Research Article

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Isoproterenol reduces the functions of human submandibular gland cells through β adrenergic receptors/gαs pathway

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Background: Human Submandibular Glands (HSG) play a crucial role in the function of secretion. Impairment of the glands would lead to series diseases like Sjögren's Syndrome (SS). However, mechanism of salivary glands secretion function hasn't been clarified completely. The sympathetic process may participate in the process of it as stress is one of the side effects of SS. Stress stimulates the sympathetic neuronal system causing functional changes of HSG. Therefore, our study was designed to investigate the effects of Isoproterenol (ISO) against human submandibular gland cells (HSG cells) and further explore the possible mechanisms concerned.

Methods and Findings: We stimulated the HSG cells by ISO (10^{-6} mol/L). Subsequently, cells morphology we observed. Two indicators, Sulfated Glycosaminoglycans (SGAG) and α-amylase concerning the cells secretion function were detected. Cells apoptosis proportions were also measured. β adrenergic receptors(β AR) and part of associated signaling molecules were measured via western blot. The present study showed that ISO could change the morphology of HSG cells. ISO significantly increased the level of SGAG proportions in HSG cells supernatant and apparently decreased the expression of α-amylase. ISO contributed to HSG cells apoptosis. Expressions of β1 AR,β2 AR and Gαs all significantly decreased. And GRK2 showed an up-regulated level under the exposure of ISO.

Conclusions: Declining functions of human submandibular gland cells induced by isoproterenol. β AR /Gαs signal pathway participates in the impairment of HSG cells.

Keywords: human submandibular gland cell • isoproterenol • α-amylase • β adrenergic receptor • gαs • Sjögren's syndrome

Abbreviations: GPCR: G Protein-Coupled Receptor; GRK2: G protein-coupled Receptor Kinase 2; HPA: Hypothalamic-Pituitary-Adrenal; HSG: Human Submandibular Gland; ISO: Isoproterenol; RA: Rheumatoid Arthritis; SGAG: Sulfated Glycosaminoglycans; SNS: Sympathetic Nervous System; SS: Sjögren's Syndrome; β AR: β Adrenergic Receptors

Introduction

Submandibular glands, parotid glands, and sublingual glands are three types of major salivary glands. They secrete lots of saliva daily, which not only contain various substances to maintain the gastrointestinal tract and oral health, but also have great significance in diseases diagnosis. Saliva play an important role in taste, mastication, swallowing, digestion of starch and the maintenance of teeth [1,2]. Dysfunction of salivary glands, especially HSG, could lead to a lot of oral diseases, such as Sjögren's syndrome, dental caries, dysphagia and oral mucositis. Sjögren's Syndrome (SS) is a prototypic chronic autoimmune disease with the salivary and lachrymal glands dysfunction progressively, leading to dry mouths and dry eyes. The main cause of the symptoms is the diminished saliva secreted by salivary glands [3,4]. Damage of salivary glands can also make the electrolyte transport and the flow of saliva in the mouth stop [5-7]. Human salivary glands that are mainly composed of ductal epithelial cells,acinar cells and myoepithelial

cells. The resting saliva is mainly produced by the submandibular gland cells [8]. Salivary gland epithelial cells are not only the target cells of the immune response but also the effector cells of maintaining the immune response in SS [9]. Thousands of patients suffer from the salivary gland hypofunction over the world in each year. However, there are no effective pharmacological tools for the treatments of salivary dysfunction. And pathophysiology of the salivary glands damage remains to be elucidated.

It was demonstrated that prognosis of autoimmune disease patients with higher stress level is poor [10]. An elevated level of stress could lead to the activation of Hypothalamic-Pituitary-Adrenal (HPA) axis and over excitation of the Sympathetic Nervous System (SNS) [11,12]. On the one hand, HPA axis provides a critical feedback mechanism for stress, but it is passivated in many autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis [13]. On the other hand, dysregulation of the autonomic nervous system could also lead to a functional somatic syndrome that is characterized by many functional symptoms [14]. The salivary gland function is associated with autonomic nervous system function. Taste and chewing induce the secretion of saliva. And secretion of saliva could also be regulated by the activation of SNS under the physiological conditions [8]. However, roles of autonomic nerve effects on human salivary glands haven't been clarified. Whether the pathophysiological mechanism of SS is associated with activation of HPA axis or over excitation of SNS are still unclear.

β adrenergic receptors (β AR) are a type of G Protein-Coupled Receptor (GPCR) that are composed of β1 AR, β2 AR and β3 AR and can be stimulated by autonomic nervous system or circulating catecholamine to regulate wide biological effects, such as cardiovascular activity, carbohydrate metabolis [15]. The classical signaling pathways that β AR participated in have played pivotal and common roles in many physiological activitise. Male Wistar rats submandibular cells responded to β AR stimuli [16]. The activation of β ARs in human submandibular gland induces secretory granules and cell membrane fusion, which in turn affects glandular secretion [17]. Increased activity of β2 AR/Gαs signaling pathways protects myocardium from Stress-induced cardiomyopathy effects [18]. Isoproterenol (ISO), a kind of non-selective β AR agonist, has been widely regarded as a tool of β AR to verify the mechanism of β AR signaling in cell immune response concerned with many areas like cardiomyocytes, adipocytes and so on. Low level of the concentration would trigger off-target effects [19]. Non-Obese Diabetic (NOD) mice, a type of spontaneous SS animal model, β AR density appeared to decline after the stimulus of ISO [20]. C-reactive protein in the rat serum that triggered the inflammatory responses has been demonstrated the increased level after injection of ISO [21]. ISO could influence the cardiomyocytes and induce the cells apoptosis [22].

In this research, ISO was used to study the potential pathogenesis concerned with HSG cells damage. Our research aimed to explore whether β AR/Gαs signal pathway participates in the process of HSG cells dysfunction induced by ISO and clarifies the relationship between SNS and dry or gland damage.

Materials and Methods

Materials

Human submandibular gland cells were purchased from Tong Pai Technology in China. Isoproterenol was obtained from Hefeng Pharmaceutical LTD (China). β1AR antagonist CGP20712A (119K4600v) and β2 AR antagonist ICI118551 (102M4619v) were provided by Sigma company (USA). High glucose of DMEM is from Multicell. Fetal bovine serum (FBS) was purchased from Clerk company. Chondroitin sulfate of shark was obtained from Qingdao Green-extract Biology Science (China), and 1,9-Dimethylmethylene Blue zinc chloride double salt (DMMB) Assay (341088-1G) is from Sigma-Aldrich (USA). Annexin V-FITC/PI Apoptosis Detection Kit is from Vazyme Biotech (China). Penicillin/streptomycin (100×), 0.25% trypsin, goat anti-mouse IgG/HRP (ZB-2305), goat anti-rabbit IgG/HRP (ZB-2301), RIPA (P00138), PMSF (ST 506-2), DAPI (C1005) were all purchased from Beyotime Technology (USA). Bull Serum Albumin (A8002) was purchased from Solarbio Life Sciences in China. Anti-β1 AR antibody (ab3442), anti-β 2 AR antibody (ab36956), anti-Gαs antibody (ab83735), anti-Salivary α-amylase antibody (ab201450) were all purchased from Abcam system (USA). Anti-GRK2 antibody (C-15) is from Santa Cruz Biotechnology (USA). Alexa Fluor 594-conjugated goat anti-rabbit IgG(H+L) (SA00006-4) was provided by Protein Tech Group (USA).

Cell lines culture and treatments

HSG cells, a kind of originated from irradiated human sub maxillary neoplastic intercalated duct cell lines of glands [23], were maintained in high glucose DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin lines. Medium was replaced 2 to 3 times a week, and 0.25% trypsin was used to change the density of the cells. ISO was used as stimuli and at the same

time, two antagonists CGP20712A and ICI118551 were used for treating the cells. The concentrations of ISO, CGP20712A, and ICI118551 were all 10^{-6} mol/L. When the stimuli were exerted; all the cells were cultured with 5% High glucose of DMEM. All conditions of the cells were incubated in 37℃ 5% CO_2.

Cells morphology observed by microscope

Cells were inoculated for 5×10^4 per well in 6-well plate. ISO was added to the well for 48 hours treatment. Morphological changes of HSG cells were observed through microscope.

SGAG measured by DMMB dye-binding assay

Preparation of standard substance: 20 mg chondroitin sulfate of shark was weighed and dissolved in 10 ml Phosphate Buffer Saline (PBS) at room temperature. Then, diluted the solution into 200, 160, 100, 80, 40, 20, 10, 5, 2.5 μg/ml in sequence. The cells were treated for 48 hours in a 6-well plate and the supernatant was collected after centrifugation at 2000 rpm for 5 minutes. The cells were lysed on ice for 30 minutes by Radio Immuno Precipitation Assay (RIPA) with 1% Phenylmethanesulfonylfluoride (PMSF) in it to extract the protein. The solution was centrifuged at 14000 rpm at 4°C for 10 minutes. Both the supernatant and the protein were stored at -80°C. 10 μl standard substance or the sample were added in 96-well plate, and then 200 μl DMMB was aspirated into every well. The absorbance was measured by Multiskan Spectrum at 525 nm wave length after 1 minute. The concentration was worked out, and the result was presented as the proportion of the supernatant in total SGAG.

α-Amylase level measured by laser scanning confocal microscopy

Adjusted the cells density to 2×10^5/ml and add 500 μl to each well with a sterile coverslip in the 24-well plate. After 48 hours of treatment, solved the cells as followed: fixed the cells with 4% paraformaldehyde for 30 minutes; permeated the cells with 0.5% Triton X-100 for 10 minutes; block the nonspecific protein with Bull Serum Albumin(BSA) or 30 minutes; incubated the anti-α-amylase antibody at the ratio of 1:900 at 4°C for a whole night; incubated the Alexa Fluor 594-conjugated goat anti-rabbit IgG (H+L) at the ratio of 1:150 at 37°C for an hour and then DAPI for 8 minutes; cover the coverslip with Fluorescence quenching agent to the glass slide. Observe the protein expression under Laser Scanning Confocal Microscopy (LSCM, Leica SP8, Germany).

Cells apoptosis proportion detected by Annexin V-FITC/PI apoptosis detection kit

Briefly, inoculated the cells for 2.5×10^5 per well in 6-well plate and the method of treatment was as mentioned. After 48 hours, transferred the cells to ep tubes using 0.25% trypsin and added binding buffer 100 μl, PI 5 μl, ANNEXIN V5 μl per tube for 10 minutes and finally added the binding buffer 200 μl to each tube. The results were measured by CytoFLEX (Beckman Coulter, USA).

Expressions of β1 AR, β2 AR, Gαs, GRK2 measured by Western blot

HSG cells were cultured and treated with the same condition in 6-well plate. After 48 hours of treatment, the cells were lysed, and the protein were extracted with the same methods as the depiction above. The protein was boiled with loading buffer (5×) at the ratio of 4:1 for 10 minutes and stored at -80°C. The methods are as followed: The protein was separated by 10% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to the Polyvinylidene Fluoride (PVDF) membranes; the membranes were blocked by 5% skim milk powder in Tween PBS; the primary antibodies (1:500) were incubated at 4°C for a whole night; the secondary antibodies were incubated at 37°C for 2 hours; the images were detected by Enhanced Chemiluminescence (ECL) regents after exposure of filters to films. The density of the bands was analyzed by the Microsoft (Image J, Broken Symmetry Software).

Statistical analysis

Data were analyzed by SPSS 16.0 software and performed with GraphPad Prism 5.0 software. The results were presented as mean ± SEM for at least 3 times experiments. All p valued less than 0.5 were considered significantly different from the control groups.

Results

Morphological features changed under exposure of ISO

HSG cells of Control group showed as spindly or fusiform shape and the edges of cells are clear. After treatment of ISO for 48 hours, the cells presented as polygonal shape or became shorter and the edges of the cells became indistinct (Figure 1).

Proteoglycans metabolism of HSG cells was disordered induced by ISO

Compared with the Control group, the proportion of SGAG in ISO group increased after 48 hours treatment. Besides, the level of ICI118551 group was decreased compared to the ISO group after 48 h

treatment of β2 AR antagonist. However, there was no significance between CGP20712A and the ISO group. Proteoglycans metabolism was disordered induced by ISO. After treatment of ICI118551, the level of SGAG outside the cells decreased. Proportions of SGAG represented for the concentration of the supernatant in the total concentration (Table 1).

α-Amylase expressed in the cells decreased under the exposure of ISO

Results have confirmed the reality that ISO could lead to the damage of HSG cells through decreasing the level of α-amylase expression in the cells compared to the Control group. Compared with the ISO group, β2 AR antagonist ICI reversed the effect by increasing the α-amylase level. But the effect of β1 AR antagonist CGP20712A on the cells didn't show any significance after 48 hours treatment (Figure 2).

Levels of apoptotic HSG cells decreased after sustained induction of ISO

The results showed that the proportion of apoptotic cells induced by ISO significantly increased compared to the Control group. β2 AR antagonist ICI118551 significantly decreased the level of HSG cells apoptosis. It may suggest that β2 AR might be involved in the process of HSG cells apoptosis (Figure 3).

Table 1. The proportion of SGAG (%) in the cell supernatant (mean ± SEM, n=5 per group).

Group	SGAG (%)
Control	2.5825 ± 0.6224
ISO	5.143 ± 0.7475*
ISO+CGP20712A	4.8079 ± 1.9100
ISO+ICI118551	3.504 ± 0.2425#

*p<0.05 versus Control group; # p<0.05 versus the ISO group

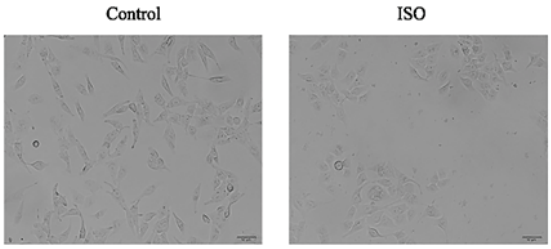
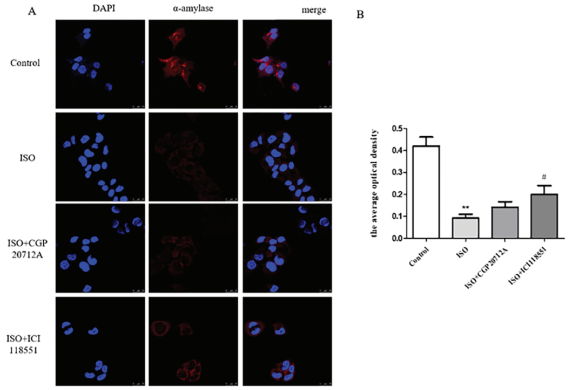
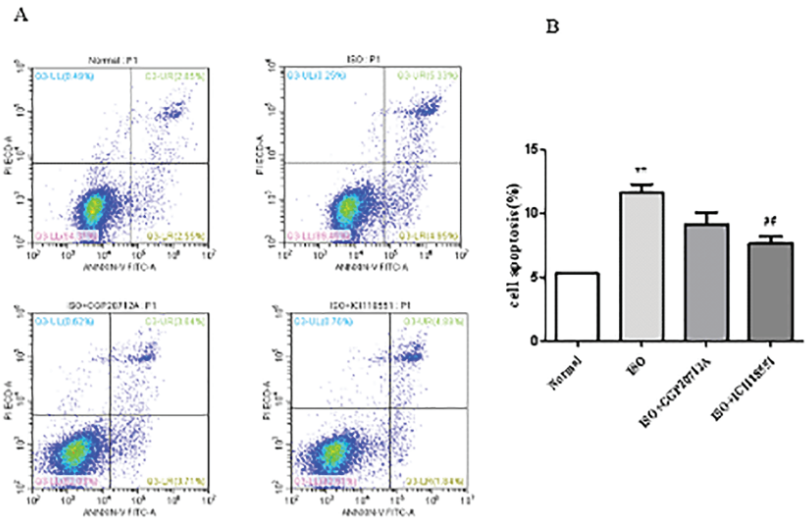


Figure 1. Morphological features changed under exposure of ISO.



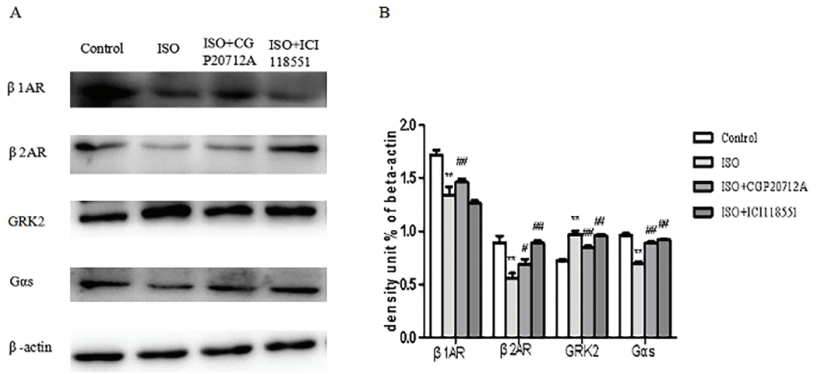
(A) The red area is the expression of α-amylase and was stained by Alexa Fluor 594-conjugated goat anti-rabbit IgG(H+L) with the cell nucleus stained by DAPI. The average optical density was measured by Image J. (B) Densitometry analysis of the images and data are presented as mean ± SEM(n=4). **p＜0.01 versus Normal group; # p＜0.05 versus ISO group.

Figure 2. Expression of α-amylase in the cytoplasm of HSG cells on the effect of ISO.



(A)The images were captured by CytoFLEX. Cells in Q3-LR area represent for apoptotic cells in the early phase and cells in Q3-UR area represent for the late apoptotic cells. The apoptotic cells proportions contain both of the cells in Q3-LR and Q3-UR areas. (B) Data analysis of the apoptotic cells proportion are presented as mean ± SEM (n=4). *p＜0.05 versus Normal group; #p＜0.05 versus ISO group.

Figure 3. Influences on HSG cells apoptosis proportion and viability induced by ISO.



(A)Images of β AR and associated signaling molecules detected by ECL (B) Band analysis of density in every indicators and data are presented as mean ± SEM(n=4). **p<0.01 versus Normal group; # p<0.05 versus ISO group; ## p<0.01 versus ISO group

Figure 4. Expression of β AR and associated signaling molecules induced by ISO.

Expression levels of β AR and associated signaling molecules decreased under the suppression of ISO

After 48 hours of ISO (1×10^{-6} mol/L) treatment, expression levels of both β 1AR and β2 AR declined compared to the Control group. The β 1AR antagonists CGP20712A and β 2AR antagonists ICI118551 showed opposite effects on HSG cells by increasing β AR expression. Meanwhile, GRK2 statistically increased in the ISO group compared to the Control group. CGP20712A and ICI118551 could both reverse the effects of ISO on HSG cells. Low expression of Gαs was demonstrated in the ISO group compared to the Control group; CGP20712A and ICI118551 might increase its expression (Figure 4).

Discussion

The secretion of salivary glands is essential for maintaining oral health and high quality of life. Except for water and some inorganic substances like metal ions, an organic substance in saliva. There are a lot of proteins consisting of oral defense system, such as lysozyme, α-amylase, mucins, lactoperoxidase, immunoglobulin, agglutinin, and others [1]. Mucins, secreted by salivary glands and distributed on the surface of the mucosa, are a type of proteoglycans and may be constituted by proteins and Sulfated Glycosaminoglycans (SGAG). As an ingredient of saliva, mucins play a role in the oral cavity such as lubrication, taste, clearance of bacteria or microorganisms [24,25]. SGAG is important in forming proteoglycans, and the metabolism has been demonstrated to be disordered in Osteoarthritis (OA) [26]. Elevated levels of SGAG in the cell supernatant suggest that the metabolism of proteoglycan in OA chondrocytes is in disorder. It also exists in saliva so that we hypothesized whether it is disordered in the damaged HSG cells. Proteoglycans metabolize into SGAG and release to the extracellular, leading to a significant increase level of SGAG extracellular. In our research, SGAG proportions of the supernatant were demonstrated an increasing level of the induction of ISO, and the effect was resisted by β 2AR antagonists ICI118551. It indicate that β2 AR may participate in secretion function of mucins.

α-Amylase is one of the important indicators to measure the function of salivary glands. In our research, the expression levels of α-amylase were demonstrated to decrease under the effects of ISO, and the effect can also be inhibited by β2AR antagonist ICI118551. The activity of α-amylase was tested to be declined in SS patients and animal models which make asitia developed [27]. It may suggest that both the activity and expression level are decrease in SS.

As for changes of HSG cells morphology, ISO changed the shape of the cell and made the cell edges blurred. The intuitive result showed that HSG cell was impaired under the exposure of ISO.

On the basis changes above, HSG cells apoptosis was measured. In our research, apoptotic cell proportions slightly elevated with the exposure of ISO and β2AR antagonist ICI118551 reversed the effects. As was known to us, apoptosis happens in many tissues in the balanced physiological context and diseases would develop if the cells lose the ability of apoptosis [28]. HSG cells apoptosis proportion was high under the stimulation of bleomycin probably through Akt/mTOR signaling pathway [29]. Many studies reported that deeper mechanisms might be involved in caspases-3 protease activity and Bax/Bcl-2 in cells apoptosis [22]. So, we hypothesize that β2 AR may be involved in the cells apoptosis through regulating caspases-3 protease

activity and Bax/Bcl-2 to play its role. The result of the increased HSG cell apoptosis levels is in accordance with the secreting functions of the impaired cells.

The above indicators suggest that functions impair probably through β AR and associated signal pathway, especially β2 AR. So we measured these indicators by western blot. Results of western blot suggest that β1 AR, β2 AR and Gαs expression all decreased induced by ISO for 48 hours. Cells with β AR antagonists presented the amelioration of all indicators compared with ISO group except for β1 AR. These results may indicate that the role of β1 AR is not so crucial in ISO-induced HSG cells. As was reported before, β1 AR and β2 AR present different roles in the heart, which β1 AR mainly promote the cells apoptosis but β2 AR has the opposite or weak effect [30,31]. Gαs protein is also involved in the process of β AR signaling pathway by activating Adenylate Cyclase (AC) to generate a series effects downstream [32]. The isoproterenol-treated rats had increased the salivary secretion of protein. However in rat parotid and submandibular glands, ISO didn't evoke obvious alterations on salivary flow and total protein secretion [33,34]. The sensitivity of different cells to ISO is not the same. High concentration of ISO (about 10^{-6} mol/L) can induce the change of Gαs, but it doesn't happen in the case of low concentration in mouse embryonic fibroblasts [35]. In AA rat model, with the agonist touching β AR, the heterotrimeric G protein would be activated. Simultaneously, G protein would be phosphorylated by G protein-coupled receptor kinase 2 (GRK2) to inhibit the coupling, and then GRK2 would translocate to the membrane to make the receptors desensitization and internalization [36]. With ISO combined with β AR, promoting the activation of G proteins and dissociating into Gαs and Gβγ submits. Subsequently, Gαs activate adenylate cyclase and the level of second messenger cyclic Adenosine Monophosphate (cAMP) was increased. In this process, GRK2 may also contribute to the impairment of HSG cells. β AR could be phosphorylated by GRK2 to inhibit further coupling, namely, receptors desensitization [37]. As was reported before, GRK2 was a high expression in many Collagen Induced-Arthritis (CIA) or Adjuvant-induced Arthritis (AA) rat model [38,39]. In Rheumatoid Arthritis (RA), GRK2 acts as an inhibitor in regulating pathway through phosphorylating the receptors to contribute to the combination with another negative regulator β-arrestin [40]. On the basis above, we hypothesize that negative regulation of GRK2 can be used as one of the targets for drug therapy.

In our body, activation of sympathetic nerve would lead to a series of symptoms, such as rapid heart rate, vasoconstriction, bronchial smooth muscle relaxation, diuretic muscle relaxation, sphincter contraction, an elevated level of the adrenal medulla and (the like). Besides, sympathetic nerve stimulation of salivary glands would result in the production of saliva with a high concentration of protein, and parasympathetic nerves excitement would make the salivary glands produce the saliva which is poor in protein [41,42]. Those changes will happen if the organs are in the physiological condition. However, continuous stimulation of the sympathetic nerve, such as β AR agonist ISO, may be able to inversely act on the salivary glands by reducing the saliva. In our research, sustained effects for 48 hours of ISO in high concentration may impair the cells through decreasing the sensitivity of sympathetic nerve system. To a large extent, in the salivary glands, impairment would also happen in the same condition.

Conclusion

Our research provided evidence that ISO can impair the function of HSG cells. β AR/Gαs pathway may be involved in the mechanism of HSG impairment as the expression of β1 AR, β2 AR, Gαs all decreased after ISO stimulated 48 hours. It helps to provide a new envisage to treat many oral diseases like SS.

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W.W. and H.W. contributed to the design of the study, served as the study coordinators and edited the manuscript. Q.Z, S.X.and P.Z designed the study, performed experiments, collected and analyzed the data and wrote the manuscript. Performed experiments and collected and analyzed the data. Q.L, X.C and F.G. helped perform experiments and analyzed data. All authors have read and approved the final manuscript.

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Declaration of competing interest

The authors declare no fifinancial or commercial conflflicts of interest.

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