Chitosan inhibits inflammation and adipogenesis of orbital fibroblasts in Graves ophthalmopathy

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Purpose: The aim of this study was to investigate the roles of chitosan in inflammation and adipogenesis of primary cultured orbital fibroblasts in Graves ophthalmopathy (GO).
Methods: Cell viability, apoptosis, and cell cycle were determined with the Cell Counting Kit-8 (CCK-8), the Annexin V-FITC/PI kit, and flow cytometry, respectively. Inflammation of orbital fibroblasts was stimulated by interleukin-1 beta (IL-1β). The levels of IL-6 and prostaglandin E-2 (PGE-2) were measured using an enzyme-linked immunosorbent assay (ELISA). The expression of cyclooxygenase-2 (COX-2) was measured with real-time PCR and western blot assay. Phosphorylation of c-Jun N-terminal kinase (JNK) was evaluated with western blot assay. An inhibitor of JNK was used to investigate the signal transduction pathway of cytokine production. Orbital fibroblasts differentiated to adipose cells in differentiation medium. Adipose cells were dyed with Oil Red O. FABP4, adiponectin, C/EBPα, PPAR-γ, and phosphorylation of AKT were evaluated with western blot assay.
Results: The results showed that IL-1β statistically significantly increased the expression of IL-6, PGE-2, and COX-2 in orbital fibroblasts. Phosphorylation of JNK was promoted by IL-1β. IL-6 and PGE-2 were modulated by the JNK signaling pathway as determined with the inhibition experiments. Chitosan downregulated expression of IL-1β-stimulated IL-6, COX-2, and PGE-2 and downregulated phosphorylation of JNK. Chitosan inhibited the production of adipose cells dyed by Oil Red O. Chitosan statistically significantly decreased the protein levels of FABP4, adiponectin, C/EBPα, and PPAR-γ with downregulation of AKT phosphorylation during adipocyte differentiation.
Conclusions: Chitosan statistically significantly inhibits inflammation and adipogenesis, as well as related signaling pathways, of orbital fibroblasts in GO. This indicates a possible therapeutic effect of chitosan on Graves ophthalmopathy.

Approximately 25–50% of patients with Graves' disease (GD) have a clinical manifestation of Graves ophthalmopathy (GO). As an autoimmune disease, GO has a global prevalence rate of 0.1–0.3% [1,2]. Inflammation and adipogenesis in orbital fibroblasts are the main characteristics of GO [3,4]. Orbital fibroblasts are located in the orbital adipose/connective tissue and the extraocular muscles. Orbital fibroblasts produce inflammatory cytokines, which are associated with conjunctival congestion in patients with GO. Orbital fibroblasts also differentiate into adipocytes to produce lipids in the orbit, which are associated with proptosis, exposure keratitis, and compressive optic neuropathy in patients with GO.

Because of the intricate mechanisms of Graves ophthalmopathy, there is no ideal treatment. Glucocorticoids are usually used to treat GO due to immunosuppression and anti-inflammatory effects. However, the side effects of glucocorticoids can seriously damage the human body. Thus, a reliably safe medicine is of paramount importance for GO treatment.

Chitosan is a natural product processed from crab shells with good biocompatibility and safety for the human body. Chitosan is used as a viscoelastic agent during cataract surgery and is also used clinically to prevent postoperative adhesion due to the product's antiproliferation and anti-inflammatory functions [5,6].

In previous studies, chitosan has been shown to have an anti-inflammatory effect by decreasing the production of prostaglandin E-2 (PGE-2) through downregulation of the c-Jun N-terminal kinase (JNK) signaling pathway in gingival fibroblasts [7]. Chitosan also inhibits adipogenesis in 3T3-L1 adipocytes [8]. Our aim was to evaluate whether chitosan could also inhibit inflammation and adipogenesis of orbital fibroblasts from GO and the relevant signaling pathways.

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In this study, chitosan inhibited the production of PGE-2 and interleukin-6 (IL-6) induced by IL-1β through down-regulation of the JNK signaling pathway of orbital fibroblasts from GO. Chitosan inhibited lipid droplet production stained by Oil Red O. The concentrations of adiponectin, C/EBPα, PPAR-γ, as well as FABP4, were decreased after the addition of chitosan with decreased phosphorylation of AKT.

METHODS

Reagents: Water-soluble liquid of chitosan was donated by Shanghai Qisheng Biologic Preparation Co., Ltd (Shanghai, China). The Cell Counting Kit-8 (CCK-8) was purchased from Biotool, Inc. (Houston, TX). Annexin V-FITC/PI apoptosis and cell cycle detection kits were purchased from BD Biosciences (Franklin Lakes, NJ). Gentamycin, penicillin, fetal bovine serum (FBS), and Dulbecco's modified Eagle's medium (DMEM) were purchased from HyClone Laboratories, Inc. (Logan, UT). SYBR Green qPCR master mix, the GoScript Reverse Transcription System, and TRIzol were purchased from Biotool, Inc., Promega (Madison, WI), and Invitrogen (Carlsbad, CA), respectively. The IL-6 and PGE-2 enzyme-linked immunosorbent assay (ELISA) kit, SP600125 (JNK inhibitor), anti-COX-2, anti-JNK, anti-adiponectin, anti-C/EBPα, anti-PPAR-γ, anti-FABP4, and recombinant human IL-1β were purchased from Abcam (Cambridge, MA).

Cell culture and adipocyte differentiation: Orbital connective tissues were obtained from three patients with GO during orbital decompression operations (Table 1). The patients with GO received no glucocorticoid therapy for 6 months before surgery. Patient thyroid function was normal. The patient clinical activity score was equal to or less than three. No patient had received prior orbit radiotherapy. Patients agreed to a written informed consent based on the protocols under the approval of the Institutional Review Board of Chongqing General Hospital and the tenets of the Declaration of Helsinki. The study was adhered to the ARVO statement on human subjects. Orbital fibroblasts were cultured from the orbital connective tissues according to previous studies [9,10]. Cells from different patients were mixed and cultured together. The orbital fibroblasts were cultured in DMEM, which contained 20 mg/ml gentamycin, 100 U/ml penicillin, and 10% FBS in a humidifying incubator at 37 °C with 5% CO_2. The cells at passages 3 to 8 were used in this study [11]. The cells were cultured in 6-well plates. After becoming confluent, the cells were cultured in adipogenic differentiation medium as described previously [9]. Orbital fibroblasts were differentiated into adipocyte in the differentiation medium after 10 days. The differentiation medium contained DMEM without serum, 33 mM biotin, 0.2 mM carba-prostaglandin, 10 mM rosiglitazone, 10 mg/ml transferrin, 0.2 nM T3, 1 mM insulin, and 17 mM pantothenic acid. The medium was added with 0.1 mM isobutylmethylxanthine, 1 mM dexamethasone, and 1 mM insulin during the first 4 days. The cells were cultured in differentiation medium for 10 days. Water-soluble chitosan was added to the differentiation medium beginning on the first day. The medium was refreshed every 3 days.

Cell viability assays: The CCK-8 assay was used to evaluate the role exerted by chitosan in orbital fibroblast activity. The orbital fibroblasts were seeded in 96-well plates with a density of 1×10^4 cells/well. The medium was removed

TABLE 1. CLINICAL FEATURES OF GO PATIENTS IN THE CURRENT STUDY.	
Character	**GO Patients (n=3)**
Average age, years (range)	52(45–56)
Gender, male/female	1/2
Smoking, yes/no	1/2
Graves' disease	
Methimazole	3
Radioactive iodine therapy	0
Surgery	0
Treatment GO	3
Prednisone	3
Surgery	3
Radiation	0
Antibodies against TSH-receptor	3
Euthyroid	3
Scores of Clinical activity (range)	1(1–2)

after the cells became confluent. The orbital fibroblasts were washed twice with PBS (1X; 137 mM NaCl, 2.7 mM KCl, 10 mM $NaPO_4$, 2 mM KPO_4, pH 7.4). Thereafter, the cells were transferred into medium without serum for 24 h. Various concentrations of chitosan (0.01, 0.1, 1, and 10 mg/ml) were added to the cells for 24 to 72 h. Thereafter, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8; 10 μl) was added to each well. Optical density (OD) at 450 nm was determined with a microplate reader (Molecular Devices, Sunnyvale, CA) [12]. Orbital fibroblasts treated without chitosan were used as the control group. Cell apoptosis after chitosan treatment was determined using the Annexin V-FITC/PI kit. Orbital fibroblasts were washed with PBS and then cultured in serum-free DMEM with various concentrations of chitosan (10, 1, 0.1, or 0.01 mg/ml) for 24 h. To detect flow cytometry, the cells (1×10^4) were stimulated at a wavelength of 488 nm, and emission was detected at 584 and 530 nm for the fluorescence of PI and Annexin V-FITC, respectively [9]. Flow cytometry was used to analyze the cell cycles. Orbital fibroblasts were fixed in 100% ice-cold methanol and incubated with RNase A (125 μg/ml) and propidium iodide (PI; 50 μg/ml) for 45 min at room temperature [5].

Orbital fibroblasts treated with chitosan after stimulation with IL-1β: Orbital fibroblasts were seeded in 6-well cultured plates and cultured in DMEM with 10% FBS. After becoming confluent, the cells were cultured with chitosan (0.1 mg/ml) in serum-free medium for 24 h. The orbital fibroblasts were cultured with IL-1β at a concentration of 10 ng/ml for 24 h to produce inflammation in vitro [8,9]. Chitosan, SP600125, and dexamethasone were applied to each group. SP600125 is a selective inhibitor of the JNK signaling pathway, which is related to the production of PGE-2 and IL-6 in fibroblasts [7]. Dexamethasone treatment served as the positive control group [13,14]. The quantity of the IL-6 and PGE-2 proteins was measured with ELISA. The cells were then collected to evaluate the expression of RNA and protein.

Orbital fibroblasts treated with an inhibitor of JNK after stimulation with IL-1β: The orbital fibroblasts were inoculated in 6-well cultured plates. After becoming confluent, the cells were cultured in serum-free medium for 24 h and incubated with 10 μM SP600125 (JNK inhibitor) for 30 min. The cells were then treated with IL-1β (10 ng/ml) for 24 h before the supernatants were collected to determine the concentrations of PGE-2 and IL-6. Human ELISA kits were used to detect the PGE-2 and IL-6 levels in accordance with the product manual.

Real-time PCR: TRIzol (Invitrogen) was used to isolate total RNA for reverse transcription to cDNA as described

previously [12]. The level of mRNA was determined with SYBR Green by real time PCR under standard thermocycler conditions (Applied Bio-systems) and a system for detecting sequences (ABI Prism 7500; Applied Bio-systems) was used to analyze results, which were normalized to human β-actin expression. PCR condition is 93 °C for 2 min, 93 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, a total of 40 circulations and the final 72 °C for 7 min. The primers were as follows: human β-actin, sense, 5′-TCA CCC ACA CTG TGC CCA T-3′, and antisense, 5′-TCC TTA ATG TCA CGC ACG ATT T-3′; human COX-2, sense, 5′-GCT CAA ACA TGA TGT TTG CAT TG-3′, and antisense, 5′-GCT GGC CCT CGC TTA TGA-3′ [8].

Western blotting: Ice-cold PBS was used to wash the orbital fibroblasts three times, and lysis solution was used to dissociate the cells as described previously [12]. Cell lysates were separated with centrifugation before the supernatants were collected to measure the protein levels using a bicinchoninic acid (BCA) protein assay. Loading buffer was added to each sample for dilution before boiling for 5 min. Subsequently, 50 μg of each sample was loaded onto a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The molecular weight was evaluated with prestained markers. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Bovine serum albumin (5%) or skimmed milk in Tris-buffered saline with Tween-20 (TBST) was used to block the membranes, which were then incubated with antibodies specific to JNK, phosphorylated JNK, COX-2, PPAR-γ, C/EBPα, adiponectin, FABP4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at a temperature of 4 °C. Horseradish peroxidase-conjugated secondary antibody and electrochemiluminescence (Pierce Chemical, Dallas, TX) were used to identify reactive bands on these membranes.

Oil Red O dye: The adipocytes were dyed with Oil Red O as previously described [9]. Oil red O (0.5 g) dry powder was fully dissolved in 100% isopropanol (100 ml). Oil red O solution was preserved at 4 °C airtight avoid light. Oil red O stock solution was added with distilled water (6:4) at room temperature for 1 h, and then filtered through a 0.2-mm filter. The adipocytes were washed twice in PBS. The cells were immobilized using 3.7% formaldehyde solution in PBS at 4 °C for 1 h. Lipid granules were stained using Oil Red O solution (300 ml) under ambient conditions for 1 h. Aqua distillate was used to wash the wells. Subsequently, the wells were visualized and photographed at 200X magnification with light microscopy (Olympus, Melville, NY). Absolute isopropanol was used to solubilize Oil Red O to quantify lipid droplets. Solution OD was determined with spectrophotometry (490 nm).

Statistical analyses: Experiments were performed three times with cells from the tissue samples (n=3). The results were analyzed with a paired-sample *t* test (SPSS 12.0, SPSS, Inc., Chicago, IL). A p value of less than 0.05 was considered statistically significant.

RESULTS

The role exerted by chitosan in orbital fibroblast activity: The results of the CCK-8 assay showed that 0.1 and 0.01 mg/ml chitosan treatment for 24 and 72 h exerted no influence on the cell viability of the orbital fibroblasts (Figure 1B,C). The percentage of apoptosis (Figure 1D), and the percentage of cells in the S phase (Figure 1E) were not affected by the 0.01 and 0.1 mg/ml concentrations of chitosan for 24 h. Therefore, the 0.1 mg/ml concentration of chitosan was used to investigate the effects on inflammation and adipogenesis in orbital fibroblasts.

The role exerted by chitosan in PGE-2 and IL-6 production by orbital fibroblasts induced by IL-1β: The results showed that the quantities of the IL-6 and PGE-2 proteins were elevated with the stimulation of IL-1β. SP600125, dexamethasone, and chitosan statistically significantly decreased the levels of the IL-1β-induced IL-6 (Figure 2A) and PGE-2 (Figure 2B) proteins. As PGE-2 is catalyzed by COX-2, we then investigated the role exerted by chitosan in COX-2 generation of orbital fibroblasts under the induction of IL-1β at 10 ng/ml. The results showed that 0.1 mg/ml chitosan statistically significantly inhibited the IL-1β-induced expression of COX-2 (Figure 2C,D) at 8 h.

Chitosan inhibits inflammation of orbital fibroblasts stimulated by IL-1β through downregulation of the JNK signaling pathway: SP600125, a selective inhibitor of JNK, reduced PGE-2 and IL-6 levels under the induction of IL-1β, indicating that the JNK signaling pathway is associated with inflammation in orbital fibroblasts. Subsequently, we investigated whether IL-1β activated the JNK signaling pathway. The results showed that phosphorylation of JNK was statistically significantly elevated with stimulation of IL-1β, notably at 45 min (Figure 2E). Chitosan (0.1 mg/ml) effectively decreased JNK phosphorylation at 45 min (Figure 2F).

The role exerted by chitosan in adipogenesis of orbital fibroblasts: The results showed that the quantity of fat granules stained by Oil Red O (Figure 3A) and the OD values of the stained cell lysates (Figure 3B) were reduced by chitosan in a dose-dependent manner. Western blot analyses were conducted with the aim of determining whether chitosan influences the expression profiles of transcription factors involved in adipogenesis and adipocyte-secreted products. Chitosan statistically significantly decreased the protein
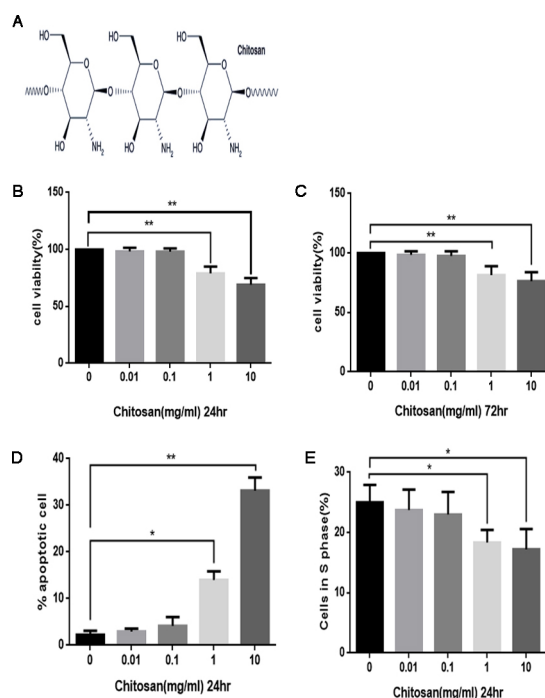

Figure 1. The role exerted by chitosan in cell activity, apoptosis, and the cell cycle of orbital fibroblasts. **A**: Chemical production of chitosan. Cell activity was detected using the Cell Counting Kit-8 (CCK-8) after the orbital fibroblasts were treated with various concentrations of chitosan for 24 h (**B**) and 72 h (**C**). Percentage of apoptosis (**D**) and cells in the S phase (**E**) in orbital fibroblasts treated with various concentrations of chitosan for 24 h. Cell activity, percentage of apoptosis, and cells in the S phase were not influenced by chitosan at the 0.01 and 0.1 mg/ml concentrations. Data are presented as the mean ± standard deviation (SD), **p<0.01 and *p<0.05 for comparison with chitosan-treated and control orbital fibroblasts. The experiments in this figure were repeated three times, and similar results were obtained.
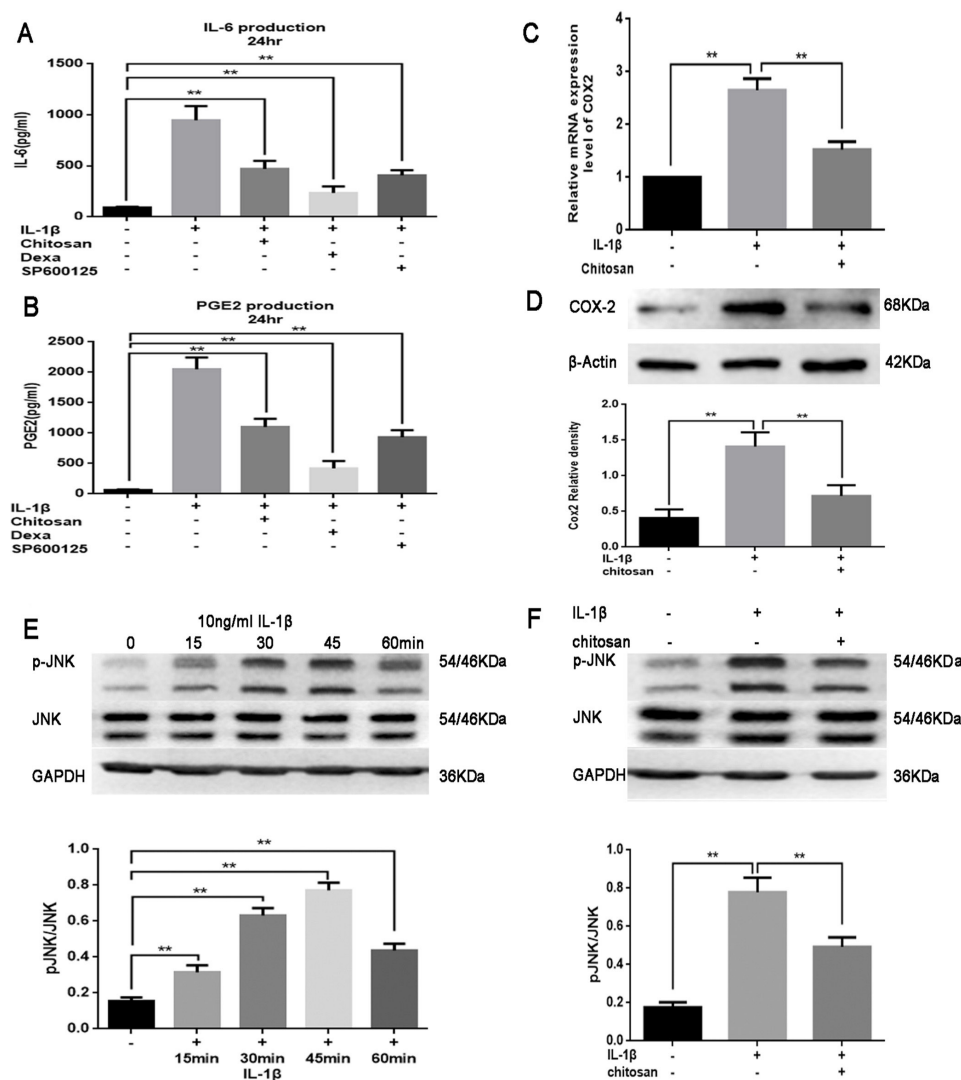
Figure 2. Chitosan inhibits inflammation induced by IL-1β in orbital fibroblasts. The cells in medium without serum were subjected to preincubation with chitosan (0.1 mg/ml), dexamethasone, and SP600125 for 30 min and then treated with interleukin-1 beta (IL-1β; 10 ng/ml) for 24 h. Enzyme-linked immunosorbent assay (ELISA) was used to examine the levels of IL-6 (**A**) and prostaglandin E-2 (PGE-2) (**B**) released into the medium. Another batch of cells was incubated jointly with 10 ng/ml chitosan and IL-1β (0.1 mg/ml) for 8 h. (**C**) Quantitative real time polymerase chain reaction (qRT-PCR) and (**D**) western blot analyses were conducted to evaluate the transcriptional and expression levels of COX-2. Human orbital fibroblasts subjected to serum starvation were treated with 10 ng/ml IL-1β, and western blot analysis was used to evaluate the concentrations of phospho-c-Jun N-terminal kinase (pJNK) concentrations after various periods of treatment. **E**: The JNK levels were used as the loading control. Human orbital fibroblasts subjected to serum starvation were exclusively treated with IL-1β or were cotreated with chitosan and IL-1β for 45 min. Western blot analysis was used to detect the pJNK quantities. **F**: The JNK levels were used as the loading control. Chitosan inhibits the production of IL-6 and PGE-2 induced by IL-1β. The expression of COX-2 and PGE-2 is also downregulated by chitosan. Chitosan downregulates the phosphorylation of the JNK pathway. Data are presented as the mean ± standard deviation (SD), **p<0.01 and *p<0.05. The experiments in this figure were repeated three times, and similar results were obtained.

levels of FABP4, adiponectin, C/EBPα, and PPAR-γ in differentiated adipocyte cells (Figure 4A).

Chitosan downregulates AKT phosphorylation in orbital fibroblasts: The P13K/AKT signal pathway exerts an essential role in adipocyte differentiation of orbital fibroblasts. Western blot analyses revealed that chitosan downregulated AKT phosphorylation in a dose-dependent manner during adipocyte differentiation of orbital fibroblasts (Figure 4B).

DISCUSSION

Proinflammatory cytokines activate the clinical progression of Graves ophthalmopathy [15,16]. Anti-inflammatory medicine is used clinically to treat Graves ophthalmopathy. As a natural medicine, chitosan exhibits anti-inflammatory activity in some fibroblasts [6,7]. Chitosan reduced IL-8 production in human nasal fibroblasts [6]. It reduced PGE-2 levels with downregulation of phosphorylation of JNK in gingival fibroblasts [7]. Our study aimed to investigate the anti-inflammatory role of chitosan in orbital fibroblasts. As chitosan shows some antiproliferative activity in other fibroblasts [5,6], the influence on orbital fibroblast activity

by chitosan in different concentrations was evaluated in this study. It was shown that at concentrations of 1.0 and 10 mg/ml chitosan inhibits the proliferation of orbital fibroblasts, although the concentration for clinical use is 20 mg/ml. We chose 0.1 mg/ml chitosan to investigate its anti-inflammatory effects.

IL-1β typically induces orbital fibroblasts to produce proinflammatory cytokines [17]. In this study, the COX-2, PGE-2, and IL-6 protein levels were increased with stimulation by IL-1β, which is in accordance with previous studies [13,18]. IL-6 upregulates the expression of thyroid-stimulating hormone receptor (TSHR) in orbital fibroblasts, which is the main autoantigen of GO [19,20]. IL-6 also induces adipogenesis in orbital fibroblasts to enlarge the orbital volume, which is associated with exophthalmos [21]. COX-2 catalyzes the synthesis of PGE-2 and mediates IL-6 expression in orbital fibroblasts [22]. Chitosan suppressed production of COX-2, PGE-2, and IL-6 induced by IL-1β in orbital fibroblasts of GO.

Signaling pathways related to anti-inflammatory effects of chitosan were reported in previous studies. Chitosan decreases PGE-2 levels through suppression of JNK phosphorylation in gingival fibroblasts [7]. IL-1β activates ERK,

p38, and NF-κB signaling pathways of orbital fibroblasts [23-25]. However, the effect of JNK on inflammation of orbital fibroblasts induced by IL-1β is unclear. The present results showed that an inhibitor of JNK (SP600125) reduced production of IL-6 and PGE-2, which was induced by IL-1β in orbital fibroblasts. Chitosan statistically significantly inhibited JNK phosphorylation, which was induced by IL-1β, indicating that the anti-inflammatory effect of chitosan was probably mediated by the JNK signaling pathway.

Chitosan inhibited adipogenesis in 3T3-L1 adipose cells in a previous study [8]. The 3T3-L1 adipose cells are typically used to simulate adipocyte differentiation in vitro. Chitosan statistically significantly inhibited lipid production and decreased PPAR-γ and C/EBPα expression in 3T3-L1 adipocytes. Chitosan also statistically significantly decreased protein expression of adiponectin, resistin, and leptin in 3T3-L1 adipocytes. In the present study, chitosan also inhibited adipogenesis in orbital fibroblasts. Orbital fibroblasts were cultured in adipocyte differentiation medium. Lipid stained with Oil Red O was decreased statistically significantly by chitosan. The expression of adiponectin and FABP4, which are adipogenic marker proteins of adipocytes, was also decreased by chitosan. PPAR-γ and C/EBPα are the key
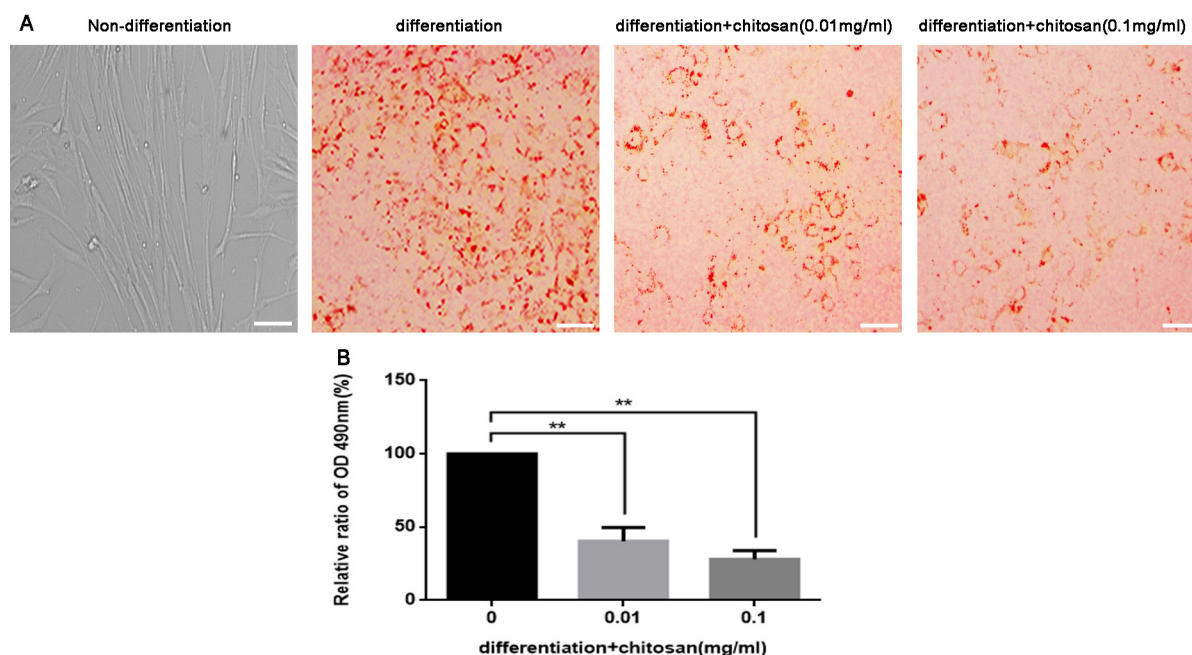

Figure 3. Chitosan inhibits the production of lipid droplets in adipocyte differentiation medium. **A**: Chitosan reduced the quantities of the fat granules dyed by Oil Red O (200X magnification) in a dose-dependent pattern. Oil Red O was dissolved in absolute isopropanol to quantify the lipid droplets. **B**: Chitosan decreased the optical density (OD) values of the dyed cell lysates as shown with the spectrophotometric analyses (490 nm). Magnification = 100X. Scale bar = 50 μm. Data are presented as the mean ± standard deviation (SD), **p<0.01 and *p<0.05. The experiments in this figure were repeated three times, and similar results were obtained.

adipogenic transcription factors. Chitosan decreased expression of C/EBPα and PPAR-γ.

AKT is the key mediator of insulin-like growth factor 1 receptor (IGF1R) signaling, which is critically important for adipogenic differentiation of 3T3-L1 adipocytes [26]. A previous study demonstrated that the P13K/AKT signaling pathway is also involved in the adipogenic differentiation of GO orbital fibroblasts [27]. Thus, protein concentrations of adiponectin and C/EBPα are reduced by downregulation of PI3K activity in the adipogenic differentiation of GO orbital fibroblasts [28]. In this study, chitosan downregulated the phosphorylation of AKT in the adipogenic differentiation of GO orbital fibroblasts. This indicates that chitosan inhibits adipogenesis and related signaling pathways in orbital fibroblasts from Graves ophthalmopathy.

Chitosan has been proven to inhibit oxidative stress in other cell types in recent studies [29-31]. As oxidative stress is involved in the development of GO, determining whether chitosan inhibits oxidative stress in orbital fibroblasts may be important [32,33].

In conclusion, this study demonstrated that chitosan inhibits adipogenesis and inflammation, as well as related signaling pathways, of orbital fibroblasts from GO. The role that chitosan exerts in orbital fibroblasts indicates a possible therapeutic effect on Graves ophthalmopathy.
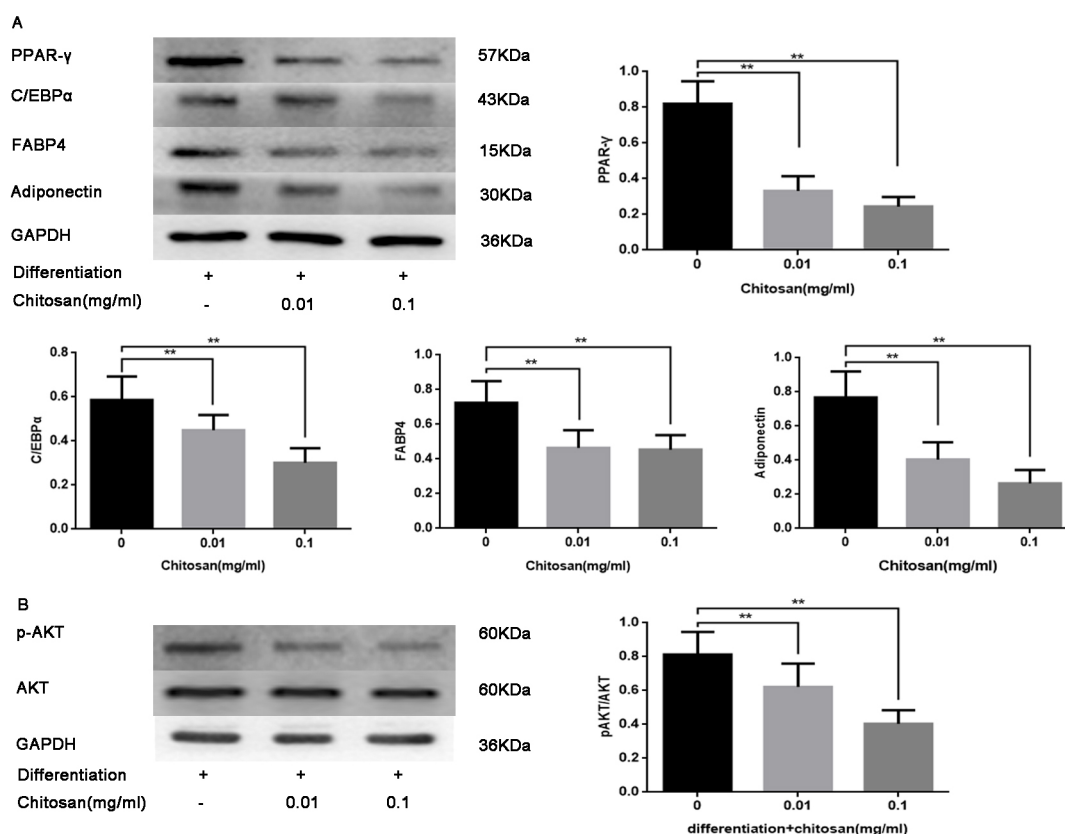
ACKNOWLEDGMENTS

Figure 4. The role exerted by chitosan in adipogenesis of orbital fibroblasts. **A**: Chitosan statistically significantly decreased the protein levels of FABP4, adiponectin, C/EBPα, and PPAR-γ in differentiated adipocyte cells as shown with western blot analysis. The role exerted by chitosan in AKT phosphorylation during the differentiation of adipose cells in orbital fibroblasts. **B**: Chitosan statistically significantly downregulated AKT phosphorylation of the orbital fibroblasts as shown with western blot analyses. Data are presented as the mean ± standard deviation (SD), **p<0.01 and *p<0.05. The experiments in this figure were repeated three times, and similar results were obtained.

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