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Evaluating Method of Cell Protection Function using Photohaemolysis

An Kwang Il, Kim Yong Nam

Abstract Haemolysis can occur without photosensitizer when erythrocytes suspension is exposed to ultraviolet lamp (30W, maximum wavelength 250nm). If erythrocytes suspension, of which the dilution is 1∶100, is put in test-tube with 40mm in diameter and put at 20cm under ultraviolet lamp, the haemolysis mainly occurs for 2 hours.

Curcumin of more than 0.005% inhibits haemolysis significantly. *Rhizoma curcumae* and *Radix salviae miltiorrhizae* exhibit clear cell protection effect in the photohaemolysis system by ultraviolet irradiation and when their concentrations are 0.3 and 0.1% respectively, synergism appears.

Key words erythrocyte, curcumin, photohaemolysis, cell protection, photosensitizer, *Rhizoma curcumae, Radix salviae miltiorrhizae*

Introduction

The great leader Comrade **Kim Jong Il** said as follows.

"Medical science should bend its energy to research on the prevention of diseases whose incidence and mortality rate are high, among them those of the cardiovascular system and cancerous diseases. It should also strive to place traditional Korean medicine on a scientific basis and combine it with modern medicine properly, prevent pollution, and increase the variety of medical supplies and appliances and improve their quality. At the same time a long-term plan should be carried out for research to advance basic medicine including virology and genetics."("ON THE FURTHER IMPROVEMENT OF THE HEALTH SERVICE" P. 18)

Radicals induced by strong light irradiation haemolyze erythrocytes, so now in some countries, evaluating methods of cell protection function of functional materials using photohaemolysis are developed and used [10, 11]. These methods are important in search of materials which have cell protection function because their material is not unstable induced chemically but alive cells, namely erythrocytes [4, 8]. But the methods used in previous studies need special devices and photosensitizer such as Rose Bengal [10] or haematophorpirin [11].

Therefore, we established photohaemolyzing method without special device or photosensitizer, and evaluated cell protection function of some plant materials by using it.

1. Materials and Methods

1.1. Materials, devices and reagents

Materials Rats(*Rattus norvegicus*, "Wistar", body weight (225±16)g), *Rhizoma curcumae*, *Radix salviae miltiorrhizae*, *Flos lonicerae*, *Herba leonuri*, *Radix scutellariae*, *Cortex phellodendri*.

Devices Centrifuge("LD4-2A"), ultraviolet lamp (30W, maximum wavelength 250nm), luxmetre("HO-116"), spectrophotometer ("UV-7504 Shimadzu")

Reagents Saline solution(0.85% NaCl, chemically pure), ethanol(analytically pure), curcumin(analytically pure)

1.2. Methods

Blood in carotid of rats was collected, weighed, mixed with saline solution (0.85%) which was 20～40 times of blood and centrifuged (1 000×g, 15min). Sunken erythrocytes were washed with saline solution more than 3 times. They were diluted with saline solution, which was 50～150 times of early blood, and shaked well to be mixed so as to become a suspension. The concentration of suspension was expressed as the ratio of blood mass (g) to diluted saline solution's volume (mL).

3mL of erythrocytes suspension was put in each test-tube with 10～40mm in diameter, and 60μL of test solutions (90% ethanol as control) was put in it. The test tubes were put in different sunlight conditions or at different distances from ultraviolet lamp (30W, maximum wavelength 250nm) in a dark room and the change of transmittancy were measured at regular intervals. Luminous intensity of sunlight was measured with luxmetre and transmitancy was measured with spectrometer at 700nm[5]. The above experiments were done in (20±1)℃.

Curcumin was used as a solution of 0.001～0.05% in 90% ethanol.

Considering the characteristics of useful components, *Rhizoma curcumae* and *Radix salviae miltiorrhizae* were heated and extracted for more than 2 hours in ethanol (more than 80%) and extra materials in water. Then the extracted solution was filtrated and concentrated to become fluid extract of which 1mL contained 1g of medicinal material.

Cell protection function of medicinal materials was evaluated by modificated method of references [10, 11] (See 2.2.)

2. Results and Discussion

2.1. Influence of some factors on photohaemolysis

It is important to select properly the kind of light source in inducing photohaemolysis.[10, 11]

After 3mL of erythrocytes suspension was added with 60μL of ethanol and put in different light conditions, the change of transmittancy was investigated at a regular interval. The result was as follows (table 1).

If erythrocytes are haemolyzed, the membrane is destroyed, so the transmittancy of suspension increases. As shown in table 1, the transmittancy hardly changed in dark or sunlight condition. Namely erythrocytes were hardly haemolyzed. When the suspension was exposed to ultraviolet lamp, the transmittancy changed significantly according to the increase of exposed time, which

Table 1. The change of transmittancy of erythrocytes suspension according to different light conditions

Light condition	The change of transmittancy according to irradiation time/%				
	Before irradiation	0.5h	1h	2h	4h
Dark	5.2	5.3	5.3	5.4	5.4
Sunlight(6 000lx)	5.7	5.7	5.7	5.8	5.9
Sunlight(50 000lx)	5.5	5.5	5.6	5.7	6.1
Ultraviolet lamp(500lx)*	5.3	6.1	23.9	39.3	74.5

Concentration of suspension: 1/50, diameter of testtube: 10mm,

* distance of ultraviolet irradiation: 30cm

shows that the erythrocytes were haemolyzed by ultraviolet rays.

Generally the shorter the wavelength of light is, the bigger the energy of photon is [3]. In previous studies, fluorescent lamp [10] or ultraviolet lamp (maximum wavelength 370nm)[11] was used as light source and photosensitizer was used, but we used ultraviolet lamp of which maximum wavelength was 250nm, so we could induce haemolysis without photosensitizer.

In table 1's condition, the haemolysis didn't end for 4 hours. In order to decrease haemolysis time, the change of transmittancy was investigated according to different concentrations of erythrocytes suspension put 30cm below ultraviolet lamp (table 2).

Table 2. The change of transmittancy according to the concentration of erythrocytes suspension

Concentration of suspension	The transmittancy according to irradiation time/%				
	Before irradiation	1h	2h	3h	4h
1/50	5.3	23.9	39.3	48.4	74.5
1/100	6.2	32.0	65.4	90.3	91.8
1/150	10.4	37.7	68.9	91.2	91.5

Diameter of testtube: 10mm

As shown in table 2, the transmittancy became to nearly saturated state for 3 hours when the concentration of erythrocytes suspension was 1/100～1/150. Namely the haemolysis occurred for about 3 hours. The concentration of erythrocytes suspension was set as 1/100 because the high beginning transmittancy is unfavourable for correct observation of haemolysis process.

Then, in order to evaluate the influence of ultraviolet irradiation amount on haemolysis, suspension was put in testtubes of different diameter, then at different distances below ultraviolet lamp and the change of transmittancy according to exposed time was investigated (table 3).

Table 3. The change of trasmittancy according to diameter of ultraviolet irradiation surface and irradiation distance

Diameter of irradiation surface/mm	Irradiation distance/cm	The transmittancy according to irradiation time/%					
		Before irradiation	1.0h	1.5h	2.0h	2.5h	3.0h
10	30	6.2	32.0	49.1	65.4	78.7	90.3
	20	5.7	34.3	53.4	70.2	82.1	90.7
	10	6.1	38.8	55.6	72.0	84.2	92.0
20	30	6.0	33.7	51.8	71.2	85.1	90.7
	20	6.0	37.2	56.0	80.0	87.3	91.0
	10	5.8	40.0	57.2	83.0	87.5	91.9
40	30	5.8	35.3	54.8	84.9	89.9	91.4
	20	5.7	40.1	59.4	89.3	91.5	91.5
	10	5.8	41.3	61.4	90.1	91.4	91.7

As shown in table 3, when the diameter of ultraviolet irradiation surface was the same, the transmittancy according to irradiation distance did not change much, but when the diameter of irradiation surface was different, the changing velocity of transmittancy differed much. Namely when the diameter of irradiation surface was 10mm, the transmittancy became saturated state after 3 hours, but when the diameter was 40mm, it became saturated state after 2.0～2.5 hours. So if the erythrocytes suspension was put in test tubes with 40mm in diameter and put at 10～20cm below ultraviolet lamp, the main haemolysis process would be observed for 2 hours.

2.2. Evaluating method of cell protection function using photohaemolysis

In previous studies[10], semi-haemolysis time, namely the time for which half of erythrocytes was haemolzed, was used as indicator for evaluation of cell protection ability by photohaemolysis.

If haemolysis inhibiter was added to photohaemolysis system, the haemolysis delays, so the measuring time becomes longer and the number of measurement increases. In order to overcome these deficiencies, we introduced new indicator based on transmittancy and evaluated cell protection ability of materials.

Generally, the change of transmittancy according to ultraviolet irradiation time in erythrocytes suspension appears as S-type curve as shown in the below Fig.

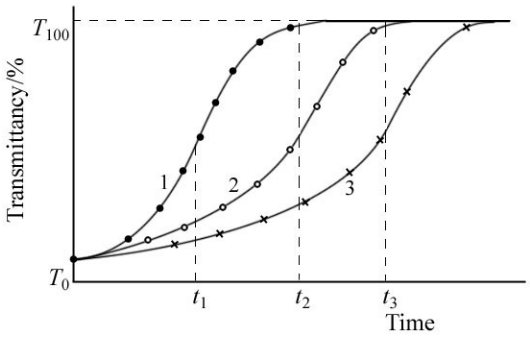

Fig. Change of transmittancy of erythrocytes suspension according to irradiation time
1－control, 2－test 1, 3－test 2

In both control and test divisions, the beginning and end values of transmittancy were almost same. The beginning value is transmittancy when erythrocytes are not haemolyzed at all and the end value is that when erythrocytes are entirely haemolyzed. If these values are represented by T_0 and T_{100} respectively and the transmittancy of any division at any time is represented by T, the haemolysis rate (percentage of haemolyzed erythrocytes to beginning erythrocytes, %) could be calculated by the following formula.

$$H = \frac{T - T_0}{T_{100} - T_0} \times 100$$

Generally, the haemolysis is done in control division for the first time, so the saturated value of the control transmittancy can become T_{100}. Then when the haemolysis rates would be compared becomes a problem. If the rates are compared at t_1 in Fig., the difference between test divisions is not clear, and if they are compared at t_3, the haemolysis inhibiting effect doesn't appear in test 1 division.

The most suitable measuring time is t_2 when the transmittancy of control becomes saturated state or just before it. Then the haemolysis inhibiting effect of test divisions appears clearly and the difference between test divisions becomes clearer.

When erythrocytes suspension (concentration 1/100) is put in test-tube with 40mm in diameter and at 20cm below ultraviolet lamp, the haemolysis measuring time (t_2) might be 2h (table 3).

On the other hand, haemolysis inhibiting ability (*IH*) was newly introduced in addition to haemolysis rate as index for evaluation of cell protection function.

$$IH = \frac{H_{\text{Control}}}{H_{\text{Test}}}$$

Using the above established method, the cell protection ability of curcumin, the main component of turmeric pigment which has already been known as cell protection substance[1, 2], was evaluated and the result was shown in table 4.

In 0.005～0.05% of curcumin, the haemolysis rate was 42.6～80.4%, which showed clear

Table 4. Haemolysis according to curcumin concentration

Curcumin concentration/%	Haemolysis rate/%	Haemolysis inhibiting ability
0	95.2±2.3	1
0.001	94.3±1.7	1.01
0.005	80.4*±2.2	1.18
0.010	53.5*±1.6	1.78
0.050	42.6*±2.0	2.23

$n=3$, * $p<0.05$

haemolysis inhibiting effect.

Like this, photohaemolysis system would be used to search substances that protect cells from damage by ultraviolet irradiation and find effective concentrations.

Compared with methods cited in the previous studies [4, 10], this method has advantage of evaluating cell protection function without photosensitizer such as Rose Bengal or haematophorpirin. However, in the future, it should be studied more how the haemolysis process would be changed using ultraviolet lamps with different wavelength and luminous intensity, and what relationship this method has with the evaluating methods using photosensitizer.

2.3. Cell protection ability of several plant materials

Using the above established method, cell protection ability of 6 species of plant materials[5－7, 9] was evaluated and compared (table 5).

Table 5. Inhibition of photohaemolysis by plant materials

Materials	Concentration/%	Haemolysis inhibiting ability
Flos lonicerae	0.15	1.03±0.08
	0.30	1.05±0.08
Herba leonuri	0.15	1.11±0.07
	0.30	1.18±0.10
Radix scutellariae	0.15	1.15±0.09
	0.30	1.24±0.08
Radix salviae miltiorrhizae	0.15	1.21±0.11
	0.30	1.28±0.12
Cortex phellodendri	0.15	1.05±0.11
	0.30	1.10±0.10
Rhizoma curcumae	0.15	1.17±0.12
	0.30	1.48±0.12

The substance which had the highest cell protection ablility among tested materials and concentrations was *Rhizoma curcumae* and 0.30%.

There are many cases when plant materials exhibit better effect if they are used together than if they are used alone[5]. So, cell protection ability was investigated when *Rhizoma curcumae* of 0.3% was added with *Radix salviae miltiorrhizae* and *Radix scutellariae* of 0.05～0.1% respectively (table 6).

If *Rhizoma curcumae* was added with *Radix salviae miltiorrhizae* or *Radix scutellariae*, the value of haemolysis inhibiting ability increased more. Especially, it was considerable that haemolysis inhibiting ability increased more than 3 times if *Rhizoma curcumae* 0.3% was added with *Radix salviae* miltiorrhizae 0.1%. Using the above synergism, medicinal materials which exhibit high cell protection effect would be manufactured even though a little plant material is used.

Table 6. Complex application effect of materials on photohaemolysis

Materials and concentration	Haemolysis inhibiting ability
Rhizoma curcumae 0.3%	1.48±0.12
Rhizoma curcumae 0.3%+*Radix salviae miltiorrhizae* 0.05%	1.95±0.23
Rhizoma curcumae 0.3%+*Radix salviae miltiorrhizae* 0.10%	4.53±0.29
Rhizoma curcumae 0.3%+*Radix scutellariae* 0.05%	1.65±0.30
Rhizoma curcumae 0.3%+*Radix scutellariae* 0.10%	2.28±0.21

But whether the cell protection materials found with the above *in vitro* method would exhibit cell protection function actually *in vivo* or not, and how high it would be should be studied more in the future.

Conclusion

Haemolysis can occur without photosensitizer when erythrocytes suspension is exposed to ultraviolet lamp (30W, maximum wavelength 250nm). If erythrocytes suspension, of which the dilution is 1:100, is put in test-tube with 40mm in diameter and put at 20cm under ultraviolet lamp, the haemolysis mainly occurs for 2 hours. The cell protection function of materials can be compared and evaluated with calculation of haemolysis rate of every division when the transmittancy of control division gets to saturation state.

Curcumin of more than 0.005% inhibits haemolysis significantly. *Rhizoma curcumae* and *Radix salviae miltiorrhizae* exhibit clear cell protection effect in the photohaemolysis system by ultraviolet irradiation and when their concentrations are 0.3 and 0.1% respectively, synergism appears.

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