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Original Article

The effect of the α7nAChR agonist on Wnt/β-catenin signaling in osteoporosis

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Abstract: The cholinergic pathway neurotransmitter acetylcholine (ACh) regulates the inflammatory cascade through a specific α7 nicotinic acetylcholine receptor (α7nAChR). However, the role and related mechanisms of α7nAChR in osteoporosis (OP) remain unclear. Therefore, this study aims to analyze the effects of α7nAChR on osteoblasts and related mechanisms. Mouse osteoblast MC3T3-E1 was cultured in vitro and divided into a control group and an α7nAChR agonist group (2.4 and 4.8 mg/kg.d). An MTT assay was used to detect the osteoblast activity, an ARS staining assay was used to analyze the formation of calcified nodules of osteoblasts, and an alkaline phosphatase (ALP) activity colorimetric assay was used to determine the ALP activity. Real-time PCR was performed to analyze the expression of RUNX2 and OPN mRNA. The inflammatory factor tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) secretions were analyzed by ELISA. The α7nAChR agonists dose-dependently promoted osteoblast proliferation, increased calcified nodules, ALP activity, RUNX2 and OPN mRNA expression, decreased inflammatory factors TNF-α and IL-6 secretion, and increased Wnt1, β-catenin mRNA and protein expression. Compared with the control group, the differences were statistically significant ($P<0.05$). α7nAChR agonists can inhibit the proliferation and differentiation of osteoblasts by regulating the Wnt/β-catenin signaling pathway, and then participate in the regulation of osteoporosis.

Keywords: Osteoporosis, α7nAChR agonist, osteoblast, proliferation, Wnt/β-catenin signaling pathway

Introduction

Osteoporosis (OP) is a systemic bone metabolic disease characterized by reduced bone mass, the degeneration of bone tissue, decreased bone strength, decreased bone toughness, and increased fragility. It is a common disease in orthopedics [1, 2]. As the population ages, the occurrence of OP is increasing [3]. Among the elderly, the incidence rate of OP is high, and the damage is severe, which seriously affects patients' quality of life. Data show that OP has the highest incidence rates in Asia and Latin America. OP patients in China account for 6.6% of the total population. By 2025, the prevalence rate could reach 13.3%. Therefore, the early prevention and treatment of OP have a significant clinical and social significance [4, 5]. There are many pathogenic factors for OP, such as age, glucocorticoids and other drugs, and menopause [6]. OP not only causes fractures and other injuries, but it also causes severe oral problems such as gingival recession, alveolar bone resorption, and root exposure, resulting in a serious decline in patients' quality of life [7, 8]. Although the clinical use of symptomatic methods such as hormone supplementation calcium supplementation, and the inhibition of bone resorption, the poor efficacy of the drugs, malabsorption, and the drug side effects have led to the poor efficacy of OP treatment [9]. Senile osteoporosis is an important manifestation of the natural decline and aging of the human body. The main pathological change is the slowing of bone loss and bone turnover. Osteoblasts have a bone formation function, osteoclasts have a bone resorption function, and the dynamic balance between the two is the key to maintaining normal bone mass [10]. Osteoblasts play a more important role in the pathogenesis of osteoporosis. The proliferation of osteoblasts in the elderly is significantly reduced, with a reduced ability to regulate osteoclasts, and a significantly degraded bone remodeling function, resulting in the low transition state of bone.

In recent years, with the development of immunology, some inflammatory factors have been found to be involved in the pathogenesis of OP.

The cholinergic anti-inflammatory pathway has been a research hotspot in the regulation of inflammation over the past decade. It embodies the regulation of the systemic inflammatory response by the nervous system [13, 14]. The vagal neurotransmitter ACh regulates the inflammatory cascade through a specific α7 nicotinic acetylcholine receptor (a7nAChR), thereby ameliorating the systemic inflammatory response [15, 16]. However, the role and related mechanisms of α7nAChR in OP have not been fully elucidated. Therefore, this study aims to analyze the effect of α7nAChR on osteoblasts and related mechanisms, and then provide a reference for its regulation of osteoblasts involved in the OP process.

Materials and methods

Main reagents and instruments

The α7nAChR agonist PNU282987 (SF9020-5 mg) was purchased from Shanghai Biyuntian Biological Co., Ltd. Mouse osteoblast MC3T3-E1 was preserved in our laboratory and stored in liquid nitrogen. Western blot related chemical reagents were purchased from Shanghai Biyuntian Biotechnology Co., Ltd., ECL reagents were purchased from Amersham Biosciences, rabbit anti-mouse Wnt1 monoclonal antibody, rabbit anti-mouse β-catenin monoclonal antibody, goat anti-rabbit horseradish peroxidase (HRP) The labeled IgG secondary antibody was purchased from Cell signaling Corporation of the United States. The RNA extraction kit and the reverse transcription kit were purchased from Axygen (USA). Alpha-MEM complete medium, fetal bovine serum (FBS), cyan, and chain double antibody were purchased from Gibco (USA). Dimethyl sulfoxide (DMSO), MTT powder was purchased from Gibco (USA). Dexamethasone, ascorbic acid, oil red O, β-sodium phosphite, and trypsin were purchased from Sigma (USA). The ALP active colorimetric quantitative detection kit was purchased from Nanjing (China). TNF-α and IL-6 ELISA kits were purchased from R&D (USA). Other commonly used reagents were purchased from Shanghai Shenggong Biological Co., Ltd. The ABI 7700 Fast Quantitative PCR Reactor was purchased from ABI (USA). The Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad Corporation of the United States. The clean workbench was purchased from Suzhou Purification Equipment Factory in Jiangsu Province.

Osteoblast MC3T3-E1 culture

Mouse osteoblast MC3T3-E1 in α-MEM medium containing 10% FBS (100 mg/l streptomycin, 100 U/ml penicillin, 2 mmol/l glutamine, 10 mmol/L β-phosphoric acid sodium phosphate, 50 μg/ml ascorbic acid) was cultured, and the 2nd-4th generation cells in the logarithmic growth phase were used for the experiments. The cultured MC3T3-E1 cells were randomly divided into 3 groups, including a control group and a high-low dose (4.8 mg/kg.d and 2.4 mg/kg.d) α7nAChR agonist group.

Growth of MC3T3-E1 cells by MTT assay

The log phase growth MC3T3-E1 cells were digested and counted and seeded in 96-well plates at 3000 cells/well. According to the above group treatment, each group was treated for 72 hours, and each group was designed with 5 replicate wells, and a concentration of 20 μl. 5 g/L MTT solution was added into each well followed by continual culture in the incubator for 4 h. After the complete removal of the supernatant, a DMSO 150 μl/well was added and followed by shaking for 10 min until the purple crystals were fully dissolved. Then, the absorbance (Absorbance, A) value was measured at a wavelength of 570 nm using a microplate reader.

Real-time PCR detection of RUNX2 and OPN expression in MC3T3-E1 cells

Total RNA was extracted with Trizol reagent, and DNA reverse transcription synthesis was performed according to the kit's instructions. The primers were designed by Primerprmier 6.0 according to each gene sequence and synthesized by Shanghai Yingjun Biotechnology Co., Ltd. (**Table 1**). Real-time PCR was performed to detect the expressions of the target genes under the following conditions: 55°C 1 min, 92°C 30 S, 58°C 45 S, 72°C 35 S, for a total of 35 cycles. GAPDH was used as a reference. According to the fluorescence quantification, the starting cycle numbers (CT) of all the samples and standards were calculated. Based on the standard CT value, a standard curve was drawn, and then the semi-quantitative analysis was carried out using the $2^{-\Delta Ct}$ method.

Determination of ALP content

After the cells reached a confluence of 70%, an osteogenic induction medium was added. The ALP content was determined according to the instructions of the ALP test kit. The cell suspen-

Table 1. Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AGTACTCCAGTGTTGCTGG	TAACGGTAGACCATGTCTGGT
Wnt1	CCAGGTAGACCATGATGT	ACGCCAGGTAGATACTCCATGC
β-Catenin	TGTGATCGACTGTCACTG	TACACTGACGTAGCGCTAA
OPN	CGTAGTGTGCTAATCGAC	TGATGACTGTAGCTGGCT
RUNX2	CTCCAGTACACTGCTCAAG	GCACTTGACGTTGTACGGATT

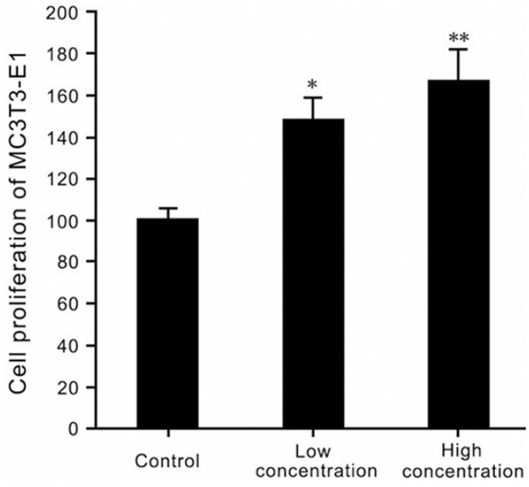

Figure 1. The effects of the α7nAChR agonist on the proliferation of MC3T3-E1 cells. *P<0.05 compared with the control group; **P<0.01 compared with the low concentration group.

sion was collected, centrifuged at 1000 rpm for 10 min, the supernatant was discarded, and the cells were collected by adding Triton-X100. After the cells were mixed, the optical density (OD) values were measured at 520 nm, and the ALP content was calculated.

ARS staining analysis of the formation of calcified nodules

After fixing the cells with 70% ethanol for 1 h, the cells were washed 3 times with double distilled water, and the ARS solution was stained for 10 min, washed three times with PBS, dried naturally, and sealed. Under the microscope, the purple-red calcium nodules were observed in the cells. A quantitative analysis of the alizarin red staining was performed by measuring the OD value at a wavelength of 570 nm using a microplate reader.

ELISA detection of TNF-α and IL-6 secretion

The TNF-α and IL-6 changes in the supernatant of each group were determined using an ELISA kit. The experimental procedure was followed according to the ELISA kit's instructions. The linear regression equation of the standard curve was calculated according to the concentration of the standard product and the corresponding OD value, and the corresponding sample concentration was calculated on the regression equation according to the OD value of the sample.

Western blot analysis of the Wnt/β-catenin protein expression

The total protein of each group was extracted using a RIPA lysis buffer and quantified using a BCA assay followed by being separated on 10% SDS-PAGE, transferred to a PVDF membrane, blocked for 1 h, and incubated with primary antibodies against Wnt, β-catenin (diluted concentrations: 1:1000, 1:1000) at 4°C overnight. After PBST washing, 1:2000 diluted HRP-conjugated goat anti-rabbit secondary antibody was added and incubated for 30 min followed by washing the membrane with PBST. After that, chemiluminescence was added for 1 min and X-ray exposure imaging was performed. X-film and strip density measurements were separately scanned using protein image processing system software and Quantity One software. The experiment was repeated four times (n=4).

Statistical analysis

All data were processed using SPSS 16.0 software. The measurement data were expressed as the mean ± standard deviation (SD). The two groups were compared using Student's *t*-test test. Differences between groups were analyzed using analysis of variance (ANOVA). P< 0.05 was considered statistically significant.

Results

The effect of the α7nAChR agonist on the proliferation of MC3T3-E1 cells

The effect of the α7nAChR agonist PNU282987 on the proliferation of MC3T3-E1 cells was determined using an MTT assay. The α7nAChR agonist significantly promoted the proliferation of osteoblasts, compared with the control group (P<0.05), and the high concentration group promoted the proliferation of MC3T3-E1 cells more significantly (P<0.01) (**Figure 1**).

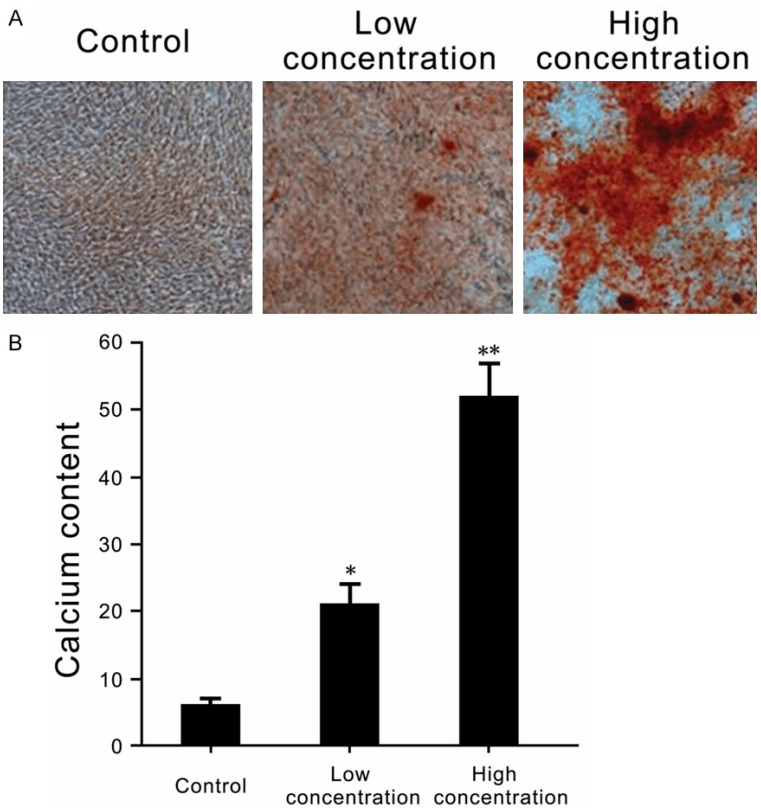

Figure 2. The effect of the α7nAChR agonist on the formation of calcified nodules in MC3T3-E1 cells. A. ARS staining analysis of the effect of the α7nAChR agonist on the formation of calcified nodules in MC3T3-E1 cells (×10); B. Calcified nodule formation quantitative analysis. Compared with the control group, *P<0.05; compared with the low concentration group, **P<0.01.

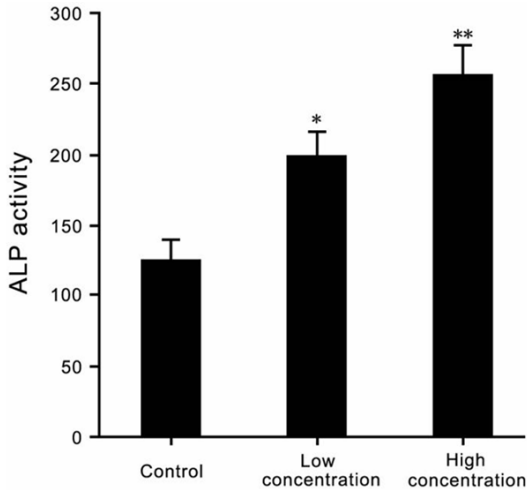

Figure 3. Analysis of the ALP activity of MC3T3-E1 cells by the α7nAChR agonist. *P<0.05 compared with the control group; **P<0.01 compared with the low concentration group.

The effect of the 2α7nAChR agonist on the formation of calcified nodules

An ARS staining assay was performed to analyze the effects of the α7nAChR agonist PNU-282987 on the formation of calcified nodules in MC3T3-E1 osteoblasts. The α7nAChR agonist significantly promoted an increase of calcified nodules in the MC3T3-E1 osteoblasts, compared with the control group (P<0.05), and the high concentration group promoted the formation of calcified nodules in the MC3T3-E1 cells more significantly (P<0.01) (**Figure 2**).

Analysis of the ALP activity of the MC3T3-E1 cells by the α7nAChR agonist

The ALP activity colorimetric assay was used to analyze the changes in the ALP activity of the MC3T3-E1 osteoblasts by the addition of the α7nAChR agonist PNU282987. The α7nAChR agonist significantly promoted an increase of ALP activity in the MC3T3-E1 osteoblasts, compared with the control group (P<0.05), and the high concentration group promoted the ALP activity of MC3T3-E1 cells more significantly (P<0.01) (**Figure 3**).

The effect of the α7nAChR agonist on the expressions of RUNX2 and OPN in MC3T3-E1 cells

A real-time PCR analysis of the effect of the α7nAChR agonist PNU282987 on the expressions of RUNX2 and OPN showed that the α7nAChR agonist significantly promoted the expressions of RUNX2 and OPN in MC3T3-E1 osteoblasts, compared with the control group (P<0.05), and the high concentration group promoted the expressions of RUNX2 and OPN in the MC3T3-E1 cells more significantly (P< 0.01) (**Figure 4**).

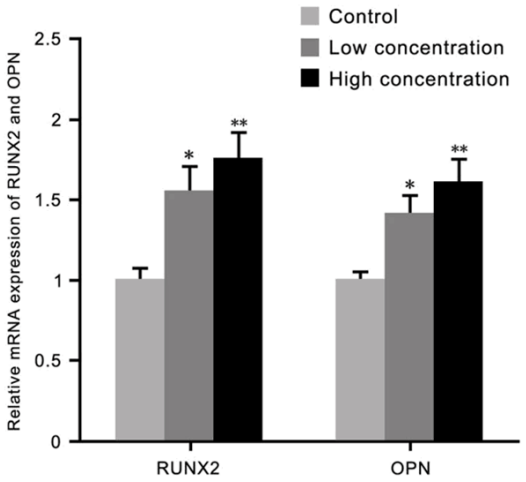

Figure 4. The effect of the α7nAChR agonist on the expression of RUNX2 and OPN in MC3T3-E1 cells. *P<0.05 compared with the control group; **P<0.01 compared with the low concentration group.

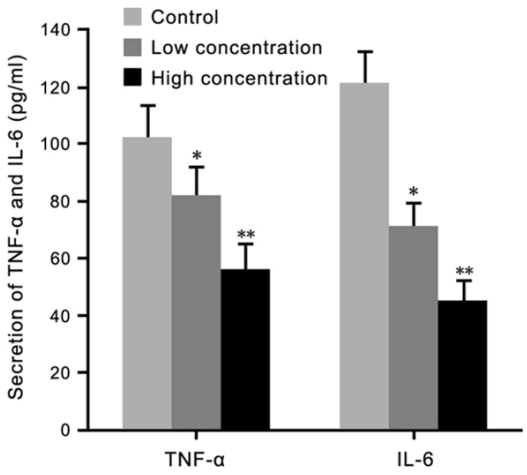

Figure 5. The effect of the α7nAChR agonist on the secretion of inflammatory factors in the MC3T3-E1 cells. *P<0.05 compared with the control group; **P<0.01 compared with the low concentration group.

The effects of the α7nAChR agonist on the secretions of TNF-α and IL-6

The effects of the α7nAChR agonist PNU282-987 on the secretions of the MC3T3-E1 osteoblast inflammatory factors TNF-α and IL-6 were analyzed using an ELISA assay. The α7nAChR agonist significantly inhibited the secretion of the inflammatory factors TNF-α and IL-6 in the MC3T3-E1 osteoblasts, compared with the control group (P<0.05), and in the high concentration group on MC3T3-E1 cell inflammation. The inhibitory effects of the TNF-α and IL-6 factor secretions were more significant (P<0.01) (**Figure 5**).

The effect of the α7nAChR agonist on Wnt/β-catenin signaling in MC3T3-E1 cells

Real-time PCR and Western blot analyses of the effect of the α7nAChR agonist PNU282987 on Wnt/β-catenin signaling showed that the α7nAChR agonist significantly promoted the expressions of Wnt1, β-catenin mRNA, and protein in the MC3T3-E1 cells, compared with control group (P<0.05), and in the high concentration group, the MC3T3-E1 cells Wnt/β-catenin signal pathway changes were more significant (P<0.01) (**Figure 6**).

Discussion

Osteoblasts not only participate in bone formation, but they also participate in the regulation of osteoclast bone resorption, inhibit osteoclast differentiation into mature multinucleated osteoclasts, and promote their apoptosis and lose their bone-breaking function, so that bone resorption can be inhibited [17]. With age, the differentiation ability of bone marrow mesenchymal stem cells (BMSCs) is gradually reduced, that is, BMSCs tend to differentiate into adipocytes, and the differentiation into osteoblasts is reduced. Studies have shown that there are significantly more fat cells in the bone marrow of elderly patients with OP. It can be seen that osteoblasts play a more important role in the pathogenesis of OP [18, 19]. Therefore, it is of great significance to study OP from the aspects of osteoblast proliferation, differentiation, and signal transduction.

The cholinergic anti-inflammatory pathway not only inhibits the production and release of the pro-inflammatory cytokines TNF-α and IL-6, but it also inhibits the production and release of high mobility histone 1 (HMGB1). Studies have confirmed that vagal stimulation can reduce the blood levels of inflammatory cytokines such as TNF-α and IL-6 and HMGB1 during the inflammatory response [20]. An increased secretion of inflammatory factors leads to increased osteoclasts and bone resorption [21]. The α7nAChR agonist has a positive effect on estrogen receptors, which may regulate the OP [22]. However, the role of α7nAChR agonists in OP has not been elucidated. Therefore, this study intends to analyze the effects of α7nAChR agonists on osteoblasts that play a key role in OP,

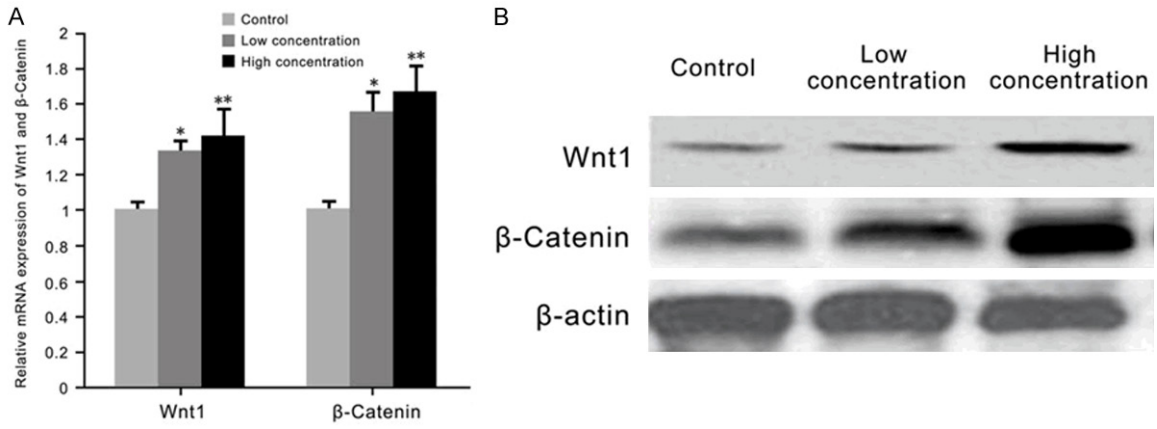

Figure 6. The effect of the α7nAChR agonist on the Wnt/β-catenin signaling pathway in MC3T3-E1 cells. A. Real-time PCR analysis of the effect of the α7nAChR agonist PNU282987 on the Wnt/β-catenin signaling pathway in MC3T3-E1 osteoblasts, compared with the control group, *P<0.05; compared with the low concentration group, **P<0.01; B. Western blot analysis of the effect of the α7nAChR agonist PNU282987 on the Wnt/β-catenin signaling pathway in MC3T3-E1 osteoblasts.

in order to uncover the possible role of the α7nAChR agonists in osteoporosis. The a7nAChR agonists are ACh, nicotine, CNI-1493, GTS-21, and PNU282987. However, since ACh can be rapidly hydrolyzed by acetylcholinesterase, nicotine cannot be used clinically due to its serious, toxic side effects, but PNU282987 is a recently developed highly selective a7nAChR agonist [23]. Therefore, this study selected PNU282987 for related research. The results of this study confirmed that α7nAChR agonists can promote osteoblast proliferation, increase calcified nodules, ALP activity, osteogenesis-related genes RUNX2 and OPN mRNA, and decrease inflammatory factor TNF-α and IL-6 secretions. As the effect is dose-dependent, the results suggest that the α7nAChR agonist can stimulate osteoblast proliferation and differentiation by inhibiting the secretions of the inflammatory factors.

Further analysis of this mechanism in this study found that α7nAChR agonists can promote the expression of Wnt1, β-catenin mRNA, and protein in osteoblasts. Various components in the classical Wnt pathway, such as the low-density lipoprotein receptor-related proteins LRP5 and β-catenin, can affect the development of osteoblasts, and have become an important target for studying osteoblast metabolism [24]. Studies have shown that the activation of LRP5 can lead to increased bone mass, and its inactivation can lead to OP. In adults, the loss of β-catenin can reduce the number of osteoblasts and increase the number of osteoclasts, which then leads to joint inflammation [25]. This result suggests that α7nAChR agonists can promote the proliferation and differentiation of osteoblasts by regulating the Wnt/β-catenin signaling pathway, which is beneficial for improving the balance of OP osteoblasts and osteoclasts and opens up new research for investigating drug therapy for OP. In further studies, it is proposed to analyze the regulation of the α7nAChR agonist on osteoporosis through an animal model of osteoporosis and provide a more substantial theoretical basis for clinical OP treatment.

Conclusion

α7nAChR agonists can inhibit the proliferation and differentiation of osteoblasts by regulating the Wnt/β-catenin signaling pathway, and then participate in the regulation of OP. This study provides a new reference for the clinical treatment of OP.

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Disclosure of conflict of interest

None.

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