

'Yang-Invigorating' Chinese Tonic Herbs Enhance Mitochondrial ATP Generation in H9c2 Cardiomyocytes

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Abstract

'Yang-invigorating' Chinese tonic herbs have shown to enhance the myocardial mitochondrial ATP generation capacity in mice *ex vivo*. In the present study, we examined the effect of treatment with the methanol extract of 'Yang-invigorating' herbs on mitochondrial ATP generation capacity in H9c2 cardiomyocytes. The effect of 'Yin-nourishing' herbs was also investigated for comparison. The results indicated that all 'Yang-invigorating' Chinese tonic herbs dose-dependently enhanced the mitochondrial ATP generation capacity in H9c2 cardiomyocytes. Three out of nine 'Yin-nourishing' herbs produced a dose-dependent stimulatory effect on ATP generation, but to lesser extent than that of Yang herbs. Results obtained from activity-directed fractionation of the three most potent 'Yang-invigorating' herbs suggested that the ATP-stimulating ingredients were rather water insoluble and largely resided in the butanol fraction. In conclusion, 'Yang-invigorating' herbs invariably stimulated mitochondrial ATP generation capacity in H9c2 cardiomyocytes. The cell-based assay of ATP generation capacity may be used as pharmacological test for 'Yang-invigorating' Chinese tonic herbs.

Keywords: Yang, Yin, Chinese Medicine, ATP, Mitochondria, Cardiomyocytes

1. Introduction

Chinese tonic herbs that can produce health-promoting action are used for the treatment of various patterns of deficiency in body function with respect to Yang, Yin, Qi, or Blood, and their combinations. These types of functional imbalance are viewed as sub-healthy conditions in modern medicine. Chinese tonic herbs are generally classified into four categories on the basis of their health-promoting actions: 'Yang-invigorating'; 'Yin-nourishing'; 'Qi-invigorating' and 'Blood-enriching' [1]. Of these four types of tonic herbs, the 'Qi-invigorating' and 'Blood-enriching' herbs are grouped under the 'Yang' family and 'Yin' family, respectively. While maintaining Yang and Yin in harmony is akin to attaining the homeostatic state in modern medicine, this phenomenon is aptly exemplified by the long-known antagonizing relationship between sympathetic and parasympathetic neural activities in the body.

According to traditional Chinese medicine theory, Yang is viewed as a manifestation of body function supported by various organs. A 'Yang-invigorating' action

therefore involves the general up-regulation of cellular activities, particularly in the heart that plays a pivotal role in fueling the vital activities in all organs. As ATP, an energy-rich biomolecule, is universally used for energizing cellular activities, particularly in the energy demanding cardiomyocytes [2], we have proposed that the 'Yang-invigorating' action may be mediated by the enhancement of mitochondrial ATP generation [3]. Recently, our laboratory has shown that treatment with the methanol extract of 'Yang-invigorating', but not 'Yin-nourishing', herbs enhanced the myocardial ATP generation capacity in mice *ex vivo* [4]. The stimulation of ATP generation was associated with an increased extent of mitochondrial electron transport [4]. However, whether 'Yang-invigorating' herbs can produce the characteristic ATP stimulatory action in cultured cardiomyocytes is yet to be determined. In the present study, we investigated the effect of treatment with the methanol extract of 'Yang-invigorating' herbs on mitochondrial ATP generation capacity in H9c2 cardiomyocytes. Methanol extracts of 'Yin-nourishing' herbs were also tested for comparison. Three most active Yang herbs, namely,

Herba Cistanches, Herba Cynomorii and Semen Cuscutae, were fractionated by differential solvent extraction with increasing polarity in order to characterize the physical property of the active ingredients.

2. Materials and Methods

2.1. Chinese Tonic Herbs

Dried Chinese herbs (see **Table 1**) used in the project were purchased from a local herbal dealer, Lee Koong Kee and voucher specimens were deposited in the Division of Life Science, Hong Kong University of Science and Technology, Hong Kong. For preliminary studies, herbs were extracted by methanol as previously described [5]. Three of the most active herbs were further subjected to differential fractionation in succession by a set of four solvents, petroleum ether, ethylacetate, butan-1-ol and water, with increasing polarity. The pooled extracts were dried by evaporating the solvents in rotavaporator under heat and reduced pressure. Dried herbal extracts were stored at 4°C until use.

2.2. Cell Culture

H9c2 cardiomyocytes, a subclone of the original clonal cell line derived from embryonic BD1X rat heart tissue and which exhibits many of the properties of skeletal muscle [6], was purchased from American Type Culture Collection. H9c2 cardiomyocytes were cultured as monolayers in Dulbecco's Modified Eagle Medium (Gibco BRL Life Technologies, Grand Island, NY), supplemented with 10% (v/v) fetal bovine serum, 100 IU/mL of penicillin (Sigma, St. Louis, MO), 100 µg/mL of streptomycin and 17 mM NaHCO₃ and were grown under an atmosphere of 5% (v/v) CO₂ in air at 37°C.

2.3. Measurement of ATP Generation Capacity *in Situ*

H9c2 Cardiomyocytes were seeded at a density of 2.5×10^4 cells/well in 24-well tissue culture plates and were incubated in humidified incubator at 37°C for 2 days to allow cell attachment and stable cell growth. After cell attachment, herbal extracts (dissolved in DMSO) were applied in the medium to achieve desired final concentrations (DMSO < 0.2%, v/v). After drug incubation, the ATP-generation capacity (ATP-GC) assay was performed. Culture medium was aspirated, and the cells were washed with phosphate-buffered saline (PBS). Digitonin (50 mg/mL) dissolved in incubation buffer (120 mM KCl, 5 mM KH₂PO₄, 2 mM EGTA, 10 mM HEPES, 0.1 mM MgCl₂, 0.5% bovine serum albumin, pH 7.4)

was added to permeabilize the cell membrane for the entry of substrates. Pyruvate (1.67 mM), malate (5 mM) and ADP (60 µM) were added for mitochondrial ATP generation *in situ*. Cellular ATP level was measured at increasing time intervals (0 to 15 min) following the addition of substrates. The ATP generation was terminated by the addition of 60 µL perchloric acid (30%, w/v). The reaction mixture was centrifuged at $540 \times g$ for 20 minutes. An aliquot (120 µL) of the resultant supernatant was then mixed with 90 µL of 1.4M KHCO₃ for neutralization. Precipitate formed after neutralization was removed by centrifugation at $2150 \times g$ for 10 min. The supernatant was subjected to measurement of ATP content by luciferase assay (ATPlite, Perkin Elmer, Boston, MA) as described [7]. The ATP-GC of untreated cells was estimated by computing the area under the curve of the graph (AUC₁) plotting ATP generated (nmol/mg protein) against time and expressed in arbitrary unit. AUC₁ values of herbal extract-treated cells were normalized to respective mean control value from untreated samples and expressed as percent control. The area under the curve (AUC₂) of the graph plotting percent control against incubation time was computed and expressed in arbitrary unit. Results were expressed as percent control.

2.4. Protein Assay

Cell lysates were prepared by adding lysis buffer (0.1% (v/v) Triton X-100 in PBS). The protein concentration was determined by Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

2.5. Statistical Analysis

The intergroup difference was analyzed by student's t test, with $P < 0.05$ being regarded as statistical significant.

3. Results and Discussion

It is believed that the up-regulation of cellular activities by 'Yang-invigoration' in Chinese medicine requires an increased supply of ATP, which is in turn largely supported by mitochondrial oxidative phosphorylation. As shown in **Figure 1**, all of the 12 tested 'Yang-invigorating' herbs increased the ATP-GC in a concentration-dependent manner in H9c2 cardiomyocytes, with the extent of stimulation at the concentration of 300 µg/mL being 18-51%. Three out of the 9 tested 'Yin-nourishing herbs (Herba Eclipae, Herba Asparagi, Semen Prinsepiae) also dose-dependently enhanced the ATP-GC in H9c2 cardiomyocytes, but to a lesser extent, with the extent of stimulation at the concentration of 300 µg/mL being

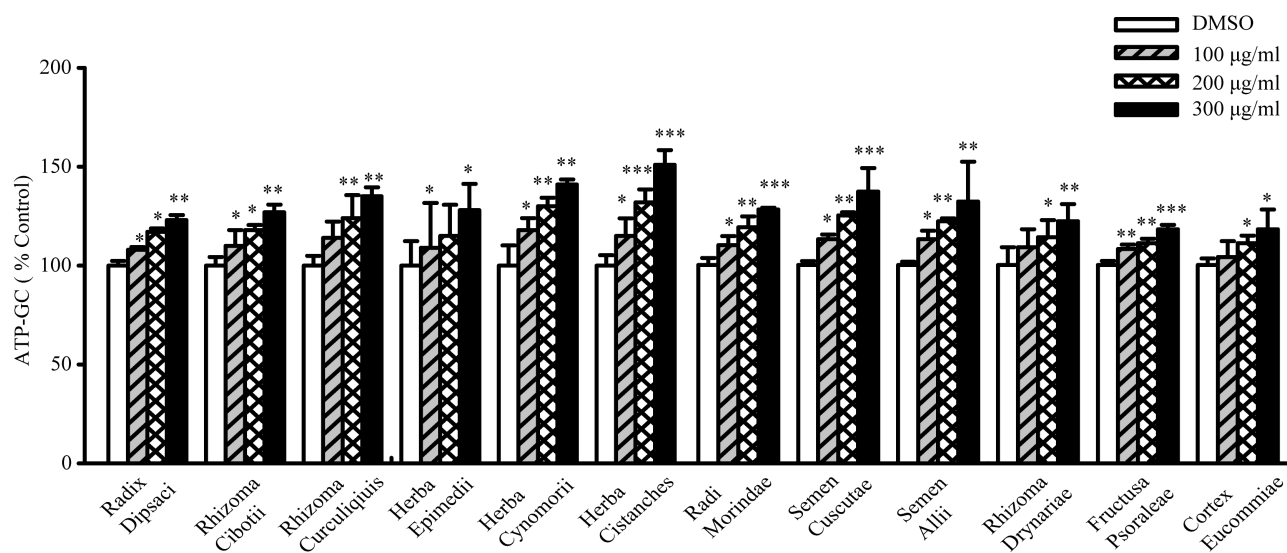
Table 1. Nomenclature of selected Yang and Yin Chinese tonic herbs.

Pharmaceutical Name	Chinese Pin Yin	Latin Botanical Name	Family	% Yield of Methanol Extract
Yang-invigorating				
Radix Dipsaci	Xu Duan	Dipsacus japonicus Miq.	Dipsacaceae	32.6
Rhizoma Cibotii	Gou Ji	Cibotium barometz (L.) J. Sm.	Dicksoniaceae	21.8
Rhizoma Curculiginis	Xian Mao	Curculigo orchioides Gaertn.	Amaryllidaceae	12.4
Herba Epimedii	Yin Yang Huo	Epimedium grandiflorum Morr.	Berbendaceae	15.1
Herba Cynomorii	Suo Yang	Cynomorium songaricum Rupr.	Cynomoriaceae	41.3
Herba Cistanches	Rou Cong Rong	Cistanches salsa (C.A. Meyer) G. Beck.	Orobanchaceae	33.6
Radix Morindae	Ba Ji Tian	Morinda officinalis How	Rubiaceae	19.8
Semen Cuscutae	Tu Si Zi	Cuscuta chinensis Lam.	Convolvulaceae	5.3
Semen Allii	Jiu Zi	Allium tuberosum Rottl.	Liliaceae	8.3
Rhizoma Drynariae	Gu Sui Bu	Drynaria fortunei (Kunze) J. Sm.	Polypodiaceae	2.1
Fructus Psoraleae	Bu Gu Zhi	Psoralea corylifolia L.	Fabaceae (Leguminosae)	23.3
Cortex Eucommiae	Du Zhong	Eucommia ulmoides Oliv.	Eucommiaceae	15.4
Yin-nourishing				
Fructus Ligustri	Nu Zhen Zi	Ligustrum lucidum Ait.	Oleaceae	25.7
Radix Oryzae	Nuo Dao Gen Xu	Oryza sativa L.	Gramineae	1.3
Herba Dendrobii	Shi Hu Cao	Dendrobium nobile Lindl.	Orchidaceae	16.8
Herba Ecliptae	Han Lian Cao	Ecliptae prostrata L.	Compositae	4.5
Radix Asparagi	Tian Dong	Asparagus cochinchinensis (Lour.) Merr.	Liliaceae	56.3
Radix Ophiopogonis	Mai Dong	Ophiopogon japonicus (L. f.) Ker-Gawl.	Liliaceae	25.8
Semen Prinsepiae	Rui Ren	Prinsepia uniflora Batal.	Rosaceae	9.5
Rhizoma Polygonati	Yu Zhu	Polygonatum odoratum (Mill.) Druce	Liliaceae	38.3
Herba Pholidotae	Shi Xian Tao	Pholidotae chinensis Lindl.	Orchidaceae	25.0

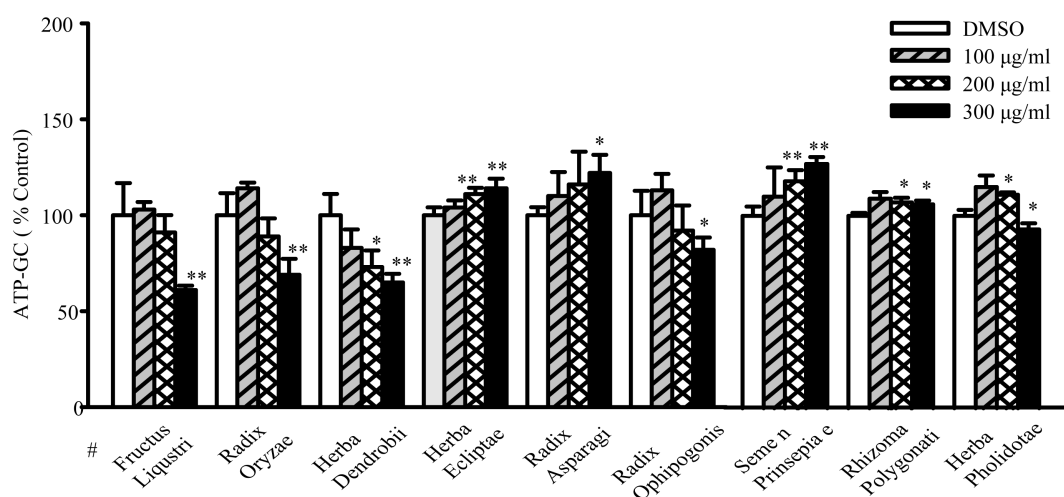
14-27%. In contrast, 5 Yin herbs (Fructus Ligustri, Radix Oryzae, Herba Dendrobii, Radix Ophiopogonii, Herba Pholidotae) produced a dose-dependent suppression of ATP-GC in H9c2 cardiomyocytes, with the degree of inhibition at the concentration of 300 µg/mL (or 80 µg/mL for Fructus Ligustri) being 18-39%. Based on the results obtained, three of the ‘Yang-invigorating’ herbs (Herba Cistanches, Herba Cynomorii and Semen Cuscutae) were selected for their high potency in stimulating ATP generation and subjected to further investigations. The three herbs were fractionated differentially by using a set of four solvents (petroleum ether, ethylacetate, butanol and water) with increasing polarity in succession. As shown in **Table 2**, butanol fractions of Herba Cistanches and Herba Cynomorii increased the ATP-GC in H9c2 cardiomyocytes, with the extent of stimulation

being 43% and 37% respectively. All four fractions of Semen Cuscutae stimulated ATP-GC in H9c2 cardiomyocytes, with the stimulation of ethylacetate fraction being most potent (53%) among them.

A recent study in our laboratory has demonstrated that ‘Yang-invigorating’, but not ‘Yin-nourishing’, Chinese tonic herbs can invariably enhance the myocardial mitochondrial ATP generation in mice *ex vivo* [4]. In the present study, we extended the observation of this characteristic pharmacological action of ‘Yang-invigorating’ herbs in H9c2 cardiomyocytes. While all tested Yang herbs enhanced the mitochondrial ATP generation in H9c2 cardiomyocytes, one-third of the tested Yin herbs also showed moderate ATP stimulatory activity at the highest concentration tested. In this regard, the cell-based *in situ* assay for mitochondrial ATP generation capacity



(a)



(b)

Figures 1. Effects of Yang and Yin Chinese tonic herbs on mitochondrial ATP generation capacity in H9c2 cardiomyocytes. (a) ‘Yang-invigorating’; (b) ‘Yin-nourishing’. (Cells were treated with herbal extracts at the indicated concentrations, as described in Materials and methods. # denotes assay concentrations of 40, 60 and 80 µg/mL. Mitochondrial ATP generation was measured in situ and the ATP generation capacity (ATP-GC) was estimated as described. The rate of ATP generation in untreated cells was 1.88 ± 0.16 (nmol/min/mg protein). Data were expressed in percent control (*i.e.* untreated), and values given are mean \pm SD, with triplicate data. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$, when compared with the untreated control, using Student’s *t* test.)

Table 2. Effects of different fractions of Yang Chinese tonic herbs on mitochondrial ATP generation capacity in H9c2 cardiomyocytes.

	Petroleum ether	Ethylacetate	Butan-1-ol	Aqueous
Herba Cistanches (30 mg/mL)#	100.0 \pm 18.0	90.0 \pm 27.6	142.8* \pm 13.2	99.6 \pm 9.6
Herba Cynomorii (20 mg/mL)	95.7 \pm 11.4	114.3 \pm 4.3	137.1* \pm 4.2	105.7 \pm 5.7
Semen Cuscutae (6 mg/mL)	131.3* \pm 16.8	153.1* \pm 4.0	145.2* \pm 7.0	112.6* \pm 3.9

Cells were treated with herbal extracts at the indicated concentrations, as described in Materials and methods. Mitochondrial ATP generation was measured in situ and the ATP generation capacity (ATP-GC) was estimated as described. Data were expressed in percent control (*i.e.* Untreated), and values given are means \pm SD, $n = 6$. * Significantly different respective control groups ($p < 0.05$), using Student’s *t* test. # Concentrations expressed as crude herb equivalence.

seemed to be less selective than the previously described *ex vivo* assay model for Yang herbs, with the latter showing no stimulatory effect of Yin herbs on myocardial mitochondrial ATP generation [4]. On the other hand, two Yin herbs, namely Radix Oryzae and Herba Dendrobii, were found to suppress the mitochondrial ATP generation in both *ex vivo* and cell-based *in situ* assays [4]. While the discrepant observation between *ex vivo* and *in situ* assays for Yin herbs remains to be explained, the moderate stimulation of ATP generation *in situ* by Yin herbs may be related to the enhancement of cellular glutathione status (data not shown), which in turn can increase the functional ability of mitochondria [8]. Interestingly, using the cell-based assay, the Herba Cistanches-induced enhancement of ATP generation was found to be suppressed by Fructus Ligustri (data not shown). This suggested the antagonistic relationship between Yang and Yin herbs in the regulation of mitochondrial ATP generation. Results from our previous and present studies suggest that the enhancement of mitochondrial ATP generation may serve as a pharmacological activity marker for 'Yang-invigorating' Chinese tonic herbs. In this regard, preliminary studies in our laboratory indicated that Chinese herbal products comprising 'Yang-invigorating' herbs were found to stimulate mitochondrial ATP-GC to varying extent in H9c2 cardiomyocytes. Results obtained from the activity-directed fractionation suggested that butanol fractions of Herba Cistanches and Herba Cynomorii contained relatively high content of ATP-stimulating active ingredients. The residence of active ingredients in relatively non-polar fractions of Semen Cuscutae might be explained by the unique characteristic of the herbs. The results indicated that the ATP-stimulating active ingredients of 'Yang-invigorating' herbs were relatively water-insoluble and may share similar structural characteristics.

The measurement of ATP generation in H9c2 cardiomyocytes *in situ* using malate and pyruvate as substrates is an indirect measure of state 3 mitochondrial respiration [9]. Under the present experimental conditions, the observation of changes in mitochondrial ATP generation by herbal pretreatment may be due to the increase in the activities of enzyme complexes in mitochondrial respiratory chain and/or the decrease in ATP consumption (or degradation) mediated by ATP-ase or other ATP-dependent enzymes. In this connection, an earlier report from our laboratory has shown that the enhancement of myocardial mitochondrial ATP generation capacity by Herba Cistanches treatment was associated with increases in complex I and III activities [10]. On the other hand, the suppression of mitochondrial ATP generation capacity, as assessed by the present *in situ* assay,

afforded by some Yin herbs may be related to the increase in ATP consumption.

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