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Osteoblastic Proliferative Activity of Extracts of *Qing'e pill* and its Disassembled Formulae

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Abstract

Qing'e pill is one of the famous traditional Chinese compound prescriptions used to treat bone diseases. It contains four botanical drugs, i.e., the cortex of *Eucommia ulmoides* Oliv., the fruit of *Psoralea corylifolia* L., the seed of *Juglans regia* L. and the rhizome of *Allium sativum* L. In this study, osteoblastic proliferation activity of extracts from *Qing'e pill* and its disassembled formulae was investigated with the osteoblast-like UMR106 cell line as a model. The extract of *Qing'e pill*, both alcohol and aqueous, stimulated cell proliferation in a dose-responsive manner. The proliferative activity of alcohol extract was more potent than that of the aqueous one with a maximal growth stimulation ratio (GSR) of 58.5% versus 38.8%. *Eucommia ulmoides* and *Psoralea corylifolia* produced a maximum proliferative promotion of 38.7% and 34.0%, respectively, when co-cultured with UMR106 cells. Neither *Juglans regia* nor *Allium sativum* showed a significant effect on osteoblastic proliferation. When *Eucommia ulmoides* and *Psoralea corylifolia* were combined, the stimulating action (GSR = 47.2%) became stronger than that of either individual drug. The enhancement effect was more marked when *Juglans regia* or both *Juglans regia* and *Allium sativum* were added. These results demonstrated the synergy of the four botanical drugs and the rationality of *Qing'e pill* prescription in a modern scientific way. This is the first time to study the stimulating osteroblastic proliferation effect of a traditional Chinese compound prescription on the basis of disassembled formulae.

Keywords: Osteoblast-like UMR106 cells, proliferation, *Qing'e pill*, *Eucommia ulmoides* Oliv., *Psoralea corylifolia* L., *Juglans regia* L., *Allium sativum* L.

Introduction

Osteoblast-like UMR106 cells are characterized extensively as osteoblast in nature, they have been widely used as a developed osteoblast model in studying the effect mechanism of anti-osteoporostic drugs on osteoblasts (Gray et al., 1987; Bankson et al., 1989). Agents that stimulate proliferation of osteoblasts may have a promoting activity in the phase of bone formation, and they may be used for anti-osteoporosis. We have successfully developed a proliferation assay with UMR106 cells for screening anti-osteoporostic agents from traditional Chinese medicines (Gao et al., 2000; Li et al., 2001; Wang et al., 2001ab).

Qing'e pill is a typical "kidney-tonifying" and "bone-strengthening" traditional Chinese compound prescription recorded in the Chinese Pharmacopoeia (2000). It contains four botanical drugs, i.e., *Eucommia ulmoides* Oliv., *Psoralea corylifolia* L., *Juglans regia* L. and *Allium sativum* L. It has been used to treat bone diseases such as osteodynia and rheumatism for hundreds of years. In recent years it has been prescribed clinically against osteoporosis. Its modified formulation was reported to have potential effect on senile osteoporosis (Shen et al., 1994a). Animal bone quantitative histomorphometric study indicated that its alcohol extract had activity in both osteo-resorption inhibition and osteo-formation promotion (Shen et al., 1994b). Wang et al. (2000, 2001b) investigated the effect of *Eucommia ulmoides* and *Psoralea corylifolia* extract, two principal drugs in *Qing'e pill*, on the proliferation of UMR106 cells and found that both the aqueous extract of *Eucommia ulmoides* and the alcohol extract of *Psoralea corylifolia* had stimulating osteoblastic proliferation activity. Hu and Wang (2001) reported that an extract of *Eucommia ulmoides* could adjust

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the metabolism of osteoblast and increase cell proliferation significantly *in vitro*. However, the effects of the other two drugs on osteoblasts are hardly known so far, neither is the coordinating effect of these four drugs. Our investigation in this paper is focused on the effect of *Qing'e pill* and the coordinating effect of these four individual drugs on osteoblastic proliferation. This is the first report to study the stimulating osteroblastic proliferation activity of a traditional Chinese compound prescription on the basis of disassembled formulae.

Materials and methods

Materials

Raw materials of the cortex of *Eucommia ulmoides* Oliv., the fruit of *Psoralea corylifolia* L., the seed of *Juglans regia* L. and the rhizome of *Allium sativum* L. were purchased from Tianyitang Chinese drug store (Shenyang, China). These drugs were identified by Fakui Chen, Professor of Pharmacognosy, Shenyang Pharmaceutical University (China). Osteoblast-like UMR106 cells were obtained from Beijing Medical University (of origin from the Massachusetts General Hospital, Boston, MA, USA). Minimum essential medium (MEM) and trypsin were obtained from Gibco (USA) and fetal calf serum (FCS) from TBD Bio-engineering Co. (Tianjin, China). MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] was purchased from Sigma (USA). All other chemicals were of analytical grade. The cell culture plates were provided by Nunc (Denmark).

Preparation of assay samples

Samples of dry *Qing'e pill* powder (9.9 g) were refluxed 3-times for 2 h with water or 75% alcohol. Each extract solution was evaporated under reduced pressure and concentrated to 100 mL.

For the study on disassembled formulae, *Qing'e* prescription was disassembled into 11 formulae as shown in Table 1. Except for the four individual drugs, the formulae were *Eucommia ulmoides* Oliv. combined with other drugs at the same ratio as in *Qing'e* prescription regulated in the Pharmacopoeia. Each extract solution of the 11 formulae was prepared with alcohol using the same method as described above. The solution was concentrated to 48 mg/mL (expressed in the weight of the cortex of *Eucommia ulmoides* Oliv. per mL). The solutions were sterilized with 0.2 µm aseptic filters (Gelmann) and stored at 4 °C. All sample solutions were diluted with MEM to the required concentration before assay. The blank control contained MEM and the same proportion of ethanol as in the test samples.

Proliferation assay

The stimulating proliferation activity of the extract solution on osteoblast-like UMR106 cells was assayed in the same procedure as described previously (Wang et al., 2001a). Briefly, the sterilized solution was diluted to the required concentrations with serum-free MEM. After UMR106 cells were co-cultured with the prepared MEM medium containing extracts for 48 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, the medium was removed, 50 µL MTT solution (1 mg MTT/mL PBS) was then added into the wells, and the incubation continued for another 4 h. Finally, MTT solution was removed from the wells, and the formed formazan was dissolved by DMSO (150 µL per well). The absorbance was recorded on an enzyme immunoassay plate reader (BIO-RAD, USA) at a wavelength of 570 nm with a reference at 630 nm. Sodium fluoride served as positive control.

Table 1. Disassembled formulae of *Qing'e pill*.

No.	Disassembled formulae
1	*Eucommia ulmoides* Oliv. (4.8 g)
2	*Psoralea corylifolia* L. (2.4 g)
3	*Juglans regia* L. (1.5 g)
4	*Allium sativum* L. (1.2 g)
5	*Eucommia ulmoides* Oliv. (4.8 g) + *Psoralea corylifolia* L. (2.4 g)
6	*Eucommia ulmoides* Oliv. (4.8 g) + *Juglans regia* L. (1.5 g)
7	*Eucommia ulmoides* Oliv. (4.8 g) + *Allium sativum* L. (1.2 g)
8	*Eucommia ulmoides* Oliv. (4.8 g) + *Psoralea corylifolia* L. (2.4 g) + *Juglans regia* L. (1.5 g)
9	*Eucommia ulmoides* Oliv. (4.8 g) + *Psoralea corylifolia* L. (2.4 g) + *Allium sativum* L. (1.2 g)
10	*Eucommia ulmoides* Oliv. (4.8 g) + *Juglans regia* L. (1.5 g) + *Allium sativum* L. (1.2 g)
11	*Qing'e pill* formulae: *Eucommia ulmoides* Oliv. (4.8 g) + *Psoralea corylifolia* L. (2.4 g) + *Juglans regia* L. (1.5 g) + *Allium sativum* L. (1.2 g)

The dosage of disassembled formulae 1–11 was calculated according to the ratio of the four drugs in the Chinese Pharmacopoeia prescription.

Statistical methods

Data were expressed as the mean ± standard deviation. Statistical differences were analyzed by using the Student's *t*-test. Linear regression analysis was performed by the correlation coefficient. Growth stimulation ratios (GSR) were calculated using the following equation: GSR% = $(A_{sample} - A_{blank})/A_{blank} \times 100$, where A is the average absorbance of six wells.

Results

Correlation between cell number and absorbance

In order to validate the cell proliferation assay, the relationship between cell number and absorbance was examined. After being counted with a hemocytomer and diluted with MEM into eight levels, cells were cultured in 96-well culture plates for 12 h at 37 °C and 5% CO_2, and then the absorbance was determined by using MTT assay as described above. A linear correlation in the range of $3.1 \times 10^3 - 4.0 \times 10^5$ cells/mL between absorbance (Y) and cell number (X) was obtained with a regression equation of $Y = 0.034 + 2.03 \times 10^{-6}X$ ($r^2 = 0.9958$, n = 6).

Effect of aqueous and alcohol extracts of *Qing'e pill* on cell proliferation

The sterilized solutions of aqueous and alcohol extracts prepared as mentioned above were diluted to various concentrations with MEM. A proportion of ethanol in MEM was controlled less than 0.75% for both control and alcohol extract samples to avoid disturbance of UMR106 cells. After being co-cultured with assay solution for 48 h, cell proliferation was assayed. As shown in Tables 2 and 3, the aqueous extracts of *Qing'e pill* promoted osteoblastic proliferation activity significantly (GSR from 23.4% to 38.8%) at concentrations from 4.8×10^{-2} to 4.8×10^{-5} mg/ml (expressed in the weight of the cortex of *Eucommia ulmoides* Oliv. per mL). The effects of alcohol extract were much more potent than that of aqueous extracts (Table 3).

Effect of alcohol extract of disassembled formulae on cell proliferation

Because the cell proliferation activity of the alcohol extract of *Qing'e pill* was much stronger than that of aqueous extract, we chose 75% alcohol as the extract solvent in the study of disassembled formulae. As shown in Table 4, the

Table 2. The effect of *Qing'e pill* aqueous extract on UMR106 cell proliferation.

Sample	Concentration	$\bar{x} \pm SD$ Absorbance	GSR (%)
Blank	0	0.552 ± 0.079	0
	4.8×10^{-6}	0.607 ± 0.064	10.0
	4.8×10^{-5}	0.731 ± 0.060	32.4**
Qing'e pill (mg/mL)	4.8×10^{-4}	0.766 ± 0.055	38.8**
	4.8×10^{-3}	0.732 ± 0.066	32.6**
	4.8×10^{-2}	0.681 ± 0.079	23.4*
	4.8×10^{-1}	0.601 ± 0.054	8.9
NaF (mol/L)	1.0×10^{-5}	0.740 ± 0.058	34.0**

* $p < 0.05$, ** $p < 0.01$, significant as compared to blank; the concentration of *Qing'e pill* was expressed as the concentration of *Eucommia ulmoides* Oliv. in the extract solution.

Table 3. The effect of *Qing'e pill* alcohol extract on UMR106 cell proliferation.

Sample	Concentration	$\bar{x} \pm SD$ Absorbance	GSR (%)
Blank	0	0.396 ± 0.048	0
	4.8×10^{-6}	0.487 ± 0.061	23.0*
	4.8×10^{-5}	0.574 ± 0.052	44.9**
Qing'e pill	4.8×10^{-4}	0.599 ± 0.044	51.3**
(mg/mL)	4.8×10^{-3}	0.629 ± 0.055	58.5**
	4.8×10^{-2}	0.526 ± 0.049	32.8**
	4.8×10^{-1}	0.449 ± 0.050	13.4
NaF (mol/L)	1.0×10^{-5}	0.548 ± 0.054	38.4**

* $p < 0.05$, ** $p < 0.01$, significant as compared to blank; the concentration of *Qing'e pill* was expressed as the concentration of *Eucommia ulmoides* Oliv. in the extract solution.

Table 4. The effect of alcohol extract of *Qing'e pill* disassembled formulae on UMR106 cell proliferation.

Formulae	GSR (%)		
	10^{-3} mg/mL	10^{-2} mg/mL	10^{-1} mg/mL
1	38.7**	28.1*	14.5
2	34.0**	22.3*	5.4
3	15.0	6.9	NAD
4	3.4	9.8	NAD
5	47.2**	25.6*	12.8
6	36.3**	24.4*	9.7
7	36.4**	20.8	8.7
8	53.5**	41.0**	19.3
9	47.8**	23.8*	12.8
10	38.9**	29.9*	18.7
11	58.8**	41.0**	20.3

* $p < 0.05$, ** $p < 0.01$, significant as compared to blank; the concentration of disassembled formulae 1–4 was calculated by Chinese Pharmacopoeia prescription, the concentration of disassembled formulae 5–11 was expressed in the concentration of *Eucommia ulmoides* Oliv. in the extracts solution.

alcohol extracts of *Eucommia ulmoides* and *Psoralea corylifolia* had marked activity stimulating proliferation. The effect of *Eucommia ulmoides* was stronger than that of *Psoralea corylifolia* at the same tested concentration. *Juglans regia* and *Allium sativum* produced insignificant effects at any tested concentrations, compared with the blank. This indicated that *Eucommia ulmoides* and *Psoralea corylifolia* were the two principal drugs, inducing the leading effect in the prescription. The joint effect of *Eucommia ulmoides* and *Psoralea corylifolia* extract solution (formula 5) on UMR106 cells was much stronger than that of either individual drug. *Eucommia ulmoides* combined with either *Juglans regia* (formula 6) or *Allium sativum* (formula 7) failed to show any increase in activity compared with *Eucommia ulmoides* extract solution (formula 1). When *Eucommia ulmoides*, *Psoralea corylifolia* and *Juglans regia* were decocted together (formula 8), the activity was further improved which was similar to *Qing'e pill* extract (formula 11). This indicated an adjuvant role of *Juglans regia* in the prescription. However, the activity of *Eucommia ulmoides*, *Psoralea corylifolia* and *Allium sativum* extract solution (formula 9) was similar to that of *Eucommia ulmoides* and *Psoralea corylifolia* extract solution (formula 5).

Discussion

Qing'e pill, a traditional Chinese compound prescription, is described as a kidney-tonifying and bone-strengthening formula, which has been clinically used for the treatment of senile osteoporosis in traditional Chinese therapy. In this study, it was found that both aqueous and alcohol extracts of this prescription significantly stimulated the proliferation of osteoblast-like UMR106 cells. This suggested that the anti-osteoporostic effect of *Qing'e pill* would be due to its ability of promoting ostoblastic bone formation. The data listed in Tables 2 and 3 demonstrated that the osteoblastic proliferative stimulating effect of alcohol extract was higher than that of aqueous extract, with a maximal growth stimulation ratio of 58.5% for the former versus 38.8% for the later. Therefore, the disassembled formulae study was performed with alcohol extracts.

According to the combination theory of traditional Chinese medicine, a compound prescription usually contains four categories of drugs that are, by virtue of their functions in the therapy, labeled as principal, subordinate, adjuvant and guide drug. The principal drug is the drug that undertakes the overwhelming capacity against diseases. The subordinate drug is the drug that is used to combining with the principal drug to reinforce mutual action besides its individual therapeutic action. The adjuvant drug is the drug that auxiliarily enhances the therapeutic effect or alleviate the potential toxicity. The guide drug is the drug that leads the active ingredients to the target. In this study, When UMR106 cells were cultured with the alcohol extract of the four individual drugs separately (Table 4). *Eucommia ulmoides* showed significant activity of stimulating proliferation, and *Psoralea corylifolia* also showed activity subordinate to that of *Eucommia ulmoides*. *Juglans regia* and *Allium sativum* did not promote osteoblastic proliferation significantly. When *Eucommia ulmoides* and *Psoralea corylifolia* were combined (formulae 5), the growth stimulation ratio was higher than that of either individual drugs. The enhancement effect was even more marked when *Juglans regia* (formulae 8) or both *Juglans regia* and *Allium Sativum* (formulae 11) were added. These results are consistent with the combination principle of this prescription, that is *Eucommia ulmoides* is the principal drug, *Psoralea corylifolia* is the subordinate drug, and *Juglans regia* and *Allium sativum* are the adjuvant drugs.

The synergy of the four botanical drugs was clearly demonstrated in this *in vitro* osteoblastic proliferation assay, although it needs to be further verified with *in vivo* experiments. The synergy effect may be due to a combined action of the components from the four botanical drugs. Another possibility is that some new compounds with higher activity come forth during the co-extraction of these materials. It is therefore worth investigating the active constituents of this compound prescription. Since very complicated constituents are contained in the four botanical drugs such as lignan glycosides, iridoids and gutta-percha in *Eucommia ulmoides* (Zang, 1989; Zhao et al., 1995), coumarin and flavone compounds in *Psoralea corylifolia* (Huang et al., 2000), fatty oil in *Juglans regia* (Zheng et al., 1998) and volatile oil compounds in *Allium sativum* (Jia et al., 1999), it would be very difficult to trace the active components. Using the developed cell proliferation assay with osteoblast-like UMR106 cells as the model, this tack would become easier by virtue of high speed and low dosage of the assay.

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