Carnosol suppresses RANKL-induced osteoclastogenesis and attenuates titanium particles-induced osteolysis

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Abstract

Osteolysis is a common medical condition characterized by excessive activity of osteoclasts and bone resorption, leading to severe poor quality of life. It is essential to identify the medications that can effectively suppress the excessive differentiation and function of osteoclasts to prevent and reduce the osteolytic conditions. It has been reported that Carnosol (Car), isolated from rosemary and salvia, has anti-inflammatory, anti-oxidative and anti-cancer effects, but its activity on osteolysis has not been determined. In this study, we found that Car has a strong inhibitory effect on the RANKL-induced osteoclast differentiation dose-dependently without any observable cytotoxicity. Moreover, Car can inhibit the RANKL-induced osteoclastogenesis and resorptive function via suppressing NFATc1, which is a result of affecting MAPK, NF- κ B and Ca²⁺ signaling pathways. Moreover, the particle-induced osteolysis mouse model confirmed that Car could be effective for the treatment of bone loss *in vivo*. Taken together, by suppressing the formation and function of RANKL-induced osteoclast, Car, may be a therapeutic supplementary in the prevention or the treatment of osteolysis.

KEYWORDS

Osteolysis, carnosol, NFATc1, MAPK, NF- κ B

1. INTRODUCTION

Bone, a dynamic tissue, experiences continuous remodeling of osteoblast formation and osteoclast resorption.^{1,2} The imbalance of osteoclastic resorption and osteoblastic bone formation may result in pathological conditions of bone structure, including osteoporosis, osteonecrosis and Paget's disease.³ Osteolytic disease occurs when the enhancement of formation and function for osteoclasts, or the relatively impairment of formation and function for osteoblasts.^{4,5} As a unique type of bone absorption cell, osteoclasts play a vital role in bone loss, which is related to the development of osteolytic damage.⁶ Therefore, inhibiting the formation and function of osteoclasts is essential to enhance the treatments for osteoporosis and osteolytic lesions, and to prevent excessive bone loss.

Osteoclasts, eventually differentiated from monocyte/macrophage progenitor cells, are multinucleated cells.^{7,8} Osteoclastogenesis depends on the activities of two essential cytokines including macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL).⁹ M-CSF is essential for the survival and proliferation of osteoclast precursor cells. It also regulates the level of RANK in the bone marrow macrophages, enabling the cells to RANKL stimulation.¹⁰ RANKL is part of the RANK/RANKL/Osteoprotegerin (OPG) signaling pathway which is essential for commitment of the osteoclast precursors to mature osteoclasts. RANKL binds to its receptor RANK, which is present on osteoclasts and their precursors, and stimulates the M-CSF-expanded precursors to commit to the osteoclast phenotype. RANKL/RANK signaling plays a crucial role in osteoclast survival, differentiation and function. Further, this signaling activates the mitogen-activated protein kinases (MAPK), NF- κ B and calcium

signaling pathways. The above-mentioned signaling pathways commit osteoclast differentiation through the activation of the master transcription factors, including NFATc1 and c-Fos.^{11,12} Therefore, studying molecular factors and signaling pathways induced by RANKL is important in the treatment of osteoclast related diseases.

Car is known as an ortho-diphenolic diterpene with hydroxyl group which located at C-11 and C-12. Rosemary and salvia miltiorrhiza contain lactones. It has been widely applied in traditional Chinese medicine (TCM) for the treatment of bone-related diseases. With numerous health benefits, it is considered to be a pharmacologically active compound with anti-inflammatory, antioxidant and anticancer effects.¹³⁻¹⁶ However, it is still unclear that how Car affects the RANKL-induced osteoclast formation and bone resorptive function at the cellular and molecular level. Currently, there is no report on whether Car has a therapeutic effect on particles-induced osteolysis. Different biomaterials in particulate form exert different types and degree of adverse effects on osteolysis.¹⁷ Some studies reported that all particulate biomaterials caused significant increases in membrane thickness compared with control (saline) air pouches, with the highest reaction seen in response to titanium particles.¹⁸ Longhofer et al found that there were significantly more cathepsin K positive osteoclast like cells in specimens from titanium particles.¹⁷

Our study investigated the regulation and potential mechanisms of Car's inhibition on RANKL-stimulated osteoclast formation from BMMs to mature osteoclasts. In addition, a particles-induced osteolysis model was used to validate the effectivity of Car in the prevention of inflammatory bone loss *in vivo*. These results demonstrated that Car inhibited osteoclast differentiation and bone resorption by suppressing the activation of MAPK, NF- κ B

and calcium signaling pathways stimulated by RANKL. These results provide strong evidence that Car can be used as a supplementary therapy for osteolytic disease.

2 MATERIALS AND METHODS

2.1 Reagents

Car (purity $\geq 98\%$) was purchased from ChemFaces (Wuhan, Hubei, China) and was dissolved in DMSO (Sigma-Aldrich, Castle Hill, NSW, Australia) to a concentration of 100 mM for storage and further working dilutions. The fetal bovine serum (FBS) and α -minimal essential medium (α -MEM) were purchased through ThermoFisher (Scoresby, Australia). Antibodies against V-ATPase d2, c-Fos, NFATc1, Integrin- β 3, CTSK, ERK, JNK, p38, I κ B- α , p-ERK, p-JNK, p-p38, and β -actin were acquired from Santa Cruz Biotechnology (Dallas, TX, USA). Luciferase assay and MTS kits were acquired from Promega (Sydney, NSW, Australia). The expression and purification of Recombinant Glutathione S-transferase (GST)-rRANKL protein were performed according to previous works.^{17,19} Recombinant human M-CSF was acquired from R&D Systems (Minneapolis, MN, USA). Anti-vinculin antibody (mouse monoclonal) was purchased from Sigma Aldrich (Sydney Australia), goat anti-mouse IgG conjugate with Alexa 488 was acquired from ThermoFisher Scientific (Scoresby, Australia), and DAPI was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2 Cell culture and osteoclastogenesis

According to the guidelines of the Animal Ethics Committee of the University of Western Australia (RA/3/100/1244), bone marrow macrophages (BMMs) were isolated from 6-week-old female C57BL/6J mice freshly. The bone marrow was flushed from the tibia and

femur and cultured in complete medium (α -MEM, 10% FBS, 100 μ g/ml streptomycin and 100 U/ml penicillin) with M-CSF (50 ng/mL) after dissecting the long bones without soft tissue. Until cells achieved fully confluent, BMMs were seeded into 96-well plates in complete medium containing 50 ng/ml MCSF. The cells were then treated with various concentrations of Car for the selected days. Culture medium was replaced every 2 days until mature osteoclasts were formed. Then, the cells were fixed by 2.5% glutaraldehyde for 10 minutes, and washed with PBS for twice. The cells were then stained for the TRAcP activity and observed under a standard light inverted microscope. The osteoclasts were identified when the positive multinucleated cells of TRAcP was calculated more than three nuclei.

2.3 Cytotoxicity assays

BMMs were seeded into a 96-well plate at 5×10^3 cells/well and cultured overnight for adhesion. The cells were exposed to different concentrations of Car on the next day for 48 hours. The MTS reagent was then added to the cells as per manufacturer's instruction and the cells were incubated for 2 hours at 37 °C. The absorbance at 490 nm was measured with a microplate reader (BMG, BMG Labtech, Mornington, Australia).

2.4 Immunofluorescence staining and confocal microscopy

For this experiment, 5×10^3 cells/well of BMMs were seeded in a 96-well plate with complete medium containing 50 ng/ml M-CSF. Starting the following day, the cells were stimulated with 50 ng/ml RANKL, with or without varied concentrations of Car, for 6 days until mature osteoclasts were formed. The cells were then fixed with 4% paraformaldehyde for 10 minutes and 1% Triton X-100 in PBS was added to permeabilize the cells. Unspecific bindings were blocked by 3% BSA in PBS. The cells were then probed with the anti-vinculin

antibody together with Rhodamine Phalloidin (ThermoFisher, Scorsby, Australia) in dark for 90 minutes to stain the podosome belt.¹⁷ The cells were incubated with a goat-anti-mouse IgG secondary antibody conjugated with Alexa 488 Fluor[®] to generate green-flourscent signals. The nuclei were visualized by DAPI. Finally, cells were observed at 100 × magnification with confocal fluorescence microscope (Nikon, A1 PLUS, Tokyo, Japan).

2.5 Hydroxyapatite resorption assay

BMMs were seeded at a density of 1×10^5 cells/well on a collagen-coated plate (BD Biocoat[™], Thermo Fisher Scientific) and were treated with M-CSF and RANKL until mature osteoclasts were visualized under the microscope.²⁰ The osteoclasts were gently harvested then plated on Corning[®] Osteo Assay Surface hydroxyapatite-coated plates(Corning Osteoassay, Corning, NY) at the same density. These mature osteoclasts were cultured with the medium containing RANKL and various concentrations of Car. After 48 hours, half of the wells were stained to detect TRAcP activity and the other half of wells were bleached to remove the cells. The absorbed areas were photographed with a standard inverted light microscope. ImageJ software (NIH, Bethesda, MD) was applied to analyse the percentage area of the hydroxyapatite surface which were reabsorbed by osteoclasts.

2.6 Luciferase reporter assay

Either an NFATc1 or NF- κ B responsive luciferase reporter construct was stably transfected to RAW 264.7 cells separately (ATCC[®], Manassas, VA, USA).²¹⁻²³ The cells were cultured in plates (48-well, 1.6×10^5 cells/well) and pretreated with Car at different concentrations for 1 hour. Cells were then stimulated with RANKL (50 ng/mL) for 24 hours (luciferase reporter gene assay of NFAT) or 6 hour (luciferase report gene assay of NF- κ B)

and then luciferase activity was determined according to the manufacturer's instruction.

2.7 Real-time PCR

Trizol reagent (ThermoFisher, Scrosby, Australia) was used to isolate the total RNA and 1 µg of the total RNA was reversely transcribed into cDNA with M-MLV reverse transcriptase with OligoDT primer (Promega, Sydney, NSW, Australia). SYBR Green qPCR Master Mix (ThermoFisher, Scrosby, Australia) was used to carry out the polymerase chain reaction amplification programmed as below: 94°C for 5 minutes, then 30 cycles of 94°C for 40 seconds, 60°C for 40 seconds, and a melting curve of 95°C for 15 sec, 60°C for 60 sec, 95°C for 15 sec. All the primers used in our experiment were listed in Table 1. The procedure was performed on the ViiA 7 Real-time PCR system (Applied Biosystems, Warrington, UK). The expression levels of genes were normalised to the average expression of *Hprt* and *Actb* to calculate the relative gene expression level. The $2^{-\Delta\Delta CT}$ method was used to analyze the data.

2.8 Western blot assay

BMMs were seeded in six-well plates (1×10^5 cells/well) and stimulated with RANKL (50 ng/mL) with or without adding Car for the selected times. Radiation immune precipitation (RIPA) pyrolysis buffer was used for the lysis of the cells and protein extraction. Protein was separated by SDS-PAGE, then transferred from gel to a PVDF membrane (GE Healthcare Life Sciences, Silverwater, NSW, Australia). Membranes were sealed for 60 minutes with 5% skim milk, and then gently shaken overnight at 4°C with various particular primary antibodies. Subsequently, membranes were gently washed, then incubated with suitable horseradish peroxidase (HRP)-conjugated secondary antibodies. Finally, the

enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was used to test the reactivity of antibody which was visualized by an Image-quant LAS4000 (GE Healthcare, Silverwater, Australia).

2.9 Ca^{2+} oscillation assay

BMMs (1×10^4 cells/well) were seeded into a 48-well plate. The cells were stimulated with RANKL (50 ng/ml) with different concentrations of Car for 24 hours. The cells were washed with assay buffer (HANKS balanced salt solution containing 1% FBS and 1mM probenecid) and subsequently stained with 4 μM Fluo4 staining solution for 50 minutes. The solution was made by dissolving Fluo4-AM (ThermoFisher, Scorsby, Australia) in 20% pluronic-F127 (w/v) in DMSO, and further diluted to working concentration in assay buffer. The cells were washed with assay buffer and incubated in dark at room temperature for 25 minutes. The fluorescence intensity was visualized by an inverted fluorescence microscope (Nikon, Tokyo, Japan) and analysed by the NIS-Elements Basic Research Microscope Imaging Software. Images were acquired at a 2-second interval for 3 minutes. Oscillating cells, which displayed more than one calcium peaks during the observation period, were counted, and the oscillation intensity was measured as the difference between the minimum and maximum of fluorescence intensities.

2.10 Preparation of titanium particle

Titanium particles were supplied by Alfa Aesar (Ward Hill, MA, USA). The titanium particles were deliquated with deionized water and filtered through Millipore filter membranes (Billerica, MA, USA) of a range of sizes (0.2, 1.2, and 10 μm). We harvested three different sizes of the particle after filtration, 0.2–1.2 μm (TI-0.2), 1.2–10 μm

and $>10\ \mu\text{m}$. The particles were washed in 70% ethanol for 24 hours to remove endotoxin and further sterilized by ethylene oxide. The particles were then suspended in PBS and modulated to $4 \times 10^8\ \text{mm}^3/\text{ml}$. The presence of endotoxin in the particle solutions was determined using limulus amoebocyte lysate assay (QCI-1000; Bio Whittaker, Walkersville, MD, USA), which was shown lower than 0.01 EU/ml. The precise sizes of the particles were determined by the image analysis system (NIKON, Tokyo, Japan), which found that there was no significant difference in size among the titanium particles.

2.11 Animal model

Twenty male C57BL/6J (11-week-old) mice were supplied by the Animal Laboratory of Sun Yat-sen University, China. The mice were housed according to the international guidelines for the care and use of experimental animals (Approval No. IACUC-DB-16-0308). Animals were adapted in the isolation zone, under the condition of local animal husbandry (24 °C, 12:12 h light-dark cycle), for one week before surgery. The surgical procedures used in this study were performed as described before.²⁴ All the mice underwent surgery at 12-week under general anesthesia. The median sagittal incision of the skull was made 10-mm to expose the periosteum in an area of $0.5 \times 0.5\ \text{cm}^2$. Mice were randomly divided into three groups (five mice per group), and received local calvaria injection of various suspensions, including 100 μl TI-0.2 particles (vehicle), 100 μl PBS (sham), and 100 μl TI-0.2 particles plus 1 mg/kg Car suspension, respectively. The incision was closed with non-absorbable suture materials. The mice were placed back in the cage when the alignment was being corrected, and allowed to drink and eat freely. None of the mice died or presented wound complications during the perioperative period and days after operation. The mice were

euthanized at two weeks postoperatively, and the calvariae were collected for micro-CT scan analysis.

2.12 Micro-CT imaging

The calvariae were scanned by a high-resolution micro-CT (μ CT80; Scanco Medical AG, Bruettisellen, Switzerland). Acquisition of radiographic projections ($n = 500$) was performed at 55 kV and 145 μ A with a 300 ms exposure time and $6 \times$ frame averaging. The 3D images were generated at voxel average size of $12 \mu\text{m}^3$ by using the reconstruction software provided by the manufacturer (Scanco Medical AG, Bruettisellen, Switzerland).

2.13 Histology

Mice calvaria were decalcified in 10% ethylene diamine tetra acetic acid (EDTA) for 21 days and then embedded in paraffin. Cross-sections ($4 \mu\text{m}$) were cut and stained with hematoxylin and eosin (H&E), and commercial tartrate resistant acid phosphatase (TRAcP) staining kit (Jiancheng Bioengineering Institute, Nanjing, China). Histological sections were analyzed using a standard high-quality light microscope (DM2000, Leica, Wetzlar, Germany). The erosion area (percentage of infiltrated fibrotic area against total tissue area) was determined by tracing the area of soft tissue between the parietal bones, including resorption pits on the superior surface of the calvaria. The presence of dark-purple-stained granules in the TRAcP staining section, located on the bone perimeter within a resorption lacuna, was defined as osteoclasts. The histomorphometric analysis was carried out by the UTHSCA Image Tool (IT version 3.0; University of Texas, San Antonio, TX).²⁴

2.14 IF Staining of Mice Calvaria

The mice calvaria was decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 21 days. Afterward, the samples were fixed in 4% formalin fixation and then embedded in paraffin blocks for 4.0- μ m serial sections. The sections were then dehydrated using an increasing concentration of ethanol wash and dewaxed using xylene. Endogenous biotin or enzyme was blocked with 3% hydrogen peroxide and antigen retrieval was performed with sodium citrate. Anti-mouse TNF- α monoclonal antibody (1:50 dilution, ab1793, Abcam) was used as the primary antibody with appropriate secondary immunofluorescent antibodies. After washing 3 times with PBS, a DAPI-containing anti-fade fluorescent fixing medium (Guangzhou HelixGen Co., Ltd., China) was added to the sections, and covered with a microscope coverslip (Fisher Scientific UK Ltd).²⁵ At last, the sections were imaged by a confocal microscopy (Zeiss LSM 710, Germany).

2.15 Statistics

All the results were obtained from at least three similar experiments. Data were expressed as mean $\pm$ SD. The One-way ANOVA with Dunnet's tests was used to compare the differences among the groups, a *p-value* < 0.05 was considered statistical significance.

3 RESULTS

3.1 Car inhibits osteoclastogenesis

In order to study whether Car can effectively suppress RANKL-induced osteoclast differentiation, BMMs were treated with RANKL and various concentrations of Car. The mature osteoclasts formed in the group of positive control after 5 days of treatment. The data showed that Car could suppress the differentiation of RANKL-induced osteoclast

dose-dependently (Figure 1A). The numbers of TRAcP-positive multinucleated cells were reduced while the concentration of Car increased from 0 μ M to 10 μ M (Figure 1B). Furthermore, MTS assay was used to determine the cytotoxicity of Car. It was shown that Car did not cause significant cell death under the concentration of Car up to 10 μ M (Figure 1C). To assess whether Car could affect the mature osteoclast morphology, the podosome belt, a crucial component for osteoclast function, was visualized by staining with anti-vinculin. Expectedly, in the Car-treated group, the number of nuclei per osteoclast and the area of podosome belt in each region were decreased (Figure 2 A-C). Furthermore, to investigate which stages of the Car affects the inhibition of osteoclast formation, Figure 3 (A-C) demonstrated that the Car inhibited osteoclast formation at all three stages and most significantly at the early stage (Day 1-3). In short, Car could suppress RANKL-induced differentiation of osteoclast in a dose-dependent fashion throughout all the stages within the non-cytotoxic concentration range.

3.2 Car inhibits osteoclastic resorption activity

In addition to looking at the effect of Car on osteoclast formation, hydroxyapatite absorption assay was used to further assess whether Car could impair cellular resorptive function of osteoclasts *in vitro*. Mature osteoclasts were treated with Car (5 or 10 μ M) for 48 hours, and the resorption area per well and the number of osteoclasts were shown in Figure 4. Despite observing some osteoclasts in the Car-treated groups, there was no statistical difference in the number of osteoclasts among all the wells. However, as the drug concentration increased, the resorption area decreased, especially at 10 μ M. This finding indicated that Car also attenuated the resorptive activity of osteoclasts.

3.3 Car attenuates osteoclast-related gene expression

To further investigate the underlying mechanisms, the expression of osteoclast-related genes was screened through the RT-PCR. The results demonstrated that genetic expression of *c-fos* and *Nfatc1* was reduced when the dose of Car reached 5 μ M (Figure 5A and B). Moreover, we investigated the regulation of Car on relevant genes of bone resorption, and the results showed that the expressions of *Ctsk* and *Acp5* treated with Car were significantly downregulated (Figure 5C and D).

3.4 Car suppresses NFATc1 activity and protein expression

RAW 264.7 cells stably transfected with the NFATc1 reporter construct was used for luciferase reporter assay,²⁶ aiming to determine the effect of Car on the activation of NFATc1. These results demonstrated that the activity of RANKL-induced NFATc1 was suppressed at the dose of 10 μ M (Figure 6A). In concordance with this finding, Car at the dose of 10 μ M significantly inhibited the protein levels of RANKL-enhanced NFATc1 on day 3 and 5, assessed by Western blot assay (Figure 6B and C). On day 1, 3 and 5, *c-fos* expression was also inhibited in the presence of Car, compared to the control group (Figure 6B and C). Moreover, other bone resorption-related genes, such as *CTSK*, *ATP6V0D2* and *ITGB3*, were down-regulated on days 3 and 5 after the treatment with Car (Figure 6B and C).

3.5 Car inhibits the RANKL-induced MAPK signaling pathway

To further investigate the mechanisms underlying the inhibitory action of Car on osteoclastogenesis, we examined the MAPK pathway (JNK, ERK, P38) with Western blotting. The phosphorylation of JNK, ERK and p38 was measured at 0, 5, 10, 20, 30, 60 minutes after RANKL stimulation in the presence or absence of Car. As illustrated in Figure 7,

the activation of ERK1/2 and P38 was suppressed with Car. After incubation with RANKL for 5, 10 and 20 minutes, Car significantly reduced the expression levels of p-ERK1/2 in BMMs. In addition, Car significantly inhibited the phosphorylation of P38 at 20, 30 and 60 minutes after RANKL stimulation. However, there was no statistically detected difference between the groups with or without Car on p-JNK expression. These results also indicated that Car suppressed the RANKL-induced MAPK signaling pathway, in particular phosphorylation of ERK1/2 and p38.

3.6 The effect of Car on RANKL-induced NF- κ B pathway and calcium oscillation

Through luciferase reporter gene assays, we found that Car treatment at 10 μ M significantly inhibited NF- κ B activation at the transcriptional level (Figure 8A). To further explore the effect of Car on NF- κ B activation, we examined the influence of Car on the degradation of I κ B- α protein after RANKL stimulation on BMMs for varied time of periods after pretreating with 10 μ M Car for 1 hour. Western blot results showed that Car had a significant inhibitory effect on the degradation of I κ B- α induced by RANKL at 10 μ M, particularly at 5, 10 and 60 minutes after RANKL stimulation (Figure 8B and C). Furthermore, because the calcium oscillation activation plays a critical role for the development of osteoclast via the transcription regulator NFATc1,²⁷ we also studied the effects of Car on intracellular calcium oscillation induced by RANKL to unveil the molecular mechanism of Car on the suppression of osteoclast differentiation and function. As illustrated in Figure 9, the results showed that RANKL-induced Ca²⁺ oscillation was significantly suppressed by Car.

3.7 Car treatment reduces titanium particles-induced osteolysis of the calvarial bone

We applied our previously published procedure to examine the effect of Car on titanium granule-induced osteolysis in vivo.²⁴ No abnormal behavior and weight loss in mice were observed during the Car-treatment period. Micro-CT analysis demonstrated that the local inflammation and osteolysis of the surface of mouse calvaria were induced by the application of titanium particles. We noticed that there was significant expansion on the width of the cranial suture. However, the osteolytic activity was not observed in the sham group. BMD, BV/TV and total porosity were measured from 3D reconstructed images. Ti particles induced significant osteolysis compared to the sham group. When Car was injected, osteolytic bone loss was prevented compared with the Ti particles group (Figure 10A-D). Further histological evaluation, H&E, TRAcP staining and immunofluorescence confirmed the inhibitory effect of Car injection on wear particle induced bone erosion and inflammatory response. Consistent with micro-CT quantification, the tissue response to Ti particles appeared to be limited in mice when treated with Car, and the erosion surface area and TRAcP-positive cells were significantly reduced. Immunofluorescence analysis showed that TNF- α is mainly localized in inflammatory cells surrounding the surface of the bone in the Ti particles group. After Car treatment, few TNF- α positive staining cells were observed (Figure 10E-G). In conclusion, these results suggested that Car reduced osteoclast-mediated osteolysis.

4.DISCUSSION

The excessive osteoclast formation and activity are featured in a wide range of osteolytic diseases,²⁸ including osteoporosis,³ periodontitis,²⁹ rheumatoid arthritis^{30,31} and the aseptic loosening of orthopedic implants.³² Nowadays, the application for treating osteolytic diseases is restricted due to the serious adverse effects, including the osteonecrosis, renal toxicity and

hormonal dysfunction,³³ making the imperative to seek novel treatments in preventing osteolytic diseases. Recently, researchers have focused drug discovery from natural compounds, a major source of drugs and medicines.^{34,35} Natural compounds with some beneficial effects such as antibacterial, anti-inflammatory and antioxidative implied its effective outcome in the treatment of osteolytic bone diseases.³⁶⁻³⁸ Herein, Car, a compound derivatively extracted from rosemary and salvia, has been extensively applied in traditional Chinese medicine. It has been reported that Car can effectively inhibit cartilage breakdown in human osteoarthritic chondrocytes via the inhibition of proinflammatory and catabolic factors.³⁹ Additionally, a study demonstrated the protective effect of Car against oxidative stress via PI3K and HO-1 pathways.⁴⁰ Furthermore, Car has showed its anti-cancer activity through the induction of oxidative stress incorporating with HSP90 inhibition which is achieved via downregulating key regulatory pathways including Notch, PI3K/Akt, mTOR, NF- κ B in cancers.⁴¹ Our study demonstrated that the inhibition of Car on osteoclast formation as well as reducing osteoclast resorption activity through the activities of transcription factors at the downstream of the MAPK as well as the NF- κ B and calcium signaling pathways. In addition, we elucidated a preventive effect of Car on the lost of bone *in vivo* applying a particle-induced osteolysis mouse model. These results collectively showed that Car has a great therapeutic potential for osteolysis.

It is well known that osteoclasts and hematopoietic mononuclear/macrophage lineages differentiate in the presence of two key factors: MCSF, inducing the proliferation of BMMs, and RANKL, inducing differentiation of osteoclast precursors into osteoclasts efficiently.^{42,43} In this study, MTS analysis revealed that Car had no statistically significant effect on the

proliferation of osteoclast precursors, while Car inhibited differentiation of RANKL-induced osteoclastogenesis throughout all the stages, particularly at the early stage, indicating that Car inhibited osteoclastogenesis during the process of osteoclast differentiation.

NFATc1 is an important protein in the formation of osteoclasts induced by RANKL and is highly phosphorylated in the cytoplasm.^{27,44} NFATc1 and c-Fos play indispensable roles in osteoclast differentiation, and the RANKL-induced NFATc1 expression has been shown to diminish in *c-Fos* knockout mice.⁴⁵ C-Fos is a part of the activated protein-1 (AP-1) transcription factor complex and is an essential factor in triggering the transcriptional regulatory cascade of NFATc1. Thus it initiates multiple target genes involved in differentiation and function of osteoclasts.⁴⁶ Previous studies have showed that bone sclerosis occurs in mice due to c-Fos deficiency, suggesting the crucial role of c-Fos in RANKL-induced osteoclast formation.^{46,47} Our results showed that Car significantly reduced the expression of *c-Fos* gene and protein. In addition, intracellular calcium level changes resulted from the interaction between RANK and RANKL, NFATc1 removes phosphate and translocates into the nucleus to activate the expression of osteoclast specific genes like *Acp5* (*TRAcP*), *ITGB3* (Integrin β 3), *CTSK* and *ATP6V0D2* (V-ATPase-d2).^{48,49} NFATc1 regulates both the above bone resorption-related genes and proteins. We identified a significant inhibition in genetic expression after Car treatment in *Acp5* (*TRAcP*), *ITGB3*, *CTSK* and *ATP6V0D2*, leading to the down-regulation of RANK-induced bone osteoclastic resorption.

ERK, JNK and P38 belong to the subfamilies of MAPKs, which regulate cellular activities such as differentiation and apoptosis.⁵⁰⁻⁵² In particular, ERK promotes osteoclast differentiation and prolongs the survival of osteoclasts,^{11,26,53} which is in accordance with

increased numbers of osteoclasts after RANKL treatment. Phosphorylated ERK translocates into the nucleus to modulate the transcription of c-Fos, while JNK applies phosphorylated c-Jun to enhance the transcription activity of AP-1.⁵⁴ Unsurprisingly, inhibition of JNK impairs osteoclast formation. P38 is involved in the control of osteoclast differentiation rather than the modulation of osteoclast function.⁵⁵ According to above-mentioned results, Car reduced the phosphorylation of ERK and P38, however, it did not significantly affect the phosphorylation of JNK. Therefore, we suggest that Car regulates the activation of AP-1 by blocking MAPK signaling pathway and suppressing the activity of NFATc1.

NF- κ B is important for RANKL-induced NFATc1 expression during early stage of osteoclast formation.²³ NF- κ B, therefore, regulates osteoclast differentiation by acting as a crucial transcriptional factor. Upon the stimulation of RANKL, followed by the degradation of its inhibitor-I κ B, the NF- κ B complex is released and translocated into the nucleus and thus initiates the transcription of multiple target genes.⁴ The degradation of I κ B- α and subsequent stimulation of NF- κ B have been observed in a amount of osteolytic lesions in which osteoclasts were overproduced.²³ I κ B- α , therefore, can be considered to reflect the activation of NF- κ B signaling molecules.^{34,56} Our results showed that Car suppressed the stimulation of NF- κ B by affecting the degradation of I κ B- α . Previous studies reported that the induction of NFATc1 is also relied on Ca²⁺-dependent calcineurin activation.¹¹ Therefore, the stimulation of NFATc1 is regulated by Ca²⁺ oscillation during RANKL-induced osteoclastogenesis.²⁷ Our study confirmed the inhibitory action of Car on Ca²⁺ oscillation and NFATc1 activation.

Osteolysis is a key pathological process in a variety of devastating skeletal disorders such as osteoporosis, arthritis, bone tumors, Paget's disease, aseptic loosening and so on.⁵⁷

Considerable evidence has shown that osteolysis is closely related to excessive osteoclastic activity and bone resorption.⁵⁸ Some studies have demonstrated that multinucleated osteoclasts are increased in titanium particle-stimulated bone tissues.⁵⁹⁻⁶¹ Given the important role the osteoclasts play in the bone destruction process, agents that can inhibit osteoclast formation and/or bone resorption are ideal therapeutic agents for preventing and ameliorating wear particle-induced osteolysis.⁶¹

There is mounting clinical evidence that particle-stimulated aseptic loosening largely cause orthopedic implants failure. Currently, the treatment of particle-induced periprosthetic osteolysis is limited in the revision surgery.^{27,62-64} Therefore, investigating the compounds as the inhibitors of particle-induced osteolysis is of interest in the enhancement of effective treatment in particle-stimulated aseptic periprosthetic loosening. From the particle-induced osteolysis mouse model, we found that Car treatment inhibited the osteoclast formation and therefore prevent bone loss around the prosthetic implants. Pharmacokinetic studies of Car will be critical in determining the potential use of Car for application as an anti-inflammatory agent.¹³ Macrophages as the progenitor cells of osteoclasts are also involved in peri-implant osteolysis and closely related to inflammation response. As they are responsible for driving inflammatory and destructive damage in bones, macrophages also appear to be promising therapeutic targets in inflammatory bone diseases.⁶⁵ Therefore, the anti-inflammatory effect of Car on macrophages may be another underlining mechanism on preventing particle-induced osteolysis which will be further explored in the future.

In conclusion, our study showed that Car can inhibit the formation and function of RANKL-induced osteoclast via NFATc1 by affecting the MAPK, NF- κ B and calcium

signaling pathways (Figure 11). Moreover, Car prevents particle-induced osteolysis *in vivo*, being in concordance with its effects *in vitro*. Therefore, Car, a natural extract from the traditional Chinese herb, appears to be a potential therapeutic supplementary for the prevention and treatment of particle-induced osteolysis, and its related osteolytic diseases.

Acknowledgements:

This project is supported in part by Guangdong Natural Science Foundation(2016A030313641), Guangzhou science and technology project (201707010460), Guangdong science and technology project (2016A020215137), Open foundation of national key laboratory of orthopedics of traditional Chinese medicine (YB12), National Natural Science Foundation of China (81672186), The outstanding doctoral dissertation project of Guangzhou University of Chinese Medicine(A1-AFD018181A59), Postgraduate international communication project of Guangzhou University of Chinese Medicine(20172101018), Traditional Chinese Medicine Bureau of Guangdong Province(20191098). This research was supported by National Natural Science Foundation of China (81501910), Guangxi Natural Science Foundation under Grant (2015GXNSFCA414001), and Guangxi Collaborative Innovation Center for Biomedicine Talent Cultivation (GCICB-TC-2017001). This study was supported in part by the Australian National Health and Medical Research Council (NHMRC, APP1107828, APP1127156, APP1163933).

Contributions:

In this study, Yongxian Li, Kai Chen, Heng Qiu, Yuhao Liu and Qian Liu performed cell

culture, PCR and signalling studies; Sipeng Lin, Junxiong Qiu, Zhenkang Wen, Jianbin Huang, Jinbo Yuan, Panjie Liu, Tengpeng Zhou, Peijie Luo, Huizhi Guo, Yanhuai Ma, Danqing Guo and Guoye Mo performed animal experiments; Yongxian Li, Kai Chen and Yongchao Tang performed histological analyses; Yongxian Li and Kai Chen wrote the manuscript; Liangliang Xu and De Liang designed animal experiments; Jiake Xu, Yue Ding and Shuncong Zhang designed and supervised the overall study and revised the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Table legend:

Table 1. Primer sequences for qPCR

Figure legends:

FIGURE 1. Car inhibited the formation of osteoclast. (A) Typical images of BMMs in TRAcP staining treated with Car with varying concentrations. (B) Quantification of osteoclasts in each experimental setting. (C) The viability of BMMs with Car evaluated by MTS assay. The data represent as mean $\pm$ SD. Scale bar represents 200 μ m. * represents as $p<0.05$ and ** represents as $p<0.01$ for statistical significance comparing the treatment group with control groups.

FIGURE 2. Car inhibited RANKL-induced osteoclast multinucleation. (A) Typical confocal images of osteoclasts positive for podosome belt and nuclei. (B) The average number of nuclei per osteoclast. (C) The average of podosome belt area per field was analyzed quantitatively (n=3). Scale bar represents 200 μ m. The data represent as mean $\pm$ SD. * represents as $p<0.05$ and ** represents as $p<0.01$ for statistical significance between the treatment and control groups.

FIGURE 3. Car significantly suppressed RANKL-induced osteoclastogenesis at the early stage. (A) Representative images of the effect of 10 μ M Car treatment in different periods of time. Osteoclast formation was induced by M-CSF (50 ng/ml) and RANKL (50 ng/ml), and Car in the designed time periods. (B) The time periods of Car treatment. (C) The counts (n=3)

of TRAcP-positive multinucleated cells (nuclei >3) with Car treatment. Scale bar = 200 μ m. * represents as $p<0.05$ and ** represents as $p<0.01$ for statistical significance between the treatment and control groups.

FIGURE 4. Car suppressed the activity of osteoclast bone resorption. (A) Typical images of the bone resorption surface of 96-well plate after removal of osteoclasts (bottom) and the osteoclasts of TRAcP stained (top). Scale bar represents 200 μ m. (B) Numbers of osteoclasts in each well (96-well plate). (C) Quantification of hydroxyapatite resorption area per osteoclast. The data represent as mean $\pm$ SD. * represents as $p<0.05$ and ** represents as $p<0.01$ for statistical significance comparing the treatment group with control groups.

FIGURE 5. Car attenuated the expression of osteoclast-specific marker genes. BMMs were treated with GSTrRANKL and M-CSF with or without designated concentrations of Car. Gene expression was standardized against *ACTB* and *HPRT*. Relative mRNA expression levels of *NFATc1* (A), *c-Fos* (B), *Ctsk* (C) and *Acp5* (TRAcP) (D) during osteoclastogenesis. The data represent as mean $\pm$ SD. * represents as $p<0.05$ and ** represents as $p<0.01$ for statistical significance comparing the treatment group with control groups.

FIGURE 6. Car reduced the protein level of osteoclast formation and bone resorption-related gene expression. (A) Stable transfection of RAW 264.7 cells with the NFATc1 reporter gene was performed by luciferase reporter assay, aiming to detect the effect of Car on activity of RANKL-induced NFATc1. (B) Typical western blot images of c-Fos, NFATc1, Integrin β 3,

V-ATPase-d2, CTSK and ACTB from BMMs cultured in the presence of M-CSF and GST-rRANKL with Car. (C) Relative quantification of the normalized expression of above-mentioned proteins against ACTB. The data represent as mean $\pm$ SD. * represents as $p < 0.05$ and ** represents as $p < 0.01$ for statistical significance between the treatment and control groups.

FIGURE 7. Car inhibited the RANKL-induced MAPK signaling pathway. (A) Representative western blot images of p-JNK1/2, JNK, p-ERK1/2, ERK, p-P38, and P38 at 0, 5, 10, 20, 30, 60 minutes stimulated by GST-rRANKL in the presence or absence of Car (10 μ M/ L). (B-F) The relative ratios of phosphorylated proteins/ unphosphorylated proteins. The data represent as mean $\pm$ SD. * represents as $p < 0.05$ and ** represents as $p < 0.01$ for statistical significance between the treatment and control groups.

FIGURE 8. Car abrogated RANKL-induced NF- κ B activity and inhibited I κ B- α degradation. (A) NF- κ B Luciferase activity. RAW264.7 cells transfected with a RANKL-induced NF- κ B luciferase construct, were pretreated with designated dose of Car. (B) Typical western blot strips of I κ B- α and β -actin protein levels of BMMs pretreated with the concentration of 10 μ M of Car for 1 hour and subsequently stimulated with GST-rRANKL (50 ng/ml) for the indicated times. (C) Relative protein levels of I κ B- α normalized to β -actin. The data represent as mean $\pm$ SD. * represents as $p < 0.05$ and ** represents as $p < 0.01$ for statistical significance between the treatment and control groups.

FIGURE 9. Car suppressed RANKL-induced Ca^{2+} oscillation. (A) Fluorescence intensity waves of Ca^{2+} oscillation without RANKL. (B) Fluorescence intensity waves of Ca^{2+} oscillation with RANKL. (C) Fluorescence intensity waves of Ca^{2+} oscillation with RANKL and Car (10 μM). (D) Intensity change of Ca^{2+} oscillation. The values of intensity change were calculated as the differences between the highest intensity and the lowest intensity (n = 6). * $p < 0.05$, ** $p < 0.01$ relative to RANKL-induced control group.

FIGURE 10. Evaluation of the influence of local Car injection on Ti particle-induced calvaria osteolysis and inflammation reaction in mice. (A) Micro-CT 3D reconstructed images. (B-D) BMD, BV/TV, and the total porosity of each sample within the ROI were measured (N = 6 per group). (E) H&E, TRAcP and immunofluorescence staining for TNF- α in mouse calvaria. (F-G) Histomorphometric analysis of the erosion area (percentage of infiltrated fibrotic area against total tissue area) and the number of TRAcP positive multinucleated osteoclasts within the ROI were measured (N = 6 per group). * $p < 0.05$, ** $p < 0.01$. All data are shown as the mean $\pm$ SD. The assays were performed at least three times for each sample.

FIGURE 11. Schematic representation of the proposed function of Car in the formation and function of osteoclasts. Car reduces the expression of *Acp5*, *c-Fos*, *NFATc1* and *CTSK* through inhibiting the MAPK, NF- κ B and calcium signaling pathways, and finally suppressing RANKL-induced osteoclast differentiation and bone resorptive function.