzyme immunoassay buffer. Tryptase & chymase concentration was determined using the specific ELISA kits, according to the manufacturer's instructions. Sera were analyzed for IgE, IL-4, IL-10, IL-12 & IL-13 contents with the commercially available ELISA kits. Values of duplicate measurements fluctuated within a very narrow margin (< 5%), and the results were expressed as means ± SD of four different experiments.

2.6. Myeloperoxidase (MPO) activity

Gingival samples (15–30 mg) were homogenized in cold hexadecyltrimethylammonium bromide (HTAB) buffer [0.5% (wt/vol) HTAB in 50 mM potassium phosphate buffer, pH 6.0, 50 mg tissue/ml] (Sigma-Aldrich, St. Louis, MO, USA) using the homogenizer. The homogenates were centrifuged at 20,000 ×*g* for 15 min at 4 °C, and the supernatant were stored at −80 °C until analysis. To 10 μl supernatant