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Effects of Ginsenosides Rg1 on Osteoblasts Cultured with Ti Particles

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Abstract

The aim of this study was to explore the role and effect of ginsenosides Rg1 on osteoblasts cultured with Ti particles. Osteoblasts from neonatal rats were cultured with particles and different doses of Rg1, the main active ingredient in ginsenosides Rg1. We found that the COX-2, PGE_2, TNF-α, IL-1, and IL -6 concentrations in the medium of cells cultured with Ti particles significantly increased as compared with that of the control cells (p<0.05 or p<0.01). In addition, cells cultured with Ti particles alone exhibited the highest concentrations of these molecules. The PGE_2, TNF-α, IL-1, and IL-6 levels in the medium of cells cultured with Rg1 were in between those of the control cells and the cells cultured with Ti particles alone. The IL-1ra level in the group cultured with Ti and medium-dose Rg1 was the highest followed by the cells cultured with Ti and high-dose Rg1 and those cultured with Ti and low-dose Rg1 (p<0.05). In conclusion, ginsenosides can reduce the levels of inflammatory cytokines produced by osteoblasts on induction with Ti particles and can prevent prosthesis loosening.

Key Words: Osteoblasts, Ginsenosides Rg1, Ti particles, Prosthesis loosening, Inflammatory factors

INTRODUCTION

Artificial joint can reconstruct the joint function with lesions. However, with the widely use of artificial joints, revision renovation gradually increased for various reasons. Many studies have focused on the mechanism and effective prevention of prosthetic loosening in order to extend the service life of prostheses. The main reason for aseptic loosening is wear particle-induced osteolysis. Wear particles can promote secretion of inflammatory cytokines, thereby increasing the activity of osteoclasts. This in turn leads to bone resorption and induces apoptosis of osteoblasts, thus accelerating osteolysis (Haynes *et al.*, 2001; Vermes *et al.*, 2001; Crotti *et al.*, 2004). Studies have revealed that wear particles can induce production of inflammatory factors, such as interleukin (IL-1, IL-6) and tumor necrosis factor α (TNF-α), by osteoblasts via autocrine or paracrine. These cytokines can result in dysfunction of osteoblasts, decreased bone matrix, weakened proliferation, and differentiation. Besides, these cytokines also have an obvious stimulating effect on the activity of osteoclasts, thus leading to osteoporosis around the prosthesis and prosthesis loosening (Jilka *et al.*, 1992; Kimble *et al.*, 1994; Pioletti *et al.*, 1999). Studies have shown that Rg1 (molecular formula, $C_{42}H_{72}O_{14}$; molecular structure shown in Fig. 1A), the main active ingredient in ginsenosides, can downregulate cyclooxygenase 2 (COX-2) expression, decrease IL-1 and IL-6 secretion, and reduce the inflammatory response (Hu *et al.*, 2006; Lahera *et al.*, 2007; Li *et al.*, 2008). However, it is not clear whether ginsenosides interfere with the inflammatory response caused by wear particles. The aim of this study was to discuss the possible inhibitory action of ginsenoside Rg1 on inflammation that is caused by wear particles, and provide a new theory for the prevention of prosthesis loosening.

MATERIALS AND METHODS

Experimental drug

The monomer purity of ginsenoside Rg1 was greater than 98% and prepared by AnHui WuHu Delta Medical Technology Co., Ltd (Batch number 2008-033).

Main reagents and instruments

Ti particles (Beijing Nonferrous Metal Company, Beijing; average diameter, 5 μm); PGE_2, TNF-α, IL-1 receptor antagonist (IL-1ra), and IL-6 ELISA kit (Xi Tang Corporation, ShangHai); TRIZOL (Invitrogen, USA); PCR primers for COX-2 and internal reference β-actin (Shanghai Boshang Biotechnology Co.,

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Ltd.); reverse transcription kit (TaKaRa, Japan); SYBR® Premix qPCR SuperMix-UDG (Invitrogen, USA); 9600 DNA amplification (PE Biosystems, USA); 7500 real-time quantitative PCR system (ABI, USA); COX-2 antibody (Abcam company, USA.); β-actin antibody (Cell Signaling, USA); WesternBreeze (Invitrogen, USA); PVDF membrane (Amersham, USA).

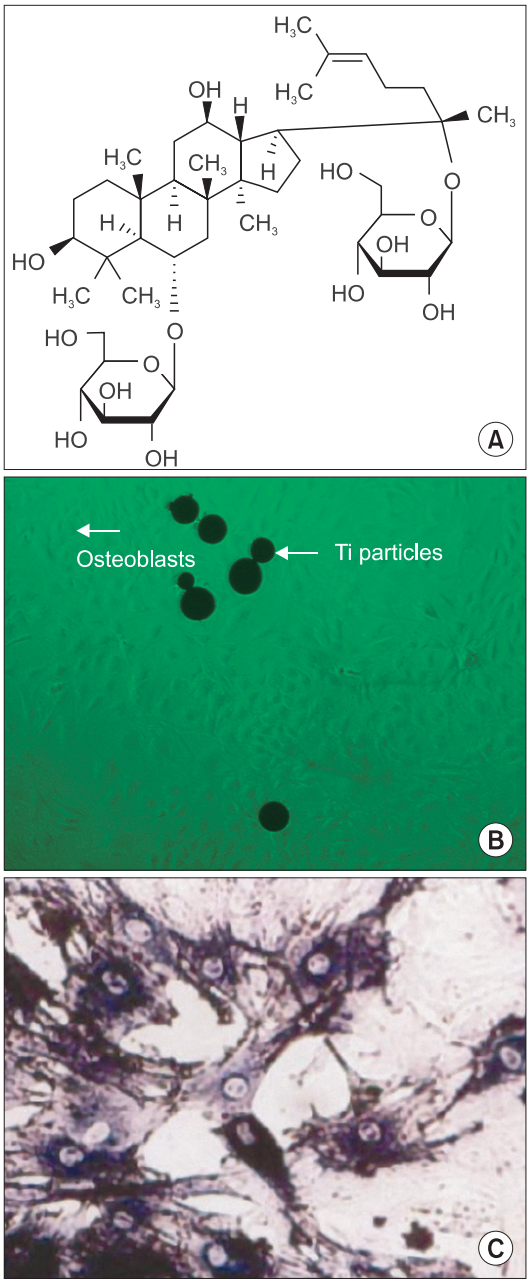

Fig. 1. (A) Molecular structure of ginsenoside Rg1 $C_{42}H_{72}O_{14}$. (B) Osteoblasts from neonatal rats were cultured with particles. Osteoblasts adhered to the Ti particles (100×). (C) Alkaline phosphatase staining was conducted using the diazol method to observe the morphological characteristics of the osteoblasts, and revealed purple-blue particles in the cytoplasm (200×).

Purification and identification of cultured osteoblasts

One neonatal Sprague-Dawley (SD) rat was provided by the animal house of Fujian Medical University. Calvarial osteoblasts were collected by enzyme digestion and passaged. They were then purified by differential adhesion. Alkaline phosphatase (AP) staining was conducted using the diazol method to observe the morphological characteristics of the osteoblasts. The cultured cells were confirmed as osteoblasts.

Preparation of Ti particle suspension

Ti particle filter based on gradient centrifugation of the Ti particle diameter of about 1 μm, scanning electron microscopy Ti particle diameter of 0.91 ± 0.65 μm. The Ti particles were soaked with ethanol for 24 h, and with 0.25% nitric acid at 70°C for 3 h, and then washed with sterilized phosphate buffer saline solution. The Ti particles were transferred into phosphate buffer (DPBS) in the preparation of 0.1% (volume ratio) suspension and sterilized at high temperature. Each milliliter of 0.1% (v/v) of the Ti particle suspension contained approximately 4.5×10^7 particles. Part of the Ti particle suspension was placed in 37°C water bath and cultured for 24 h. after centrifugation at 1,000 r/min for 10 min, the supernatant (the extract) was used as the endotoxin samples.

Bacterial endotoxin test

The bacterial endotoxin test was performed in accordance with the USP bacterial endotoxin test, using an ET-201 toxinometer (Wako Pure Chemical Industries, Ltd.) equipped with a thermostat (37 ± 1°C). In this test, the change of the sample solution containing limulus ambocyte lysate (LAL) into a gel, induced by endotoxins, was regarded as a change in the opacity. The time needed to reach a transmitted light ratio of 94.9%, i.e., the time for gelation (Tg), was measured with the toxinometer (turbidimetric method). A standard curve was obtained from the reference endotoxin solutions diluted with sterile water (0.12, 0.06, 0.03 and 0.015 EU/ml).Ti particle suspension or standard solution was mixed with 50 μl of the endotoxin solution (0.625 EU/ml) to obtain an endotoxin concentration of 0.0313 EU/ml. These solutions, and others to which no endotoxin had been added, were tested. Endotoxin solution diluted with sterile water served as the control.

Ginsenoside intervention

Third-generation cells (1×10^5/ml) were seeded in culture flasks and passaged. They were divided into 5 groups and cultured with different growth media as follows: control group: DMEM supplemented with 10% fetal bovine serum. Ti particle group: 0.1% of the volume ratio of Ti with titanium particles DMEM+10% FBS medium. Ti+high-dose Rg1 group: the volume ratio of 0.1% Ti particles+high dose (final concentration 100 μg/ml) ginsenoside Rg1+10% FBS DMEM medium. Ti+ medium-dose Rg1 group: the volume ratio of 0.1% titanium particles+in the dose (final concentration 50 μg/ml) ginsenoside Rg1+10% FBS DMEM medium. Ti+low-dose Rg1 group: the volume ratio of 0.1% titanium particles+low dose (final concentration 25 μg/ml) ginsenoside Rg1+10% FBS DMEM medium. All the cells were continuously cultured for 24 h.

Makers and methods

PGE_2, TNF-α, IL-1, IL-1ra, IL-6 levels: Cell supernatants were collected after 24 h of intervention and tested in 96-well plates. A total of 100 μl of standard or sample was added to each well, and the plate was incubated for 30 minutes at 37°C. HRP-coupling liquid, substrate, and 50 μl stopping solution were added serially and the plate was analyzed at 450 nm.

Detection of protein by western blotting: Total protein was extracted from osteoblasts and the protein concentration was determined using the bicinchoninic acid (BCA) assay. A total of 30 μg protein added to each lane of a 12% gel and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Then, the protein was transferred to a polyvinyl difluoride (PVDF) membrane. The membrane was blocked for 30 min at room temperature. Antibody against the target protein was diluted 1:5000 and the membrane was incubated with 10 ml primary antibody for 1 h. The membrane was washed with 40 ml lotion for 6 times: two 1-min washings; two 20-min washings; and two 5-min washings. Then, AP-labeled secondary antibody was used to block the membrane for 30 min at room temperature followed by washing of the membrane as mentioned above. The AP chemiluminescent substrate was reacted with the membrane for 5 minutes at room temperature. Exposure and development of the X film (kodak) were conducted in a dark room. The Phoretix 1D electrophoresis biological image analysis system was used for analyzing the target band on the film. The optical density value of every band was automatically read and recorded by the computer.

Real-time quantitative SYBR GREEN for COX-2 and TNF-α gene expression: After the 24-h intervention, the cells were collected for detection of COX-2 and TNF-α expression. The COX-2 primers were as follows: forward 5′ TCGGTGGAGAGGTGTATCCT 3′, reverse 5′ TGCTGGTTTGGAACAGTCG 3′; the length of the product was 227 bp. The following were the TNF-α primers: forward 5′ CTCAGCCTCTTCTCATTCCT 3′, reverse 5′ CTGCTTGGTGGTTTGCTAC 3′; the length of product was 207 bp. The following were the internal reference β-actin primers: forward 5′ AGGCTGTGTTGTCCCTGTA 3′, reverse 5′ ATGTCACGCACGATTTCC 3′; the length of product was 193 bp. Total RNA was extracted using Trizol. Extracted RNA was added along with bromophenol blue to the agarose gel for electrophoresis. The bands were analyzed by using the gel electrophoresis image analysis system (BioRed), and no degradation of RNA was noted. The absorbance value of RNA was measured and the RNA concentration, calculated. After the volume of RNA was calculated on the basis of the concentration, 500 ng RNA was used for reverse transcription, which was performed according to manufacturer's instructions. After reverse transcription, mRNA was amplified using the ABI7500 according to the manufacturer's instructions. Amplified curves and curves of threshold cycle (CT) values were obtained. The values for the COX-2,TNF-α and β-actin genes were introduced into the respective standard curve and converted out of their initial template amount. β-actin was used as a reference gene for RNA correction of all samples. The corrected value was obtained by dividing the quantitative results of the COX-2,TNF-α gene with those of the β-actin gene. COX-2 ,TNF-αmRNA expression in the negative control group was considered as "1," the relative amount of mRNA expression in the other groups was calculated in terms of the correction value. The relative amount of mRNA expression was compared between the groups.

Statistical analysis

The experimental data was analyzed by using SPSS 13.0. The data was shown in mean ± standard deviation. Multigroup comparison was conducted with the F test and q test in order. $p<0.05$ was considered as significant.

RESULTS

Bacterial endotoxin test

The measured sensitivity of LAL 0.06 EU/ml, the value of the label between 0.5-2.0λ, can be used to detect endotoxin. Interfere with test results Es=0.06, at 0.5-2.0λ (including 0.5 and 2.0λ); meanwhile Et=0.071-0.085, at 0.5 Es-2.0 Es (including 0.5 Es and 2.0 Es), is that the sample of the TAL and endotoxin response interference effect. 5 sample tube with the negative control tube (1) result is negative, the positive control sample tube (1) and the positive control tube (2) the result is positive, the sample of titanium particles bacterial endotoxin <0.06 EU/ml. Sample endotoxin content conform to USP provision.

Morphological observation and identification of osteoblasts

Osteoblasts from the skull bones of neonatal rats were digested and isolated with enzyme several times and observed under inverted phase contrast microscope. Adherent cells gradually stretched and assumed a spindle-shape, triangular shape, or irregular polygonal shape. After 2 passages, the cells proliferated quickly with cell body enlargement and development of slender processes. The nucleus was large and mostly round or oval-shaped and was located at the center or at one side of the cell. Osteoblasts adhered to the Ti particles

Table 1. PGE_2, TNF-α, IL-6 concentrations in the cell supernatants (x ± s, μg/ml)

Group	n	PGE_2	TNF-α	IL-6
Control	10	$31.687 \pm 0.466^{\S}$	$40.055 \pm 0.471^{\ddagger}$	$57.550 \pm 0.610^{\ddagger}$
Ti particle	10	$53.362 \pm 0.307^{\dagger}$	50.121 ± 0.532 *	80.537 ± 0.883*
Ti+high Rg1	10	$41.0475 \pm 0.431^{\dagger,\S,\dagger\dagger}$	$49.6750 \pm 0.336^{*,\ddagger,**}$	$70.975 \pm 0.945^{*,\ddagger,**}$
Ti+medium Rg1	10	$38.9975 \pm 0.234^{\dagger,\S,\P}$	$46.4313 \pm 0.245^{\dagger,\ddagger,\|}$	$68.154 \pm 0.745^{*,\ddagger,\|}$
Ti+low Rg1	10	$32.5012 \pm 0.124^{\S,\P,\dagger\dagger}$	$42.5205 \pm 0.513^{*,\ddagger,\|,**}$	$63.335 \pm 0.845^{*,\ddagger,\|,**}$

*$p<0.05$, †$p<0.01$, *vs.* control group; ‡$p<0.05$, §$p<0.01$, *vs.* Ti particle group; ‖$p<0.05$, ¶$p<0.01$, *vs.* Ti+high-dose Rg1 group; **$p<0.05$, ††$p<0.01$, *vs.* Ti+medium-dose Rg1 group.

(Fig. 1B); AP staining revealed purple-blue particles in the cytoplasm (Fig. 1C). Five visual fields under the microscope were selected randomly and 100 cells were counted in every field. Osteoblast purity was 90.2% (3.64%), which was consistent with the culture requirement.

Determination of PGE_2, TNF-α, IL-1, IL-1ra, and IL-6 concentrations in the cell supernatants

The PGE_2, TNF-α, IL-1, and IL-6 contents in the groups treated with Ti particles increased significantly as compared with levels of these substances in the control group, except for PGE_2 in the Ti+low-dose Rg1 group (p<0.05 or p<0.01). The Ti particle group exhibited the highest concentration of these substances. The PGE_2, TNF-α, IL-1, and IL-6 levels in groups treated with Rg1 were in between those of the control group and the Ti particle group. The PGE_2, TNF-α, IL-1, IL-6 levels decreased as the concentration of Rg1 decreased, and a significant difference was noted among the groups (p<0.05). The IL-1ra levels in groups treated with Ti particles decreased significantly as compared with the control group. The IL-1ra levels in the Ti+medium-dose Rg1 group was the highest followed by the Ti+high-dose Rg1 group and the Ti+low-dose Rg1 group (Table 1, 2).

COX-2 and TNF-α mRNA expression by osteoblasts

Some amplification curves obtained by fluorescence quantitative RT-PCR for β-actin, COX-2, and TNF-α and the dissolution curves showed a single peak. This indicated specific amplification, which suggested that the quantitative PCR system and the conditions met the requirements of quantitative PCR. The results showed that after 24 hours of intervention, the expression of COX-2 and TNF-α mRNA was the highest in the Ti particle group and these values were significantly higher than those of the control group (p<0.05). The expression levels of COX-2 and TNF-α mRNA in the groups treated with Rg1 were in between the levels in the control group and the Ti particle group. In the case of the 3 groups treated with Ti and Rg1, expression of COX-2 and TNF-α mRNA was the highest in the Ti+high-dose Rg1 group followed by the Ti+medium-dose Rg1 group and the Ti+low-dose Rg1 group. A significant difference was noted among the groups (p<0.05)(Fig. 2).

COX-2 protein expression by osteoblasts

COX-2 protein expression was similar to COX-2 mRNA expression. COX-2 protein expression in the Ti particle group was the highest, followed by the Ti+high-dose Rg1 group, Ti + medium-dose Rg1 group, Ti+low-dose Rg1 group, and the control group. In the 3 groups treated with Ti+Rg1, the highest COX-2 expression was noted in the Ti+high-dose Rg1 group followed by the Ti+medium-dose Rg1 group and Ti+low-dose Rg1 group. A significant difference was noted among the groups (p<0.05) (Fig. 3).

DISCUSSION

Many factors contribute to the loosening of artificial joints. Many studies have revealed that particles were produced with the use of artificial prostheses due to wear, corrosion, or col-

Table 2. IL-1, IL-1ra concentrations in the cell supernatants (x ± s, μg/ml)

Group	n	IL-1	IL-1ra
Control	10	37.579 ± 3.526§	268.019 ± 3.87‡
Ti particle	10	83.106 ± 4.413†	64.111 ± 1.36*
Ti + high Rg1	10	59.506 ± 1.294†,§,††	116.3508 ± 5.43*,‡, **
Ti + medium Rg1	10	56.881 ± 3.561†,§,¶	229.7675 ± 3.54*$^{,\ddagger,\parallel}$
Ti + low Rg1	10	45.081 ± 3.459§,¶,††	207.2032 ± 2.43*$^{,\ddagger,\parallel}$,**

*p<0.05, †p<0.01, *vs.* control group; ‡p<0.05, §p<0.01, *vs.* Ti particle group; $^{\parallel}$$p$<0.05, ¶$p$<0.05, *vs.* Ti+high-dose Rg1 group; **p<0.05, ††p<0.01, *vs.* Ti+medium-dose Rg1 group.

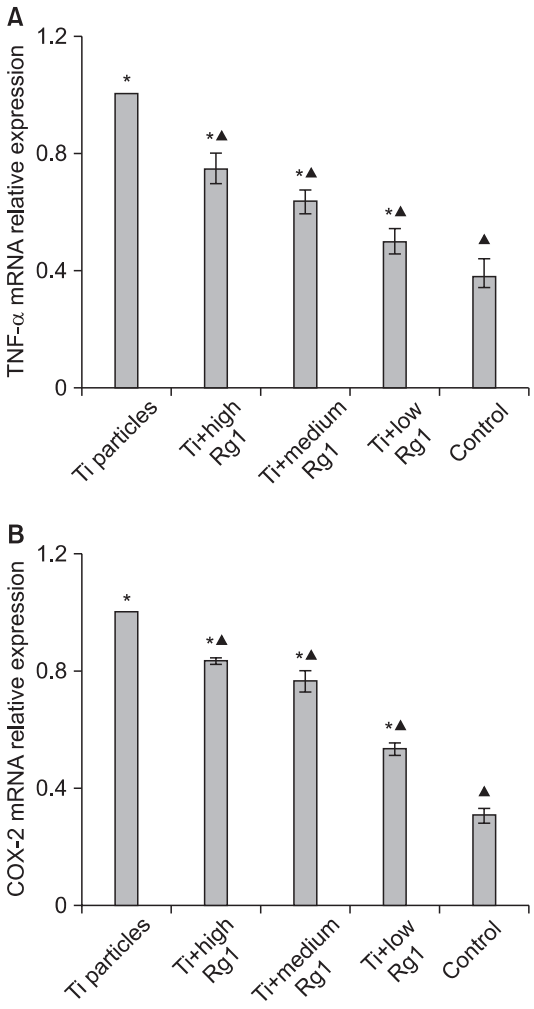

Fig. 2. Expression of TNF-α and COX-2 gene in different groups. TNF-α and COX-2 mRNA relative expression were investigated by Real-time quantitative SYBR GREEN. (A)TNF-α mRNA relative expression in control group, Ti particle group, Ti+high-dose Rg1 group, Ti+medium-dose Rg1 group, Ti+low-dose Rg1 group is 0.376 ± 0.063, 1, 0.743 ± 0.056, 0.634 ± 0.039 and 0.495 ± 0.047. (B) COX-2 mRNA relative expression in control group, Ti particle group,Ti+high-dose Rg1 group, Ti+medium-dose Rg1 group, Ti+low-dose Rg1 group is 0.304 ± 0.028, 1, 0.832 ± 0.015, 0.764 ± 0.035 and 0.532 ± 0.028. Bar graphs with error bars represent mean ± SD (n=5). *p<0.05 vs control group. ▲p<0.05 vs Ti particle group.

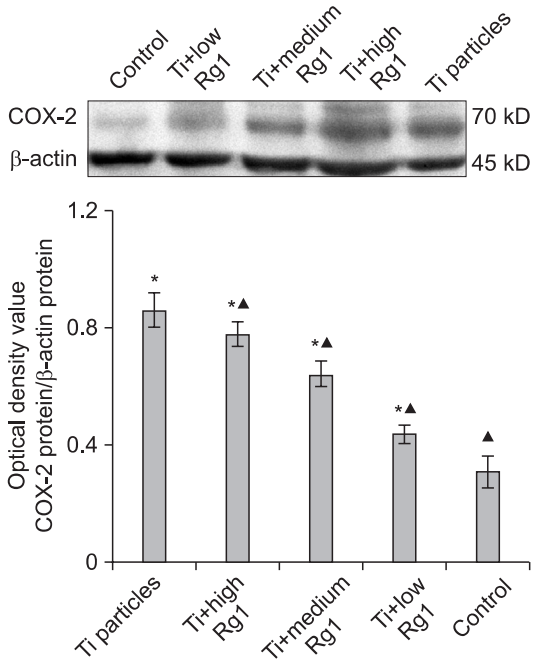

Fig. 3. COX-2 protein expression in different groups. COX-2 protein expression was investigated by western blotting. The optical density value of COX-2 protein/β-actin protein in control group, Ti particle group, Ti+high-dose Rg1 group, Ti+medium-dose Rg1 group, Ti+low-dose Rg1 group is 0.304 ± 0.058, 0.854 ± 0.067, 0.774 ± 0.045, 0.634 ± 0.054 and 0.433 ± 0.032. Bar graphs with error bars represent mean ± SD (n=5).*p<0.05 vs control group.▲p<0.05 vs Ti particle group.

lisions of material. Most prostheses are made of metal, polyethylene, bone cement, and surface-coating material. These substances trigger a series of biological responses that can cause prosthetic loosening (Fisher *et al.*, 2004; von Knoch *et al.*, 2004). Studies on aseptic loosening of prostheses have shown that wear particles stimulate mononuclear cells, fibroblasts, and osteoblasts to produce inflammatory cytokines, such as TNF, IL, and PGE_2, thus activating osteoblasts and causing osteolysis (Mohanty,1996; Gallo *et al.*, 2002; Jacobs *et al.*, 2001). If the level of cytokines produced by osteoblasts can be reduced effectively, loosening of prostheses may be prevented in theory. The results of this study show that the PGE_2, TNF-α, IL-1, and IL-6 concentrations in the culture fluid of Ti particles and osteoblasts increased significantly as compared with the concentration of these molecules in the control group (p<0.05); this implies that Ti particles could stimulate the production of PGE_2, TNF-α, IL-1, and IL-6 by osteoblasts and these cytokines had osteolytic activity.

Studies have shown that osteoblasts can produce IL-1, which binds to osteoblasts. Intracellular cAMP synthesis and GTP activity are thus enhanced, and PGE_2, IL-6, and TNF-α are produced by osteoblasts as a result (Pacifici *et al.*,1987). IL-1ra (Interleukin-1 Receptor Antagonist, IL-1ra) is a unique cytokine regulatory molecules, which, IL-1 homology, but with different gene coding. IL-1Ra with IL-1 receptor specific binding, to prevent excessive inflammation caused by elevated IL-1 into a bone cell damage, inhibition of IL-1 induction of prostaglandin PGE_2 bone cells and tissue degradation enzymes (Dinarello,1993; Burger *et al.*,1995). Abrahamsen's study of bone biopsy showed that the IL-1ra mRNA/IL-1 mRNA value was inversely proportional to the rate of bone loss in postmenopausal women, suggesting that IL-1ra and IL-1 expression levels influence bone metabolism (Abrahamsen *et al.*, 2000). In this study, by comparison of the IL-1 and IL-1ra levels in different treatment groups, we found that IL-1ra with IL-1 receptor and inhibit the biological activity of IL-1, after intervention by Rg1 in the culture medium of osteoblasts increased IL-1ra to block IL-1 receptor in osteoblasts with the combination of Inhibition of TNF, PGE_2 and other inflammatory cytokine expression.

PGE_2 is the product of arachidonic acid metabolism induced by COX-2 (Dickens *et al.*, 2002). Tissue samples around loosening prosthesis that were cultured in vitro released high concentrations of PGE_2 and showed higher bone resorption activity. Osteoblasts can release PGE_2 under the action of TNF-α and stimulate maturation and differentiation of osteoclasts, thus leading to osteolysis. Golding confirmed that tissue samples around loosening prostheses that were cultured in vitro released high concentrations of PGE_2 and exhibited higher bone resorption activity. Some researchers believe that wear particles may first stimulate PGE_2 production and thus induce IL-6 release; however, PGE_2 could promote the release of TNF-α and IL-1, which regulate bone resorption (Chen, 1996; Lü *et al.*, 2004).In addition, TNF-α can induce the release of collagenase and PGE_2 by osteoblasts, which are associated with bone resorption. TNF-α can also inhibit the synthesis of osteocalcin and impact new bone formation (Chen and Yan, 2001). We speculated that under the conditions of this experiment, Ti particles can promote secretion of COX-2, PGE_2, TNF-α, IL-1, and IL-6 by osteoblasts, and thereby affect the activity of osteoblasts, decrease osteoblast function and bone formation, damage bone formation - absorption, and increase bone resorption.

Ginsenoside has anti-inflammatory and antioxidant effects. Rg1 can significantly reduce contents of COX-2, PGE_2, TNF-α, IL-1 and IL-6 in vivo to inhibit the infernal circle induced by inflammatory and oxidant stresses (Lei *et al.*, 2000; Li, 2005). Results of this study showed that after osteoblasts co-cultured with Ti particles were intervened with Rg1, PGE_2, TNF-α, IL-1 and IL-6 levels in the cell culture fluid were significantly lower than single Ti particle group but were higher than the normal control group, and COX-2 and TNF-α mRNA expressions and COX-2 protein expression in osteoblasts also had the similar changing trend. This suggested that Rg1 had certain effects of inhibiting inflammatory factors and protecting osteoblasts. This study also found that COX-2, PGE_2, TNF-α, IL-1 and IL-6 levels were significantly lower in the Ti+high-dose Rg1 group than in the Ti+low-dose Rg1 group (p<0.05). Combing with existed studies of Rg1, it was considered to be closely related with evident pro-apoptotic effect of Rg1 on abnormal cells (Hela cell, HL-60 cell, C6 glioma cells and human heptoma carcinoma cells SMMC-7721) and the positive correlation of this effect with concentrations (Ge *et al.*,1997; Kim *et al.*,1998; Wang *et al.*, 2003). Osteoblasts showed significant changes in cell shape, ultrastructurs and differentiations after co-culturing with Ti particles (Fleury *et al.*, 2006; Dai *et al.*, 2011). However, the detailed mechanism of Rg1 on osteoblasts and the relationship with drug concentration wait to be further investigated.

This provides an objective basis for preventing prosthesis loosening with ginsenosides, indicating the prospect of clini-

cal application of ginsenoside for prevention and treatment of prosthesis loosening.

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