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The serum of rats administered flavonoid extract from *Epimedium sagittatum* but not the extract itself enhances the development of rat calvarial osteoblast-like cells *in vitro*

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The flavonoid extract from *Epimedium sagittatum* (FES) has been found by us to be effective in preventing osteoporosis induced by ovariectomy in rats. In the present study, the effect of FES on the development of rat calvarial osteoblast-like (ROB) cells was investigated. No appreciable effect was observed when ROB cells were exposed to FES *in vitro*. However, serum isolated from rats administered FES orally was able to significantly stimulate the proliferation as well as the osteoblastic differentiation of ROB cells compared to serum from control rats. The results indicate that the serum of rats administered FES contains active metabolites of FES that enhance the development of osteoblasts, while the original form of FES itself is inactive.

1. Introduction

Epimedium sagittatum has now become a well-known traditional Chinese medicine in America and Europe as an aphrodisiac. The herb, also named horny goat weed, has long been used to treat impotence, improve erectile function and increase sexual desire in man as well as in woman. However, other curative effects of the herb may also contribute to its reputation. For example, it is one of the herbs most frequently used in treating osteoporosis in traditional Chinese medicine, based on the knowledge that the herb has the function of strengthening bones. Modern researches have shown that the aqueous extract of *Epimedium sagittatum* can prevent osteoporosis induced by orchidectomy or glucocorticoid in rats by suppressing bone resorption and enhancing bone formation (Li et al. 1993; Li et al. 1996). We and others have found that the flavonoid extract of *Epimedium sagittatum* (FES) prevents osteoporosis induced by ovariectomy or retinoid acid in rats and therefore inferred that the extract contains anti-osteoporosis constituents (Ji et al. 2001; Ma et al. 2002a). However, in the present study, FES itself was found to have no effect on cells, but the serum of rats administered FES was found to promote the development of rat calvarial osteoblast-like (ROB) cells.

2. Investigations, results and discussion

FES was directly added at 0.1, 1, 10 and 100 μg/ml respectively to the culture medium of ROB cells, in order to observe its effect on cell proliferation and differentiation. Neither an inhibitory or a stimulatory effect on cell proliferation was found, nor any influence on the activity of alkaline phosphatase, an early marker of osteoblastic differentiation. We then turned to the field of serum pharmacology, a popular pharmacological testing method for herbal medicines in China and Japan (Hiroko et al. 1987; Masaomi et al. 1988). The serum of rats administered FES orally (SES) was obtained from blood collected 1.5 h after administration, when the absorbed and metabolized FES reach peak concentrations in the blood. The serum of rats administered the vehicle of FES was also collected as a control. The rat serum was added at 2.5%, 5% and 10% respectively to the culture medium of ROB cells. The cell proliferation was assayed by reduction of MTT 48 h after

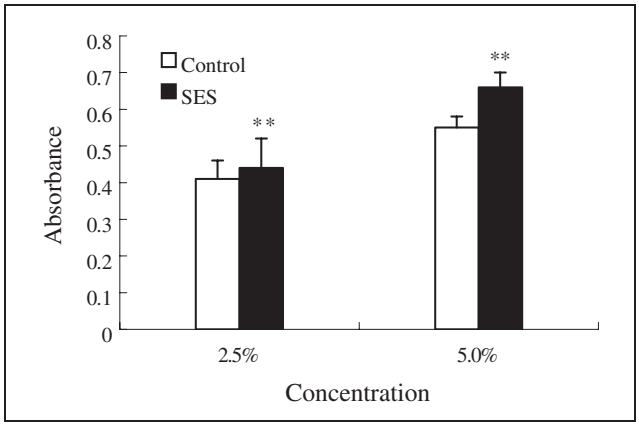

Fig. 1: Stimulant effect of the serum of rats administered flavonoid extract of *Epimedium saggitatum* (SES) on the proliferation of rat calvarial osteoblast-like cells at different concentration. Serum of rats administered vehicle of the extract was used as control. Proliferation was assessed by MTT assay after 48 h of incubation and expressed by the absorbance value at 570 nm. The results are mean values of six parallel cultures. Standard deviation (S.D.) of the mean is shown by vertical bars. ** $P < 0.01$ vs control.

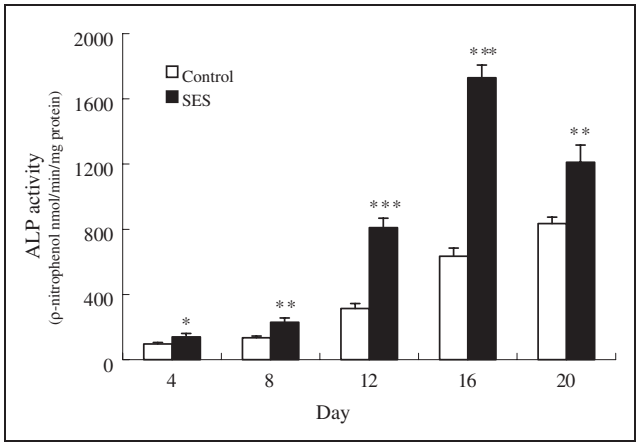

Fig. 2: Increase of intracellular alkaline phosphatase (ALP) activity in rat calvarial osteoblast-like cells induced by addition of 5% serum from rats administered flavonoid extract of *Epimedium sagittatum* (SES) for 20 days. Equal concentration of serum from rats administered vehicle of the extract was used as control. The results are the mean of triplicate cultures. Standard deviation (S.D.) of the mean is shown by vertical bars. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control.

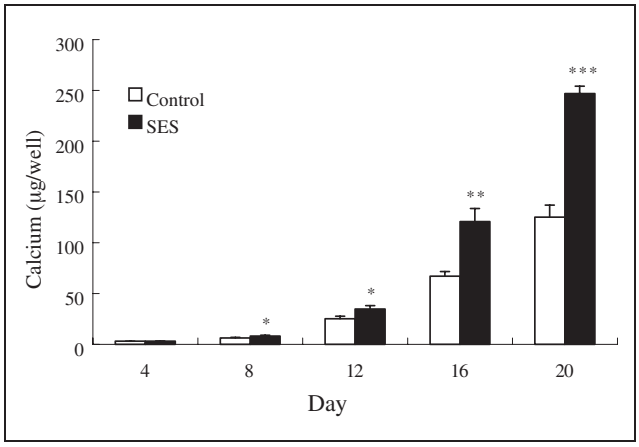

Fig. 4: Increase of calcium deposition in rat calvarial osteoblast-like cells induced by addition of 5% serum from rats administered flavonoid extract of *Epimedium sagittatum* (SES) for 20 days. Equal concentration of serum from rats administered vehicle of the extract was used as control. The results are the mean of triplicate cultures. Standard deviation (S.D.) of the mean is shown by vertical bars. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control.

addition. As shown in Fig. 1, the absorbance values at 570 nm, which correspond to the numbers of living cells, were significantly higher in the 2.5% and 5% SES groups than in their respective controls, indicating that SES has a significantly stronger ability to stimulate the proliferation of ROB cells than the control serum. However, 10% rat serum seemed to be toxic because it caused cell death and detachment.

The effect of SES on cell differentiation was investigated in the 5% SES-supplemented medium containing 5% fetal bovine serum, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. The intracellular alkaline phosphatase (ALP) activity in the SES group was significantly higher than that of the control as early as the 4th day, and rose to about 2 to 3 times higher than that of the control on the 12th and 16th days respectively, and then declined on the 20th day (Fig. 2).

Osteocalcin is the most abundant non-collagen protein in bone, being produced by osteoblasts and often used as a marker of bone formation (Lee et al. 2000). The osteocalcin synthesized and released by ROB cells into the medium of the SES group was significantly more than that of the control during every 4-day period of the incubation except on days $0 \sim 4$ (Fig. 3), indicating that SES effectively promoted the synthesis of bone matrix protein.

SES also significantly promoted calcium deposition. The quantity of Ca^{2+} in the SES group was significantly higher than that of the control after the 8th day, and increased markedly with exposure time in the culture (Fig. 4). The number of mineralized bone nodules, the nodular aggregates of mature osteoblasts entrapped in bone matrix protein and minerals (Fig. 5), was 1.5-fold higher in the SES group than in the control on the 20th day (Table).

All the results showed that SES strongly stimulates the proliferation and differentiation of ROB cells, while FES itself has no appreciable effect on the cells. It indicates that the serum contains the active metabolites of FES that enhance the development of ROB cells, but the original form of FES is inactive. Researchers have paid great attention to the metabolism of flavonoids, especially dadizen and ipriflavone, the natural isoflavone and its derivative, for the treatment of osteoporosis (Yasud 1994; Rohatagi and Barrett 1997). Ipriflavone, for example, has at least five major metabolites, and two of them have bioactivity (Rohatagi et al. 1997). The metabolism of icariin, one of the most abundant flavonoids in *Epimedium sagittatum*, has also been studied. At least four metabolites including

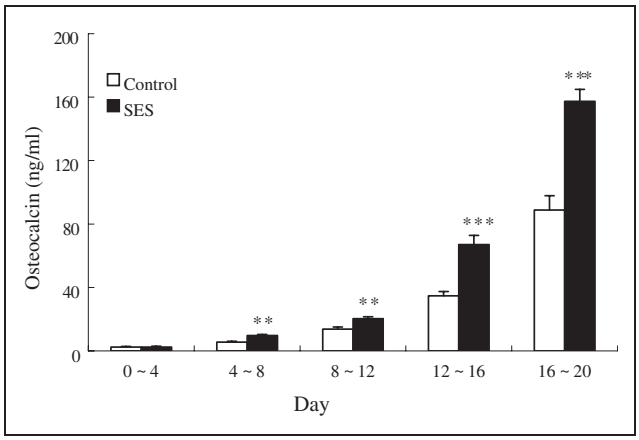

Fig. 3: Increase of osteocalcin accumulation in the culture medium of rat calvarial osteoblast-like cells induced by addition of 5% serum from rats administered flavonoid extract of *Epimedium sagittatum* (SES) for 20 days. Equal concentration of serum from rats administered vehicle of the extract was used as control. The results are the mean of triplicate culture. Standard deviation (S.D.) of the mean is shown by vertical bars. ** $P < 0.01$, *** $P < 0.001$ vs control.

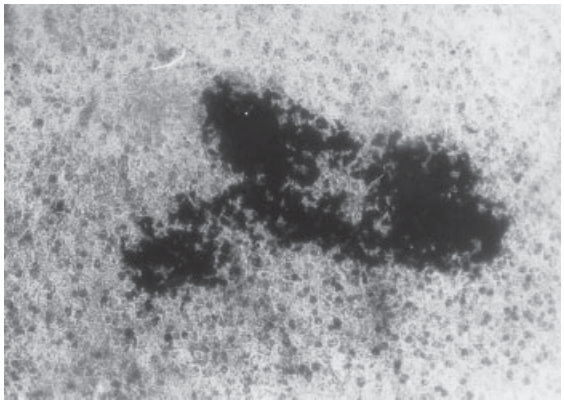

Fig. 5: Mineralized bone nodules visualized by von Kossa staining (Magnification = 100×)

Table: Number of mineralized bone nodules on the 20th day

Group	Number
SES	$237 \pm 19^{**}$
Control	152 ± 13

mean ± SD, n = 3
** $P < 0.01$ vs control group

icaritin, icariside I, icariside II and icariside II 7-O-Glc UA were found in bile and urine after it was administered orally in rats, however, little original molecules of icariin could be detected (Qiu et al. 1999). More than eighty flavonoids have been isolated, identified and named in the *Epimedium* genus, the most common including icariside II, ikarisosid C, epimedoside A, epimedin B, epimedin C, and baohuoside I, besides icariin (Li et al. 1994; Li et al. 1995; Li et al. 1996; Liang et al. 1997a; Liang et al. 1997b). Only a few of them have been studied with respect to their metabolism and pharmacological functions.

Our results further demonstrate the possibility of developing an alternative therapy for osteoporosis from *Epimedium sagittatum*, especially from the flavonoids contained in the herb. However, in the course of purification and screening for bioactive compounds with anti-osteoporosis activity, their metabolites must be considered.

3. Experimental

3.1. Plant material

Dried aerial parts of *Epimedium sagittatum* (Siebold & Zucc.) Maxim were purchased from Lanzhou Pharmaceutical Company Ltd., Gansu, China, having been collected in Bozhou, Anhui, China. The same herb was authenticated by Associate Professor Xun Pu of the Botany Department, School of Life Sciences, Lanzhou University.

3.2. Preparation of FES extract

FES was prepared as described previously (Ma et al. 2002b).The herb was milled and soaked in 10 × weight of water, boiled for 1.5 h, and extracted twice. The water extract was successively filtered, concentrated and defatted by petroleum ether 40–60 °C. The water soluble fraction was extracted three times by ethyl acetate. The three parts of the ethyl acetate soluble fraction were combined and distilled. The crude extract was then purified twice with methanol. The final extract contained 58.63% total flavonoids as determined by ultraviolet spectrometry using icariin as standard. It was dissolved in 0.5 N NaOH and adjusted to pH 7.8 by 0.5 N HCl.

3.3. Preparation of SES

Twenty Wistar rats at 6 months of age were purchased from the Experimental Animal Center of Gansu Provincial Academy of Medical Science. They were divided randomly into two groups and were given FES or equivalent volume of vehicle. FES was administered orally at 0.5 g/kg body weight once a day for three days. 1.5 h after the last administration, blood of the rats was collected from the abdominal aorta under ether anesthesia. Blood from the same group was mixed and centrifuged to obtain serum. The serum was heat-inactived at 56 °C and sterilized by membrane filtration before use. The serum of rats administered with vehicle alone was also prepared as a control.

3.4. Cell culture

Rat calvarial osteoblast-like cells were isolated enzymatically from calvariae of newborn Wistar rats (within 48 h) based on the method of Hagiwara (Hagiwara et al. 1999). Ten calvariae were dissected out and all adhering soft tissue was removed. They were cut into pieces and subjected to three sequential 20-min digestions with 5 ml of an enzyme mixture containing 1 mg/ml collagen II and 0.5 mg/ml trypsin. The pieces were then digested twice with 1 mg/ml collagen II for 60 min. The released cells were collected by centrifugation and combined. They were plated in 75-cm^2 dishes and grown in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 in air at 37 °C. The medium was replaced every four days. After reaching confluence, the cells were detached by treatment with 0.25% trypsin and replanted into tissue culture plates (DMEM, fetal bovine serum and trypsin were purchased from Gibo BRL, collagen II is a Sigma product).

3.5. Cell proliferation assay

ROB cells were plated in 96-well tissue culture plates at 3×10^3 cells/well and allowed to grow for 24 h. The medium was then freshly changed and supplemented with FES at 0.1, 1, 10 and 100 μg/ml respectively, in the presence of 10% fetal bovine serum. The equivalent volume of vehicle was added to the control cultures. When SES was assayed, it was added to the medium at 2.5%, 5% and 10% respectively, in the presence of 5% fetal bovine serum. An equal concentration of control serum was used as a control. After 48 h, cell proliferation was assessed by MTT assay. 10 μl of 5mg/ml MTT solution was added to each well and incubated for another 2 h. 100 μl DMSO was added to dissolve the formazan crystals. The absorbance at 570 nm was measured (Xu et al. 2001).

3.6. Cell differentiation assay

ROB cells were plated in 6-well tissue culture plates at 1×10^5 cells/well. After 3 days, the medium was changed and supplemented with FES at 0.1, 1, 10 or 100 μg/ml, in the presence of 10% fetal bovine serum, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. The equivalent volume of vehicle for FES was used as the control. SES was added to the medium at a concentration of 5%, in the presence of 5% fetal bovine serum, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. A 5% concentration of control serum was used as control. The intracellular alkaline phosphatase activity, osteocalcin concentration of medium and mineral deposition of the cell layer were assayed every 4 days until the 20th day.

3.6.1. Measurement of alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was measured according to the method of Cheng (Cheng 1994). The cells were collected into 0.5 ml of 50 mM Tris (pH 7.4, containing 0.1% Triton X-100 and 2 mM $MgCl_2$), and sonicated (Ultrasonic crasher Ty92–2, SCIETZ, Ningbo, China). The ALP activity of the sonicate was determined by Sigma kit 104-LL using ρ-nitrophenyl phosphate as substrate, and the rate of nitrophenol production was measured at 405 nm. The enzyme activity was expressed as nmol ρ-nitrophenyl/min/mg protein. The protein content was determined by the Bio-Rad DC protein assay kit (Bio Rad, Hercules, CA, USA).

3.6.2. Osteocalcin assay

The culture medium was collected when it was freshly changed on the 4th, 8th, 12th, 16th and 20th days, and stored at −30 °C until assay. The osteocalcin concentration of the medium was determined by a rat osteocalcin EIA kit (Biomedical Technologies, Inc, Stoughton, MA, USA) (Yamamoto et al. 2002).

3.6.3. Calcium assay

The cell layer was rinsed twice with PBS and incubated in 1 ml of 1 N HCl overnight with gentle shaking. The Ca^{2+} in the sample was quantified by the O-cresolphthalain complexone method with Sigma kit 587-A (Jaiswal 1997). Results were expressed as μg/well.

3.6.4. Mineralized bone nodule assay

The presence of mineralized bone nodules on the 20th day was demonstrated with von Kossa staining (Jaiswal 1997). The cell layer was rinsed once with PBS and fixed with 3.7% formaldehyde − 90% ethanol solution for 10 min. Freshly prepared 1% silver nitrate was added and exposed to ultraviolet light for 30 min. The mineralized bone nodules were counted manually.

3.7. Statistics

Values are represented as mean ±S.D. Statistical analyses were performed using Student's t-test.

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