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Cell-Based Biological Markers for Blood-Enriching Chinese Herbs: Erythropoietin Production in HepG2 Cells and Nitric Oxide Release in Human Umbilical Vein Endothelial Cells (HUVECs)

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Abstract

Chinese tonifying herbs, which are classified into four functional categories (namely, Yang, Qi, Yin, Blood) are commonly used for restoring normal body function and the prevention of diseases. To explore cell-based biological markers for Blood-enriching Chinese herbs, we investigate the effect of 11 commonly used Blood-enriching herbs on erythropoietin (EPO) production in HepG2 cells. Herbs for nourishing Yin were tested for determining the specificity of Blood-enriching herbs in inducing EPO production. In addition, the effects of Blood-enriching herbs on nitric oxide (NO) production in both HepG2 cells and human umbilical vein endothelial cells (HUVECs) were also investigated. The results indicated that methanolic extracts of Blood-enriching herbs (but not Yin-nourishing herbs) showed characteristic pharmacological activity in inducing EPO production in HepG2 cells and NO release in HUVECs. The experimental findings, therefore, support the use of cell-based EPO production and NO release as biological markers for Blood-enriching Chinese tonifying herbs.

Keywords

Blood-Enriching Chinese Herbs, Erythropoietin, Nitric Oxide

1. Introduction

Traditional Chinese Medicine (TCM) has a long history of development and usage in China, which dates back several thousand years [1]. With the strong in-

fluences of ancient Chinese philosophy and various schools of thought, TCM has been developed into a medical system distinct from Western medical science, with its unique theories, techniques, and pharmaceutical systems [2] [3] [4]. According to the Yin-Yang theory in TCM, the Yin-Yang balance within the body is crucial for maintaining optimal health. As such, the restoration of the Yin-Yang balance is essential for the prevention and treatment of diseases. The vast diversity of plants and animals in China has enabled the development and usage of a wide variety of herbal preparations for the practice of TCM. While herbal drugs can be divided into two main classes, namely, preventative and therapeutic (for treating diseases), preventative herbal medications (also known as Chinese tonifying herbs) are subdivided into four functional categories according to their mode of action: Yang-invigorating, Yin-nourishing, Qi-invigorating, and Blood-enriching [5] [6] [7]. In theory, Qi herbs belong to a sub-category under Yang and Blood herbs and constitute a sub-category of Yin.

Our laboratory recently demonstrated the characteristic pharmacological activity of Yang/Qi-invigorating and Yin-nourishing herbs [8] [9]. However, the specific pharmacological properties of Blood-enriching herbs have yet to be determined. According to TCM theory, Blood nourishes and moisturizes all parts of the body, and it is indispensable in the manifestation of body functions. Blood deficiency is causally related to insufficient red blood cell production, which is known as anemia in modern medicine [10], and/or inadequate capillary blood perfusion to organs/tissues [11]. With regard to red blood cell production, erythropoietin (EPO) is a growth factor that can stimulate the production of red blood cells (*i.e.*, erythropoiesis) from bone marrow [12] [13]. Under physiological conditions, EPO is mainly synthesized in the kidney and then secreted into the bloodstream. A small amount is also produced by the liver [14]. With respect to capillary blood perfusion, nitric oxide (NO), which is released by endothelial cells of blood vessels, can cause the relaxation of vascular smooth muscle and hence increase blood flow and thereby enhance capillary blood perfusion [15].

To explore cell-based biological activity markers for Blood-enriching Chinese tonifying herbs, we investigated the effect of 11 commonly used Blood-enriching herbs on EPO production in HepG2 cells. Herbs for nourishing Yin were tested for determining the specificity of Blood-enriching herbs in inducing EPO production. The effects of Blood-enriching herbs on NO production in both HepG2 cells and human umbilical vein endothelial cells (HUVECs) were also investigated.

2. Materials and Methods

2.1. Reagents

Fetal bovine serum (FBS), minimum essential medium (MEM), F-12K nutrient mixture (Kaighn's modification) medium, and large vessel endothelial supplement (LVES, at a concentration of 50X) were obtained from ThermoFisher Scientific Inc. (Waltham, MA, USA). Sodium heparin, vanadium (II) chloride

(VCl_3), N-(1-Naphthyl) ethylenediamine (NED), sulfanilamide, sodium nitrite and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Sodium pyruvate and bovine serum albumin (BSA) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Bio-Rad protein assay dye reagent concentrate was obtained from Bio-Rad (Hercules, CA, USA). Human erythropoietin ELISA kits were purchased from Wuhan Huamei Biotech Co., Ltd. (Wuhan, HB, P.R. China). Herbal materials were obtained from Lee Hoong Kee Limited, CRCare Store, or Eu Yan Sang. All other chemicals were of analytical grade.

2.2. Cell Culture

The HUVEC cell line used was an endothelial cell line derived from the vein of the human umbilical cord and the HepG2 cell line originated from the human liver and was purchased from American Type Culture Collection (Rockville, MD, USA). HUVECs were cultured in F12-K medium supplemented with 10% (v/v) heat-inactivated FBS (HIFBS), LVES (at 1X concentration), and 0.1 mg/mL sodium heparin. HepG2 cells were cultured in MEM supplemented with 10% (v/v) heat-inactivated FBS (HIFBS) and 1 mM sodium pyruvate. All cells were cultured in an atmosphere of 5% CO_2 at 37˚C in a 100 mm culture plate. The medium was replaced every 2 - 3 days and cells were split at a sub-cultivation ratio of 1:10 2 - 3 days following medium replacement.

2.3. Herbal Extraction

Herbal materials (100 g) were cut into small pieces and soaked overnight in methanol in a ratio of 1:3 - 1: 20 (weight: volume, g: mL), depending on the physical property of the herbal material. The herbal material was then heated under reflux in methanol at 60˚C for 2 h. This procedure was repeated once. The pooled methanol extracts were dried by evaporating the solvent under reduced pressure, and the herbal extracts were obtained at various yields (Table 1).

2.4. NO Release Assay

Herbal extract pre-incubation and NO release assay.

HUVECs and HepG2 cells were seeded in 6-well plates at cell concentrations of 60,000 cells/2mL and 600,000 cells/2mL, respectively. After stable attachment, the cells were pre-incubated for 5 hours (HepG2 cells) or 48 hours (for HUVECs) with a medium containing 1 - 300 μg/mL of herbal extract [dissolved in DMSO, 0.2% (v/v) final concentration]. After the incubation, supernatants of the culture medium were collected for the measurement of NO release, and the attached cells were rinsed with phosphate-buffered saline (PBS-A) and lysed with 0.1% (w/v) Triton-X in PBS-A. The lysates were used to measure protein concentrations by the Bradford method using BSA as the standard.

The amount of NO released into the medium from herbal extract-incubated cells was measured using the Griess assay. The Griess assay is a spectrophotometric

Table 1. The percentage of yield of methanolic extract of Blood-enriching and Yin-nourishing herbs.

	Pharmaceutical name	Chinese name	Yield (%)
Blood-enriching herb	Polygoni Multiflori Radix	首烏片	0.8
	Polygonati Rhizoma	黃精	38.7
	Longan Mesocarp	龍眼肉	52.0
	Angelicae Sinensis Radix	當歸	10.0
	Equus Derma	阿膠	3.4
	Preparata Rehmanniae Radix	熟地	27.6
	Glycine Max Pericarp	烏豆衣	2.4
	Loranthi Herba	桑寄生	5.9
	Mori Fructus	桑椹子	6.1
	Sesami Semen	胡麻仁	2.7
	Lycii Fructus	枸杞子	35.2
	Ligustri Lucidi Fructus	女貞子	8.0
	Coicis Semen	內仁肉	38.3
Yin-nourishing herb	Ophiopogonis Radix	麥冬	2.1
	Dendrobium Pachyrhiza Rhizoma	石斛草	5.4
	Pholidotae Chinensis Herba	石仙桃	10.2
	Asparagi Radix	天冬	7.7
	Oryzae Glutinosae Radix	糯稻根須	1.9
	Polygonati Odorati Rhizoma	玉竹	4.9
	Ecliptae Herba	旱蓮草	3.8

method based on a diazotization reaction for the detection of nitrite (NO_2^-) formed as a result of the oxidation of NO [16]. Griess reagent solutions were prepared as follows and sodium nitrite was dissolved in distilled deionized water (double-distilled (dd)H_2O) and used as standard:

1) VCl_3 reduction solution: 80 mg VCl_3 was partly dissolved in a small amount of ddH_2O (~2 mL), 0.84 mL hydrochloride acid (HCl, 37%) was added, and the solution diluted to 10 mL with ddH_2O;

2) NED solution (0.2%): 1 mg NED was dissolved in 1 mL ddH_2O;

3) Sulfanilamide solution (2%): 0.02 g sulfanilamide was dissolved in 1 mL 10% (v/v) HCl;

4) Working Griess solution: 5 parts A + 1 part B + 1 part C.

One hundred and twenty microliters of supernatant/standard/medium blank were pipetted into 96-well plates. Working Griess solution (100 μL) was applied to the corresponding wells. The sample mixtures were incubated at 45˚C for 60

min. The absorbance of sample mixtures was measured at 550 nm following incubation. The amount of NO released from the cells was expressed in nmol/mg protein [17].

2.5. EPO Assay

HepG2 cells were seeded as described above, and following stable attachment, cells were incubated for 48 hours with a medium containing 300 μg/mL of herbal extract. After incubation, cells were rinsed with PBS-A and lysed with 0.1% (w/v) Triton-X in PBS-A. The lysates were used to measure protein concentration and EPO levels with the ELISA kit [18].

2.6. Statistical Analysis

Data were expressed as mean ± SEM ($n \geq 3$) and analyzed by one-way ANOVA, followed by Tukey's test to determine significant differences between groups at a level of $p < 0.05$.

3. Results

Methanol extraction of Blood-enriching and Yin-nourishing herbs produced dried extracts at yields ranging from 0.8% to 52% relative to the dried weight of the raw herbs (**Table 1**). With the primary objective of examining whether or not the herbal extracts could induce EPO production, an optimal and non-toxic concentration of 300 μg/mL was uniformly adopted for incubation, regardless of differences in extraction yield. **Figure 1** shows that incubating methanolic extracts of Blood-enriching herbs at a concentration of 300 μg/mL with HepG2 cells for 48 hours induced the production of EPO to varying extents, with the degree of stimulation in descending order being Glycine Max Pericarp > Angelica Sinensis Radix, Equus Derma, Preparata Rehmanniae Radix, Loranthi Herba, Lycii Fructus > Polygoni Multifori Radix, Logan Mesocarp, Sesami Semen >> Mori Fructus.

In addition to the induction of EPO production, the incubation with methanolic extracts of Blood-enriching herbs also caused NO release in HepG2 cells, with the stimulatory effect of Polygoni Multiflori Radix being the most prominent (**Figure 2**).

When the effect of methanolic herbal extracts was examined in HUVECs, all tested extracts except those of Longan Mesocarp and Loranthi Herba (which inhibited cell growth) were found to induce NO release (**Table 3**). While the optimal concentrations for inducing NO release varied from 1 to 300 μg/mL, the degree of stimulation (regardless of the effective concentration) was in descending order: Equus Derma >> Glycine Max Pericarp, Polygonati Rhizoma, Angelicae Sinensis Radix, Polygoni Multiflori Radix > Preparata Rehmanniae Radix, Mori Fructus, Sesami Semen, Lycii Fructus. Results obtained from the present study showed that when compared with HepG2 cells, NO release was more prominent in HUVECs, under both basal and stimulated conditions.

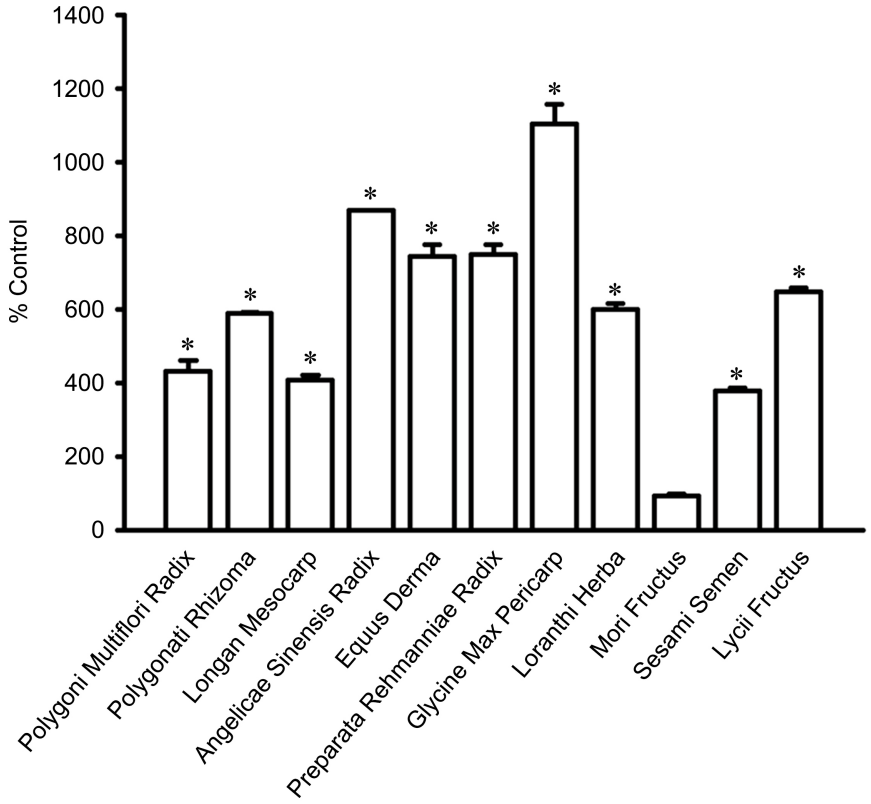

Figure 1. The effect of methanolic extracts of Blood-enriching herbs on EPO production of HepG2cells. EPO and protein concentrations of cell lysates were measured as described in Materials and Methods. Values given are the percent control, when compared with the sample without drug incubation. The control value (mIU/mg protein): 170.7 ± 6.05. *Significantly different from the control. On the other hand, incubation with methanolic extracts of Yin-nourishing herbs at their respective optimal/non-toxic concentrations did not produce any detectable stimulation of EPO production (Table 2).

Table 2. The effect of methanolic extracts of Yin-nourishing herbs on EPO production of HepG2. The control value (mIU/mg protein): 202.8 ± 3.62. *Significantly different from the control.

Pharmaceutical name	Concentration (μg/mL)	Yield (%)
Ligustri Lucidi Fructus	25	93.6 ± 4.47
Coicis Semen	50	75.0 ± 2.53*
Ophiopogonis Radix	50	95.0 ± 6.90
Dendrobium Pachyrhiza Rhizoma	25	91.6 ± 7.48
Pholidotae Chinensis Herba	12.5	92.2 ± 6.18
Asparagi Radix	300	95.5 ± 4.97
Oryzae Glutinosae Radix	12.5	87.4 ± 2.95
Polygonati Odorati Rhizoma	300	92.5 ± 2.49
Ecliptae Herba	75	106.7 ± 3.18

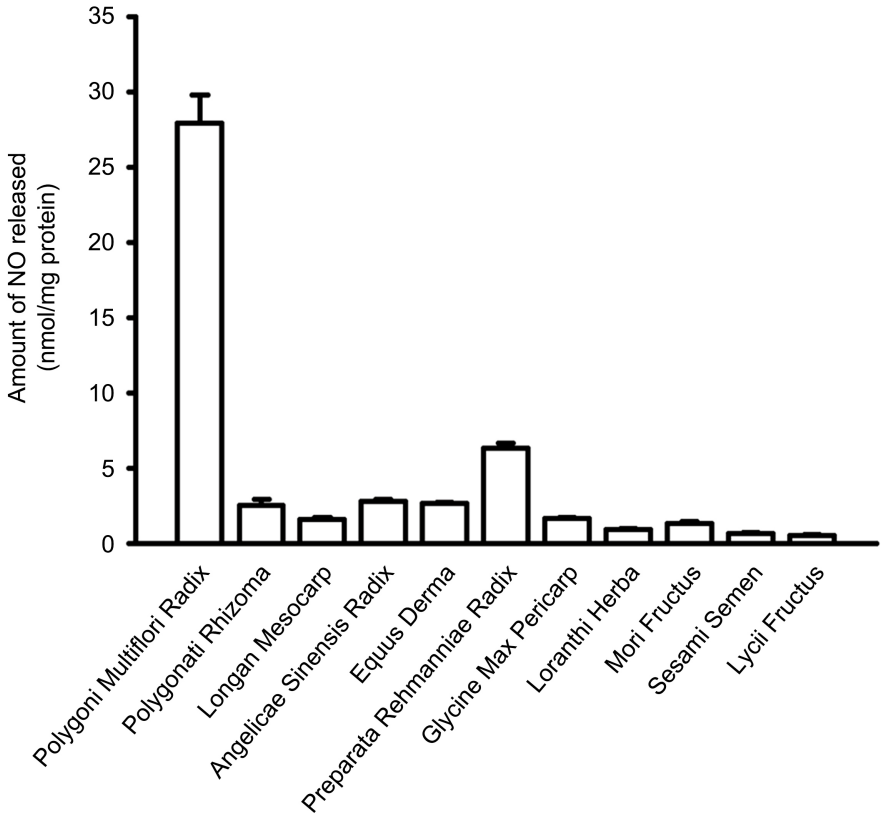

Figure 2. NO released from HepG2 cells following the incubation with methanolic extract of blood-enriching herbs. The control value was undetectable.

Table 3. NO released from HUVECs following the incubation with methanolic extract of blood-enriching herbs. The control value (noml/mg protein) = 33.05 ± 0.94. *Significantly different from the control.

Pharmaceutical name	Concentration (μg/mL)	Yield (%)
Polygoni Multiflori Radix	300	140.9 ± 2.67*
Polygonati Rhizoma	300	158.6 ± 2.26*
Longan Mesocarp	3 - 300	86.4 ± 2.71 - 104.6 ± 1.48
Angelicae Sinensis Radix	30	147.4 ± 3.90*
Equus Derma	30	227.9 ± 9.15*
Preparata Rehmanniae Radix	1	124.1 ± 0.54*
Glycine Max Pericarp	300	168.2 ± 8.10*
Loranthi Herba	30 - 300	Growth inhibition
Mori Fructus	10	123.3 ± 3.25*
Sesami Semen	30	125.4 ± 3.32*
Lycii Fructus	1	116.1 ± 1.74*

4. Discussion

In the present study, all tested Blood-enriching herbal extracts were found to induce EPO production in the human hepatocyte cell line (HepG2). The absence of EPO induction activity in all tested Yin-nourishing herbal extracts suggested the appropriateness of the HepG2 cell-based assay of EPO induction for assessing the biological activity of Blood-enriching herbs. Our finding of EPO induction by Blood-enriching herbs has not been reported at least in a systematic manner. This study will provide a functionally relevant biological activity marker for Blood-enriching herbs. EPO is indispensable to red blood cell production [13]. Prenatally, the liver is the primary site of EPO synthesis but at birth production switches to the kidneys [14]. In this connection, fetal hepatocytes and specialized fibroblast-like peritubular cells located in adult kidneys are the primary producers of EPO [14] [19]. Nevertheless, the use of HepG2 cells rather than specialized kidney cells provides a convenient means for assessing the EPO induction activity even though the detection sensitivity may be lower. Red blood cell production is mainly regulated by the hypoxia-inducible transcription factor (HIF)-EPO axis, wherein hypoxia promotes the availability of HIFs which stimulate the EPO enhancer [19]. Under normoxic conditions, various HIFs are inactivated by enzymatic hydroxylation. In this regard, three HIF-α prolyl hydroxylases can initiate proteasomal degradation of HIF-α, thereby suppressing EPO production [19]. It has been demonstrated that a Chinese herbal formulation (Jian Pi Yi Shen) stimulates HIF-2α and EPO protein expression in both the kidney and liver of rats with chronic kidney disease [20]. A novel HIF prolyl hydroxylase inhibitor (TP0463518) was found to induce dose-dependent EPO production from the liver in healthy volunteers and in patients with chronic kidney disease [21]. It seems possible that chemical constituents present in Blood-enriching herbs may activate HIF-2α and/or produce an inhibitory effect on HIF prolyl hydroxylase, with a resultant increased production of EPO.

The observation of more prominent induction of NO release by Blood-enriching herbs in HUVECs than HepG2 cells is consistent with the fact that NO is continuously synthesized in endothelial cells by the constitutive calcium-calmodulin-dependent enzyme nitric oxide synthase [22]. One early known biological property of NO is the modulation of vascular dilator tone that enables effective blood perfusion of tissues [22]. Regarding the stimulated NO release in endothelial cells, the involvement of the nuclear factor erythroid-2 related factor 2 (Nrf2)/heme oxygenase 1 (HO-1) signaling pathway is well established. Oxidative stress can trigger the Nrf2/HO-1 pathway, with the resultant increase in NO production and endothelial protection. In addition, natural herbal medicines were found to protect vascular endothelial cells against oxidative challenges *via* the activation of the Nrf2/HO-1 signaling pathway [23] [24]. On the other hand, a recent study has shown that all tested herbs (such as Salviae Miltirrhizae Radix and Notoginseng Radix) reveal NO bioactivity through their inherent nitrite and nitrate content and their ability to reduce nitrite to NO [25]. The mechanism

underlying the stimulatory effect of Blood-enriching herbs on NO production in HepG2 cells and HUVECs remains to be investigated.

5. Conclusion

Methanolic extracts of Blood-enriching herbs (but not Yin-nourishing herbs) showed characteristic pharmacological activity in inducing EPO production in HepG2 cells and NO release in HUVECs. Glycine Max Pericarp and Equus Derma, two commonly used Blood-enriching Chinese herbs, are more effective in inducing both EPO production and NO release. Our results support the use of cell-based EPO production and NO release as biological markers for Blood-enriching Chinese tonifying herbs.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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