Supporting information

Title page:

Sophocarpine attenuates wear particle-induced implant loosening by inhibiting osteoclastogenesis and bone resorption via suppression of the NF-κB signaling pathway in a rat model Supplemental Method

Supporting Methods

Flow cytometry (FACS) analysis

Rat bone marrow cells were isolated and cultured in complete α-MEM containing 30ng mL^{-1} M-CSF for 2 days. Then cells were collected and washed twice with PBS containing 2% BSA, following by being resuspended in FACS staining buffer (Biolegend, San Diego, CA) at a concentration of 5×10^5 cells/100 μL. Cells were subsequently incubated with primary antibody against CD11b-Phycoerythrin (PE) (ab25533) (Abcam, Cambridge, UK) for 1h and washed twice, and finally resuspended in 0.1mL PBS. Flow cytometry analysis was performed using BD FACSCANTO II (BD Biosciences, Mt. View, CA). The results were measured using FlowJo 10 software.

Primary rat osteoblast isolation and osteogenic differentiation

Rat primary osteoblasts (OB) were isolated from the neonatal rat calvarial bone as reported previously (Xie et al., 2016), and cultured in complete α-MEM at a 37°C humidified incubator containing 5% (v/v) CO2. OBs were seeded into plate until reaching 70-80% confluence and subsequently cultured the osteoinductive medium (complete α-MEM containing 10 mM β-glycerophosphate and 0.1 mM L-ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA)) for 7 or 14 days.

ALP and mineralization assay.

OBs were cultured in osteoinductive medium supplemented with SPC (0, 0.25, 0.50, or 1.00 mM). On day 7, cells were fixed with 4% PFA and stained with

commercial ALP staining kit according the manufacturer's protocol. ALP activity was quantified using an ALP Color Development Kit (Beyotime, Shanghai, China) and OD value was measured at 405 nm using an ELX800 absorbance microplate reader. On day 14, cells were fixed and stained with Alizarin Red S (ARS) (Cyagen Biosciences, Guangzhou, China). 10% cetylpyridinium was added to destain the mineralized nodules and the calcium concentration was measured at 562nm.

Determination of NO

Rat BMMs were seeded into 24-well plate at a density of 5×10^4/well in complete α-MEM containing 30ng mL^{-1} M-CSF overnight. Medium containing SPC (0, 0.25, 0.50, or 1.00 mM) with or without Ti particles (0.1mg mL^{-1}) were used to culture cells for another 24h. The concentration of Ti particles was determined by the wear debris retrieved from peri-implant tissues (von Knoch *et al.*, 2004). The NO levels were measured using the Griess reagent according to the manufacturer's protocol.

ELISA for cell supernatant

BMMs were cultured in complete α-MEM containing 30ng mL-1 M-CSF overnight. After pretreated with medium containing SPC (0, 0.25, 0.50, or 1.00 mM), Ti particles (0.1mg mL^{-1}) or PBS were added for another 24h. The supernatant was collected and the release of PGE_2 was detected using ELISA kits according to the manufacturer's protocol.

Supporting Reference

von Knoch M, Jewison DE, Sibonga JD, Sprecher C, Morrey BF, Loer F*, et al.* (2004). The effectiveness of polyethylene versus titanium particles in inducing osteolysis in vivo. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* **22**(2)**:** 237-243.

Xie YF, Shi WG, Zhou J, Gao YH, Li SF, Fang QQ*, et al.* (2016). Pulsed electromagnetic fields stimulate osteogenic differentiation and maturation of osteoblasts by upregulating the expression of BMPRII localized at the base of primary cilium. *Bone* **93:** 22-32.

Supporting Figure Legends

Figure S1

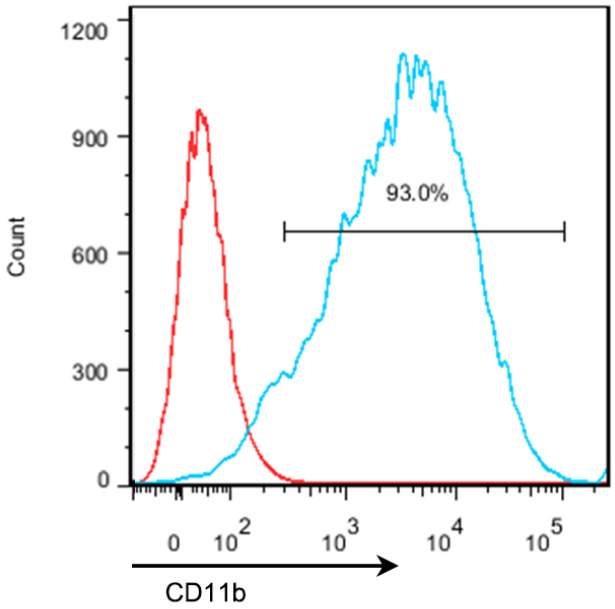
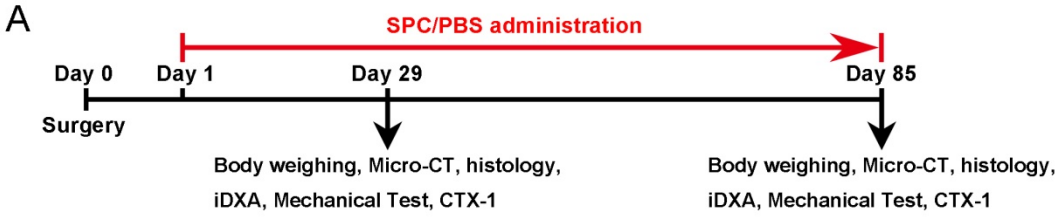


Figure S1. Flow cytometry analysis of CD11b positive expression was measured on BMMs. 91.3 ± 3.1% BMMs are CD11b positive cells. Data on graph are the mean ± SD of five independent experiments.

Figure S2



B

Samples size for each time point

Body weighing (N=10/group)
Femurs for histology and immunhistochemistry (N=5/group)
Femurs for Micro-CT and iDXA (N=11/group for iDXA, including N=7/group for micro-CT)
Femurs for mechanical tests (N=6/group)
Elisa for Serum CTX-1 (N=10/group)

Figure S2. The schematic diagrams showed the design of the study and animal size for each time point in each group. (A) A schematic diagram for the timeline of the study was showed. (B) The table showed the detail tests for the study and the animal size for each tests at each time point.

Figure S3

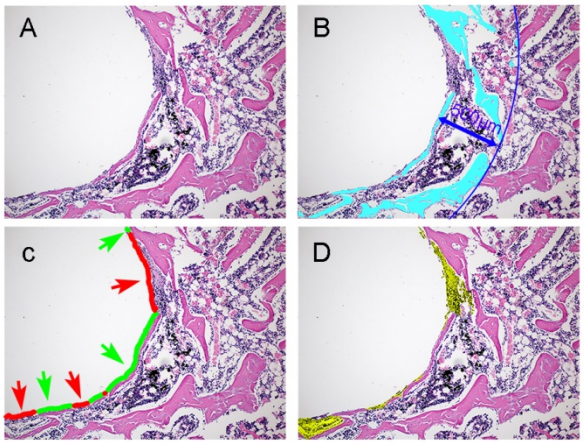


Figure S3. The schematic diagrams showed the details for the quantification of B.Ar/T/Ar, BIC and the mean thickness of pseudomembrane. (A) An image of a slice for H&E staining was showed. (B) The image showed the ROI and bone area (blue) for the histological analysis of B.Ar/T.Ar. (C) This image showed the bone-implant contact part (red line) and uncontact part (green line). (D) This image showed the area of the pseudomembrane around the implant (yellow).

Figure S4

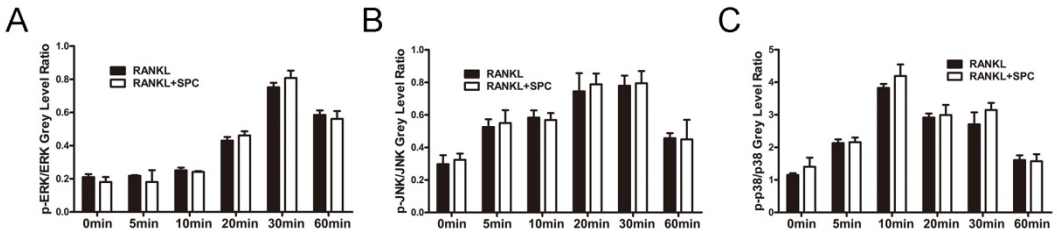


Figure S4. The quantification for SPC on the activation of MAPK signaling pathway were measured. (A) The grey levels of phospho-ERK were quantified by being normalized to total ERK. (B) The grey levels of phospho-JNK were quantified by being normalized to total JNK. (C) The grey levels of phospho-p38 were quantified by being normalized to total p38. Experiments were performed at least 3 times and values are expressed as mean ± SD.

Figure S5

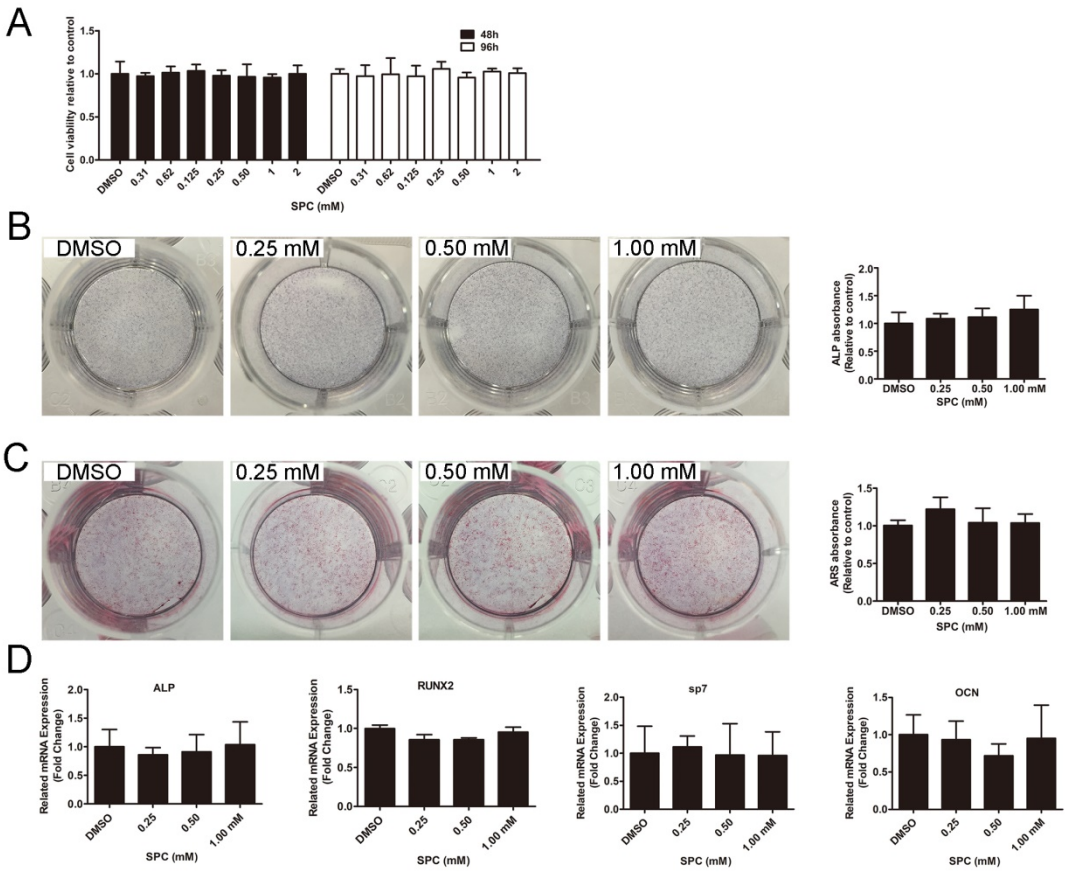


Figure S5. SPC has no effects on osteogenic differentiation in vitro. (A) Rat primary osteoblasts were culture in a 96-well plated and treated with various concentrations of SPC (0.31-2mM) for 48h or 96h. The viability of cells was measured by the CCK8 kit. (B) Osteoblasts were cultured in osteoinductive medium in the presence of indicated dilutions of SPC for 7 days. ALP staining was performed and ALP activity was measured. (C) Osteoblasts were cultured in osteoinductive medium with different dilutions of SPC for 14 days. ARS staining and quantification were performed. (D) The mRNA expression of ALP, RUNX2, sp7 and OCN in the primary osteoblasts were quantified after osteogenic differentiation for 14 days in the presence of different concentrations of SPC. Experiments were performed at least 3 times and values are expressed as mean ± SD.

Figure S6

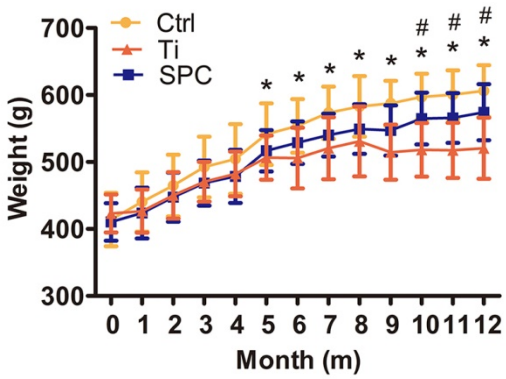


Figure S6. SPC prevented the diminishment of weight gain in a rat model of aseptic implant loosening. Body weight of all rats was measured every week after SPC administration. n = 11 animals per group, Values are expressed as mean ± SD; *p < 0.05 versus the control group; #p < 0.05 versus the Ti group.

Figure S7.

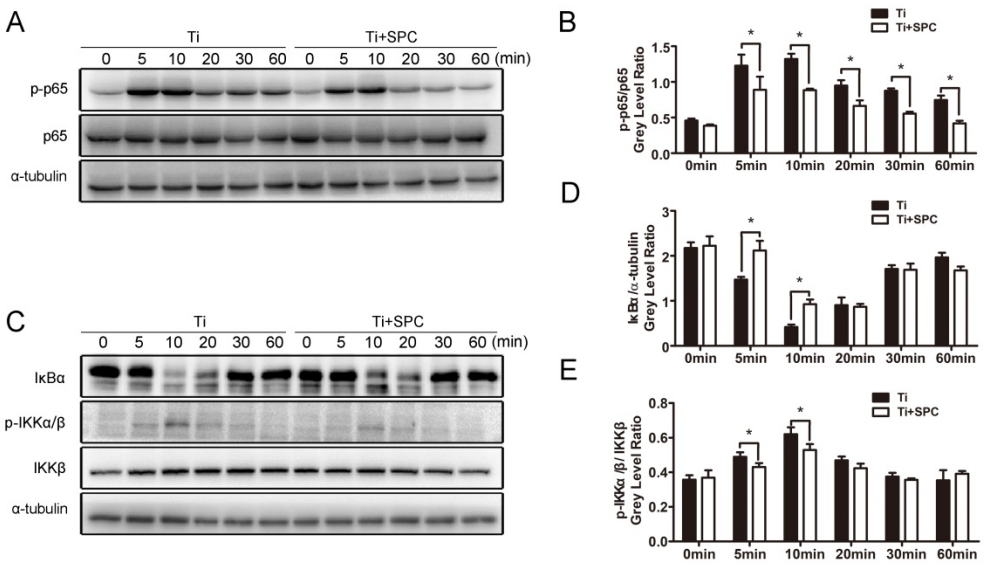


Figure S7. SPC impaired Ti-induced activation of NF-κB signaling pathway in vitro. (A, C) After 4h pretreatment with 1mM SPC or DMSO, BMMs were treated with 0.1mg mL^{-1} Ti for 0, 5, 10, 20, 30 and 60 min. Cells were collected and cell lysates were analyzed by Western blotting using primary antibodies specific to phospho-p65, p65, IκBα, phospho-IKKα/β, IKKα/β， and α-tubulin. (B, D, E) The grey levels of phospho-p65 and phospho-IKKα/β were analyzed by being normalized to total p65 and IKKα/β. IκBα was normalized to α-tubulin. Three independent experiments were performed for the data analysis and values are expressed as mean ± SD; *p < 0.05versus the control group.

Figure S8

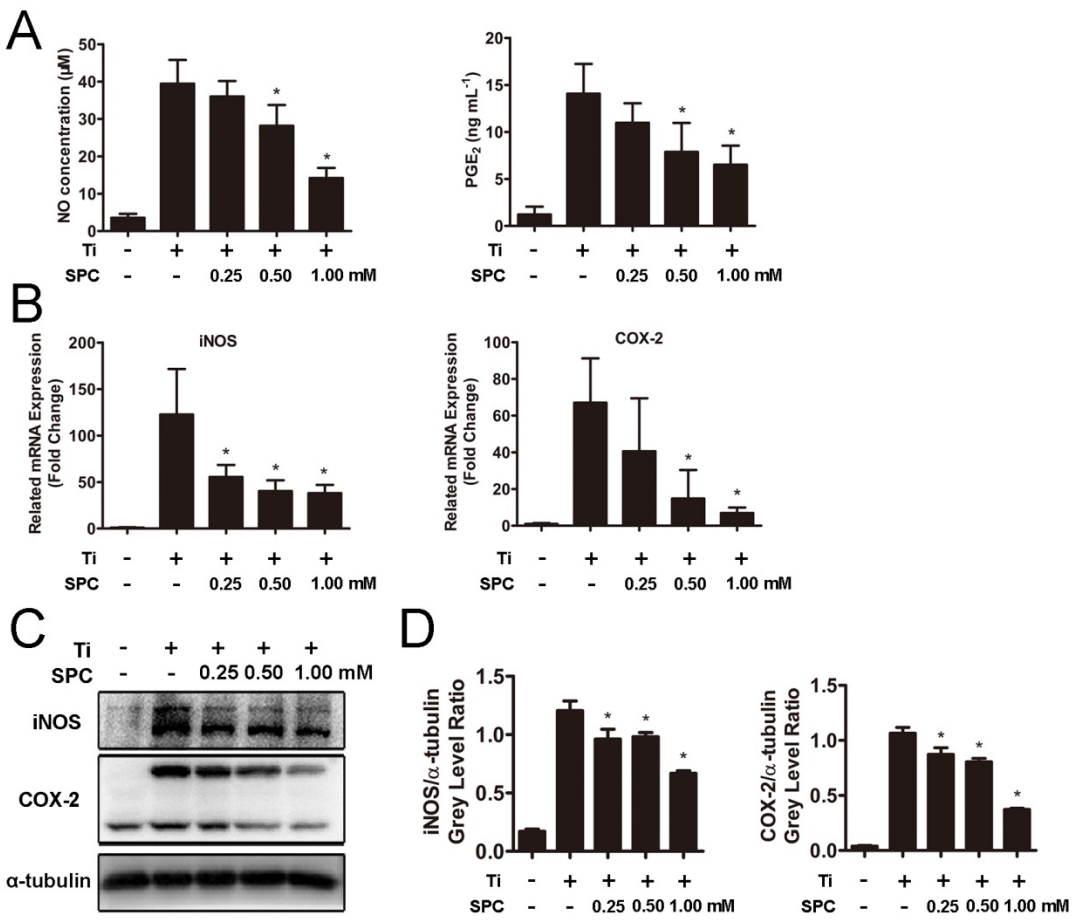


Figure S8. SPC inhibited Ti-induced NO and PGE_2 production, as well as the expression of iNOS and COX-2. (A) Rat BMMs were pretreated with different concentrations of SPC (0, 0.25, 0.50 or 1.00mM) for 4h and subsequently treated with 0.1mg mL^{-1} Ti or vehicle for 24h. Supernate was collected and the production of NO and PGE_2 were measured. (B) After being pretreated with indicated concentrations of SPC for 4h, BMMs were treated with or without 0.1mg mL^{-1} Ti for another 4h. The mRNA expression of iNOS and COX-2 were measured by RT-PCR. (C) BMMs were pretreated with various dilutions of SPC for 4h, following treated with 0.1mg mL^{-1} Ti particle or vehicle for another 24h. The protein expression of iNOS and COX-2 were explored using Western Blotting. (D) The grey levels of iNOS and COX-2 were normalized relative to α-tubulin using Image J Software. Data were analyzed by three independent experiments and values are expressed as mean ± SD; *$p < 0.05$ versus Ti

particles alone.

Figure S9

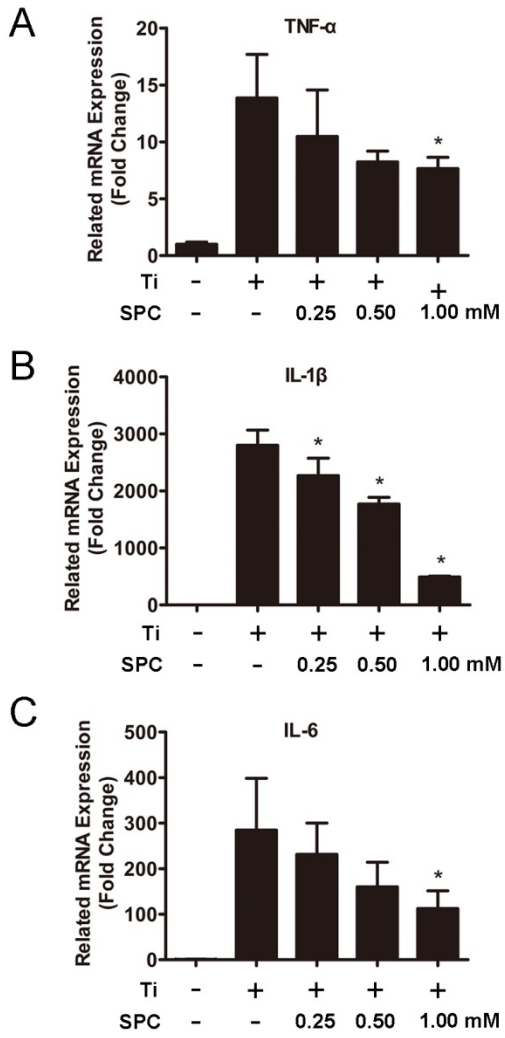


Figure S9. SPC suppressed Ti-induced proinflammatory cytokine expression in rat primary macrophages. (A, B and C) After being pretreated with 30ng mL^{-1} M-CSF and indicated dilutions of SPC for 4h, rat primary BMMs were treated with or without 0.1mg mL^{-1} Ti for another 4 h. Real-time PCR was performed to analyze the mRNA expression of TNF-α, IL-1β and IL-6 and all results were normalized to the expression of GAPDH. All experiments were performed 5 times and values are expressed as mean ± SD; *$p < 0.05$ versus Ti particles alone.