Total coumarins from fruits of *Cnidium monnieri* inhibit formation and differentiation of multinucleated osteoclasts of rats[1]

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ABSTRACT

AIM: To determine the effects of TCFC (total comarins from the fruits of *Cnidium monnieri*) on the activity of osteoclasts *in vitro*. **METHODS:** Osteoclasts isolated from rat marrow cells were co-cultured with osteoblasts under the 1,25-dihydroxyvitamine D_3. The tartrate-resistant acid phosphatase (TRAP) stain was used to identify osteoclast morphology. The activity of TRAP was measured by *p*-nitrophenyl sodium phosphate assay. The resorption pit area on the bone slices formed by osteoclasts was measured by computer image processing. Calcium concentration in the medium of co-culture of bone slices and osteoclasts was determined by atomic absorption spectra. **RESULTS:** TCFC 2.5-25 mg/L inhibited osteoclast formation and differentiation. TCFC 0.25-25 mg/L inhibited TRAP activity of osteoclasts and TCFC 25 mg/L decreased the TRAP activity by 26.3 % and 24.1 % after 48 h and 72 h, respectively. TCFC 25 mg/L decreased the osteoclastic bone resorption pit area by 25.05 % and Ca^{2+} release from bone slices by 41.73 %. **CONCLUSION:** TCFC reduced the bone lose by decreasing the osteoclast formation, its TRAP activity, and osteoclastic bone resorption.

INTRODUCTION

Osteoporosis characterized by loss of bone mass is a major health problem, especially in elderly women. The etiology of human osteoporosis is multifactorial and is influenced by heredity, hormone excess or deficiency, dietary components, and physical activity. An increased frequency in osteoporosis correlates with longer life expectancy. Bone loss has been attributed to the imbalance between the bone formation and bone resorption. Many agents such as calcium, estrogen, calcitonin, vitamine D_3, and ipriflavone have been used clinically in the treatment of this disease[1].

The fruit of *Cnidium monnieri* L Cuss (umbelifera) is an important drug in traditional Chinese medicine for the treatment of inflammation and gynecological disease such as vaginitis. Several investigations have shown that total coumarins from the fruits of *Cnidium monnieri* (TCFC) have antiosteoporotic effects. In ovariectomized rats, TCFC inhibited the high bone turnover and reversed the bone loss at early menopause[2]. In prednisolone-treated rats, TCFC increased the femoral bone density[3]. But what is the mechanism of TCFC's effect on bone metabolism remains unknown. In our

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previous study, we found that TCFC significantly promoted osteoblast proliferation, alkaline phosphatase activity, and collage synthesis[4]. In order to further understand the mechanism of the action of TCFC on osteoclastic cells, we investigated the effects of TCFC on osteoclasts induced by 1,25-dihydroxyvitamine D_3 from rat marrow cells.

MATERIALS AND METHODS

Drugs and reagents TCFC was extracted from the fruits of *Cnidium monnieri*, composed of osthole, imperatorin, and bergapten. The content of coumarins in TCFC was 80 %. TCFC was dissolved in absolute ethanol at a concentration of 250 mg/L and diluted to the appropriate concentration using culture medium. RPMI-1640 medium, α-MEM medium, and fetal calf serum (FCS) were purchased from Gibco company. 1,25-Dihydroxyvitamin D_3, dexamethasone, naphthol AS-BI phosphate, and pararosaniline were purchased from Sigma company. Ipriflavone (IP) was donated by Prof WENG Ling-Ling in Department of Osteoporotic Drug Research, West-China Medical University. IP was dissolved in ethanol at the concentration of 10 mmol/L and diluted to the appropriate concentration using culture medium. The ethylene glycol methyl ether, potassium sodium tartrate, *p*-nitro-disodium phenylphosphate, Triton X-100, and *p*- nitrophenol were domestic AR grade products.

Animals and cultures of multinucleated osteoclasts Wistar rats in 3–4-day old were purchased from Experimental Animal Center of Second Military Medical University. Primary osteoblastic cells were prepared according to Liu *et al*[5]. Osteoblastic cells were isolated from the calvaria of 3–4-day newborn rats. Twenty to thirty calvaria were collected and digested using a solution of phosphate buffered saline (PBS) containing 0.25 % trypsin and collagenase 1 g/L. Cells isolated in fractions 3 and 4 were combined and cultured in RPMI-1640 medium containing 10 % FCS at 1×10^8 cells/L for 7 d. These cells had typical properties of osteoblasts such as alkaline phosphatase activity. All cultures were maintained at 37 °C in a humidified atmosphere of 5 % CO_2. Rat marrow cells were collected as described by Udagawa *et al*[6]. Briefly, 3-4 d old Wistar rats were killed, the femurs were disarticulated, the ends were removed. The bone marrow cells were flushed out using a syringe and a ten gauge needle with α-MEM medium. A single cell suspension was prepared by repeated pipetting with a 5-mL pipette. Primary osteoblastic cells (1×10^8 /L) and bone marrow cells (1×10^9/L) were co-cultured in α-MEM medium containing 10 % FCS, 1,25-dihydroxyvitamine D_3 (10 nmol/L) and dexamethasone (100 nmol/L) at 37 °C in a humidified atmosphere of 5 % CO_2 for 10 d in 6-well culture dishes (1.5 mL per well). Prior to plating the cells, a cover glass or bone slices were put into culture dishes. The formation of osteoclast-like MNC (multinucleated osteoclasts) was confirmed by the staining of TRAP and resorptive pit formed on bone slices.

Preparation of bovine cortical bone slices Bovines cortical bones were cut into 40-μm thick with a cutting machine (LEITZ 1600, German). The slices were treated by ultrasonic for 30 min and rinsed in distilled water, then sterilized with 75 % ethanol, dried, and left under ultraviolet light for 15 min before use.

Staining of tartrate-resistant acid phosphatase[7] The cells were fixed with 2.5 % glutaral (v/v) for 30 min and stained at 37 °C for 60 min with staining solution (0.2 mol/L, acetic acid buffer 18 mL, pH 5.0, 62 mmol/L azopararosaniline 1 mL, naphthol AS-BI phosphatase 1 mL, potassium sodium tartarate 282.22 mg), rinsed with distilled water, sealed with glycerol-gelatin, then observed under microscope.

Counting of the positive-TRAP MNC osteoclast Primary osteoblast (1×10^8/L) and marrow cells (1×10^9/L) were suspended in α-MEM medium containing 10 % FCS, 1,25-dihydroxyvitamine D_3 (10 nmol/L), and dexamethasone (100 nmol/L). A 100 μL aliquot of cell suspension was added to 96-well culture dishes and cultured at 37 °C in a humidified atmosphere of 5 % CO_2. After incubation for 24 h, the medium was replaced with fresh medium containing either TCFC (0.0025-25 mg/L) or ipriflavone (1 μmol/L). The cells were cultured for 10 d, stained for TRAP, and the positive-TRAP MNC osteoclasts were counted.

Measurement of TRAP activity[8] As described previously, 100 μL suspension of primary osteoblast (1×10^8/L) and marrow cells (1×10^9/L) were co-cultured in α-MEM medium containing 10 % FCS in the presence of 1,25-dihydroxyvitamine D_3 (10 nmol/L) and dexamethasone (100 nmol/L) at 37 °C in a humidified atmosphere of 5 % CO_2 for 8 d. The medium was replaced with fresh medium containing TCFC (0.0025-25 mg/L) or IP (1 μmol/L). Then cells were cultured for 24, 48, and 72 h. The TRAP activity was measured after rinsing cells twice with PBS. Briefly, 10 μL 0.1 % Triton X-100 was added for 10 min to lyse the cells. Then 100 μL substrate solution (preparation of

reactive solution: resolve 0.4 g *p*-nitro-disodium phenylphosphate in deionized water, and add 2.0 g potassium tartrate, then add deionized water to 150 mL to resolve it adjusting to pH 3.5 with HCl 1 mol/L, finally add deionized water to 200 mL) was added and incubated at 37 °C for 30 min. To stop the reaction, 100 μL NaOH 1 mol/L was added to each well. The samples and standards were diluted in NaOH 20 mmol/L, and the absorbance was measured at 405 nm. The nanomolar number of *p*-nitrophenol in each well was calculated.

Observation and assay of pit formation Primary osteoblasts (1×10^8 cells/L) and marrow cells (1×10^9 cells/L) were suspended in α-MEM medium containing 10 % FCS, 1,25-dihydroxyvitamine D_3 (10 nmol/L) and dexamethasone (100 nmol/L). A 100 μL aliquot of cell suspension was plated in a 96-well culture dish, bone slices were placed into each well of 96-well culture dish, and cultured at 37 °C in a humidified atmosphere of 5 % CO_2. After 24-h incubation, the media was discarded, the fresh media with or without TCFC or IP were added into culture dish. The cells were continuously cultured for 10 d. The bone slices was soaked in NH_4OH 0.25 mol/L solution to remove attached cells, fixed with osmium and glutaral, dried at critical point of CO_2, and coated with gold. The resorption pit on bone slices was observed under scanning electronic microscope. In addition, bone slices were rinsed with distilled water and stained with Eosin solution. Resorbing pits were observed and the pit area was quantified with a computer image analysis system (Apollo DN 3500) linked to the light microscope.

Calcium concentration measurement As described previously, bone slices were placed into each well of 96-well culture dish. Primary osteoblasts and marrow cells were co-cultured for 8 d. Then medium was discarded. The fresh media with or without TCFC or IP 1 μmol/L were replaced. Cells were continuously cultured for 48 h and 72 h. Calcium concentration in culture medium was measured with atom absorption spectra (PE company, 2100 atom absorption spectra instrument). The calcium concentration was used to express bone resorptive activity of osteoclast.

Statistical analysis Each series of experiments was repeated at least 5 times. The results of typical experiment were expressed as mean±SD. Significance was evaluated using Student's *t*-test.

RESULTS

Identification of osteoclast Two distinct types of cells were present in cultures, the monolayer of osteoblast-like cells and the free, large cells growing on them. The first type cells were in close side by side contact with each other and with occasional cellular junctions. The second type cells were free, sub-round cells characterized by process and pseudopodia, numerous nucleus and vacuole in cytoplasm. After the cytochemical staining for TRAP, the top sub-round cells were positive with red sediment formed in their cytoplasm, and the osteoblast-like cells were negative (Fig 1). After primary osteoblast and marrow cells were co-cultured for 10 d on the bone slices in the presence of 1,25-dihydroxyvitamine D_3 and dexamethone, numerous resorptive pits appeared on the surface of bone slices. The resorption pits were roundish, elliptical, and irregular, their margins were rodent, and bottoms were rough under scanning electronic microscope (Fig 2).

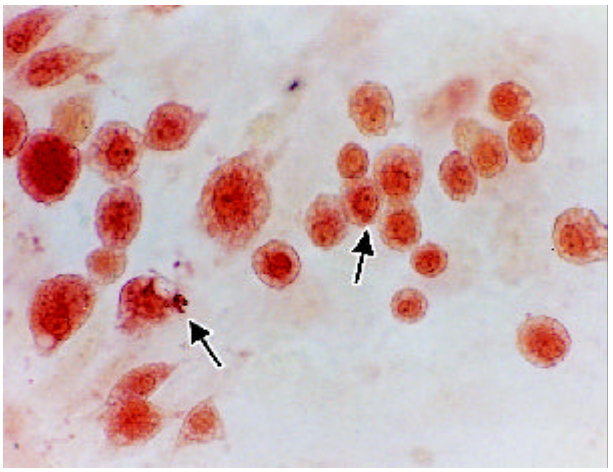

Fig 1. Staining for tartrate-resistant acid phosphatase of osteoclast under light microscope (×200). Arrows indicate the multinucleated osteoclastic cells.

Effects of TCFC on TRAP-positive multinucleated osteoclast formation The formation and differentiation of osteoclast were inhibited by TCFC at 2.5-25 mg/L. IP 1 μmol/L also inhibited the formation of osteoclast (Tab 1).

Effects of TCFC on TRAP activity of osteoclast The TRAP activity of osteoclast gradually increased during 24-72 h and were concentration-dependently inhibited by TCFC at 0.25-25 mg/L from 24 to 72 h. IP 1 μmol/L inhibited the activity of TRAP from 48 to 72 h (Tab 2).

Effects of TCFC on bone resorption activity of osteoclast After osteoclasts were treated with TCFC

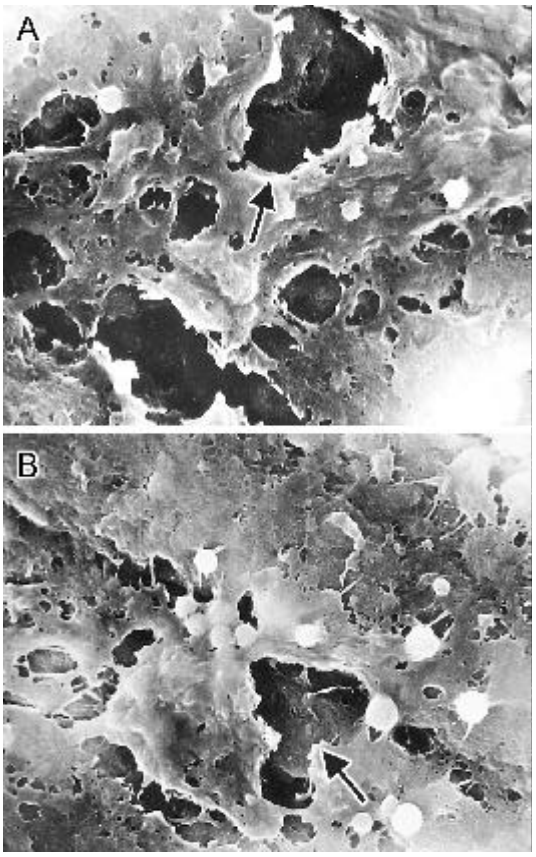

Fig 2. Resorption pits on bone slices formed by osteoclast under scanning electronic microscope (×7000). Arrows indicate resorption pits formed by osteoclast on bone slices. A) Control; B) TCFC 25 mg·L^{-1}.

Tab 1. Effects of total coumarins from the fruits of *Cnidium monnieri* (TCFC) on TRAP-positive multinucleated osteoclast formation. *n*=5. Mean±SD. $^{c}P<0.01$ *vs* control.

Group	Number of TRAP-positive MNC/well
Control	364±14
IP 1 μmol/L	200±10^{c}
TCFC/mg·L^{-1}	
0.0025	354±20
0.025	351±11
0.25	306±18
2.5	276±19^{c}
25	270±4^{c}

at 0.0025-25 mg/L for 10 d, the bone resorption pit areas on the surface of bone slices were reduced respectively by 15.54 %, 17.56 %, 18.64 %, 19.98 %, and 25.05 %, compared with that of control (Fig 2).

Tab 2. Effects of TCFC on TRAP activity of osteoclasts in cultures. *n*=5. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

Group	TRAP activity/μmol·g^{-1}·min^{-1}		
	24 h	48 h	72 h
Control	2.09±0.03	2.84±0.20	3.17±0.13
IP 1 μmol/L	2.00±0.05	2.08±0.11^{b}	2.20±0.13^{b}
TCFC/mg·L^{-1}			
0.0025	2.06±0.04	2.56±0.24	3.12±0.33
0.025	2.03±0.09	2.79±0.39	3.11±0.24
0.25	2.01±0.07^{b}	2.79±0.35	3.07±0.13^{b}
2.5	1.96±0.05^{b}	2.36±0.09^{b}	2.64±0.12^{b}
25	1.93±0.03^{b}	2.09±0.03^{c}	2.41±0.13^{c}

IP also reduced the osteoclastic pit area on the surface of bone slices by 18.10 % (Tab 3).

Tab 3. Effects of total coumarins from the fruits of *Cnidium monnieri* (TCFC) on the resorption pit area on bone slices formed by osteoclast in cultures. *n*=5. Mean±SD. $^{b}P<0.05$ *vs* control.

Concentration	Area/μm^{2}
Control	2007±164
IP 1 μmol/L	1644±38^{b}
TCFC/mg·L^{-1}	
0.0025	1695±186
0.025	1654±91^{b}
0.25	1633±19^{b}
2.5	1606±33^{b}
25	1504±168^{b}

After treatment of osteoclast with TCFC 0.0025-25 mg/L for 48 h, the calcium released from bone slices was reduced, but the difference was not significant compared with that of control. TCFC 0.025-25 mg/L for 72 h reduced the calcium release from bone slices by 25.55 %, 36.53 %, 37.39 %, and 41.73 %, respectively (Tab 4).

DISCUSSION

Zambonin-Zallone *et al*[9] and Oursler *et al*[10] developed the methods to obtain osteoclasts from the peritrabecular bone marrow of medullary bone of laying hens kept for 7 d on a low calcium diet. However,

Tab 4. Inhibitory effects of total coumarins from the fruits of *Cnidium monnieri* (TCFC) on the Ca^{2+} release from bone slices induced by osteoclast in cultures. *n*=5. Mean±SD. $^{b}P < 0.05$, $^{c}P < 0.01$ *vs* control.

Group	Ca^{2+} concentration/mmol·L^{-1}	
	48 h	72 h
Control	1.55±0.15	3.23±0.15
IP 1 μmol/L	1.10±0.03^{c}	1.75±0.13^{c}
TCFC/mg·L^{-1}		
0.025	1.63±0.10	2.73±0.50
0.25	1.43±0.08	2.40±0.15^{c}
25	1.38±0.05	2.05±0.43^{b}

avian osteoclasts possess no or a very small number of calcitonin receptor[11]. Therefore, it seems that avian osteoclasts are not the suitable source for studying bone resorption. To obtain mammalian osteoclasts, mechanical disaggregation of newborn rat long bones has been generally used[12,13]. However it is difficult to obtain a large number of osteoclasts using this method. Akatsu *et al* developed a useful method to induce the mouse osteoclast-like multinucleated cells *in vitro*[14]. Briefly, after primary mouse osteoblast cells and bone marrow cells were co-cultured in the presence of 1,25-dihydroxyvitamine D_3 for 6 d, tartrate-resistant acid phosphatase (TRAP)-positive osteoclast-like cells were induced, and these cells expressed abundant amounts of calcitonin receptors and formed resorptive pits on the surface of bone slices[15, 6]. Since MNC possess osteoclast-like characteristics, they have been regarded as osteoclastic cells. We used this method to obtain a large number of osteoclasts to study the antiosteoporotic effects of TCFC.

In experimental osteoporosis of ovariectomized rat, TCFC not only depressed the osteoclast resorption but also partly stimulated the osteoblast function[2]. TCFC inhibited both the formation of osteoclasts and the bone-resorption activity of osteoclasts *in vitro*. Osteoclastic bone resorption is thought to be mediated by two different processes: one is the formation of new osteoclasts, and the other is the resorption activity of osteoclasts.

Firstly, we investigated the effect of TCFC on osteoclasts formation using the co-culture system of rat osteoblastic cells and bone marrow cells. It was reported that bone resorbing agents such as 1,25-dihydroxyvitamine D_3, PTH, and IL-1 markedly stimulated the formation of osteoclasts[16,17]. TCFC also inhibited 1,25-dihydroxyvitamine D_3-stimulated osteoclasts formation. The 1,25-dihydroxyvitamine D_3 is thought to stimulate osteoclast formation by a common mode involving prostaglandin E_2 (PGE_2), which itself promotes osteoclast formation[18]. So the inhibitory effect of TCFC on osteoclast formation may be due at least partly to the depression of PGE_2 production.

It is pertinent to consider that the inhibitory activity of TCFC on osteoclast formation is sometimes a result of toxic effects. However, osteoblastic cells are essential for osteoclast formation. We investigated the cytotoxic effect of TCFC on osteoblastic cells using MTT assay, TCFC showed no cytotoxicity, and promoted the proliferation of osteoblasts[4]. This suggests that TCFC may affect the differentiation of osteoclasts rather than affecting the participation of osteoblastic cells in osteoclast differentiation.

Secondly, effects of TCFC on the resorbing activity of osteoclasts were examined by pit formation and calcium release from bone slices assay. It has been reported that the decrease in bone resorption associated with the decrease in the number of osteoclasts can be seen *in vivo*, and this causes a cessation of bone resorption during remodel[19]. The resistant tartrate acid phosphatase is one of the marker enzymes of osteoclast and is directly related to activity of bone resorption. Therefore, we examined the TRAP activity of osteoclasts. TCFC decreased the TRAP activity of osteoclasts. Similar results have been obtained in osteoclasts treated with ipriflavone. It was reported that IP could inhibit the TRAP activity of osteoclastic FLG29.1 cells[20].

In conclusion, TCFC inhibited the formation and differentiation of multinucleated osteoclasts, decreased the activity of TRAP and bone resorption *in vitro,* which led to the prevention of experimental osteoporosis in ovariectomized rats.

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