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Original Article

Anti-inflammatory effect of peroxisome proliferator-activated receptor-γ (PPAR-γ) on non-obese diabetic mice with Sjogren's syndrome

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Abstract: Objective: To investigate the anti-inflammatory effect of peroxisome proliferator-activated receptor-γ (PPAR-γ) on non-obese diabetic mice (NOD mice) with Sjogren's syndrome. Methods: 22 eight-week-old female NOD mice were randomly divided into 2 groups. Rosiglitazone and normal saline were administered in the PPAR-γ group and the control group respectively. At the age of 9, 12 and 15 weeks, one mouse in each group was sacrificed respectively, and the remaining mice were sacrificed at the age of 18 weeks. Blood were obtained by cardiac puncture, and salivary glands were resected. The degree of salivary gland damage and infiltration of lymphocytes were examined by H&E staining. The level of IL-1β, IL-4, IL-6 and TNF-α in serum were measured by ELISA. The mRNA expression level of IL-1β, IL-4, IL-6 and TNF-α in MSG were detected by Real-time PCR. Expression of PPAR-γ in the salivary glands was detected by Immunohistochemistry. Results: Compared with the control group, mice in the PPAR-γ group showed that (1) histopathologic changes in the salivary glands were significantly ameliorated; (2) at the age of 18 weeks, IL-6 [(25.86 ± 7.32) vs (37.41 ± 11.34)] and TNF-α [(56.88 ± 22.19) vs (78.61 ± 20.76)] were expressed significantly lower and IL-4 [(25.76 ± 12.65) vs (12.11 ± 3.70)] was expressed significantly higher in serum ($P < 0.05$); (3) the expression of TNF-α was significantly decreased and the expression of IL-4 was significantly increased in MSG ($P < 0.05$). Conclusion: PPAR-γ ameliorates Sjogren's syndrome on NOD mice effectively. The mechanism may be related to the reduction of Th1 cytokines and change of T helper cell balance from Th1 to Th2.

Keywords: Sjogren's syndrome, peroxisome proliferator-activated receptors, non-obese diabetic mice, cytokines

Introduction

Sjogren's syndrome (SS) is the second most common autoimmune disease in which periductal and perivascular lymphocytic infiltration develops in the exocrine glands and leads to acinar cell destruction and loss of secretory function [1, 2]. The etiology and mechanism of SS are undefined, but many studies have shown that a variety of pro-inflammatory cytokines play an important role within the involvement of auto-immune responses in salivary and lacrimal glands, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α [3, 4]. However, up to date, no universally effective therapy is available for this disease, controlling the development of the disease and reducing system involvement are the main goals of the treatment. The ideal measures for controlling the development of SS remains to be further explored.

Several mouse models have been used to investigate the pathogenesis of SS, such as the non-obese diabetic (NOD) mouse model, the MRL/lpr mouse model, the NZB/W F1 mouse model, the NOD-scid mouse model and the NFS/sld mouse model. Among these, the NOD mouse model is a well established reliable model, because development of adenitis is accompanied by decreased secretory function in the salivary and lacrimal glands [5]. At the

Table 1. Primer sequences for real-time PCR

Gene	GeneBank accession	Primer sequence (5'-3')	PCR Product (bp)
IL-1β	NM_008361	F CGCAGCAGCACATCAACAAGAGC	111
		R TGTCCTCATCCTGGAAGGTCCACG	
IL-4	NM_021283	F CCTGCTCTTCTTTCTCGAATGT	346
		R TTTCAGTGATGTGGACTTGGAC	
IL-6	NM_031168	F CACAAGTCCGGAGAGGAGAC	141
		R CAGAATTGCCATTGCACAAC	
TNF-α	NM_013693	F CCACCACGCTCTTCTGTCTAC	103
		R AGGGTCTGGGCCATAGAACT	
β-actin	NM_007393	F CAGGCATTGCTGACAGGATGCA	122
		R GGCCAGGATGGAGCCACCGATC	

age of 8 weeks, mononuclear cells infiltration of the salivary gland is first detected in NOD mouse, accompanied by the reduction in saliva secretion and the production of multiple antibodies [6-8].

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of transcription factors that can be activated by lipophilic ligands [9]. PPARs have three isoforms, α, β and γ. PPAR-γ regulates genes involve in the β-oxidative degradation of fatty acids, but also promote adipocyte differentiation and glucose homeostasis [10]. PPAR-γ expresses at high levels in adipose tissue and has also been detected in macrophages, endothelial cells, T cells and vascular smooth muscle cells [11-13]. PPAR-γ agonists have been shown anti-inflammatory activity by inhibiting cytokines production in a variety of mouse models for chronic inflammatory disease and autoimmune disease, including multiple sclerosis (MS) [14], rheumatoid arthritis (RA) [15] and systemic lupus erythematosus (SLE) [16]. Beauregard et al. found PPAR-α and PPAR-γ can inhibit IL-1β-induced NO production in cultured lacrimal gland acinar cells, so they raised the conclusion that PPAR agonists may be useful therapeutic agents for preventing dry eye disease due to NO-mediated lacrimal gland damage [17]. However, the effect of PPAR-γ on progression of SS is not clear.

Because of the anti-inflammatory and anti-proliferative actions of PPAR-γ agonists, we hypothesized that PPAR-γ ligand, rosiglitazone, could reduce the levels of inflammatory cytokines and histological manifestations of NOD mice with SS. In this study, we investigated the anti-inflammatory effect of PPAR-γ in non-obese diabetic mice with sjogren's syndrome, so as to provide therapeutic benefit in the treatment of SS.

Materials and methods

Animals and animal procedures

Female 8-week-old NOD mice were purchased from Shanghai laboratory animal center (China). They were placed in a specific-pathogen-free barrier environment at Anhui Medical University and maintained with food and water ad libitum. At 9 weeks of age, 22 mice were randomly divided into 2 groups: Rosiglitazone group, which received rosiglitazone (Avandia, GlaxoSmithKline, Tianjin, China) at a daily dose of 20 mg/kg by oral gavage, and control group, which were administered with saline. At the age of 9 weeks, 12 weeks and 15 weeks, one mouse in each group was sacrificed respectively. At the age of 18 weeks, the others were sacrificed. Blood samples were collected by cardiac puncture and the serum were harvested and stored at -80°C. The salivary glands of all mice were removed at sacrifice, and were vertically divided into two halves. One half was immediately snap frozen in liquid nitrogen, then stored at -80°C until use, while the other half was fixed in 10% (w/v) buffered formalin for histological examination. The research protocol was reviewed and approved by the Animal Care and Use Committee of Anhui Medical University.

Histology

The salivary glands fixed in 10% phosphate-buffered formalin were blocked with paraffin, sectioned into 2 μM slices, and stained with hematoxylin-eosin staining for evaluation of the degree of salivary gland damage and infiltration of lymphocytes. The focus score was used to describe the frequency of inflammation in salivary glands (the number of foci containing ≥50 mononuclear cells/mm^2 of glandular tissue). All histological/macroscopic evaluations were done in a double blind manner. Stained sections were observed at ×100 magnification for glandular structure and Lymphocyte infiltration,

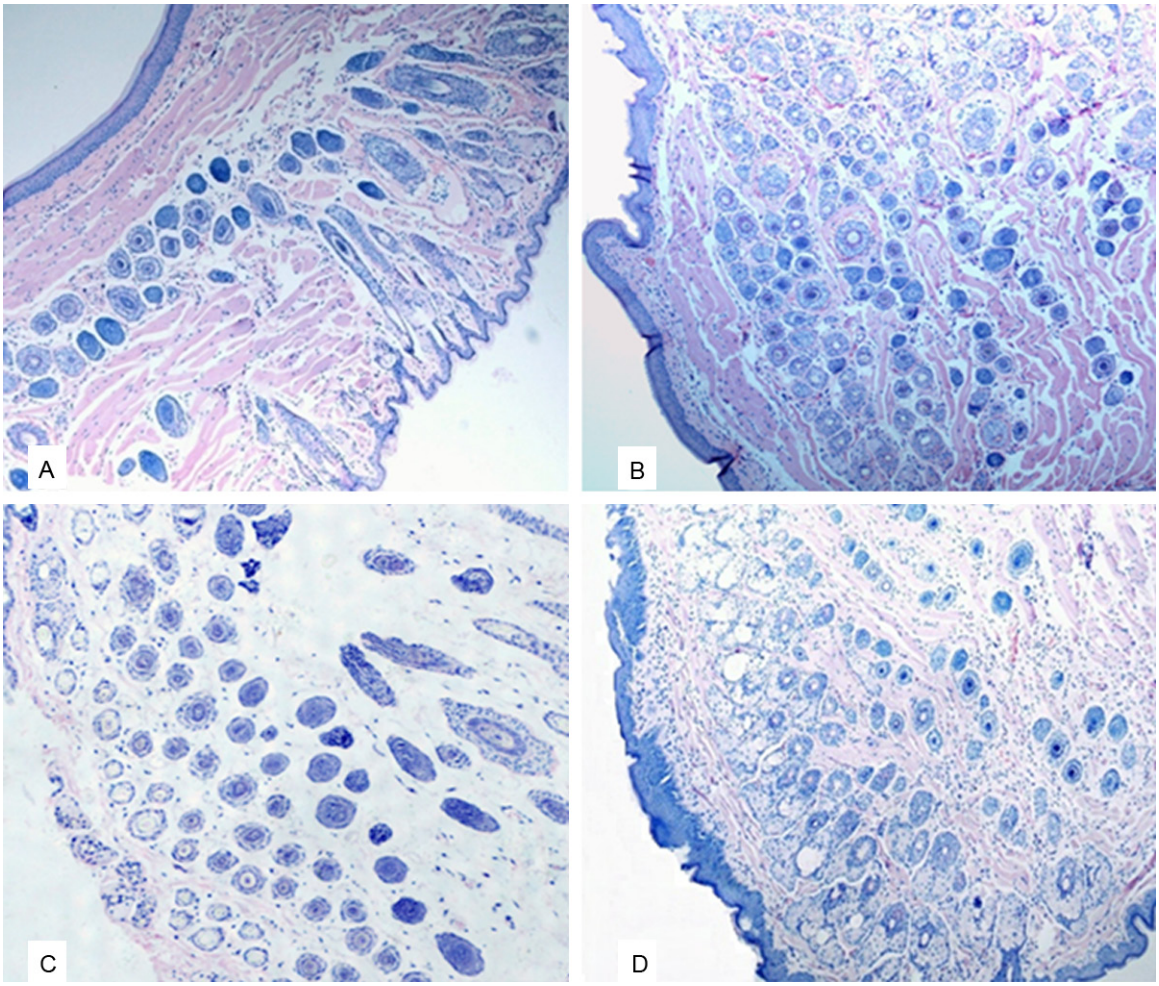

Figure 1. Hematoxylin and eosin-stained sections of the salivary gland tissue. A. Infiltration of scattered lymphocytes was present in interstitial tissue of salivary gland in the PPAR-γ group treated with rosiglitazone at the age of 12 weeks. B. The infiltration of small lymphocytes and ductal dilatation were shown in salivary gland in the controls at the age of 12 weeks. C. The infiltration of small lymphocytes in ductal wall and structural destruction were found in salivary gland in PPAR-γ group at the age of 18 weeks. D. The infiltration of moderate lymphocytes in interstitial tissue and ductal wall was shown in salivary gland in controls at the age of 18 weeks, accompanied by ductal dilatation, while obvious focal lymphocytic infiltration was undetected. Original magnification ×100.

and photographed with a microscope equipped with a digital camera (Eclipse E400 with a DMX 1200; Nikon, NY).

Cytokine ELISA

Levels of IL-1β, IL-4, IL-6 and TNF-α were quantified by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (R&D Systems, UK).

Quantitative real-time PCR

RNA extraction from the frozen salivary gland was performed following the manufacturer's instruction (RNeasy Mini Kit, Qiagen Inc., Tokyo). 5 ul RNA was used as template to generate cDNA using Reverse Transcription Kit (Invitrogen, UK) according to manufacturer's recommendations. Real-time PCR was done using QuantiTect SYBR Green PCR kit (Takara, China). The 20 μl reaction system contains 10 μl of SYBR premix Ex Taq II, 0.4 μl of Rox Reference Dye II, 2 μl of cDNA, o.5 μM primers and distilled water. The PCR conditions were denaturation 30 s at 94°C, annealing 34 s at 56°C (IL-1β and TNF-α), 58°C (IL-6 and β-actin) and 60°C (IL-4), extension 40 s at 72°C followed by 40 cycles. Sequences of primer sets for Real-time PCR are shown in **Table 1**. Samples were processed on an ABI7500 HT system (Applied Biosystems, Inc., Foster City, CA).

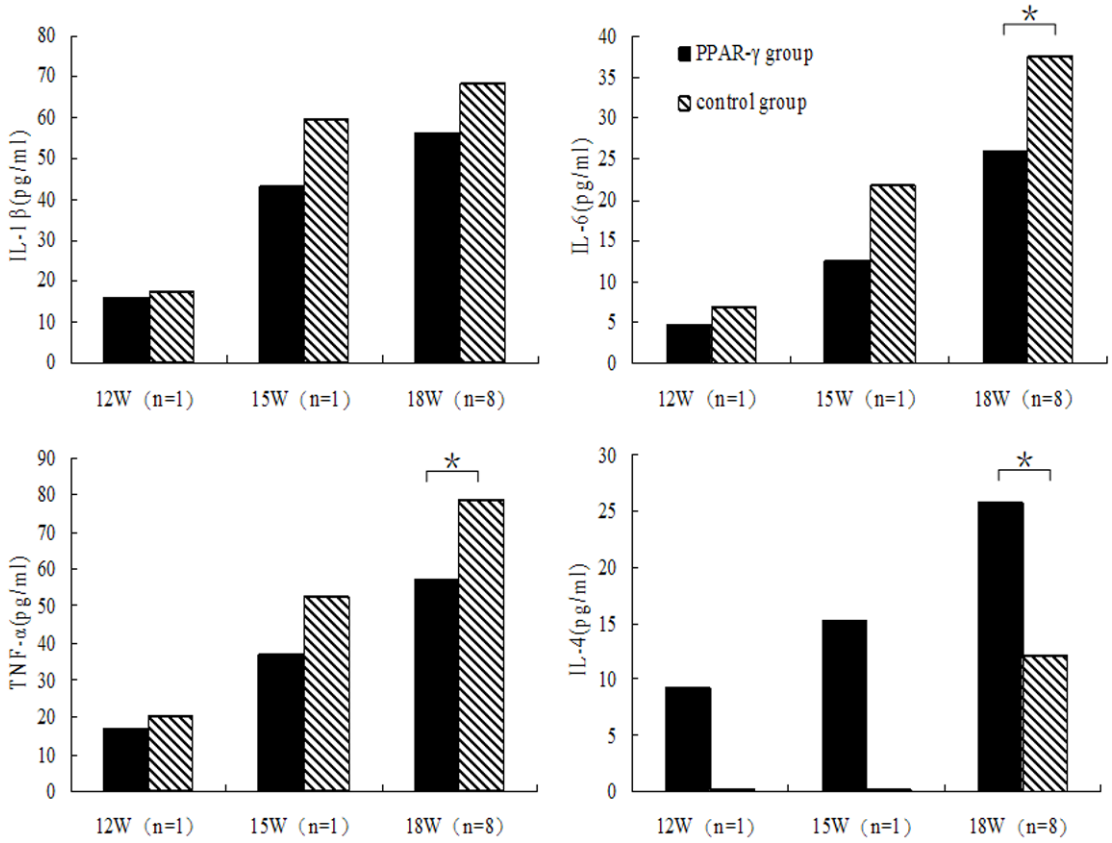

Figure 2. Cytokine Levels in serum of NOD mice with SS.

Immunohistochemistry

Formalin-fixed, paraffin-embedded, the salivary glands were sectioned into 2 μM slices, deparaffinized in xylene and rehydrated in a graded series of ethanol concentrations. Slides were heated to unmask antigen sites. Endogenous peroxidase activity was inhibited by 1% hydrogen peroxide. Sections were blocked with 10% goat serum for 60 minutes and then incubated overnight at 4°C with antibody against PPAR-γ (1:40 dilution; sc-7273; Santa Cruz biotechnology, Inc.). Sections were washed and incubated with a common secondary biotinylated antibody for 20 minutes and then incubated with diaminobenzidine (DAB) for 2 minutes before counterstaining with hematoxylin. The slides were air dried, then cleared in xylene and mounted in pertex.

Statistic analysis

The results were expressed as mean ± SD. Data was compared by using the unpaired Student's *t*-test. A *P* value of less than 0.05 was considered significant. All statistical analyses were performed using Statistical Package for Social Science version 10.01 software.

Results

Down-regulation of lymphocytes infiltration in the salivary glands of NOD mice with SS following treatment with PPAR-γ

To investigate lymphocytes infiltration in the salivary glands of NOD mice with SS, the salivary glands were removed from mice after 8 week treatment with rosiglitazone or saline and were stained with H&E for analyzing lymphocytes infiltration. Infiltration of lymphocytes in salivary glands was gradually increased in control group while inhibited in treated group (**Figure 1**).

Effect of PPAR-γ on the levels of cytokines in serum

The levels of IL-1β, IL-6 and TNF-α were gradually increased in PPAR-γ group and control

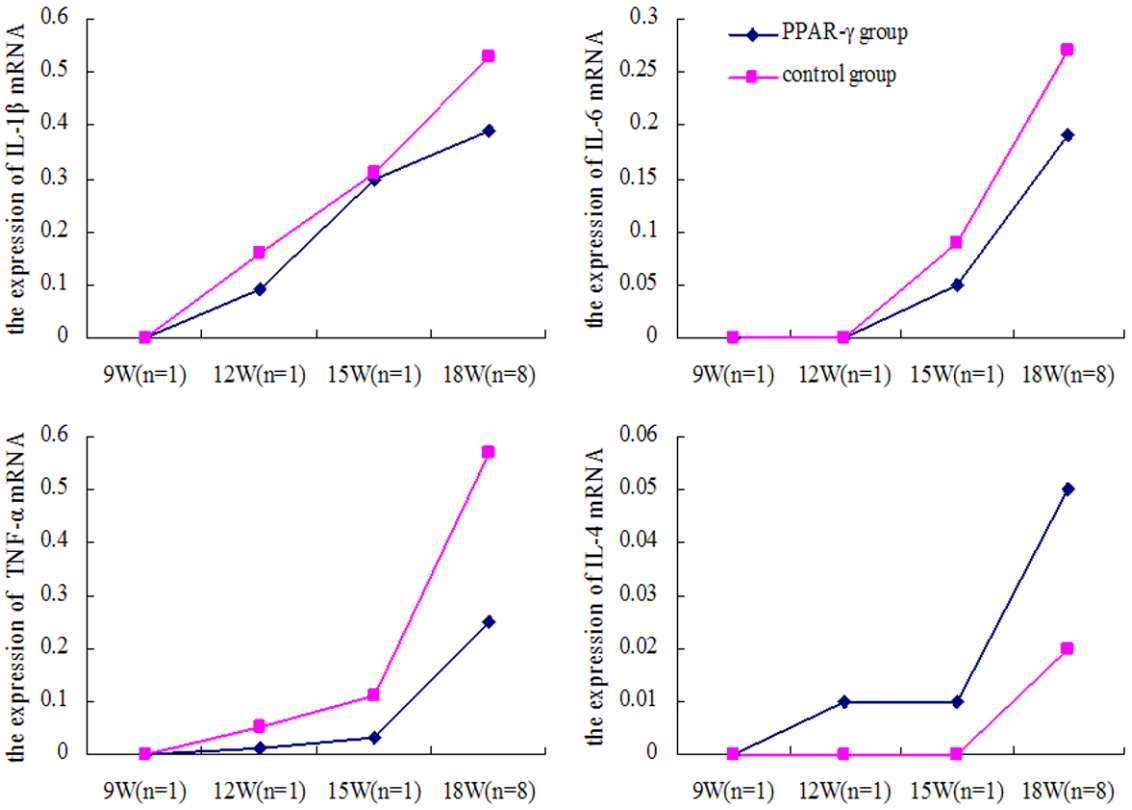

Figure 3. Expression of cytokines in the salivary glands of NOD mice with SS.

group and the level of IL-4 was gradually increased in PPAR-γ group with age. However, IL-4 was not detected in the serum of 12-week-old and 15-week-old mice in control group.

The treatment of rosiglitazone reduced the levels of IL-1β, IL-6 and TNF-α in serum sample. At the age of 18 weeks, the levels of IL-6 [(25.86 ± 7.32) vs (37.41 ± 11.34) pg/ml] and TNF-α [(56.88 ± 22.19) vs (78.61 ± 20.76) pg/ml] were significantly lower. The level of IL-4 was significantly higher in the PPAR-γ group compared to the control group [(25.76 ± 12.65) vs (12.11 ± 3.70) pg/ml] ($P < 0.05$) (**Figure 2**).

Effect of PPAR-γ on the mRNA expression of cytokines in the salivary glands

The mRNA expressions of IL-1β, IL-6 and TNF-α were gradually up-regulated in both PPAR-γ group and control group, and the mRNA expression of IL-4 was gradually up-regulated in PPAR-γ group. However, IL-4 was only detected in salivary glands in controls at the age of 18 weeks.

The salivary gland mRNA expression of TNF-α in the PPAR-γ group was markedly down-regulated ($P < 0.05$), mRNA expressions of IL-1β and IL-6 were slightly down-regulated and mRNA expression of IL-4 was significantly up-regulated at the age of 18 weeks ($P < 0.05$) (**Figure 3**).

Expression of PPAR-γ in the salivary glands

To identify the localization of PPAR-γ in the salivary glands we performed immunohistochemical analysis of PPAR-γ protein. The expression of PPAR-γ was observed in mononuclear cells. Its levels were increased by the treatment with rosiglitazone at the age of 18 weeks, while not changed in controls (**Figure 4**).

Discussion

The NOD mouse strain exhibits immunological, histopathological and physiological characteristics of SS with focal mononuclear cell infiltration of the exocrine glands associated with reduced secretory flow from approximately 8 weeks of age. NOD mouse is more similar with human SS, so it has unique advantage to be

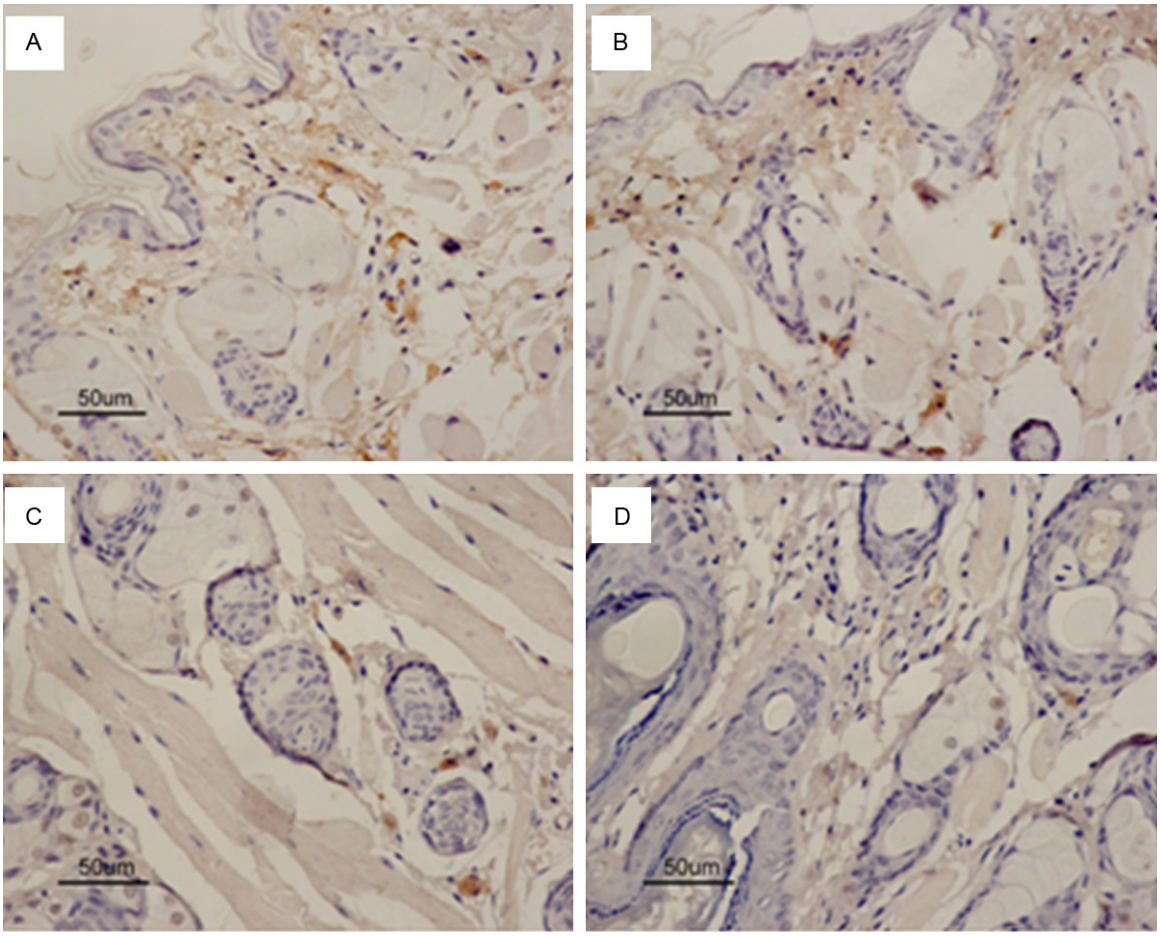

Figure 4. Immunohistochemical analysis of PPARγ in the salivary glands (original magnification ×400). The expression of PPAR-γ were more strongly by the treatment with rosiglitazone at the age of 18 weeks (C) than at the age 9 weeks (A), control at the age 9 weeks (B) and controls at the 18 weeks (D).

used in SS research. In this study, we found that the NOD mice developed Sjogren's syndrome-like autoimmune inflammation in the salivary glands. Inflammation developed spontaneously in the salivary glands of these mice at the age of 12 weeks. Lymphocytic infiltration in salivary gland and the destruction of salivary gland in NOD mice were gradually increased with age.

The pathogenesis of SS is generally considered that autoimmune response led to the infiltration of a large number of lymphocytes, plasma cells and mononuclear cells within the exocrine glands through a variety of cytokines and inflammatory mediators, resulting in the destruction and dysfunction of the exocrine glands. IL-1β and TNF-α are recognized as inflammatory cytokines, and play important role in chronic inflammation. In patients with SS the number of cells spontaneously secreting IL-1β and TNF-α were increased in comparison with controls, and positively correlated with the disease duration [3]. The expression of IL-1β in salivary glands was also increased in patients with SS [18]. The levels of TNF-α in peripheral blood and the expression of TNF-α in lymphocytes infiltrated in salivary gland were markedly increased [3, 18, 19]. IL-1β and TNF-α can promote local infiltration of inflammatory cells which can release inflammatory mediators and increase the production of collagenase [20], and promote acinar cells to synthesize matrix metalloproteinases (MMP)-II which increased the destruction of acinar cells structure [21]. IL-1β, TNF-α and other pro-inflammatory cytokines mRNA expression in serum, saliva and salivary gland tissue of NOD mice with SS were also significantly higher than that of normal mice, and the levels of which were gradually

increased with age [6, 22]. The over-expression of cytokines mentioned above may be involved in regulation of autoimmune reaction in salivary gland. B cell activity is elevated in patients with SS, and the activation of polyclonal B cells can promote the production of a large number of organ-specific or non-specific autoantibodies, and result in macroglobulinemia. IL-6 is not only a pro-inflammatory cytokine, but also an important B cell differentiation factor which can promote the production of autoantibodies. IL-6 was found highly expressed in peripheral blood, saliva and labial gland biopsies of SS patients, and high levels of IL-6 correlated with the degree of lymphocytes infiltration in the salivary gland and the number of extraglandular symptoms [23, 24]. Elevated level of IL-6 may be one of the reasons of high activity of B cells in patients with SS. In this study, with age, the levels of Th1 cytokines IL-1β, IL-6 and TNF-α were gradually increased in serum, and their mRNA expression was also gradually up-regulated in the salivary glands. These results indicate that the levels of IL-1β, IL-6 and TNF-α in local salivary gland and systemic were closely related to the degree of disease activity of SS.

Our study is consistent with the results reported by other investigators [25, 26] suggesting that the expression of IL-4 is lower in serum and salivary gland in NOD mice, and is not detected in the early stages of the disease. IL-4 is a classical anti-inflammatory cytokine and the key cytokine in a Th2 type immune response, involved in B cell activation, proliferation and differentiation, and has no or low expression in mucosal biopsies of SS patients. Moreover, the ratio of the pro-inflammatory cytokine IFN-γ to IL-4 is higher in the salivary gland and lower in the peripheral blood of patients, which reflects a skewed immune pattern towards a Th1 response locally in the salivary gland [27].

PPAR-γ is a member of the nuclear hormone receptor superfamily of transcription factors. PPAR-γ ligand thiazolidinediones (TZDs) has been used for the treatment of type 2 diabetes. In recent years, several studies found that PPAR-γ also has immunomodulatory and anti-inflammatory effect. In this study, immunohistochemistry results showed that no immunoreactivity or little week immunoreactivity for PPAR-γ was detected in early stage of NOD mice with SS. At the age of 18 weeks, the expression of PPAR-γ was up-regulated in both PPAR-γ group and controls. However, compared with control group, the expression of PPAR-γ was significantly increased after the PPAR-γ agonist rosiglitazone treatment. These results suggest that PPAR-γ may play an active role in the pathophysiology of SS, and PPAR-γ ligands may have the potential to suppress inflammation of salivary gland in SS.

PPAR-γ agonists, contributing to homoeostatic control of T cell proliferation and differentiation, as well as, induction of T cell apoptosis, suggest the inhibitory role of T cell mediated immune response and anti-CD3-induced TNF-α secretion [28]. PPAR-γ agonists can also induce B cell apoptosis and inhibition of B cell activity and proliferation [29]. PPAR-γ was suggested to reduce the expression of IL-1β, IL-6 and TNF-α by interfering with the transcription factors involved in pro-inflammatory signal transduction pathways, like activator protein-1 (AP-1), signal transducers and activators of transcription-1 (STAT-1), nuclear factor-κB (NF-κB) and nuclear factor of activated T cells [11], as well as IL-6 and TNF-α contribute to up-regulation of NF-κB activation by significantly increased dissociation of NF-κB and its inhibitive factor I κB -α. With respect to anti-inflammatory cytokines, PPAR-γ agonists can also induce IL-4 production, indicating an additional protective role for the receptor in inflammatory reactions [30, 31]. In the present study, administration of PPAR-γ ligand ameliorated the NOD mice with SS. The result suggests that PPAR-γ ligand has the potential to suppress the inflammation of salivary glands in NOD mice with SS. PPAR-γ ligand significantly reduced the levels of Th1 cytokines IL-6 and TNF-α and increased the level of Th2 cytokine IL-4 in serum. Moreover, PPAR-γ ligand treatment induced the mRNA expression of IL-4 and inhibition of TNF-α mRNA expression in salivary glands. Taken together, PPAR-γ treatment ameliorated the NOD mice with SS may, at least partly, be due to the suppression of inflammatory cytokines and the change of the helper T cell balance from Th1 to Th2.

Overall, our study suggested PPAR-γ can ameliorated NOD mice with SS through inhibition of the expression of Th1 cytokines IL-6 and TNF-α and down-regulation of the level of Th2 cytokine IL-4 in peripheral blood and (or) salivary gland. It to some extent controlled the progression of SS in NOD mice. PPAR-γ ligand such as

rosiglitazone may have the potential to modulate human autoimmune disease of SS.

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Disclosure of conflict of interest

None.

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