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Article

Quantitative analysis of effects of salvianic acid a combined with hydroxy safflower yellow a on rat endothelial cells after hypoxic injury using the combination index method

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Cerebrovascular disease is the second most serious disease in the world. It has the features of high morbidity, high mortality and recurrence rate. Numerous research on the compatibility of Chinese medicine with effective ingredients of cerebral ischemia has been made during the past decades. The purpose of this study is to quantitatively analyze the combined pharmacological effect of effective ingredients in Danshen and Honghua (Dan Hong) on rat microvascular endothelial cells after gradually oxygen-glucose deprivation. The experimental concentration range for the compatibility of two effective ingredients were determined in the preliminary experiments by Cell Counting kit-8 (CCK-8) method. Drugs were added to rat brain microvascular endothelial cells at a non-toxic dose level. After that, the cells were cultured for 12 h, and placed in a hypoxic environment. Finally, the cell survival rate was used as a measure of drug effect. In order to determine synergism or antagonism, the combination index (CI)-isobologram method was performed to analyze the data from the experiments. Based on this theory, the potencies of each drug and the shapes of their does-effect curves are both taken into account. The results show that the synergism or the antagonism between two effective ingredients compatibility change with different proportion and dosage. Furthermore, it can be seen from the results of these experiments that when these drugs are used in combination, the dosage required to achieve the same therapeutic effects is greatly reduced compared with the case of single one. It is worth mentioning that our experiments also prove that the median-effect equation and the CI method can be applied in the field of traditional Chinese medicine.

Keywords: Salvianic acid A. Hydroxy safflower yellow A. Combination index. Cerebrovascular disease. Hypoxic injury.

INTRODUCTION

Ischemic encephalopathy refers to the blood supply focal brain dysfunction, determined by insufficient blood supply temporary. Seriously, it has high morbidity and mortality(Wu *et al*., 2016).Improving ischemia area with the blood supply is pivotal to treat this disease. Meanwhile, cerebrovascular endothelial cell has extensive physiological functions such as anticoagulation, maintaining wall permeability, antithrombosis and fibrinolysis. As the first barrier between tissue and blood, cerebrovascular endothelial cell is the first to be deprived of oxygen(Dickey, Long, Hunt, 2011). Cerebrovascular endothelial cell injury is the early pathological and basic cause of cerebral ischemia damage. Therefore, effective protection of cerebrovascular endothelial cells is the key to prevent and treat cardiovascular and cerebrovascular diseases.

At present, traditional Chinese medicine (TCM) and its effective ingredients have been received wide attention, due to their stable therapeutic effects and extensive usage. Radix et Rhizoma Salviae Miltiorrhizae (*Salvia miltiorrhiza* Bge., Labiatae, Danshen in Chinese) and

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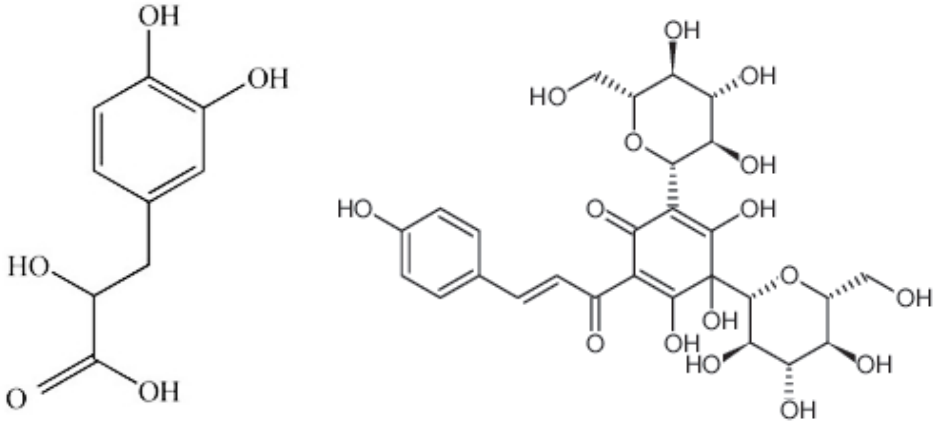
Flos Carthami (*Carthamus tinctorius* L., Compositae, Honghua in Chinese), which have been used in China for thousands of years, are effective drugs used to promote blood circulation and eliminate cerebral congestion (Lam *et al*., 2008; Siow *et al*., 2000). Salvia miltiorrhiza is slightly cold in nature and bitter in taste, which has the functions of promoting blood circulation and regulating menstruation, removing blood stasis and hemostasis. The studies showed that the main chemical constituents of Salvia miltiorrhiza were water-soluble phenolic acid and lipid soluble phenanthraquinone. Salvianolic acid A is one of the main water-soluble components in Salvia miltiorrhiza. Pharmacological studies have shown that salvianolic acid A has a variety of biological activities, which can reduce ischemia-reperfusion injury, cerebral infarction, and has a protective effect on oxidative damage (Wan *et al*., 2020). Safflower is a kind of traditional Chinese medicine for activating blood circulation and dredging menstruation. It is often used in the treatment of dysmenorrhea, chest pain, abdominal pain and traumatic injury. In addition, it can be used in the treatment of various cardiovascular and cerebrovascular diseases, such as coronary heart disease, cerebral infarction and myocardial ischemia. Hydroxy safflower yellow A is one of the most effective water-soluble bioactive components in Carthamus tinctorius, which has the effects of inhibiting platelet aggregation and thrombosis, treating cardiovascular and cerebrovascular diseases, anti-inflammatory and antioxidant (Dong *et al*., 2014).

Salvia miltiorrhiza and Carthamus tinctorius are famous blood activating drug pairs, which are common drug pairs in modern Chinese medicine prescriptions. Both of them are commonly used drugs for promoting blood circulation and removing blood stasis. After hundreds of years of clinical medication verification, they are still in use today and are enduring. Clinically, the combination of these two drugs is often used to treat coronary heart disease and ischemic encephalopathy. Additionally, it can improve the indexes of cerebral hemodynamics in elderly patients with chronic cerebral insufficiency and cerebral infarction, and reduce blood viscosity. So we selected SAA and HSYA as the experimental drugs.

It is noted that, these two drugs are often combined for the treatment of cerebrovascular diseases, and therefore, they are called Danshen and Honghua (Dan Hong) compatibility. In order to facilitate the study of Dan Hong compatibility and its specific effects, the representative Chinese medicine ingredients salvianic acid A(SAA)(Liu *et al*., 2021; Wang *et al*., 2007; Zhao, Li, Fang, 2020) and hydroxy safflower yellow A (HSYA)(Cui *et al*., 2019; Dai *et al*., 2020; Li *et al*., 2015b) were selected to carry out the experiments. The chemical constructions of these two effective ingredients are shown in Figure 1.



A **B**

FIGURE 1 - Chemical constructions of two effective ingredients.

A: chemical structure of SAA, molecular formula: $C_9H_{10}O_5$;
B: chemical structure of HSYA, molecular formula: $C_{27}H_{32}O_{16}$.

The aim of our study is to quantitatively assess the synergism or the antagonism of two conventional drugs (SAA and HSYA) for treating cerebral vascular diseases caused by ischemia in vitro.

Under normal circumstances, the synergism or antagonism of two drugs can be determined by statistical methods. In this way, different statistical criteria result in ambiguity and diversity in the definition of synergism and antagonism(Chou, 2006). However, the necessary conditions to determine synergism or antagonism are the explicit definition of additive effect. Because antagonism effects and synergy effects are relative to additive effects. For example, synergistic effects are enhanced additive effects, not just additive effects. A equation have been made in the past decades to derive the general models for the additive effect, in order to chiefly facilitate the data analyses(Chou, 2010; Ashton, 2015).

The median-effect equation and the CI method base on the mass-action law(Webb, 1963; Chou, Talaly, 1977; Chou, Talalay, 1984; Chou, 1976) have been received a wide range of comments (Chou, 2010; Chou, Talalay, 1984; Goldman *et al*., 1991; Han *et al*., 2005). Moreover, a large number of literatures(Chou, 2006; Chou *et al*., 1994; Chou *et al*., 2003; Chou *et al*., 2002; Gooley *et al*., 2001) have proved that this method can quantitatively analyze the specific synergism or antagonism of different drug combinations.

In this paper, the synergism or antagonism of the two drugs (SAA and HSYA) in the vitro environment were determined quantitatively by the median-effect equation and the CI method. As a general rule, the dosage of drugs can be reduced due to the synergistic effects of drugs. It can reduce the toxicity of the whole drug combination and achieve the same therapeutic effects. Therefore, the overall treatment results can be improved.

MATERIAL AND METHODS

Chemicals and Regents

HSYA (purity: ≥98%) was bought from Tianjing Phytomarker Co. Ltd. (Tianjin, China); SAA (purity: ≥98%) was purchased from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China); Dimethyl sulfoxide (DMSO) and Cell counting kit 8 (CCK-8) were purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China); D-Hank's buffer solution was got from Hangzhou Haotian Biotechnology Co., Ltd. (Hangzhou, China); 0.25% trypsin-EDTA (0.2%) mixed digestion solution was bought from America Gibco Co., Ltd. (New York, USA); Mycoplasma free fetal bovine serum was got from Zhejiang Tianhang Biotechnology Co., Ltd. (Hangzhou, China). Culture medium of various volumes were Sigma products. DMEM/F12 and Trizol were GIBCO Company products.

Instruments

High speed centrifuge (Sigma Co., Ltd., Germany); Type 3111 carbon dioxide incubator (Thermo Fisher Scientific, Inc (TMO)), USA); SX-500 autoclaves pot (Tomy Digital Biology Co., Ltd, Japan).

Cells and Cell Culture

Rat brain microvascular endothelial cells lines were supplied by BeNa Culture Collection Co., Ltd. (Beijing, China). The cells were maintained in DMED/F12 medium (GIBCO BRL, Grand Island, N.Y.) containing 10% heat-inactivated fetal bovine serum at 37 °C in a 5% CO_2-humidified atmosphere.

Brain Microvascular Endothelial Cells Hypoxia Injury Model

Brain microvascular endothelial cells of rat were cultured for 12 h in vitro. The endothelial cells with good growth were used for the experiment. The original culture medium was completely removed before oxygen deprivation. The culture dishes were washed with the culture medium three times and the culture medium were added into the dishes. After the inoculation of cells, all the culture plates were placed in an oxygen-deficient device that is a homemade device made with a microwave oven container box (Ethylene, the volume of 2L). There were two small holes in the top of the left and right sides. We placed the medical three-way pipe end to insert into the holes and seal fixed. Finally, the other end was connected to the silicone tube that the mixture of 5% CO_2, 94% N_2

and 1% O_2 was imported into the device. After 30 min, the inlet and outlet of the hypoxic device were occluded in order to simulate the process of ischemia in vivo.

Survival rate of cells detected with CCK-8 method

Rat brain microvascular endothelial cells were cultured in medium for 24 h at 37°C in a 5% CO_2-humidified atmosphere. Subsequently, the cells were incubated with CCK-8 for anther 4 h at 37°C in a 5% CO_2-humidified atmosphere. After the above treatment was finished, optical density(OD) was measured at 450nm.

Groups and Treatment

According to the cytotoxicity test results from drug toxicity experiments, non-cytotoxic concentration of SAA and HSYA are 0-1000 μg/mL and 0-1500 μg/mL, respectively. When the drug concentrations exceed the above ranges, the cell growth and reproduction will be inhibited. Two parts of experiments were designed to research the effects of single drug and two drugs combination on cells, respectively. The first part of experiments analyzed the effects of two drugs used separately on cells proliferation. The specific dosages are shown in Table I.

TABLE I - Drugs concentration of SAA and HSYA used as single drug*

Groups	1	2	3	4	5	6	7	8	9
Dose (SAA)	0.010	0.020	0.030	0.040	0.050	0.060	0.070	0.080	0.090
Dose (HSYA)	0.010	0.020	0.030	0.040	0.050	0.060	0.070	0.080	0.090

Values are means of three replicates± SE. Within a column, mean values followed by the same letter are not significantly different according to Tukey's test ($p<0.05$).

The second part of experiments was conducted to study the effects of two drugs combination on cells proliferation. The drugs were administered to Dose(SAA):Dose(HSYA)=1:1 and Dose(SAA):Dose(HSYA)=10:1, respectively. The reasons for choosing these two proportions are based on the relevant requirements of CI theory and the proportions in traditional Chinese medicine prescriptions. According to CI theory, the combination ratio was approximately equal to the D_m ratio of the component drugs. In this experiment, the D_m ratio of these two drugs was approximately 1. However, in the traditional Chinese patent medicine Danhong, the ratio of SAA to HSYA was approximately 10:1, so the experiment was conducted in accordance with these two ratios. The specific dosages are shown in Table II.

TABLE II - Drug combination concentration of SAA and HSYA in different proportions*

Proportions (SAA:HSYA)	Groups	1	2	3	4	5	6	7	8
1:1	Dose (SAA)	0.010	0.020	0.030	0.040	0.050	0.060	0.070	0.080
	Dose (HSYA)	0.010	0.020	0.030	0.040	0.050	0.060	0.070	0.080
10:1	Dose (SAA)	0.010	0.020	0.030	0.040	0.050	0.060	0.070	0.080
	Dose (HSYA)	0.001	0.002	0.003	0.004	0.005	0.006	0.007	0.008

* The unit of dosage is *mg/mL*

Median-Effect Theory for Dose-Effect Analysis

The multiple drug effect analysis of Chou and Talalay(Ashton, 2015; Webb, 1963; Chou, Talaly, 1977; Chou, Talalay, 1984) which is based on the median-effect theory, was used to calculate the combined drug effects. Dose-effect curves for each agent and their combinations in multiply diluted concentrations can be plotted by using the median-effect equation(Chou, 2010; Ashton, 2015) as follows.

$$\frac{fa}{fu} = (\frac{D}{D_m})^m \quad [1]$$

In Equation 1, D is the drug dose, D_m is the drug dose required for 50% effect, fa is the true indicator of whether dose (D) affects efficacy (e.g., 0.9 if cell growth is promoted by 90%), fu is the unaffected efficacy (therefore, fa = 1-fu), and m represents the shape characteristic coefficient of the dose-effect curve. Corresponding to the ground, m = 1, m > 1 and m < 1 represent hyperbolic, sigmoidal, and negative sigmoidal dose-effect curves, respectively, for an inhibitory drug. Therefore, both the potency (D_m) and shape (m) parameters were taken into account in this method. Rearranged Equation 1, and then, one got the following result:

$$D = D_m \times (\frac{fa}{1-fa})^{\frac{1}{m}} \quad [2]$$

The D_m and m values can be easily calculated through the relation between x = log(D) and $y = \log(\frac{fa}{fu})$, according to the median-effect plot. It can be known that the slope is m and the vintercept in the median-effect plot is log (D_m). We can readily manifest the consistency of the data to the median-effect theory by the linear correlation coefficient (r) of the median-effect plot.

Combination Index for Determining Synergism and Antagonism

The combination index CI-isobologram equation (Chou, 2010; Ashton, 2015) is shown below.

$$\mathrm{CI} = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} \quad [3]$$

Where CI < I, CI = 1 and CI > 1 indicate synergism, additive effect, and antagonism, respectively(Chou, 2010; Ashton, 2015). In Equation 3, $(D)_1$ and $(D)_2$ respectively represent the dosage of the first and second drugs used in a drug combination experiment, and the final drug effects of this experiment is x%. $(D_x)_1$ and $(D_x)_2$ in the denominators of Equation 3 are the doses of drug one and drug two alone, respectively, that also inhibit x%.

The Name and Version of the Software

All the data was processed by MATLAB 2018a.

RESULTS

Data Acquisition and Basic Processing

Hypoxic culture for 12 h was used as a unified experimental condition. Then, the survival rates of cells in each group after 12 h of hypoxic culture, which were used as a physiological index to determine the strength of drug effect, were measured by CCK-8 method. Each group of experiments was repeated 5 times, and the average value of 5 experiments was taken as the final result. Therefore, all data in this research were normalized with the maximum cell survival rate as the upper bound.

Parameters of Drugs used alone

The dose-effect relationships of SAA and HSYA were subjected to the median-effect plot with the data of efficacy indicator when different doses of drugs used in rat brain microvascular endothelial cells. In order to make dose-effect relationship more accurate and closer to the real situation, the parameters, (i.e, potency (D_m), shape (m), and conformity (r)) have to be determined. The final calibration results are given in Table III.

TABLE III - Dose-effect relationship parameters of various agents against the growth of rat brain microvascular endothelial cells in vitro*

Ingredient	D_m	*m*	*r*	*n*
SAA	0.0329	4.7842	0.9912	5
HSYA	0.0321	4.7010	0.9658	5

*The parameters *m*, D_m and *r* are the slope, antilog of J-intercept, and the linear correlation coefficient of the median-effect plot, which signifies the shape of the dose-effect curve, the potency (CI_{50}), and conformity of the data to the mass-action law(Chou, Talalay, 1984; Chou, 2010; Webb, 1963), respectively;*n* is the number of sets of dose-effect relationship experiments that were carried out, respectively.

The dose-effect curves of these two drugs are shown in Figure 2 and Figure 3, respectively.

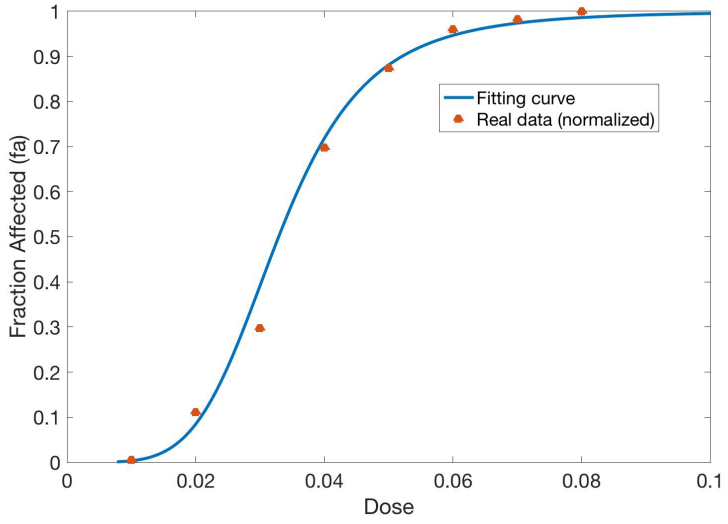


FIGURE 2 - The dose-effect curve of SAA.

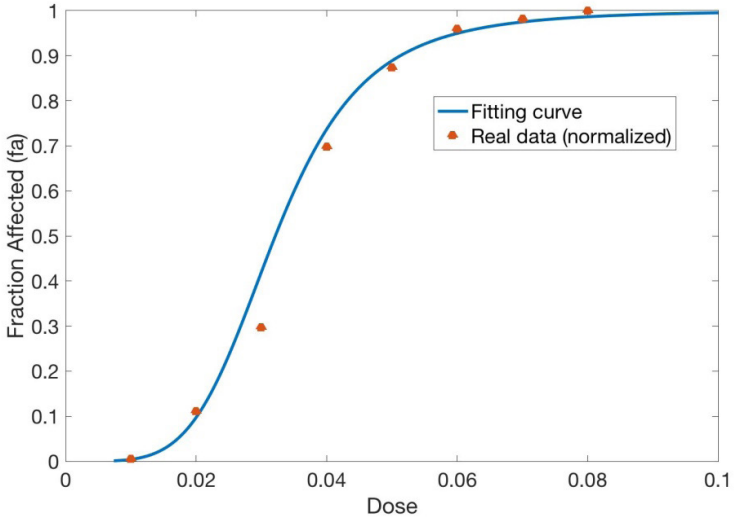


FIGURE 3 - The dose-effect curve of HSYA.

The *r* values of SAA and HSYA are 0.9912 and 0.9658, respectively. According to the *r* values, the dose-effect curves of these two drugs are in suitable agreement with the logistic model. Therefore, the effects of these two drugs on rat brain microvascular endothelial cells meet the application requirements of CI theory. As can be seen from the data in Table 1 and the curve trend in Figure 2 and Figure 3, both drugs have significant effects on cells within a certain range. The synergism effects or antagonism effects can be calculated out based on the CI equation by using the D_m and *m* values for single drugs and for their combination mixtures.

Two-Drug Combinations

Two parts of experiments were designed to research the effects of two drugs combination on cells in two different ratios. In the first part, two drugs were proportioned according to the ratio of 1:1, and in the second part, two drugs were proportioned according to the ratio of 1:10. In each part of the experiment, 8 groups with different concentrations were set up gradient. Table IV summarizes the CI values calculated from data of two experiments.

TABLE IV - Example of experimental design and dose-effect relationships of SAA and HSYA, and their two-drug combinations on growth inhibition of rat brain microvascular endothelial cells in vitro *

Drug			Parameter			
SAA	**HSYA**	**Fractional inhibition, *fa*****	***m***	***D_m***	***r***	**CI$**
$(D)_1$						
0.010		4.191				
0.020		14.52				
0.030		23.958				
0.040		36.069				
0.050		59.664				
0.060		68.442				
0.070		72.567				
0.080		75.834	2.162	0.045	0.982	
$(D)_2$						
	0.010	7.590				
	0.020	14.454				
	0.030	26.631				
	0.040	52.767				
	0.050	64.251				
	0.060	69.828				
	0.070	71.247				
	0.080	72.468	1.900	0.042	0.959	
$(D)_1 + (D)_2 = 1:1$						
0.010	0.010	0.202				0.910
0.020	0.020	0.297				1.412
0.030	0.030	0.400				1.688

TABLE IV - Example of experimental design and dose-effect relationships of SAA and HSYA, and their two-drug combinations on growth inhibition of rat brain microvascular endothelial cells in vitro *

Drug			Parameter			
SAA	**HSYA**	**Fractional inhibition, *fa*****	***m***	***D_m***	***r***	**CI$**
0.040	0.040	0.640				1.385#
0.050	0.050	0.814				1.109
0.060	0.060	0.892				0.972
0.070	0.070	0.954				0.721
0.080	0.080	0.988	4.392	0.048	0.987	0.418
$(D)_1 + (D)_2$ = 10:1						
0.010	0.001	0.380				0.310
0.020	0.002	0.407				0.587
0.030	0.003	0.440				0.826
0.040	0.004	0.614				0.792
0.050	0.005	0.645				0.930
0.060	0.006	0.657				1.089
0.070	0.007	0.720				1.106
0.080	0.008	0.736	3.272	0.035	0.9509	1.218

*The incubation conditions for this result are defined in the "Materials and Methods" section.

**The fa data in this table have been normalized. The processing method is defined in the "Materials and Methods" section. Attention:

CI < 1, CI - 1 and CI > 1 indicate synergism, additivity, and antagonism, respectively. As based on the classic isobologram equation, CI can be calculated by equation 3: $CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2}$, where $D_x = D_m \times (\frac{fa}{1-fa})^{\frac{1}{m}}$.

#Simple calculation for the CI value of 0.04mg/mL SAA+0.04mg/mL HSYA that promoted rat brain microvascular endothelial cell growth by 64.0% (fa = 0.640). On the basis of equation 2, for SAA alone to promote cell growth by 64.0% would require, $[D_{0.64}]_{SAA} = (D_m)_{SAA} \times (\frac{0.640}{1-0.640})^{\frac{1}{2.162}} = 0.045 \times 1.378 = 0.062 mg/ml$ and for HSYA alone to promote cell growth by 64.0% would require, $[D_{0.64}]_{HSYA} = (D_m)_{HSYA} \times (\frac{0.640}{1-0.640})^{\frac{1}{1.900}} = 0.042 \times 1.4402 = 0.0605 mg/ml$.

Therefore, $CI = \frac{0.04 mg/ml}{0.062 mg/ml} + \frac{0.04 mg/ml}{0.0605 mg/ml} = 1.3847$

The CI-dose curves of two experiments are shown in Figure 4 and Figure 5, respectively.

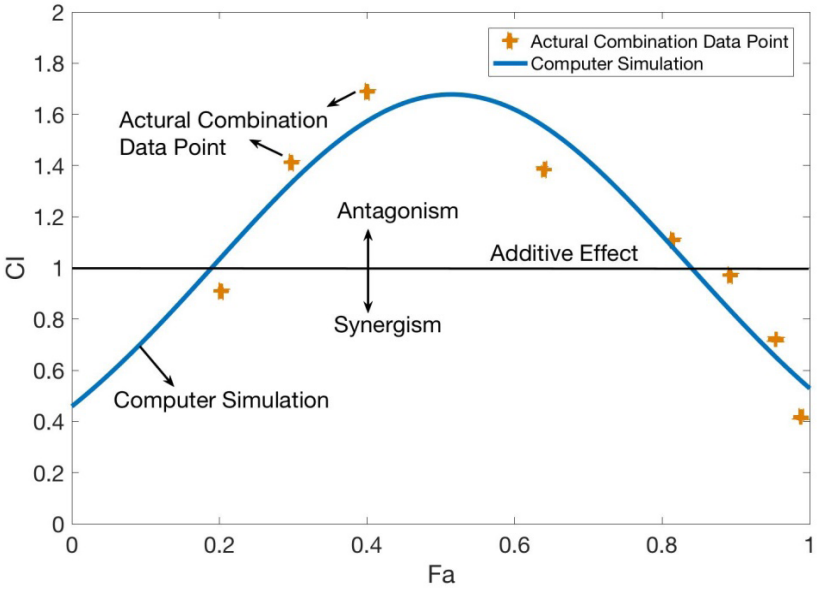


FIGURE 4 - The Fa-CI plot when the ratio of SAA and HSYA is 1:1.

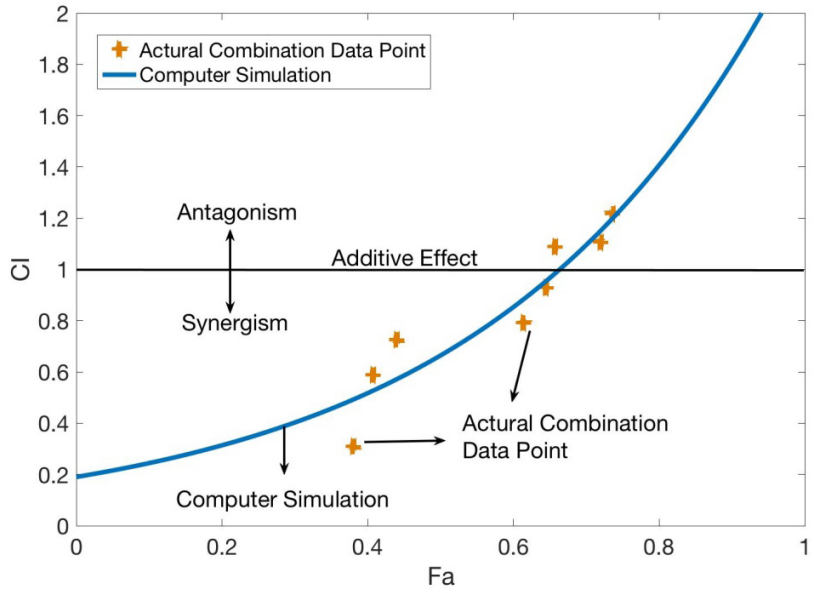


FIGURE 5 - The Fa-CI plot when the ratio of SAA and HSYA is 10:1.

It can be seen from both the figures and the data that these two drugs were not always synergistic or antagonistic under the condition of the same ratio. In the experiment with drug administration ratio of 1:1, when the drug concentration was low, the CI index was close to 1, and the pharmacological action of the two drugs was approximately additive effects. When the specific concentration of the two drugs exceeded nearly 0.020mg/mL for SAA and 0.020mg/mL for HSYA, CI > 1. It means the pharmacological effects of two drugs were antagonistic. Moreover, when the concentration of SAA and HSYA reached 0.030mg/mL and 0.030mg/mL, respectively, the CI value reached 1.688, which almost reaching the maximum value. It indicates that in this case, the two drugs had the strongest antagonism. Then as the concentration of the two drugs continued increasing, the degree of antagonism between

the two drugs gradually decreased. Until the concentration of both drugs were 0.060mg/mL, the CI index reduced to 1 again, and the pharmacological effects of the two drugs changed from antagonism to synergism. Finally, as the concentration increasing, the CI index decreasing, and the synergistic effect of the two drugs increasing.

However, when the ratios of the two drugs were changed, the interaction between the two drugs was quite different (Figure 5, the dosage ratio changes to HSYA: SAA=1:10). As the concentration of the two drugs increased proportionally, the CI index kept increasing, and the pharmacological effects of the two drugs changed from synergistic effects to antagonistic effects. When the concentrations of SAA and HSYA reached 0.055mg/mL and 0.006mg/mL, respectively, the CI index was close to 1, which is near a critical point. This implies that the pharmacological effects of the two drugs reached to the additive effects. After that, the antagonistic effects between the two drugs would be stronger with the increasing of drug concentration.

By the data and curves, it can be seen that the two drugs in the pharmacological effects changed based on the different ratios of drugs, and different drug concentration. It cannot simply rely on the qualitative experiments to determine the interaction relationship of two drugs. Furthermore, the results prove the importance and scientific nature of CI index.

The Effect of Individual Drugs on Overall Outcomes

In order to give a more intuitive view of the effects, we constructed the coordinate system with $\frac{(D)_1}{(D_x)_1}$ as the X-axis and $\frac{(D)_2}{(D_x)_2}$ as the Y-axis. Then, the values of $\frac{(D)_1}{(D_x)_1}$ and $\frac{(D)_2}{(D_x)_2}$ from the data for each experiment were obtained. After that, every dot was denoted in the coordinate system. As can be seen from the above, CI < 1, CI = 1 and CI > 1 indicate synergism, additive effect and antagonism, respectively. Therefore, it is reflected in the coordinate system that $x + y < 1$, $x + y = 1$ and $x + y > 1$ reveal synergism effects, additive effects and antagonism effects, respectively. Such a graph is called as normalized isobologram figure. The normalized isobologram figures of these two parts of experiments are exhibited Figure 6 and Figure 7, respectively.

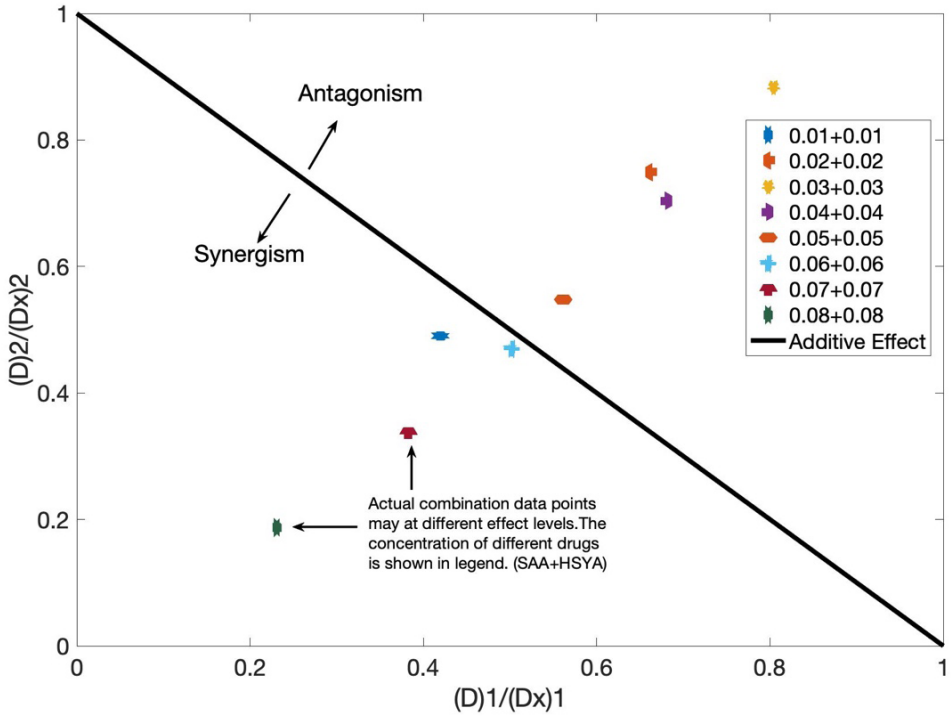


FIGURE 6 - The normalized isobologram figure when the ratio of SAA and HSYA is 1:1.

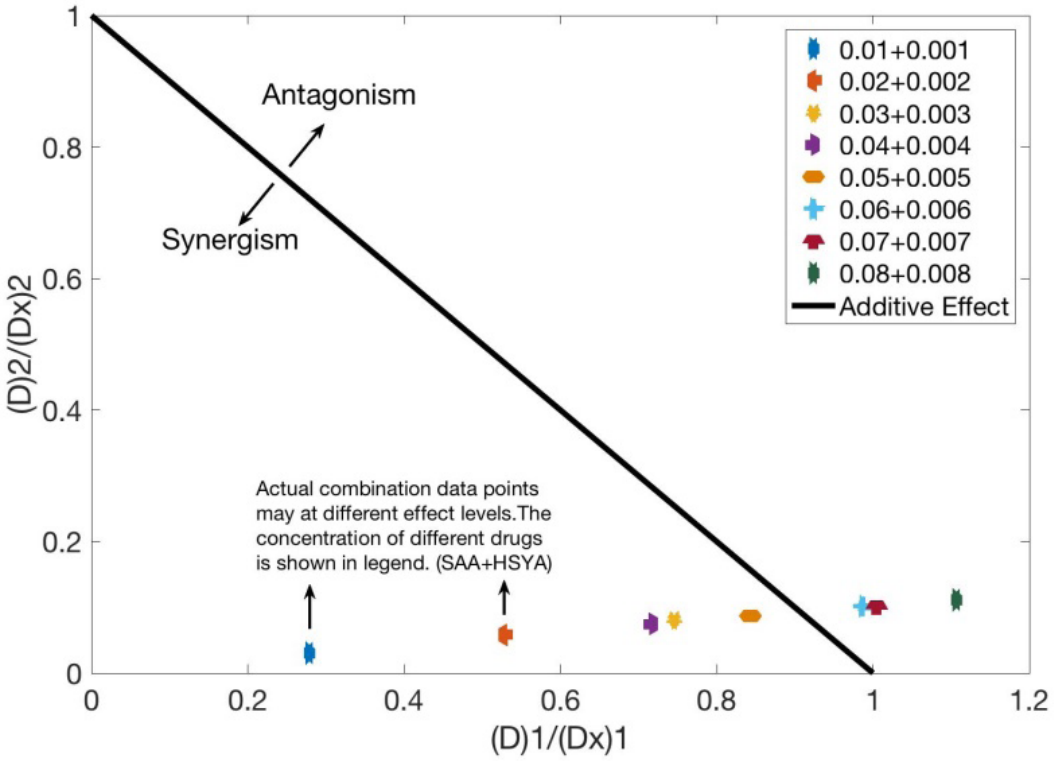


FIGURE 7 - The normalized isobologram figure when the ratio of SAA and HSYA is 10:1.

It follows from FIGURE 6 and FIGURE 7, that the concentration of drug combination above the black solid line are actually antagonistic, whereas the drug combination concentration below the black solid line are actually synergistic.

In particular, in Figure 7, all points were located closer to the X axis. The reason is that the values of $\frac{(D)_2}{(D_x)_2}$ for the points in FIGURE 7 are always close to 0, no matter how much concentration (within a certain range). It shows that the effect of HSYA is very significant in this ratio. Moreover, SAA can promote HSYA in a certain range.

It is indeed that, there are two points (the dosage are 0.07mg/mL+0.007mg/mL and 0.08mg/mL+0.008mg/mL, respectively), whose *x* values are all greater than 1 in Figure 7. This shows that HSYA not only has no promoting effect on SAA, but also counteracts the efficacy of SAA itself in such a case. That is to say, in this case, the efficacy of HSYA is better than the efficacy of the two drugs combination. This situation is the worst, and it is also the negative effect which should be avoided in clinical medication.

DISCUSSION

In recent years, the role of cerebrovascular endothelial cells in a variety of cardio-cerebrovascular disease is becoming more and more serious. The blood brain barrier is an important ingredient in maintaining the integrity of the blood-brain barrier. The central nervous system plays an important role in cerebral blood flow to keep the stability of internal environment(Hankey, Warlow, Sellar, 1990; Zeng, Chao, Luan, 2006).

Danshen and Honghua both are drugs used in promoting blood circulation and eliminating cerebral congestion, which are often combined for treating disease. Among them, the salvianic acid A (SAA)(Lam *et al*., 2008) used in the experiment is a representative water-soluble active ingredient in salvia miltiorrhiza. It has been demonstrated to expand blood vessels, improve blood flow and coagulation, stabilize mitochondrial membrane potential, resist oxidation, and resist inflammation(Li *et al*., 2015b; Yang *et al*., 2008). Another drug used in this study is HSYA, which is a chalcone

made from carthamus, meanwhile, the highest content in safflower yellow(Siow *et al*., 2000), with the brain mitochondria injury induced by ischemia protection, enhances the ability of the body, removes hydroxyl free radicals, and inhibits the effect of peroxide and neuron apoptosis(Chou, Tan, Sirotnak, 1993; Wei *et al*., 2005; Zhu *et al*., 2005b). Most importantly, these active ingredients have the ability to repair cerebral ischemia(Li *et al*., 2015b).

In the study of Danshen and Honghua, they are often used to treat cerebral ischemia disease together, and the traditional statistical analysis method can qualitatively analyze the two drugs(Li *et al*., 2015a; Zhu *et al*., 2005a). Contrary to our general belief, if two drugs are combined to produce a better effect than if they applied alone, nonetheless, it does not mean that the two drugs must have synergistic effects. The reason is that each drug has its own effect, and adding up the dosage will definitely result in the additive state of effect (Ashton, 2015; Chou, 2010; Webb, 1963; Chou, Talaly, 1977; Chou, Talalay, 1984). For example, if drug one or drug two each inhibits the cell growth by 60%, and these two drugs are additive effects, the combined effect cannot be 120% inhibition, because the maximum effect can only be 100%. We must know that additive effects are not a simple arithmetic sum of the effects of two drugs.

There was another alternative method of Webb(Webb, 1963) to calculate additive effect, but which can lead to false conclusions, the reason is that this method only considers data points one by one at a time, but the shape and trend of the dose-effect curve were not took into account(Ashton, 2015; Chou, 2010; Chou, Talaly, 1977; Chou, Talalay, 1984).

This situation is normally termed "therapeutic synergy" when applied to treatment studies. Due to the inherent complexity of drug combination, the performance evaluation of a drug often lacks dose effect parameters and adopts non-quantitative subjective evaluation methods. Therefore, in the course of treatment, it is uncertain whether drug combination can actually produce synergistic effects(Chou, 2010; Wei *et al*., 2005). In some cases, when we show that there seems to be synergy, the real situation is not synergy, maybe just additive effect, maybe even weak antagonism.

The median-effect equation and the CI method have been already using in many territories, such as anti-cancer and anti-human immunodeficiency virus agents. Moreover, it was also invested used to clear leukemia cells for autologous bone marrow transplantation, antimicrobial agents, and the multiple immunosuppressant for organ transplants. So far, a lot of scholars have adopted this method for experimental design and data analysis. Their work has been published in hundreds of biomedical journals(Marty *et al*., 2005; Pietras, 1998; Simon *et al*., 1997; Chen *et al*., 2005; Cheson *et al*., 2004).

In particular, in the field of Chinese medicine research, this method is also very practical, the reason is that the TCM in clinic often delivered as a form of a variety drugs combination (such as TCM prescription). However, the existing statistical methods cannot quantitatively analyze the pharmacological effects between drugs. Therefore, a quantitative method is urgently needed to study the specific synergistic antagonism between drugs in detail. The median-effect equation and the CI method is the most suitable mathematical models to study this problem. On the basis of this method, the pharmacological effects of two or more drugs in a same prescription can be researched clearly under different dosage concentrations and different ratios. This method can be widely used in the detailed pharmacological effects among drugs in TCM.

CONCLUSION

The synergistic and antagonistic effects of effective ingredients in Dan Hong on cerebral ischemia were quantitatively analyzed by the median method and the CI-effect theory. The analysis results illustrate that the active ingredients of SAA and HSYA did synergize within a given dose. And through the study of CI theory, it can be seen that this research method is suitable for the quantitative analysis of a variety of TCM effective component combination of collaborative antagonism effect in the future. Compared with the traditional statistical analysis of the results, the analysis results in this method are more scientific and detailed. Therefore, this method is worth promoting in the pharmacodynamics study of the combined use of TCM.

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