Gallic Acid Derivative Stimulate Osteogenic Differentiation by Regulating Autophagy via JNK/mTOR Signaling Pathways in vitro

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ABSTRACT. *This paper was to demonstrate the effects and mechanism of methylated-based gallates 4-O-methylgallic acid on osteoblast differentiation. Cells were treated with different concentrations of 4-O-methylgallic acid.Then researchers evaluated the overall effects of 4-O-methylgallic acid on osteoblast by cell proliferation bioassay, ALP activity assay, Alizarin red S staining and detection the expression level of osteogenic-related gene including runt-related transcription factor 2 (RUNX2), bone sialoprotein (BSP), osteocalcin (OCN), alpha-1 type I collagen (COL1A1).Subsequently, cells were treated with ZXHA-TC with or without the JNK inhibitor SP600125 and the autophagy inhibitor 3-methyladenine (3-MA).Then western blotting and cell transfection were used by two researchers explore the potential relationship and signaling pathway between autophagy and the differentiation of osteoblast mediated by 4-O-methylgallic acid.The results of the present study demonstrated that 4-O-methylgallic acid stimulated cell viability, increased ALP activity, upregulated the expression of osteogenic genes, evoked the expression of autophagy markers. A range of 6.25×10^{-3} μg/ml to 6.25×10^{-1} μg/ml were recommended, within which 6.25×10^{-2} μg/ml showed the best performance. SP600125 and 3-methyladenine suppressed the stimulating effects of 4-O-methylgallic acid on osteogenic differentiation By blocking JNK/mTOR signaling pathway.This study indicated that 4-O-methyl gallic acid stimulated osteoblast differentiation by regulating autophagy via JNK/mTOR signaling pathways.*

KEYWORDS: *4-O-methyl gallic acid, osteoblast differentiation, autophagy, JNK/mTOR signaling pathways*

1. Introduction

Osteoporosis is a systemic skeletal disease charaterized by low bone mass, bone fragility, and increased fracture risk [1,2]. Every year, approximately 2 million fractures are attributed to osteoporosis in America, which resulted in more than 432,000 hospitalizations and nearly 2.5 million visits [3]. Gradually, osteoporosis has evolved into a seriously global health issues [4,5]. Therefore, there is an urgent need to develop new therapeutic options for preventing and treating osteoporosis. And many orthopedist focused on bone repairing and remodeling [6].

Over the past years, pervious studies have made significant advances in cell molecular biology of osteoporosis. For example, autophagy was considered to be involved in the pathogenesis of osteoporosis [7,8]. When osteoblast were treated by the autophagy inducer rapamycin, its differentiation were upregulated [9]. Meanwhile, a negative effect could be noted between Atg7 gene deficiency and the differentiation of osteoblast [10]. Similary, a reduction of bone volume, cortical thickness and bone mineral density could be observed after rats knocked out the Atg7 gene [11]. In addition, JNK signaling pathway has been reported to regulate autophagy by activating Atg7 gene [12].Therefore, the current realization demonstrated that through activating Atg7 gene, the JNK signaling pathway can promote autophagy and further maintain the function of osteoblast.

Gallic Acid was a group of polyphenol compounds with multifunction, and played a crucial role in anti-osteoporosis [13,14]. However, this effect may be influenced by the strong acidity and hydrophilicity of gallic acid [15,16]. Santamaria et al. proposed that modification by sulfonamide groups may enhance the hydrophobicity and bioactivity of gallic acid and therefore support the growth of cells [17].

Based on the hypothesis that 4-O-methylgallic acid acted as an accelerant in osteogenic differentiation, we explored the effects and mechanism of methylated-based gallates 4-O-methylgallic acid on osteoblast differentiation. This study may be pointed out a novel therapeutic target for osteoporosis.

2. Methodology

2.1 4-O-methylgallic

Acid.4-O-methylgallic acid was prepared from GA and methoxybenzoic acid. The molecular formula was presented in Fig. 1. In this study, the 4-O-methylgallic acid was purchased from biological company (SHANGHAI ZZBIO CO., LTD, Shanghai, China).

2.2 Extraction and culture of primary osteoblasts

Researchers extracted primary osteoblasts from the bilateral parietal bone of 3 to 7 days' newborn Sprague Dawley rats by enzymatic digestion. 6 newborn Sprague Dawley rats were purchased from the Animal Resources Center of Guangxi Medical University(Nanning, China). After rats were executed by cervical dislocation, the bilateral parietal bones were stripped clearly with sterile gauze in a sterile environment, cut into pieces about 1×1 mm in a sterile vial and digested with 0.25% trypsin (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China) for 30 min at 37˚C. After centrifugation at 1000 rpm for 5 min, bilateral parietal bones were digested again with 2 mg/ml collagenase type II (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China) for 4 h at 37˚C. Then, cells were resuspended following centrifugation at 1000 rpm for 5 min in α-MEM basal culture medium containing 20% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotic mixture (100 U/ml penicillin, 100 U/ml streptomycin). Cultures were maintained in a 5%-CO2 incubator (Thermo ScientificTM Forma Series II Water-Jacketed, Santa Ana, CA, USA) at 37°C with the culture medium changed every other day. At 80–90% confluence after about 7 days of culture and cells were passaged at 1:2 upon. Finally, third generation cells with good growth were used for the following experiments.

2.3 Cells treatment

4-O-methylgallic acid was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) and diluted in PBS to reach a final concentration of 6.25 mg/ml. The stock solution was stored at 4˚C for one week and diluted with culture medium immediately before the experiment. Our previous studies have show that gallic acid derivative ranged from 6.25×10^{-3} to 6.25×10^{-1} μg/ml significantly promoted cell growth [18,19]. Therefore，cells were treated with the culture medium containing various concentrations of 4-O-methylgallic acid(0 μg/ml as control, 6.25×10^{-3}, 6.25×10^{-2} and 6.25×10^{-1} μg/ml) for the following researches. Meanwhile, other cells were respectively treated with the culture medium combined 4-O-methylgallic acid with JNK inhibitor SP600125 (10M) or the autophagy inhibitor 3-methyladenine(10mmol).

FDA/PI staining.To demonstrate the potential effects between 4-O-methylgallic acid and osteoblast viability, an PDA/PI staining was performed at 2, 4 and 6 days was used to determine the viability of cells in the samples. Researchers respectively added 5 μmol/l fluorescein diacetate (FDA; (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China)) and 2 μg/l propidium iodide (PI; (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China) stock solutions to the cells. Further, the cells were incubated in the dark for 5 min at 37˚C. Images were captured under a laser scanning confocal microscope (Nikon A1; Nikon Corporation, Tokyo, Japan).

2.4 MTT assay

To demonstrate the potential effects of 4-O-methylgallic acid on primary osteoblast, a MTT assay which performed at 2, 4 and 6 days was used to determine the cytotoxicity in the samples. Cells were seeded into 24-well plates at a density of 5×10^3 cells/well. The culture medium was replaced with various concentrations of 4-O-methylgallic acid (0, 6.25×10^{-3}, 6.25×10^{-2} and 6.25×10^{-1} μg/ml) After culturing for 24 hours at 37˚C. Then 1 ml 0.5 mg/ml MTT (Beijing Solarbio Science & Technology Co.,

Ltd, Beijing, China) was added to each well for 4 h at 37˚C. The formed formazan crystals were then dissolved in 1 ml dimethyl sulfoxide. After thoroughly and evenly mixing the samples, 200 μl was randomly extracted from three parallel wells at each 4-O-methylgallic acid concentration and transferred to a 96-well plate. Sample absorbance values were measured at 570 nm using a microplate reader (Multiskan™ GO Microplate Spectrophotometer; Thermo Fisher Scientific, Inc.). Results are presented as optical density absorbance values.

2.5 ALP activity assay and staining

To demonstrate the potential effects of 4-O-methylgallic acid on osteoblast differentiation,cells were seeded at a density of 1x104 on to coverslips of 24-well plate and cultured in media with various concentrations of 4-O-methylgallic acid. The expression of ALP were detected by using an ALP assay kit after 2, 4, 6 days' culturing.(Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China).

2.6 Alizarin red staining

To demonstrate the potential effects of 4-O-methylgallic acid on osteoblast differentiation, the alizarin red staining were performed at 2, 4, 6 days. Cells were seeded at a density of 1x104 on to coverslips of 24-well plate and cultured in media with various concentrations of 4-O-methylgallic acid. On day 2, 4, 6, the cells were washed with distilled water and fixed in 70% ethyl alcohol for 1 hour at 4˚C. Then, cells were rinsed twice with deionized water and stained with Alizarin red S solution (40 mM, pH 4.2; Beijing Solarbio Science & Technology Co, Ltd, Beijing, China) for 10 min at room temperature. Staining was observed and images were captured with a light microscope (Nikon Corporation).

2.7 Reverse transcription-quantitative polymerase chain reaction(RT-qPCR)

To measure the osteogenic gene expression in cells cultured with various concentrations of 4-O-met hylgallic acid, the autophagy inhibitor 3-methyladenine (5mmol) or a combination of both, the reverse t ranscriptio-quantitative polymerase chain reaction(RT-qPCR) were performed at 2 days. According to t he manufacturer's instructions, total RNA was extracted with the RNA isolation kit (Beijing Solarbio S cience & Technology Co., Ltd, Beijing, China). Subsequently, 300ng of total RNA was reversely transc ribed into cDNA by using RT system (Promega Corporation, Madison, Wi, USA). The total qPCR was performed with SYBR Green master mix (Beijing Solarbio Science & Technology Co., Ltd, Beijing, C hina) under the thermal cycling conditions as follows: at 95℃ for 10 minutes, and then 40-cycles whic h were at 95℃ for 15 seconds and at 60℃ for 1 minute. The marker gene expression was analysed by 2 -ΔΔ using CT method with a standardized control of β-actin. Each sample of each gene was repeated th ree times.The sequences of the primers used were thefollowing:RUNX2,Forward5'-CCAAGTGGCCA GGTTCAACG-3',Reverse5'-GGGATGAGGAATGCGCCCTA-3';BSP,Forward5'-CCGGGAGAAC AATCCGTGCC-3',Reverse5'-AAAGCACTCGCCATCCCCAA-3';OCN,Forward5'-CAGGTGCAA AGCCCAGCGAC-3',Reverse5'-TGGGGCTCCAAGTCCATTGTT-3';COL1A1, Forward5'-CATGA GCCGAAGCTAACCC-3',Reverse5'-CTCCTATGACTTCTGCGTCTGG-3';GAPDH,Forward5'-CC CATCTATGAGGGTTACGC-3',Reverse 5'-T TTAATGTCACGCACGATTTC-3'.

2.8 Western blot

Cells were cultured with 6.25×10-2μg/ml 4-O-methylgallic acid alone or combined with 5mmol 3-methyladenine and SP600125 for 2 days, researchers extracted the proteins by splitting cells in RIPA ly sis buffer solution (Beijing Soleboard Technology Co., Ltd, Beijing, China). The protein concentrations were measured with BCA protein assay kit (Beijing Soleboard Technology Co., Ltd, Beijing, China). 5 0μg protein was separated by 12% SDS-PAGE and imprinted on a 0.22μm nitrocellulose membrane. T he membranes were sealed with 5% milk-TBS at room temperature for 2 hours and incubated overnight with the primary antibody at 4 ℃. Then the membranes was washed with TBST and incubated with th e secondary antibody at 37℃for 1 hour. Furthermore, researcherd analyzed the optical density of the ta rget protein level by densitometry using Image-Pro Plus 6.0(Bio-Rad Laboratories，Inc，Hercules, C A，USA). The primary antibody included rat polyclonal antibody-microtubule-associated light chain 3 (LC3) II/I (1：1,000), rat polyclonal anti-Beclin1 (1：1,000) and rat polyclonal anti-autophagy relate

d 7 (ATG7; 1：1,000), rat polyclonal anti-JNK (1：1,000), rat polyclonal anti-phosphorylation (p) - J NK (1：1,000)and ratmonoclonal antibody glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5,00). The secondary antibody was conjugated with appropriate horseradish peroxidase (goat anti rat; 1：1,500). The above antibodies were purchased from abcam company.

2.9 Autophagy by cell transfection

Stub-RFP-LC3 lentivirus was supplied by Shanghai Jikai Gene Chemical Technology Co, Ltd. According to the manufacturer's instructions, GFP-LC3 lentivirus was transfected into osteoblasts. Then the cells were treated with various concentrations of 4-O-methylgallic acid for 48 hours. And intracellular fluorescence expressions were observed under a fluorescence microscope (Nikon Corporation).

2.10 Statistical analysis

GraphPad Prism 5 (GraphPad Software, Inc, La Jolla, CA, USA) was used for statistical analysis. Data were expressed as mean±standard deviation. An unpaired two-tailed student T-Test were used for data from two groups, and one-way ANOVA and Bonferroni were used for more than two groups. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1 Cell viability and cytotoxicity

FDA/PI staining was used to detect cell viability at 2, 4, 6 days after incubation with different concentrations of 4-O-methylgallic acid. In the FDA/PI staining imaging (Figure 2 A-L), live cells were stained green and dead cells were stained red. Figure 2M presented the statistical analysis of live cell numbers from Figure 2A - Figure 2L. The reslut showed that more live cells were found over time in all groups. And at the same time point, more live cells were found in the 4-O-methylgallic acid-treated group, especially in the concentrations of $6.25 \times 10^{-2} \mu g/ml$ ($P < 0.05$). MTT assay was used to detect cytotoxicity at 2, 4, 6 days after incubation with different concentrations of 4-O-methylgallic acid. As showed in Figure 2N, primary osteoblast treated with 4-O-methylgallic acid produced a dose-dependant increase on proliferation, especially in the concentrations of $6.25 \times 10^{-2} \mu g/ml$ ($P < 0.05$). The FDA/PI staining and MTT assay both indicated that 4-O-methylgallic acid had a positive effect on osteoblast growth, among which the concentrations of $6.25 \times 10^{-2} \mu g/ml$ worked best.

3.2 Cell differentiation

ALP activity assay was used to detect cell differentiation at 2, 4, 6 days after incubation with different concentrations of 4-O-methylgallic acid. Figure 3A showed that as time elapsed, ALP activity increased from day 2 to day 4 and decreased from day 4 to day 6. And at the same time point, all 4-O-methylgallic acid-treated groups showed higher ALP activity than control group's, especially in the concentrations of $6.25 \times 10^{-2} \mu g/ml$ ($P < 0.05$).The expressions of RUNX2、BSP、OCN、COL1A1 genes were used to detect the pro-osteogenic effects of different concentrations of 4-O-methylgallic acid at 2, 4, 6 days (Figure 3B-D). Compared with the control group, the expression of those genes were obviously up-regulated in 4-O-methylgallic acid-treated groups, especially in the concentrations of $6.25 \times 10^{-2} \mu g/ml$ ($P < 0.05$).These two quantitative assays both indicated that 4-O-methylgallic acid promoted osteogenic differentiation, among which the concentrations of $6.25 \times 10^{-2} \mu g/ml$ perfomed outstandingly.

3.3 Cell mineralization

Alizarin red staining was used to detect cell mineralization at 2, 4, 6 days after incubation with different concentrations of 4-O-methylgallic acid. In the alizarin red staining imaging (Figure 4 A-L), the mineralization area were stained red. Figure 4M presented the statistical analysis of mineralization area from Figure 4A - Figure 4L. The reslut showed that staining was markedly strengthened over time

in all groups. Cell treated with 4-O-methylgallic acid exhibited stronger staining compared with the control, particularly when treated with 6.25x10-2μg/ml 4-O-methylgallic acid.

3.4 Autophagy

Western blot and RT-qPCR were used to detect the expression of autophagy associated protein and genes at 2 days after incubation with different concentrations of 4-O-methylgallic acid. The relative genes included LC3、Beclin1 and ATG7. And the relative proteins included LC3II/I、Beclin1 and ATG7. The assays showed that the expression of these genes and proteins were significantly up-regulated following 4-O-methylgallic acid treatment, particularly when treated with 6.25x10-2μg/ml 4-O-methylgallic acid (Figure 5 A,C,D). This finding was further verified by cell transfection. As showed in Figure 5B, cells transfected by GFP-LC3 lentivirus exhibited stronger staining in 4-O-methylgallic acid groups, particularly when treated with 6.25x10-2μg/ml 4-O-methylgallic acid. Meanwhile, this effect could be blocked by the autophagy inhibitor 3-methyladenine. And when cells were treated in the media of combining 4-O-methylgallic acid and 3-methyladenine, the relative expression of osteogenic genes were obviously much lower than that in 4-O-methylgallic acid alone group (Figure 6). The result indicated that 3-methyladenine significantly inhibited 4-O-methylgallic acid-induced autophagy and osteogenic differentiation.

3.5 JNK/mTOR Signaling Pathways

Western blot were used to detect the expression of JNK/mTOR pathways associated protein (JNK、P-JNK) at 2 days after incubation with different concentrations of 4-O-methylgallic acid. The qualitative and quantitative assays both showed that the expression levels of these proteins were significantly up-regulated following 4-O-methylgallic acid treatment, particularly when treated with 6.25x10-2 μg/ml 4-O-methylgallic acid (Figure 7 A,C). And this effect could be blocked by JNK inhibitor SP600125(Figure 7 B,D). Meanwhile, when cells were treated in the media of combining 4-O-methylgallic acid and SP600125, the expression of autophagy associated protein and genes were significantly inhibited. And the relative expression of osteogenic genes were obviously much lower than that in 4-O-methylgallic acid alone group (Figure 8). The result indicated that SP600125 significantly inhibited 4-O-methylgallic acid-induced autophagy and osteogenic differentiation.

In this study, we demonstrated the anti-osteoblast effect of methylated-based gallates(4-O-methylgallic acid) by providing osteogenic differentiation in the in-vitro cell experiments. Overall, this study indicated that 4-O-methylgallic acid promoted osteoblast differentiation in a time-dependent manner, which extremely depended on regulating autophagy via JNK/mTOR signaling pathways.

The results from in-vitro cell studies indicated that 4-O-methylgallic acid acted as a accelerant in osteogenic differentiation, and may be a novel therapeutic target for the treatments for osteoporosis. Some enzymes and complex reflected the process of osteogenesis, including alkaline phosphatase (ALP) and calcium combined with Alizarin Red S. ALP was an enzyme, which increased during the differentiation stage and decreasd when mineralization occurred. Therefore, ALP was commonly used as a marker of osteogenesis because of the susceptibility [20-22]. In our study, ALP activity in 4-O-methylgallic acid group was elevated in a time-dependent manner. This was further verified by Alizarin red S staining, which provided the information about mineralization process during cells differentiated. when Alizarin Red S combined with calcium in osteoblasts, a specific colors would occur [23-25]. And the more obvious mineralization appeared, the more active osteogenic differentiation was. Here, our study showed that treatment with the dose of 4-O-methylgallic acid ranging from 6.25×10-3 μg/ml to 6.25×10-1 μg/ml enhanced osteoblast activity, among which 6.25×10-2 μg/ml worked best.

Osteoblast differentiation was improved in the 4-O-methylgallic acid group, which probably related to autophagy. Autophagy was a series of biochemical processes on cells self-digestion, contributed to degrade harmful components in cells and maintain the stability of intracellular environment [26,27]. In our study, treatment with 4-O-methylgallic acid significantly increased the expression of key autophagic molecules LC3II/I, Beclin1 and ATG7. Meanwhile, when 4-O-methylgallic acid acted with osteoblasts carrying the Stub-RFP-LC3 gene, LC3 II was highly expressed. On the other hand, collagen I, SPP1, OCN and RUNX2 gene were promoted in 4-O-methylgallic acid but inhibited in autophagy inhibitor 3-MA, which indicated its stimulative effects on autophagy. Consequently, we hypothesized that 4-O-methylgallic acid promoted osteoblast differentiation by regulating autophagy.

JNK/mTOR signaling pathways transmitted extracellular signals into cells and participate in many physiological and pathological reactions in the body [28-30]. Over the past several years, there have been many studies foucus on exploring the potential association between JNK/mTOR signaling pathways and autophagy [31-33]. HuiXia Li et al demonstrated that JNK/mTOR signaling pathways regulated autophagy by activating Atg7 gene, and the bone mass in Atg7 knockout mice is significantly lower than that in the control group [10]. Currently, the JNK/mTOR signaling pathways was known as a autophagy activator and has been found to play an important role in maintaining osteoblast function. In our study, the expression of key autophagic molecules LC3II / I, Beclin1 and ATG7 were inhibited by J JNK/mTOR signaling pathways inhibitor SP600125. All these results indicated that 4-O-methylgallic acid enhanced osteoblast autophagy by JNK/mTOR signaling pathways.

Compared with previous researches, our study has the following strengths. First, it was the first study to explore the anti-osteoporosis effects of 4-O-methylgallic acid. In the past years, 4-O-methylgallic acid was widely used as a biomarkers of polyphenol intake [34]. However, rare studies foucesed on its pharmacological effects, whithin which majority of published studies were about the antioxidant effects [35-38]. This study offered a new idea about the pharmacological effects of 4-O-methylgallic acid. Second, various research methods were used in this study to comprehensively demostrate the pharmacological mechanism of 4-O-methylgallic acid, including MTT assay, RT-qPCR, wastern blot and cell transfection. Third, the best concentration of 4-O-methylgallic acid were found in this study. It provided the theoretical foundation for further researches. Meanwhile, the limitation of this study should be taken into account. In this study, anaimal experiment were lacking. Therefore, more experiments in vivo were needed to verify this finding.

4. Conclusion

In summary, Our study indicated that 4-O-methyl gallic acid stimulated osteoblast differentiation by regulating autophagy via JNK/mTOR signaling pathways. The recommended dose of 4-O-methylgallic acid for enhancing osteoblast activity was $6.25 \times 10\text{-}2 \mu g/ml$. This study provided an insight into cell therapy of osteoporosis. For further clinical application, more related experiments in vivo were needed.
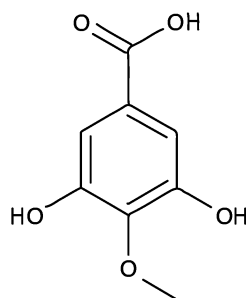

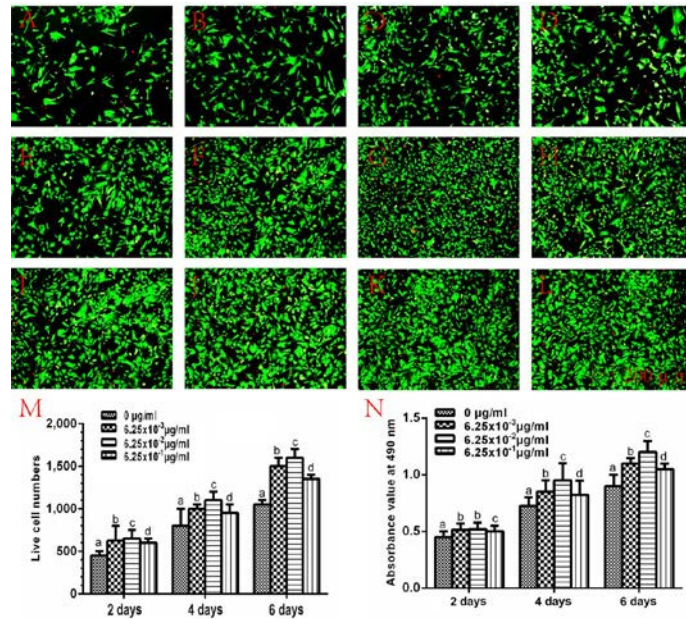*Figure 1: The molecular formula of 4-O-methylgallic acid*

Figure 2: Cell viability and cytotoxicity were determined by FDA/PI staining and MTT assay. (A-D) Staining of primary osteoblasts treated with various concentrations of 4-O-methylgallic acid at 2 days. (E-H) Staining of primary osteoblasts treated with various concentrations of 4-O-methylgallic acid at 4 days. (I-L) Staining of primary osteoblasts treated with various concentrations of 4-O-methylgallic acid at 6 days. (M) Statistical analysis of the data from the staining pictures from A-L (n=3). (N) the effect of 4-O-methylgallic acid at concentrations on primary osteoblasts. The bars with different letters at the same time are significantly different from each other (p<0.05; n=3), and those with similar letters show no significant difference. Scale bar=200 μm.
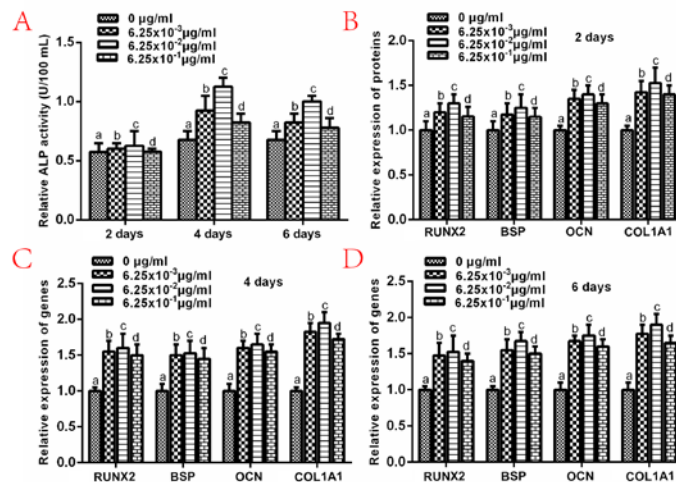

Figure 3: Cell differentiation were determined by ALP activity assay and RT-qPCR. (A) Time-course of ALP activity of osteoblasts treated with various concentrations of 4-O-methylgallic acid. Relative ALP activity (units/mg protein) was expressed as mean ± standard deviation (n=3). (B-D) The pro-osteogenic effects of 4-O-methylgallic acid at various concentrations on osteoblasts. The bars with different letters at the same time are significantly different from each other (p<0.05; n=3), and those with similar letters show no significant difference.
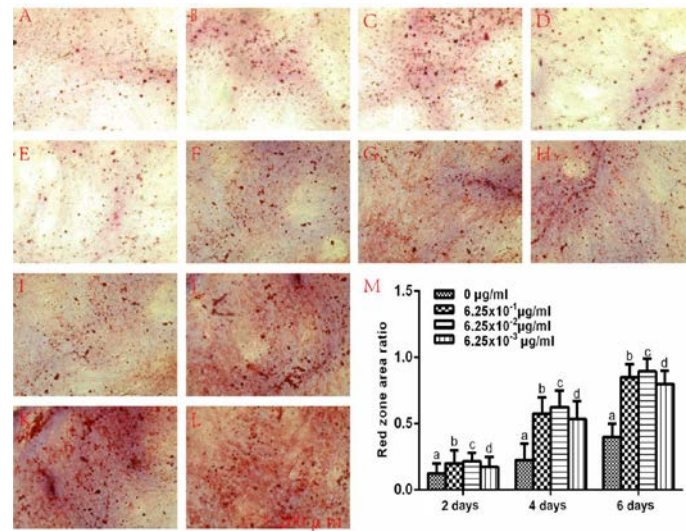
Figure 4: Cell mineralization were determined by Alizarin red staining. (A-D) Staining of primary osteoblasts treated with different concentrations of 4-O-methylgallic acid at 2 days. (E-H) Staining of primary osteoblasts treated with different concentrations of 4-O-methylgallic acid at 4 days. (I-L) Staining of primary osteoblasts treated with different concentrations of 4-O-methylgallic acid at 6 days. (M) Statistical analysis of the data from the staining pictures from A-L (n=3). The bars with different letters at the same time are significantly different from each other (p<0.05; n=3), and those with similar letters show no significant difference. Scale bar=200 μm.
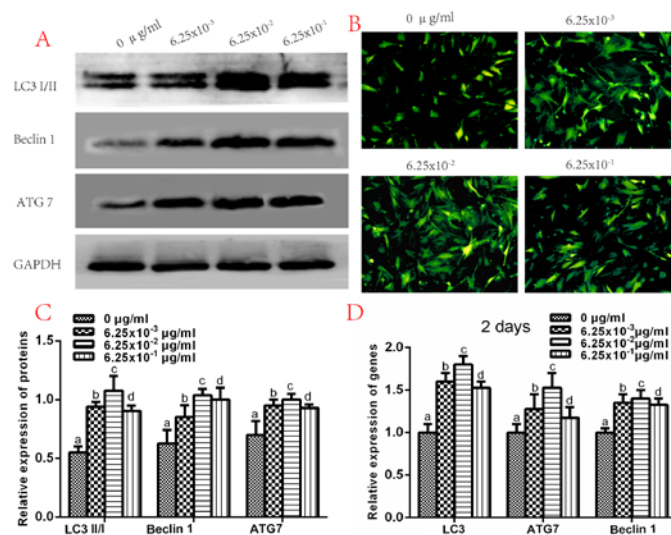

Figure 5: The expression of autophagy associated proteins and genes were determined by western blot and RT‑qPCR at 2 days after incubation with different concentrations of 4-O-methylgallic acid. (A) The expressions of LC3II/I, Beclin1, ATG7 and GAPDH proteins on different concentrations of 4-O-methylgallic acid at 2 days by western blot. (B) Staining of GFP-LC3 lentivirus transfected osteoblasts treated with concentrations of 4-O-methylgallic acid at 2 days. (C) Statistical analysis of the data from

Figure 2A. (D) The expressions of LC3II/I、Beclin1 and ATG7 proteins on different concentrations of 4-O-methylgallic acid at 2 days by RT-qPCR. The results were expressed as mean ± standard deviation. The bars with different letters at the same time are significantly different from each other (p<0.05; n=3), and those with similar letters show no significant difference. Scale bar=50 μm.
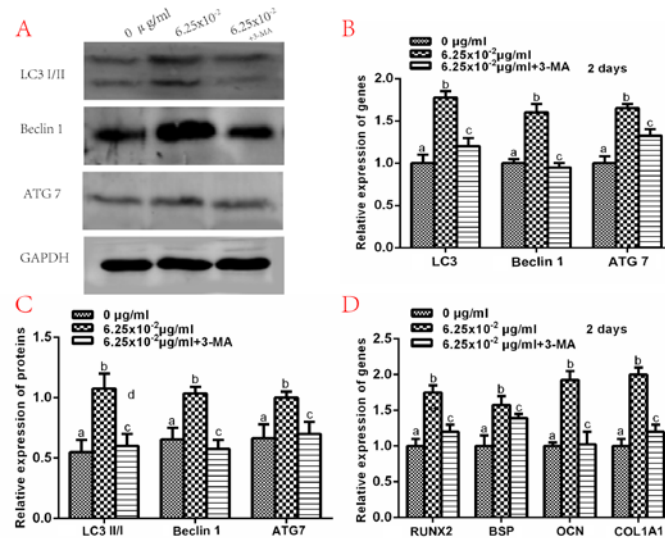

Figure 6: The expression of autophagy associated proteins and genes were determined by western blot and RT-qPCR at 2 days after incubation with 4-O-methylgallic acid and 3-methyladenine. (A) The expressions of LC3II/I、Beclin1, ATG7 and GAPDH proteins after incubation with 4-O-methylgallic acid and 3-methyladenine by western blot. (B,C) The expressions of LC3II/I、Beclin1 and ATG7 genes and proteins by RT-qPCR. (D) The expressions of RUNX2, BSP, OCN and COL1A1 genes by RT-qPCR. The results were expressed as mean ± standard deviation. The bars with different letters at the same time are significantly different from each other (p<0.05; n=3), and those with similar letters show no significant difference.
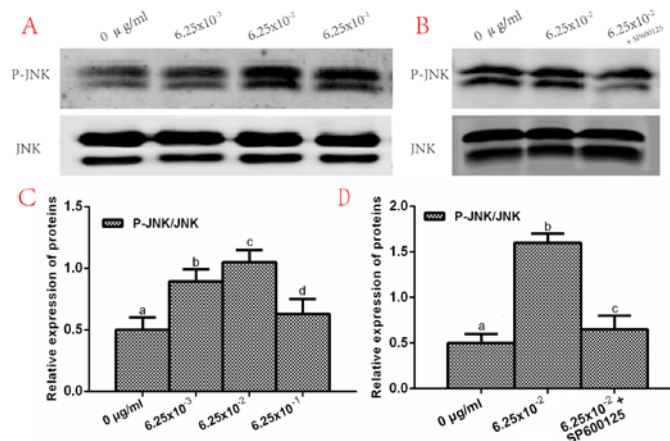

Figure 7: The expression of JNK/mTOR pathways associated protein were determined by western blot and RT-qPCR at 2 days after incubation with different concentrations of 4-O-methylgallic acid and SP600125. (A) The expressions of p-JNK and JNK proteins on different concentrations of 4-O-methylgallic acid by western blot. (B) The expressions of p-JNK and JNK proteins after incubation with 4-O-methylgallic acid and sp600125 by western blot. (C) The quantitative analysis of the data from Figure 7A. (D) The quantitative analysis of the data from Figure 7B. The results were expressed as mean ± standard deviation. The bars with different letters at the same time are significantly different from each other (p<0.05; n=3), and those with similar letters show no significant difference.
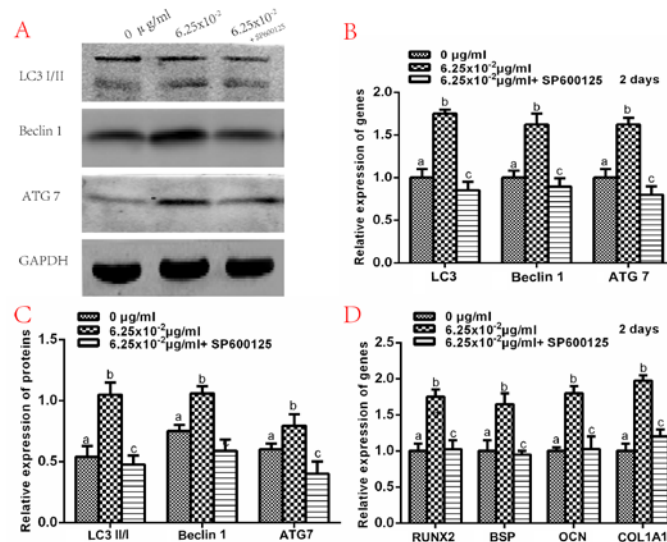
Figure 8：*The expression of autophagy associated proteins and genes were determined by western blot and RT-qPCR at 2 days after incubation with 4-O-methylgallic acid and SP600125. (A) The expressions of LC3II/I、Beclin1, ATG7 and GAPDH proteins after incubation with 4-O-methylgallic acid and SP600125 by western blot. (B,C) The expressions of LC3II/I、Beclin1 and ATG7 genes and proteins by RT-qPCR. (D) The expressions of RUNX2, BSP, OCN and COL1A1 genes by RT-qPCR. The results were expressed as mean ± standard deviation. The bars with different letters at the same time are significantly different from each other (p<0.05; n=3), and those with similar letters show no significant difference.*

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