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Effects of the Chungsimyonjatang Water Extract on the Rat Myocardial Cells in Cultures

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Abstract

淸心蓮子湯 煎湯液이 培養 心筋細胞에 미치는 影響

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ADR유발성 심근독성에 대한 심근세포의 손상기전을 규명하기 위해 ADR의 독성을 MTT정량, NR정량, LDH활성도 및 심박동을 측정하였다. 배양된 심근세포에서 청심연자탕 전탕액의 심근세포 보호효과는 LDH 활성도 측정과 심박동 측정을 통해 관찰할 수 있었다. 이 실험을 통해 다음과 같은 결과를 얻을 수 있었다.

1. ADR은 배양심근세포에서 세포의 생존능력을 떨어뜨렸고, LDH의 활성도를 높였으며, 심박동수를 감소시켰다.
2. 청심연자탕 전탕액은 배양심근세포에서 ADR에 의해 증가된 LDH 활성도를 유의하게 감소시켰다.
3. 청심연자탕 전탕액은 배양심근세포에서 ADR에 의해 감소된 심박동을 유의하게 증가시켰다.

이상의 결과를 통해 ADR은 신생 마우스에서 적출해낸 배양 심근세포에서 독성효과를 나타냈음을 알 수 있었으며, 청심연자탕 전탕액은 ADR에 의해 유발된 심근세포독성에 매우 효과적으로 방어효과를 나타냄을 알 수 있었다.

key word : Chungsimyonjatang, MTT assay, NR assay, LDH activity assay, Heart beating rate, Adriamycin(ADR), Myocardial cell

I. INTRODUCTION

According to [Dongyi-soose-bowon][1] Chungsim-yonjatang(CYT) is known as a remedy for interior-overheated-disease of taeumin. Also, it is a medication for heart related diseases such as fright, continuous violent palpitation, nocturnal emission, according to modern medical research.

Research for CYT were followed; Kim, the effect of the CYT on the myocardial ischemia[2]; Kim, the influence of immune and hypo-allergenic reaction[3]; Hong and others, the effect of anti- stress[4]; Ok and others, the

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effect of cerebral neuron[5]; Park and others, effect on blood pressure and local blood mass of cerebrum of rats[6]; Park and others, clinical experiment in the use of CYT[7].

However, this paper emphasizes the application between the CYT and the heart disease symptom, which conducted in the artificially damaged myocardial cells.

An anti-cancer medication such as adriamycin (ADR) interferes the growing and the separation of the cancer cell by control of composition of DNA, RNA and protein[8-10]; however, these anti-cancer medicine gives serious damage to the heart and the results are supported by myocardial cell system test.[11-12]

To examine the mechanism of myocardial damage against adriamycin(ADR)-induced myocardiotoxicity, cytotoxicity of ADR was observed by MTT assay, NR assay, LDH activity and heart beating rate. The cardioprotective effect of CYT water extract in cultured myocardial cells are investigated in LDH activity assay and heart beating rate.

II. Materials and Methods

1. Materials

1) Experimental Animal

ICR types of healthy rats were used.

2) Prescription of Experiment

Prescription of CYT was based on [Dongyi-soosebowon] The description and amounts were followed:

Botanical Name	Weight(g)
Radix Ophiopogonis	4
Rhizoma Dioscoreae	8
Rhizoma Acori Graminei	4
Radix Scutellariae	4
Semen Zizyphi Spinosae	4
Semen Biotae	4
Arillus Longan	4
Radix Asparagi	4
Semen Raphani	4
Radix Polygalae	4
Semen Nelumbinis	8
Flos Chrysanthemi	1.3
Total amount	53.3

Prescription of CYT

2. Methods

1) Cell Cultivation

The myocardial cell of the heart tissue was treated for 20 minutes, after it washed Ca, Mg free Hank's balanced salt solution (HBSS, Gibco). Put above product in the mixture of Eagle's minimum essential medium (MEM, Gibco), 10% fetal bovine serum (FBS, Gibco), and penicillin G (25 unit/ml). Then set 96-multiwell plate (Gibco) in 1x10 cell/well. The cells were exchanged with new cultured fluid within three days and compared with none adriamycin cultured fluid.

2) Making of Water Extract

First, prepare 197.2g of CYT and 1.8 L of third distilled water in round-based flask. Second, after cooling down above fluids for 3 hours, centrifuge at 3,000 rpm for 20 minutes. Third, decompressional concentration with vaccum concentration. Fourth, lyophilization for 24 hours.

Final Product: powder sample of 45.29g

3) The Use of ADR

Prepare the 1 mg/ml, 100 g/ml, 10 g/ml and 1 g/ml of adriamycin (ADR, Sigma) in the refrigerated storage and use them whenever they need. To study the effect of ADR in cultured myocardial cells, rinse the myocardial cell 3 times with phosphate buffered saline (PBS) and culture for 9 - 72 hours in the cultured solution of 1 to 100 g/ml of ADR.

4) The Use of CYT Water Extract

Set up various concentrations of CYT Water Extract. Before exposure to cultured myocardial cells in ADR, be treated CYT Water Extract for 3 hours. After the exposure to ADR solution for 36 hours and investigate the effect of CYT Water Extract in cultured myocardial cells.

5) Cardiotoxicity and Protection Effect

(1) MTT Assay

Rinse the MTT <3-(4,5-dimethylthiazol-2-yl) -2,5- diphenyltetrazolium bromide (Sigma)> and cultured myocardial cell of ADR or CYT Water Extract with PBS 3

times. Pour 50 mg/ml MTT in above solution and treat them at 37C, 5% CO. After the completion of cultivation, process dimethylsulfoxide(DMSO, Merk) and measure fluorometer with spectro-photometer at 590 nm.

(2) NR Assay

Rinse 3 times myocardial cells with various concentrations of CYT Water Extract in PBS. After the complete reaction, pour 5 mg/ml NR in previous solution and culture for 3 hours at 37C, 5% CO. Rinse the cultured cell with PBS, and process in 1% formalin and 1% glacial acetic acid. Finally, measure and evaluate fluorometer with microelisa reader at 540nm.

(3) Lactate Dehydrogenase(LDH) Activity

Place the enzyme substance of kit (Japan) in 1.0 ml tube (Palcon) and combine them with cultured solution. Study the reaction of the combination at 37 C. After complete reaction, compound them with 3.0 ml inhibitor solution of dilution. Measure fluorometer with spectrophotometer at 540nm.

(4) Heart Beating Rate (BR)

For heart beating rate compare the cultured myocardial cell and 36 hours of cultured ADR solution. To analyze the effect of CYT Water Extract, process the cultured myocardial cells in various concentrations of CYT Water Extract for 3 hours and expose them in the ADR solution.

(5) Statistics

The statistic results are based on ANOVA and Tukey-Kramer Multiple Comparison Test.

III. Results

1. Toxic Consequence of Adriamycin (ADR)

1) Cell Viability Analysis

(1) MTT Assay

For the effect of ADR, cultured myocardial cells are treated with various concentrations (1 g/ml to 45 g/ml) of ADR for 36 hours. ADR-induced cardiotoxicity is measured by MTT assay in cultured myocardial cells. Cell viability is decreasing, as the ADR concentration is getting bigger. Especially, when cultured myocardial cells are exposed to 30 g/ml ($p<0.05$) and 45 g/ml ($p<0.01$) ADR, the decreasing rates are similar compare to other results (Table 1, Fig. 1).

For the time-response relationship of ADR, cultured myocardial cells are treated with 30 g/ml ADR, the value of MCV, for 9 to 72 hours. ADR-induced cardiotoxicity is measured by MTT assay in cultured myocardial cells. As time goes cell viability are decreasing; in particular, when the cultured cells are exposed for 36 hours ($p<0.05$) and 72 hours ($p<0.01$), the decreasing rates are similar compare to other time intervals (Table 2, Fig. 2).

Table 1. Absorbance (% of control) at 590nm Wavelength for the MTT assay on ADR in Cultured Myocardial Cells

ADR(㎍/㎖)	MTT absorbance(590nm)	Decrease rate of cell viability(%)
0	1.75±0.18	-
1	1.44±0.16	17.3
15	1.29±0.09	26.3
30	0.92±0.08*	47.3
45	0.57±0.04**	67.4

Cultured myocardial cells were treated with various concentrations of ADR for 36 hours. The values are the mean±SE for 6 experiments. Significant differences from the control are marked with asterisks. *$p<0.05$; **$p<0.01$

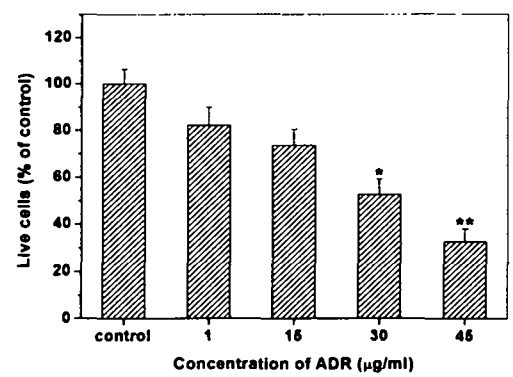

Fig. 1. Dose-dependency of ADR.

ADR-induced cardiotoxicity was measured by MTT assay in cultured myocardial cells. Cultures were exposed to 1, 15, 30 and 45 ㎍/㎖ ADR for 36 hours, respectively. Other legends are the same as table 1. *$p<0.05$; **$p<0.01$

Table 2. Time-response Relationship of ADR by MTT assay in Cultured Myocardial Cells

ADR (μg/mℓ)	MTT absorbance(590nm)				
	0 hr	9 hr	18 hr	36 hr	72 hr
0	1.47± 0.19	1.46± 0.16	1.43± 0.18	1.36± 0.17	1.32± 0.15
30	1.42± 0.15	1.14± 0.12	1.04± 0.08	0.70± 0.05*	0.31± 0.02**

Cultured myocardial cells were treated with 30 μg/mℓ ADR for various time intervals. The values are the mean±SE for 6 experiments. Significant differences between groups are marked with asterisks. *p<0.05; **p<0.01

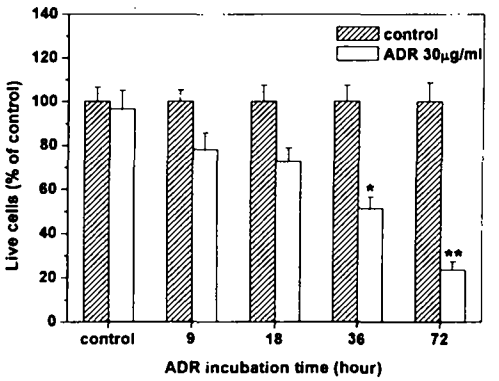

Fig. 2. Time-dependancy of ADR in cultured myocardial cells.

Cultures were exposed to 30 μg/mℓ ADR for 9, 18, 36 and 72 hours, respectively. Other legends are the same as table 2. *<0.05; **p<0.01

(2) NR Assay

While the myocardial cells are cultured, rinse them with Ca, Mg free Hank's Balanced Salt Solution (HBSS, Gibco) 3 times. Cultured myocardial cells are grown in media containing various concentrations of ADR for 36 hours. Decreasing cell viability is captured as ADR concentration was increasing. Specially, cultured cells that are exposed to 30 g/ml and 45 g/ml are showed 50.3% ($p<0.05$) and 28.1%m ($p<0.01$) of similar decrease (Table 3, Fig. 3).

To study the time-response relationship of ADR, cultured myocardial cells are incubated with 30 g/ml ADR for 9 to 72 hours. It shows the decreasing rate of cell viability response to various time intervals; particularly, ADR incubation time of 36 and 72 hours demonstrates the similar decreasing rates (Table 4, Fig. 4).

Table 3. Absorbance (% of control) at 540nm Wavelength for the NR assay on ADR in Myocardial Cells

ADR(μg/mℓ)	NR absorbance(540nm)	Decrease rate of cell viability(%)
0	1.53±0.16	-
1	1.21±0.15	20.9
15	1.15±0.12	24.8
30	0.77±0.07*	49.7
45	0.43±0.04**	71.9

Cultured myocardial cells were grown in media containig various concentrations of ADR for 36 hours. The values represent the mean ±SE for 6 experiments. Significant differences from the control are marked with asterisks. *p<0.05; **p<0.01

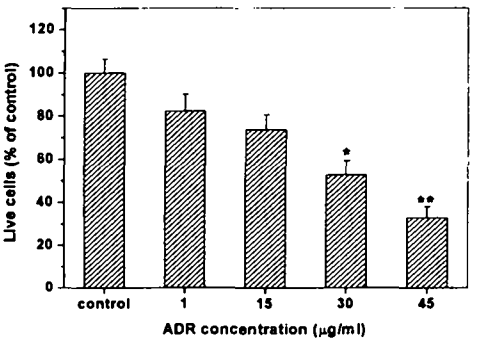

Fig. 3. Dose-response relationship of ADR in cultured myocardial cells.

Cytotoxicity was measured by NR assay. Cultures were exposed to 0(control), 1, 15, 30 and 45 μg/mℓ ADR for 36 hours, respectively. Other legends are the same as table 3. *p<0.05; **p<0.01

Table 4. Time-response Relationship of ADR by NR assay in Cultured Myocardial Cells

ADR(μg/mℓ)	NR absorbance(540nm)				
	0 hr	9 hr	18 hr	36 hr	72 hr
0	1.38± 0.14	1.34± 0.11	1.31± 0.13	1.27± 0.15	1.25± 0.12
30	1.29± 0.15	0.97± 0.08	0.89± 0.07	0.63± 0.06*	0.52± 0.02**

Cultured myocardial cells were incubated with 30 μg/mℓ ADR for various time intervals. The values represent the mean±SE for 6 experiments. Significant differences between groups the control are marked with asterisks. *p<0.05; **p<0.01

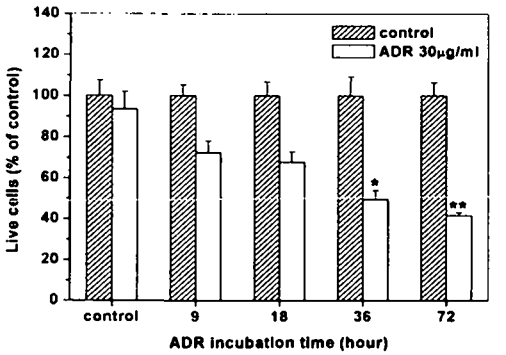

Fig. 4. Time-dependancy of ADR in cultured myocardial cells.

Cultures were exposed to 30 μg/mℓ ADR for 9, 18, 36 and 72 hours, respectively. Cell viability was measured by NR assay. Other legends are the same as table 4. *p<0.05; **p<0.01

2. Effect of CYT Water Extract

1) LDH Activity

(1) ADR-induced Cardiotoxicity

To measure the LDH Activity in various concentration of ADR cultured mouse myocardial cells are exposed to various concentrations of ADR for 36 hours. As a result, the concentration of ADR and LDH activity are increased proportionally and indicated the cardiotoxicity; specifically, when the cultured cells are compared to none ADR-treated cell, they showed a similar increasing rate in 35 and 50 g/ml. The value of MCV (midcytotoxicity) presented during 35 g/ml ADR process (Table 5, Fig. 5).

Table 5. Dose-response Relationship of ADR on LDH activity in Cultured Mouse Myocardial Cells

ADR(μg/mℓ)	control	5	20	35	50
Amount of LDH Release	12.8± 1.2	16.8± 1.5	18.3± 2.1	20.6± 2.8*	24.7± 3.6**

Cultured mouse myocardiac cells were exposed to various concentrations of ADR for 36 hours. LDH release was measured at wavelength of 540nm. The values are the mean±SE for 5 experiments. Significant differences from the control are marked with asterisks. *p<0.05; **p<0.01

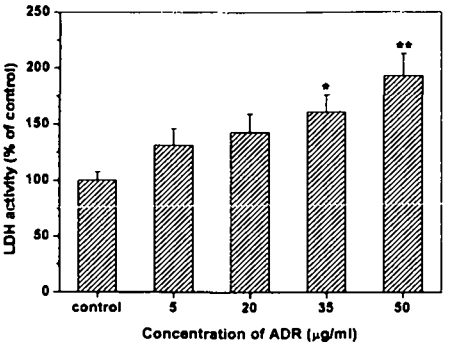

Fig. 5. Dose-response relationship of ADR on LDH activity in cultured mouse myocardiac cells. Other legends are the same as table 5. *p<0.05, **p<0.01.

(2) The Protection of CYT

To report the relationship between the ADR-induced cardiotoxicity and the effect of CYT Water Extract respect to the LDH Activity, cultured mouse myocardial cells were preincubated with various concentrations of CYT water extract for 3 hours, and then exposed to 35 g/ml ADR for 36 hours.

As a result, the cell that only treated with CYT Water Extract showed nontoxic compare to the cell that treated in ADR. In the case of preincubated CYT Water Extract, the concentration of ADR and LDH Activity rates were diminishing proportionally. Statically, the cell that treated in 40 g/ml ($p<0.05$), 60 g/ml ($p<0.01$), 80 g/ml ($p<0.01$) CYT Water Extract revealed similar diminishing effect compare to the cell that only treated in ADR (Table 6, Fig. 6).

Table 6. Dose-response Relationship of CYT Water Extract for LDH activity in Cultured Mouse Myocardial Cells

ADR(μg/mℓ)	Amount of LDH Release				
	Concentration of CYT(μg/mℓ)				
	0 μg/mℓ	20 μg/mℓ	40 μg/mℓ	60 μg/mℓ	80 μg/mℓ
0	10.6± 1.0	10.4± 0.8	10.1± 0.6	9.6± 0.5	9.3± 0.7
35	16.6± 0.8	6.1± 0.7	5.1± 0.5*	4.4± 0.3**	3.3± 0.4**

Cultured mouse myocardial cells were preincubated with various concentrations of CYT water extract for 3 hours, and then exposed to 35 μg/mℓ ADR for 36 hours. LDH release was measured at wavelength of 540nm. The values represent the mean±SE for 5 experiments. Significant differences from the ADR-treated group are marked with asterisks. *<0.05; **p<0.01

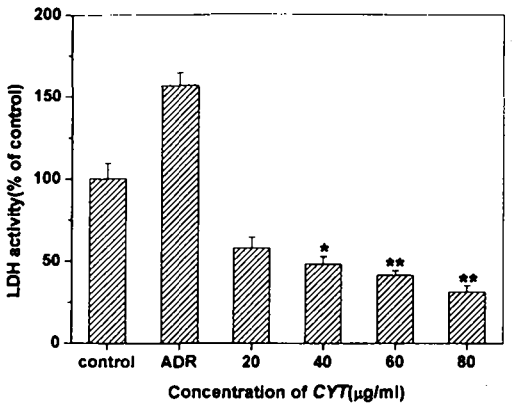

Fig. 6. Dose-response relationship of CYT water extract for LDH activity in cultured mouse myocardial cells.

Significant difference from the ADR-treated group are marked with asterisks. Other legends are the same as table 6. *<0.05; **p<0.01

2) Heart Beating Rate

(1) ADR-induced Cardiotoxicity

To determine heart-beating rate, cultured mouse myocardial cells were treated with various concentrations of ADR for 36 hours. As for the result, the beating rate was decreasing at 1 g/ml and 10 g/ml; however, they did not show the statistical similarity. In the process of 30 g/ml and 50 g/ml ADR they indicated the similar decreasing rate of 47.5% ($p<0.05$) and 26.3% ($p<0.01$). The value of MCV (midcytotoxicity) presented during 35 g/ml ADR process (Table 7, Fig. 7).

Table 7. Dose-response Relationship of ADR on BR in Cultured Mouse Myocardiac Cells

ADR(μg/mℓ)	Beating rate (Number/min)	Decrease of Beating rate (% of control)
0	118±15	-
1	99±8	16.1
10	89±5	24.6
30	56±3*	52.5
50	31±6**	73.7

Cultured mouse myocardial cells were treated with various concentrations of ADR for 36 hours. BR was measured by count of beating number per minute, compared with control. The values are the mean±SE for 5 experiments. Significant differences from the control are marked with asterisks. *p<0.05; **p<0.01

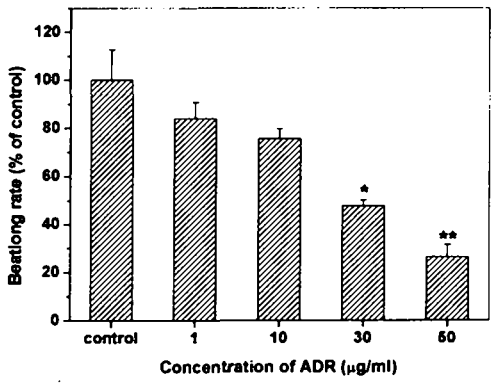

Fig. 7. Dose-response relationship of ADR on beating rate in cultured mouse myocardiac cells.

Other legends are the same as table 7. *p<0.05, **p<0.01.

(2) The Protection of CYT

To report the relationship between heart beating rate of ADR and the effect of CYT Water Extract respect to the beating rate, cultured mouse myocardial cells were preincubated with the concentrations of 40 to 100 g/ml of CYT water extract for 3 hours, and then exposed to 30 g/ml ADR for 36 hours.

As a result, the cell that only treated with CYT Water Extract did not showed significant difference compare to the cell that treated in ADR. In case of preincubated CYT Water Extract, the concentration of ADR and the beating rate are escalating proportionally. Statically, the cell that treated in 60 g/ml ($p<0.05$) and 80 g/ml ($p<0.01$) CYT Water Extract reveals similar escalating effect compare to the cell that only treated in ADR (Table 8, Fig. 8).

Table 8. Dose-response Relationship of CYT Water Extract for BR in Cultured Mouse Myocardial Cells

ADR (μg/mℓ)	Beating rate (Number/min)				
	Concentration of CYT(μg/mℓ)				
	0 μg/mℓ	40 μg/mℓ	60 μg/mℓ	80 μg/mℓ	100 μg/mℓ
0	120±11	122±14	121±13	123±12	124±15
30	56±4	76±6	84±8	101±10*	116±12**

Cultured mouse myocardial cells were preincubated with various concentrations of CYT water extract for 3 hours, and then exposed to 30 μg/mℓ ADR for 36 hours. BR was measured by count of beating number per minute. The values represent the mean±SE for 5 experiments. Significant differences from the ADR-treated group are marked with asterisks. *p<0.05, **p<0.01

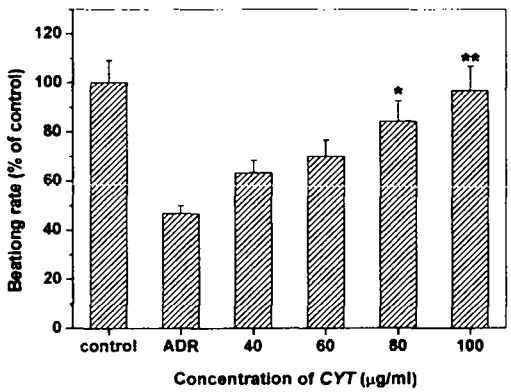

Fig. 8. Dose-response relationship of CYT water extract for BR in cultured mouse myocardial cells.

Significant difference from the ADR-treated group are marked with asterisks. Other legends are the same as table 8. *$p<0.05$, **$p<0.01$

Ⅳ. Further Research

According to [Dongyi-soose-bowon] CYT was known as a remedy for interior-overheated-disease of taeumin. However, the book did not disclose the prescription or symptoms. One of the recipes for the CYT is semen nelumbinis which is not a regular formula for taumin and uses for palpitation, fidgetiness and insomnia, involuntary ejaculation, leucorrhea.[13] In addition, eight other recipes of CYT such as radix ophiopogonis, rhizoma acori graminei, radix scutellariae, semen zizyphi spinosae, semen biotae, arillus longan and radix polygalae are used as a treatment for various heart diseases.

While many other studies are reported in heart diseases fields, Kim declared the relationship between the myocardial blood and CYT.[2] He stated that the benefit of CYT as the thrombocyte growth, the amount of fibrinogen increase, and the reduction of prothrombin time.

Therefore this paper considered different point of view from other dissertations. In this paper the myocardial cell was treated in the CYT Water Extract, then examined the protection of CYT Water Extract against the damaged myocardial cell.

The toxic induced objects in the myocardial cells are a heavy metal, ADR, dichloromethane and a chemical.[10, 17] ADR is the one of anthracycline and obtaind from the streptomyces peucetius var. caesius. It has a same toxic influence in the myocardial cell such as dichloromethane and causes the heart failure and the myocardial infraction.[18-21]

As the know-how in the cultivation fields are established in many areas, numerous destructive or nontoxic objects are discovered and explored.[22-25] Also, many studies are examined using the myocardial cell as a model of various diseases to search for mechanism, process, method of treatment.[10,26,27]

Therefore this paper analyzed the CYT Water Extract against the ADR damaged myocardial cell.

First of all, it exposed the cultured mouse myocardial cell to various time and concentration of ADR. Cell viability was measured by MTT assay and NR assay. As a result, cell viability was decreasing as the concentration of ADR and ADR incubation times were increasing (Table 1-4, Fig. 1-4). This result proved that ADR induced the cardiotoxicity that reported in Chung and others.

To explore the prevention of CYT in ADR-induced cardiotoxicity, cultured myocardial cells were preincubated with the concentrations of 0 g/ml, 20 g/ml, 40 g/ml, 60 g/ml, and 80 g/ml of CYT water extract for 3 hours, and then exposed to ADR for 36 hours. From the result, LDH activity and heart beating rate were determined.

LDH activity experiment indicated the proportional relationship between the concentration of ADR and cardiotoxicity. Among the results, ADR concentration of 35 g/ml and 50 g/ml increased in similar rate compare to none ADR treated cell. The value of MCV presented during 35 g/ml ADR process (Table 5, Fig. 5). For heart beating rate when the concentration of ADR increased, the heart beating rate decreased. Statistical similarities in ADR concentration were 30 g/ml and 50 g/ml, respectively. The value of MCV presented during 35 g/ml ADR process (Table 7, Fig. 7). The conclusion

corresponded with Keeper's study28) that stated ADR treated cell restrained the cell pulse beat and caused the exposure of LDH.

Cultured myocardial cells were preincubated with various concentrations of CYT water extract; the result was LDH activity diminishing; specifically, the cell that treated in 60 g/ml and 80 g/ml CYT reveals similar decreasing effect compare to the cell that only treated in ADR (Table 6, Fig. 6). In addition, heart-beating rate that exposed to ADR was increasing as the concentration was increasing; statistically 60 g/ml and 80 g/ml showed similar increasing effect (Table 8, Fig. 8).

The conclusion demonstrates CYT Water Extract has the preventive effect of ADR-induced cardiotoxicity caused by LDH activity and heart beating. Based on empirical study in this paper, it is clear that the CYT Water Extract is effective remedy for the heart disease such as fright and palpitation.

V. Conclusion

To clarify the mechanism of myocardial damage against adriamycin(ADR)-induced myocardiotoxicity, cytotoxicity of ADR was examined by MTT assay, NR assay, LDH activity, and heart beating rate. The cardioprotective effect of CYT water extract in cultured myocardial cells were investigated with LDH activity assay and heart beating rate.

The results of these experiments were obtained as follows

1. ADR induced the decrease of viability, the increase of LDH activity, and the decrease of heart beating rate in cultured myocardial cells.
2. CYT water extract has the significant effect of decreasing LDH activity increased by ADR in cultured myocardial cells.
3. CYT water extract has the significant effect of increasing heart beating rate decreased by ADR in cultured myocardial cells.

From the results, it is suggested that ADR shows toxic effect in cultured myocardial cells derived from neonatal mouse and CYT water extract are very effective in the prevention of ADR-induced cardiotoxicity.

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