Pharmacological Studies on Tonifying Herbs

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1. Abstract

Chinese tonifying herbs are used for the treatment of various patterns of deficiency in body function. They are generally classified into four categories on the basis of their health-promoting actions: Ying-nourishing; Yang-invigorating; Qi-invigorating; and Blood-enriching (Geng and Su, 1991). A delicate balance between Yin and Yang is akin to attaining homeostasis in modern medicine. This phenomenon is aptly exemplified by the long-known antagonizing relationship between parasympathetic and sympathetic neural activities in the body. ROS are chemically highly reactive and mitochondrial DNA, proteins and lipids are vulnerable to oxidative damage (Leeuwenburgh et al., 1999). The oxidative damage can result in generalized organelle dysfunction, defective mitochondrial biosynthesis and poor energy metabolism (Chandwaney et al., 1998). Recent studies in Prof. Robert Ko's laboratory showed that while Yang tonifying herbs enhanced myocardial ATP-generation capacity in mice (Ko et al., 2006) and Yin tonifying herbs produced antioxidant activities in mice *ex vivo* (Ko et al., 2007). When a functional imbalance between ROS production and antioxidant capacity caused by various disease states and/or aging occurs, age-related disorders such as cancer, cardiovascular diseases, brain dysfunction, and cataract may occur (Finkel & Holbrook, 2000). Adenosine triphosphate (ATP) is a molecule universally used for energizing cellular activities, particularly in cardiomyocytes (Jacobus, 1985). It has been hypothesized that the 'Yang-invigorating' action may be mediated by the enhancement of mitochondrial ATP generation (Ko et al., 2006) whereas 'Yin-nourishing' action may be related to antioxidation. To test the hypothesis, using H9c2, a cell line derived from mouse embryonic cardiac cells, the effects of methanoic extracts of 'Yang-invigorating' or 'Yin-nourishing' herbs on ATP-generation capacity and menadione-induced oxidative injury were examined and compared.

2. Materials and Methods

2.1 *Chemicals*

ATP, ADP, pyruvate, malate, digitonin, perchloric acid (PCA) and menadione were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Luciferase enzyme was obtained from Fluka (Switzerland). All other chemicals were of analytical grade.

2.2 *Herbal Preparation*

While Chinese herbs were traditionally extracted by water for oral consumption, methanol was used in the present experiment for the sake of convenience in processing and storage of samples (Ko et al., 2006). Eleven dried Chinese tonifying herbs (Yang and Yin) (Table 1) were selected. They were cut into small pieces and then extracted by heating under reflux in methanol at 60°C for 2 hours. The procedure was repeated twice. The pooled extracts were dried by evaporating the solvent under reduced pressure and in a warm water bath.

Table 1. Yang and Yin Chinese tonifying herbs

Pharmaceutical name	**Yield of methanol extract (%)**	**Plant part used**
Yang-invigorating		
Herba Cynomorii	25.2	Stem
Herba Cistanchi	37.8	Whole plant
Herba Epimedii	13.0	Leaves
Fructus Psoraleae	14.8	Fruit
Rhizoma Drynariae	1.1	Rhizome
Semen Cuscutae	3.0	Seed
Yin-nourishing		
Radix Asparagi	53.6	Root
Fructus Ligustri	10.1	Fruit
Herba Dendrobii	6.8	Whole plant
Herba Ecliptae	6.2	Whole plant
Radix Oryzae	2.2	Root

2.3 *Cell Culture*

H9c2 was used as cell model for examining the effect of herbal extract treatment on the ATP generation capacity (ATP-GC) *in situ*. H9c2 cells were cultured in Dulbecco's modified Eagles medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 0.1 mg/mL streptomycin in a humidified incubator at 37°C. For ATP-GC assay, H9c2 cells was seeded into each wells of a 24-well plate at a cell density of 50000 cells/ml. After the cell attachment, the methanolic extracts of Yang and Yin herbs [dissolved in DMSO with a final concentration of DMSO <0.2% (v/v)] were added and incubated with the cells for 24 hours. The control group was treated with DMSO only (Leung, 2006).

2.4 *ATP-GC Assay*

After the drug treatment, cell culture medium was first aspirated. H9c2 cells in each well were rinsed with200 μL of 1X phosphate buffered saline (PBS), then the cells were incubated with digitonin (50 μg/mL) for 3 minutes at 37°C. The perforated cells were mixed with 200μL of substrate solution (containing 5mM pyruvate and 15mM malate), followed by 100μL of ADP (0.18mM), and the reaction mixtures were incubated for increasing periods (0, 5, 10 and 15 min) of time at 37°C. The reaction was terminated by the addition of 60μL of PCA (30%, w/v), and the reaction mixtures were then centrifuged at 1500rpm for 20 min at 4°C. An aliquot (120μl) of the supernatant was mixed with 90μL of 1.4M $KHCO_3$ for neutralization. The mixtures were mixed by vortexing and then centrifuged again at 3000 rpm at 4°C. The resultant supernatants were analyzed for ATP content by bioluminescence assay. After mixing 50μL each of the supernatant and luciferase solution (5-fold dilution from stock) in a white micro-titer plate, bioluminescence was measured at 28°C using Victor^2 Multi-Label Counter (Model 1420, Perkin Elmer, manufactured by Wallac, Turku, Finland). The ATP content was estimated from a standard calibration curve (Leung, 2006).

The mitochondrial ATP-GC of cells was estimated by computing the area under the curve of the graph (AUC_1) plotting ATP generated (nmol/mg protein) against time (0, 5, 10 and 15 min) and expressed in arbitrary unit (Figure 2.4.1). AUC_1 values for experimental group of increasing incubation times were normalized to a respective mean control value from control group and expressed as percentage control. Then the percentage control values were plotted against increasing incubation time (0 to 15 min), and the area under the curve was computed and expressed in arbitrary unit known as AUC_2 (Figure 2.4.2) (Leung et al., 2005). The two-step data processing was aimed to minimizing the inter-assay variabilities under the present experimental conditions (Leung et al, 2005).

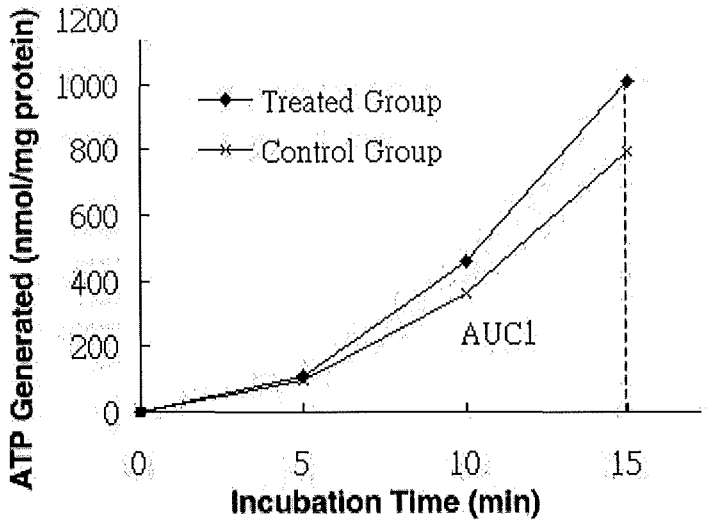


Figure 2.4.1 Measurement of ATP generation capacity in cells

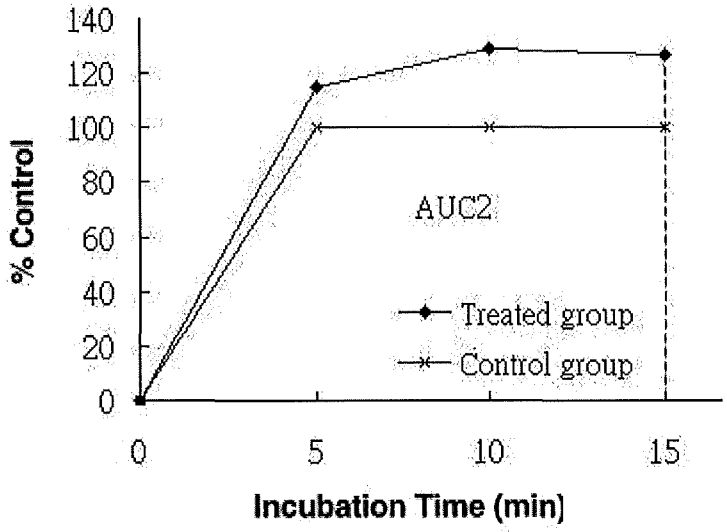


Figure 2.4.2 The extent of stimulation on ATP-GC was estimated by AUC_2

2.5 *Oxidative Challenge by Menadione-induced LDH leakage*

H9c2 cells were seeded in each well of a 12-well plate at a cell density of 37500 cells/mL. They were divided into two experimental groups, namely menadione and vehicle (i.e. absolute ethanol) treatment. Twenty-four hours after the herbal treatment, menadione (or vehicle) was added at a final concentration of 12.5μM (in 750μl cell culture medium), and then incubated with the cells at 37°C for 4 hours. After the incubation, an aliquot of culture medium was saved for later analysis of lactate dehydrogenase (LDH) activity. Attached cells were rinsed with 200μL PBS

and then were lysed by adding 300μL 0.1% Triton-X and incubating for 20 min at 4°C. An aliquot (60 or 20 μL, respectively) of medium or lysate [diluted with 40μL LDH buffer (0.1M sodium phosphate buffer, pH8.0)] was assayed for LDH activity. The reaction was initiated by the addition of 140μL reaction mixture (10μL 20mM pyruvate, 10μL 3mM NADH and 120μL LDH buffer). Absorbance changes of the reaction mixture in a final volume of 200μL were monitored spectrophotometrically at 340nm for 5 min (4 cycles) at 37°C. The enzyme activity was estimated by using an extinction coefficient for reduced form of NAD at 340 nm of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$ and expressed as mU/L of sample. One unit (U) represents the activity of enzyme that can catalyze the oxidation of 1μmol NADH per min.

LDH Released (mU/mL):

$$= \frac{[(\text{slope}/1000000) \times 60 \times (\text{total volume in ml})]}{6.22 \times (\text{sample volume in ml}) \times 0.707}$$

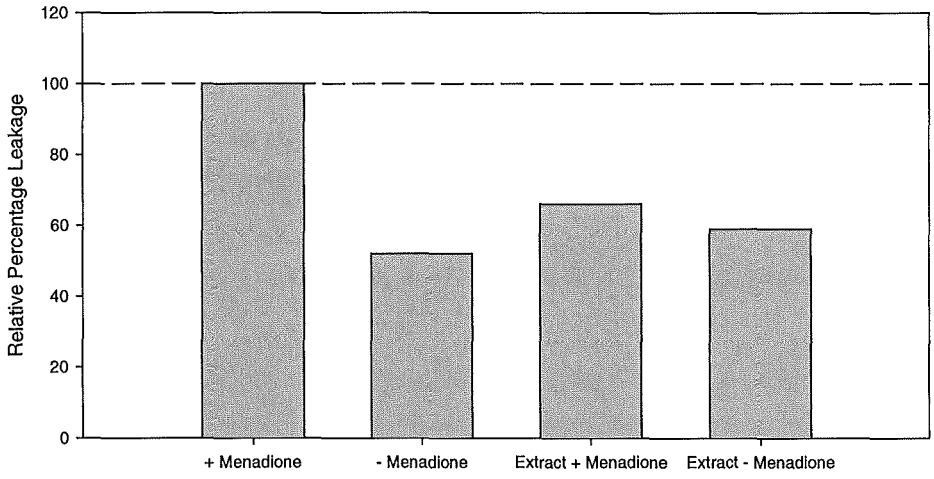
$LDH_{\text{medium or lysate}} = LDH_{\text{medium or lysate}} \times \text{sample volume in ml}$

$LDH_{\text{total}} = LDH_{\text{medium}} + LDH_{\text{lysate}}$

The percentage of LDH release was estimated as follow:

$$\%\ LDH\ release = \frac{LDH_{\text{medium}}}{LDH_{\text{total}}}$$

Figure 2.5.1 Relative Percentage Leakage with Corresponding Treatments



The degree of protection against menadione-induced oxidative injury was estimated by the decrease in the extent of LDH leakage, which indicates less damage induced by menadione.

2.6 *Protein Assay*

Protein concentration was determined using a Bio-Rad protein assay kit. An aliquot (300μL) of 0.1% Triton-X-treated cells was mixed with 600μL water to achieve a 3-fold dilution. Then an aliquot (10μL) of diluted sample was added into the well of transparent 96-well micro-titer plate followed by the addition of 200μL of diluted (5-fold) Bio-Rad assay reagent. The mixture was stood at room temperature for 5 min. The absorbance of the mixture at 570nm was measured. Protein concentration was determined from a calibration curve using bovine serum albumin as standard (Leung, 2006).

2.7 *Statistical Analysis*

All data were expressed as mean + standard error of the mean (SEM), unless otherwise specified. They were analyzed by one-way analysis of variance (one-way ANOVA) and inter-group difference was detected by Duncan's multi-range test when $p < 0.05$ (Leung, 2006).

3. Results

As shown in Fig. 3.1, all tested Yang-invigorating herbs drugs stimulated ATP generation in cultured H9c2 cells, with the degree of stimulation ranging from 4-39% and the relative potency being in a descending order of *Rhizoma Drynariae*, *Fructus Psoraleae*, *Semen Cuscutae*, *Herba Cynomorii*, *Herba Cistanchi*, *Herba Epimedii*. In contrast, all tested Yin-nourishing herbs showed inhibitory effect on ATP generation, with the degree of inhibition ranging from 15-73% and the relative potency being in a descending order of *Herba Ecliptae*, *Radix Asparagi*, *Herba Dendrobii*, *Radix Oryzae*, *Fructus Ligustri* (Fig. 3.2).

Table 2 shows the effect of Yang/Yin herbs on menadione-induced oxidative injury in H9c2 cells, an indirect measure of *in vivo* antioxidant activity. Three out of 6 tested Yang-invigorating herbs exhibited *in vivo* antioxidant activity, as evidenced by the protection against menadione-induced cytotoxicity, with the degree of protection by *Rhizoma Drynariae* being the most potent. However, none of the tested Yin-nourishing herbs showed positive response.

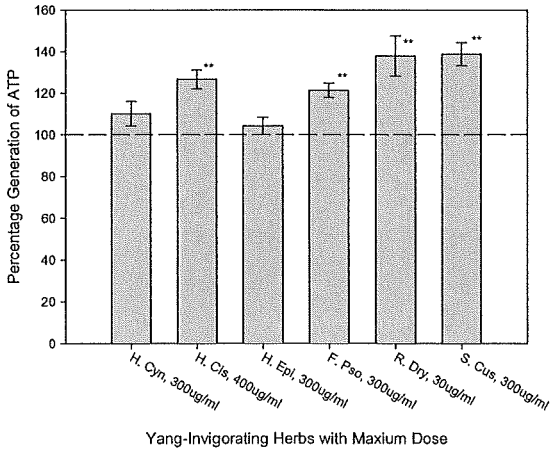


Figure 3.1 Effects of Yang-Invigorating Herbs on ATP Generation Capacity (**p-value<0.01)

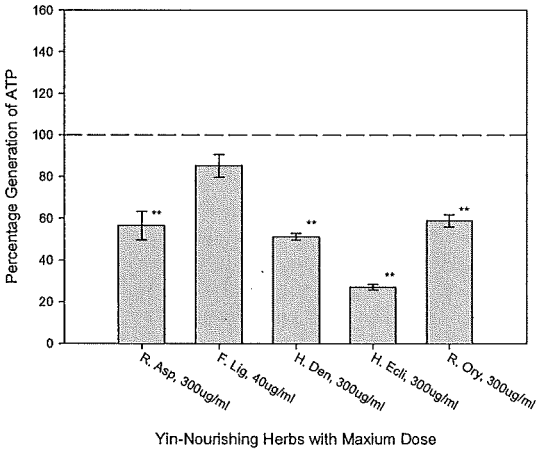


Figure 3.2 Effects of Yin-Invigorating Herbs on ATP Generation Capacity (**p-value<0.01)

Table 2 Effects of Yang/Yin herbs on menadione-induced cytotoxicity in H9c2 cells

	In Vivo Antioxidant Activity
Yang-Invigorating Herbs	
Herba Cynomorii	+
Herba Cistanchi	+
Herba Epimedii	–
Fructus Psoraleae	–
Rhizoma Drynariae	+++
Semen Cuscutae	–
Yin-nourishinbg herbs	
Radix Asparagi	–
Fructus Ligustri	–
Herba Dendrobii	–
Herba Ecliptae	–
Radix Oryzae	–

H9c2 cells were pretreated with the methanolic extract of herbs at a final concentration of 12.5μM *for 24 h. Then the cells were challenged by menadione. The relative potency of in vivo antioxidant activity was ranked by noting the percent of inhibition on LDH leakage.*

4. Discussion

Experimental results indicated that all tested Yang-invigorating herbs enhanced ATP generation capacity to varying extents. The stimulation of ATP generation capacity was associated with *in vivo* antioxidant activity in some Yang-invigorating herbs, as assessed by the protection against menadione toxicity. On the other hand, Yin-nourishing herbs suppressed the ATP generation capacity and produced undetectable *in vivo* antioxidant activity. The finding of differential effect of Yang and Yin herbs on ATP generation capacity in cultured H9c2 cells was consistent with the previous observation using mice for assessing the effect of Yang/Yin herbs on myocardial ATP generation capacity (Ko et al., 2006). The use of H9c2 cells may be more convenient for assessing Yang-invigorating activity in herbal extracts.

When ATP generation is boosted up, the generation of reactive oxygen species (ROS) from mitochondria increased in parallel. The increased production of ROS can trigger the cellular antioxidant system in order to restore the balance between oxidants and antioxidants. As such, cells treated with Yang-invigorating herbs may up-regulate the cellular antioxidant defense by virtue of its stimulatory action on mitochondrial ATP generation. This offers protection against oxidative stress-induced cellular injury, as in the case of menadione toxicity. The observation that not all Yang herbs exhibited *in vivo* antioxidant activity may be due to the presence of compounds in the herbal extract that can produce a negative influence on the antioxidant system. The inability of Yin-nourishing herbs to produce *in vivo* antioxidant activity suggests that the antioxidant properties of Yin herbs may be attributed to free radical scavenging components, which may not be available (or being absorbed) intracellularly to counteract the menadione-induced generation of oxidants.

In conclusion, using cultured H9c2 cells, Yang-invigorating but not Yin-nourishing herbs were found to stimulate mitochondrial ATP generation. While Yin-nourishing herbs possessed undetectable *in vivo* antioxidant activity, the enhancement of ATP generation capacity by Yang-invigorating herbs was sometimes associated with a parallel increase in *in vivo* antioxidant activity.

5. Reference

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