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Effects of total coumarins of Fructus Cnidii on skeleton of ovariectomized rats[1]

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ABSTRACT Total coumarins of Fructus Cnidii (TCFC), 5 g·kg^{-1} by intragastric gavage, 6 d /wk, ×7 wk, was effective for prevention of bone loss in ovariectomized (OVX) rats. In comparison to aging control rats, the proximal tibia of placebo-treated OVX rats were characterized by an increase in eroded perimeter (+298 %), label perimeter (+77 %), osteoid perimeter (+47 %), mineral apposition rate (+32 %) and bone formation rate (+130 %). These changes indicated a high bone turnover in OVX rats leading to a rapid bone loss (−44 %) in proximal tibial metaphysis. In contrast, the TCFC-treated OVX rats showed an increase of cancellous bone area (+41 %) compared with placebo-treated OVX rats and decrease in all the above indices of bone turnover to near aging control levels except that of the osteoid area (+88 %) which was higher than that in aging control, but mineralization lag time did not show significant changes. The results suggested that the TCFC inhibited the high bone turnover and reversed the bone loss at early menopausal stage.

KEY WORDS Fructus Cnidii; coumarins; ovariectomy; osteoporosis; bone development; bone resorption; estrogens

Postmenopausal osteoporosis is a serious problem in elderly women, for which estrogen replacement is the only widely accepted therapy. Total coumarins extracted from Fructus Cnidii, the dried ripe fruit of Chinese herb *Cnidium monnieri* (L) Cuss, showed estrogen-like effects in this aspect[1]. Total coumarins contained osthol and total linear furocoumarins.

Ovariectomized (OVX) rats exhibited similar signs[2-4] and estrogen replacement suppresses the bone turnover[5-6]. The present study was to characterize histologically the effects of TCFC on cancellous bone mass and turnover in OVX rats.

MATERIALS AND METHODS

Rats Twenty-four Sprague-Dawley ♀ rats (Guangdong Animal Experimental Center), aged 3 months and weighing 200±s 17 g, were anesthetized by ip sodium pentobarbital 40 mg·kg^{-1}. Bilateral ovariectomies were performed in 16 rats through dorsal approach. The remaining 8 rats were subjected to sham surgery in which the ovaries were exteriorized but not removed. Eight OVX rats were treated with the TCFC at daily dose of 5 g·kg^{-1}, starting ig on the day of surgery, 6 d per week, for 7 wk. The other 16 rats were ig with water housed at 25 ℃ with food (Animal Center, Guangdong Medical College) and water *ad lib*. Rats were injected ip with tetracycline (Xin-Ya Pharmaceutical Factory, Shanghai) 25 mg·kg^{-1} on d 10 and d 2 before killing. The regimen resulted in deposition of a double labeling at bone surface that was actively mineralizing throughout the injection period.

After 7 wk of treatment with the TCFC or placebo rats were killed by exsanguination under sodium pentobarbital anesthesia. Success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by marked atrophy of uterine horns. The

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proximal tibiae were stripped of muscles and placed in 10 % phosphate-buffered formalin for fixation for 48 h, then pro-stained with Villanueva bone stain.

Total coumarins of Fructus Cnidii (Zhangjiang Pharmaceutical Factory) contained osthol (2 % of *Cnidium monnieri* (L) cuss) λ_{max}^{EtOH} 322 nm, total linear furocoumarins (0.8 % of *Cnidium monnieri* (L) cuss) λ_{max}^{EtOH} 249 nm.

Bone histomorphometry After staining, the *proximal tibiae were dehydrated in graded concentrations* of ethanol and defatted in acetone, then embedded in methyl methacrylate (Shanhu Chemical Factory, Shanghai). Longitudinal sections of proximal tibia at 230 μm thickness were cut using a low-speed metallurgical saw then sections were ground to a thickness of 20 μm and coverslipped for morphometric measurements.

A digitizing analysis image system was used on the 20-μm sections for histomorphometric measurements of the proximal tibial metaphysis area from the growth plate-metaphyseal junction down 1 mm in zone I and zone II (Fig 1).

The total tissue area, trabecular area and perimeter, osteoid perimeter, width and area, eroded perimeter, single and double labeled perimeter and interlabeling width were measured. These parameters were used to calculate the trabecular area, trabecular thickness, trabecular number, trabecular separation, eroded perimeter, osteoid perimeter and area, labeled perimeter, mineralization lag time, mineral appositional rate, and bone formation rate. Analyses were performed on a semiautomated digitizing image analyzer at 39.63 and 313× magnification. The system consisted of a light or epifluorescent microscope and digitizing pad (Summagrephic, Fairfield CT, USA), coupled to an Apple Macintosh Computer and a morphometry program named "Stereology" (KSS Computer Engineers, Magna UT, USA). Static and dynamic measurements and calculations of the proximal tibia were *performed* (Tab 1)[7].

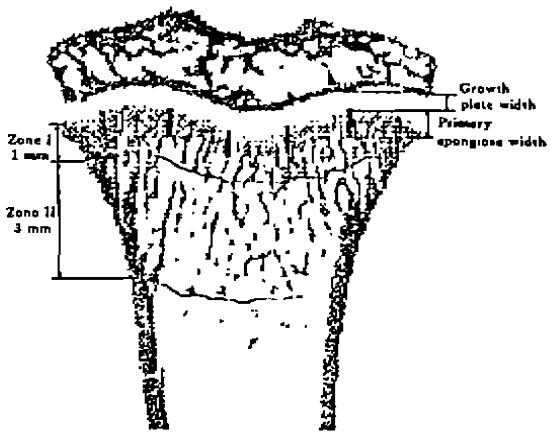

Fig 1. Measured area (Zone II) of the proximal tibia for trabecular bone.

Statistical analysis Data were expressed as $\bar{x} \pm s$. The significance of the difference between the 2 means within the same group was analyzed by t test for paired data.

RESULTS

Body weight All rats gained weight during the study. OVX rats treated with water or the TCFC weighed heavier than the sham-OVX rats. The final mean body weight for the former 2 groups of OVX rats were 288 ± 20 g and 262 ± 16 g, respectively. In contrast, the sham-OVX rats weighed 250 ± 24 g ($P < 0.05$ both).

Cancellous bone In comparison with sham-OVX control rats, the water-treated OVX *rats were characterized by a decreased* cancellous bone area (−44 %) and increased indices of bone turnover such as label perimeter (+77 %), eroded perimeter (+298 %), osteoid perimeter (+47 %), mineral apposition rate (+32 %), and bone formation rate (+130 %). In contrast, the TCFC-treated OVX rats showed an increase of cancellous bone area (+41 %) compared with water-treated OVX group and decreased all the above indices of bone turnover to near aging control levels except that of the osteoid area, which was higher (+88 %) than the watertreated OVX rats and also higher (+100 %) than the aging control rats, but mineralization lag time did not exhibit significant changes (Tab 2).

Tab 1. Histomorphometric measurements and calculations of proximal tibial metaphysis.

Parameter	Code	Unit	Description
Tissue area	T. Ar	mm^2	Measured area including bone and marrow tissue
Trabecular area	Tb. Ar	mm^2	Area of cancellous bone within T. Ar
Trabecular perimeter	Tb. Pm	mm	Perimeter of cancellous bone within T. Ar
Single label perimeter	sL. Pm	mm	The length of trabecular surface singly label with demeclocycline
Double label perimeter	dL. Pm	mm	The length of trabecular surface double label with demeclocycline
Interlabel width	Ir. L. Wi	mm	The distance between double demeclocycline labels
Eroded perimeter	E. Pm	mm	The length of trabecular surface with Howship's lacuna
Osteoid perimeter	O. Pm	mm	The length of trabecular surface covered with osteoid
Osteoid width	O. Wi	μm	Width of osteoid

Derived cancellous bone histomorphometry

Parameter	Code	Unit	Formula
Percent trabecular area	Tb. Ar	%	Tb. Ar/T. Ar × 100
Trabecular thinckness	Tb. Th	μm	(2000/1.100) × (Tb. Ar/Tb. Pm)
Trabecular number	Tb. N	no/mm	(1.199/2) × (Tb. Pm/T. Ar)
Trabecular separation	Tb. Sp	μm	(2000/1.199) × (T. Ar−Tb. Ar)/Tb. Pm
Percent eroded perimeter	E. Pm	%	E. Pm/Tb. Pm × 100
Percent osteoid perimeter	O. Pm	%	O. Pm/Tb. Pm × 100
Osteoid area	O. Ar	mm^2	O. Pm × O. Wi
Percent osteoid area	O. Ar	%	(O. Pm × O. Wi/1000)/T. Ar × 100
Percent labeled perimeter	L. Pm	%	(dL. Pm+sL. Pm/2)/Tb. Pm × 100
Mineral apposition rate	MAR	μm	IrL. Wi/Interval
Mineralization lag time	MLT	d	O. Wi/MAR
Bone formation rate	BFR	%/Year	(dL. Pm+sL. Pm/2) × MAR/Tb. Ar/1000 × 365 × 100

DISCUSSION

The ovariectomy-induced bone loss rat model is widely accepted for studying the prevention and treatment of postmenopausal osteoporosis. We confirmed that OVX induces massive cancellous bone loss associated with an increase in a higher bone turnover with bone resorption exceeding formation are consistent with finding reported previously by Wronski *et al*[18].

The new findings from the present study demonstrated that bone loss due to OVX can be prevented by the TCFC. Our histomorphometric data clearly indicated that the TCFC can slow down the bone turnover and restored the bony substance in OVX rats. The advantages of the TCFC were that the drug not only depressed the osteoclast resorption but also partly stimulated the osteoblast function.

As the fundamental tissue problem in postmenopausal osteoporosis is the lack of trabeculae, correcting this defect would be the therapeutic basis for curing the disease. We now acknowledge that estrogen replacement is a widely accepted therapy for this skeletal disorder. The mechanism of the protective effect of estrogen against postmenopausal bone loss apparently involves a suppression of bone turnover. The TCFC has analogous effect as

Tab 2. Histomorphometric indices in the proximal tibial metaphysis. $\bar{x}\pm s$. [a] $P>0.05$, [b] $P<0.05$, [c] $P<0.01$ *vs* **Sham control.** [d] $P>0.05$, [e] $P<0.05$, [f] $P<0.01$ *vs* **OVX+water** % **derived from formula of** $X_2\div X_1\times 100-100$.

Parameters ($n=8$ rats)	Group 1 Sham control	Group 2 OVX+water	Group 3 OVX+TCFC
Trabecular area/%	28.7±2.8	15.7±2.3 %−44[c]	22.3±1.8 %−20[c] 41[f]
Trabecular perimeter/mm	68±14	45±7 %−34[c]	65±5 %−5[a] 45[f]
Trabecular thickness/μm	58±8	54±5 %−6[a]	53±4 %−4[a] −2[d]
Trabecular number/per mm	4.95±0.70	2.90±0.32 %−43[c]	4.22±0.25 %−4[a] 46[f]
Trabecular separation/μm	148±24	295±43 %99[c]	185±13 %25[a] −37[f]
Labeled perimeter/%	4.2±1.6	7.4±1.8 %77[c]	5.2±1.0 %25[a] −29[f]
Eroded perimeter/%	0.42±0.24	1.67±0.7 %298[c]	0.69±0.22 %65[a] −59[f]
Osteoid area/%	0.31±0.11	0.33±0.18 %6.5[a]	0.62±0.23 %100[c] 88[e]
Mineral apposition rate/$\mu m\cdot d^{-1}$	1.70±0.29	2.25±0.21 %32[c]	2.04±0.50 %20[a] −9[d]
Bone formation rate/$\mu m\cdot d^{-1}$	7.3±3.6	16.9±5.0 %130[c]	11.0±4.6 %9.50[a] −35[d]
Mineralization lag time/d	7.1±2.2	6.7±1.6 %−4[a]	8.2±2.7 %15[a] 21[d]

estrogen[17], our data were consistent with previous works on estrogen in OVX rats[15]. Further study showed that estrogen receptors[19] have been detected on rat osteoblast-like cell in vitro suggesting a direct action on the osteoblast. The TCFC may has the similar effect. On the other hand, estrogen inhibited the osteoclastic bone resorption, our data showed the same inhibition. However, the mechanism by which estrogen inhibits bone resorption is yet unknown.

It should be emphasized that the current study did not provide insight into the skeletal effects of the TCFC during the later stages of estrogen deficiency when the rates of bone loss and bone turnover both have subsided. Furthermore, our findings indicated that the TCFC effectively prevent bone loss in early estrogen-deficient state, (20 % less than that in sham-OVX control), it remains to be determined whether TCFC can substantially reverse established osteopenia in a late estrogen-deficient state.

In summary, our results indicated that treatment with the TCFC depresses bone turnover and prevents the development of osteopenia at early menopousal stage as a substitute to estrogen.

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蛇床子总香豆素对去卵巢骨骼的影响[1]

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摘要 蛇床子总香豆素（TCFC）5 g·kg^{-1}·d^{-1} ig，每周6次，持续7 wk. 不脱钙骨制片测量. 结果：(1)去卵巢喂水模型组胫骨骨小梁明显减少（−44 %），出现骨吸收大于形成的骨高转化率. (2)用 TCFC 治疗的去卵巢组与(1)比胫骨骨小梁的面积增加（+41 %），并降低骨高转化率的指标使基本接近对照组（除类骨质外，但矿化延迟时间不变）. 本文提示 TCFC 可能防止绝经早期由骨高转化率引起的骨丢失.

关键词 蛇床子；香豆素；卵巢切除术；骨质疏松；骨发育；骨质吸收；雌激素类