

REDUCING TOXICITY AND INCREASING EFFICIENCY: ACONITINE WITH LIQUIRITIN AND
GLYCYRRHETINIC ACID REGULATE CALCIUM REGULATORY PROTEINS IN RAT
MYOCARDIAL CELL

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Abstract

Background: Compatibility of *Radix Aconiti Carmichaeli* and *Liquorice* is known to treat heart diseases such as heart failure and cardiac arrhythmias. This work answers the question that whether the active components (Aconitine, Liquiritin and Glycyrrhetic Acid) of *Radix Aconiti Carmichaeli* and *Liquorice* could result in regulating intracellular calcium homeostasis and calcium cycling, and thereby verifies the therapeutic material basis.

Materials and Methods: The myocardial cells were divided into twelve groups randomly as control group, Aconitine group, nine different dose groups that orthogonal combined with Aconitine, Liquiritin and Glycyrrhetic Acid, and Verapamil group. The myocardial cellular survival rate and morphology were assessed. The expression of calcium regulation protein (RyR2, NCX1, DHPR- α 1) in the myocardial cell by Western-blotting.

Results: The results exhibited that Aconitine (120 μ M) significantly damaged on myocardial cell, decreased the survival rate and expression of Na⁺/Ca²⁺ exchangers (NCX1) and dihydropyridine reducta- α 1 (DHPR- α 1), and increased the expression of ryanodine receptor type2 (RyR2) obviously. The compatibility groups (Aconitine, Liquiritin and Glycyrrhetic Acid) all could against the damage on the myocardial cell by Aconitine at different levels.

Conclusion: Aconitine with Liquiritin and Glycyrrhetic Acid may regulate the expression of calcium-regulated proteins to protect myocardial cells from damage.

Keywords: Aconitine, Liquiritin, Glycyrrhetic Acid, myocardial cell, calcium regulatory

Introduction

Radix aconiti carmichaeli, the sub-root of *Aconitum carmichaeli*. debx, with widely clinical application in yang depletion syndrome and yang deficiency syndromes, is the preferred drug when rescuing someone to prevent the likely collapse of yang. *Radix aconiti carmichaeli* has positive properties of cardiogenic, antinociceptive and anti-inflammatory (Lai et al., 2011; Wang et al., 2014) and it is also a well-known Chinese medicinal herb used to treat chronic heart failure. Aconitine is the main component of *Radix aconiti carmichaeli*, which is responsible for the pharmacological activity. Aconitine, however, has high cardiotoxicity, which could result in severe arrhythmias even death (Sujata et al, 2015; Li et al, 2010; Lin et al, 2011; He et al, 2013). Meanwhile, the previous studies demonstrate that its effective dose is close to the toxic dose (Zhang et al, 2012), and its dose range should be carefully controlled. All these reasons limit its extensively clinical application.

Liquiritin and Glycyrrhetic Acid are the major effective components of *Liquorice*, which is the root of

Glycyrrhiza uralensis Fisch., *Glycyrrhiza inflata* Bat. or *Glycyrrhiza glabra* L. Based on the prescription features of Traditional Chinese Medicine (TCM), the compatibility of *Radix aconiti carmichaeli* with *Liquorice*, and Aconitine with Liquiritin and Glycyrrhetic Acid plays the major role in the effect-enhancing and toxicity-reducing (Karoline et al, 2013). As their active ingredients, Aconitine with Liquiritin and Glycyrrhetic Acid are the pivotal studying targets undoubtedly. Nevertheless, the mechanism behind these effects is absent.

The process of excitation-contraction coupling of heart is that the myocardial cells are excited by electrical stimulation and are contracted by intracellular Ca^{2+} transportation. Ca^{2+} is the direct activator of contraction of myocardial cells as the second messenger, and plays the key role in electrical activity of myocardial cells. Notably, it is proved that calcium-regulated proteins play an important part in maintaining the cell calcium homeostasis and calcium cycle (Guo et al, 2008). Recently, there are some emerging studies about the effect of calcium regulation of proteins in myocardial cells on heart failure and arrhythmia (Bers, 2002). Moreover, preliminary studies have showed that arrhythmogenesis toxicity of Aconitine was closely linked to Ca^{2+} signals (Zhou et al, 2013; Zhang et al, 2007).

In this study, we select Aconitine, Liquiritin and Glycyrrhetic Acid, which are the main active ingredients of *Radix aconiti carmichaeli* and *liquorice*, respectively. Aconitine with the certain dose causes damage on myocardial cells significantly, while the compatibility of Aconitine with Liquiritin and Glycyrrhetic Acid on calcium-regulated proteins of the passage of rat myocardial cell at the molecular level indicates that it could regulate the expression of calcium-regulated proteins to inhibit the cytotoxicity of myocardial induced by Aconitine, enhance the calcium regulatory adaption of myocardial cell, and maintain the function of heart diastole and systole. In the course of the experiment, various doses were designed by the method of orthogonal testing, and the best combination is conducted for the guidance of reasonable and safe clinical prescription in vitro.

Materials and Methods

Drugs and Reagents

Aconitine, Tianjin ChemStandard Biotechnology Company Ltd, purity $\geq 98\%$, Lot Number: PCM-WT-001-20111220; Liquiritin, Tianjin ChemStandard Biotechnology Company Ltd, purity $\geq 98\%$, Lot Number: PCM-GC-008-20110809; Glycyrrhetic Acid, Tianjin ChemStandard Biotechnology Company Ltd, purity $\geq 98\%$, Lot Number: PCM-GC-002-20110529; Verapamil injection, Shanghai Harvest pharmaceutical Co., Ltd, Lot Number: 101201.

MTT, USA Biosharp company, purity $\geq 98\%$, Imported Fetal Bovine Serum (FBS), Shanghai Pufei Biotech Co., Ltd, Lot Number: 11A0801A; DMSO, USA Amresco company, Lot Number: 3607B037; 0.25%pancreatin +0.02%EDTA, Hangzhou Jinuo bio-medical technology Ltd, Lot Number: 11112901; DMEM medium with high glucose, Hangzhou Jinuo bio-medical technology Ltd, Lot Number: 12050402; Cell culture dish, 96-well plates, 6-well plates, Nunc company; BCA Protein Quantitation Kit, Beijing Dingguo Changsheng Biotechnology Co., Ltd; First Antibody, RYR2, molecular weight: 240-250kDa, Type: Rabbit pAb, Item No: 19765-1-AP, PTG; First Antibody, NCX1, molecular weight: 120kDa, Type: Mouse mAb, Item No: ab6495, Abcam; First Antibody, DHPR alpha 1, molecular weight: 240kDa, Type: Mouse mAb, Item No: ab84814, Abcam; Second Antibody, Goat Anti-Rabbit, Item No: SA00001-2, PTG.

Cell line

The rat myocardial H9c2(2-1) cell lines of the 18th generations (Shanghai Institutes for BiologicalSciences cell bank, CAS).

Instrument

Nikon eclipse TE2000-S Inverted Fluorescence Microscope, Japan's Nikon; SANYO CO₂ cell incubator, Japan's Sanyo; LDZ5-2 Low Speed Autobalancing Centrifuge, Beijing Jingli centrifuge Co., Ltd; Model-680 Enzyme-linked Immunometric meter, Bio-Rad Laboratories Ltd, TU-1900 Double Beam UV-visible light Spectrophotometer, Beijing Persee general instrument Co., Ltd; Clean bench, Suzhou purification Antai Co.,Ltd.

Experimental Drug

Dissolved Aconitine, Liquiritin, Glycyrrhetic Acid and Verapamil 10mg separately on the basis of molecular weight in serum-free DMEM medium with a small amount of DMSO (the concentration is below 0.5%) to connected into the 1mL stock solution, which stored in the condition of -20°C after filtration for getting rid of bacterium.

Cytotoxicity Test of the Myocardial Cells

To detect and select cells viability of gradient concentration of Aconitine, Liquiritin, Glycyrrhetic Acid and Verapamil through cytotoxicity test by MTT.

We set the series of gradient concentration of Aconitine, Liquiritin, Glycyrrhetic Acid and Verapamil: 5, 15, 30, 60, 120, 240, 480, 720, 960 μM. Each concentration was repeated three times.

Subculture the myocardial cells with trypsin digestion, and seed in 96- well plates at the density of 1×10⁵/mL. After 48 h, discard the culture solution and rinse twice with PBS solution. Add serum-free DMEM medium into 96- well plates, and culture for 12 h. Then discard the solution to 96- well plates. Add different concentration drugs solution and culture for 4 h.

Set the well with equal volume of medium, MTT and DMSO as the zero well group, the well with cells, the same concentration of drug dissolution medium, culture medium, MTT and DMSO as the control group, and set five same wells for each group. Discard the supernate of cultured myocardial cells that affected by the drugs for 4 h, then culture with adding 180μL fresh DMEM medium and 20 μL MTT (5 g/L) for each well. Discard the supernate after 4 h, add 150 μL DMSO per well, oscillate 15 min at the condition of 37°C until the sediment was totally dissolved. Then test OD values at 490nm wavelength by Elisa and calculate cell survival rates.

$$\text{Cell survival rate\%} = (\text{OD}_{\text{drugs group}} - \text{OD}_{\text{zero group}}) / (\text{OD}_{\text{control group}} - \text{OD}_{\text{zero group}}) \times 100\%$$

Experiments and Treatment Groups Designed by Orthogonal Experimental Design

Orthogonal experimental design was adopted in the study. Orthogonal experimental design is a reliable method used for studying multiple factors and multiple levels. It can select a representative point from the comprehensive test according to the orthogonality-arrangement test by taking full advantage of the standardization orthogonal table. It is a scientific design method to reduce the number of tests, shorten the test cycle, and quickly find the optimal solution, which actually reduces the amount of works (Xu et al, 2002). Orthogonal design method is widely used in various fields on behalf of high efficiency, rapid and economical experimental design methods (Allison et al, 2016; Raquel et al, 2012; Guo et al, 2012; Wang et al, 2011).

Based on the results of MTT, cells were divided into twelve groups randomly as follows: control group, Aconitine group, nine combination groups of Aconitine with Liquiritin and Glycyrrhetic Acid using orthogonal table L₉(3⁴), and Verapamil group. Each group was repeated six times.

Major treatment methods: (1) control group: added the equal volume of DMEM solution; (2) Aconitine group: the concentration of Aconitine was drawn from the cell survival rate test; (3)-(11) orthogonal combination groups: the concentration of Aconitine, Liquiritin and Glycyrrhetic Acid was drawn from the cell survival rate test; (12) Verapamil group: (the concentration of Verapamil was drawn from the cell survival rate test).

Morphological Changes of Myocardial Cells

Observe the growth conditions and morphological characteristics of each group myocardial cells with invert microscope at 4 h separately. Compare and analyze pictures of each group cells when drugs have worked 4 h.

Cells viability of 12 groups

We determined the cells viability of control group, Aconitine group, nine orthogonal combination groups and Verapamil group, after 4h on the myocardial cells by MTT. The methods and steps of determination of cell survival rate were the same to the previous experiment. Each group was repeated six times.

Expression of Calcium-regulated Proteins (NCX1, RyR2, DHPR-a1) with Western Blotting

We seeded the subcultured myocardial cells into 6- well plates at the density of 1×10^6 /mL, cultured the cells with drugs for 4 h and collected the cells by the centrifugation method. Lysed cells with RIPA lysis solution, and detected the protein concentrations by LOWRY method, detected RYR2, NCX1, DHPR-a1 by Western blotting method. Protein samples were run SDS-PAGE gel electrophoresis, and then printed on the PVDF membrane. Blocked the membrane for 1 h by using TBST buffer solution containing 5% nonfat dry milk, incubated with the first antibody for the night, rinsed with TBST for four times, added goat anti-rabbit antibody and worked for 30 min at room temperature, colored and exposed by ECL chromogenic reagent kit. The protein signal band images were obtained from the film though Tanon 1600 gel image scanner, analyzed the signal band though Tanon Gis image analysis software, and calculated the changes of each protein with β -Actin as internal inference. Each group was repeated six times.

Statistical analysis

Data were expressed as $\bar{x} \pm s$, and analyzed with SPSS17.0 statistic software. One-way ANOVA analysis was used to compare between these groups. The significant differences were shown as $P < 0.05$, and the extremely significant differences were shown as $P < 0.01$.

Results

Selective Drug Action Concentration by Cytotoxicity Test of the Myocardial Cells

After 4h on the myocardial cells with the individually increase of concentration of Aconitine, Liquiritin, Glycyrrhetic Acid and Verapamil, the myocardial cell survival rates gradually decreased, which showed that this process is dose-dependent. This decline was not obvious at the beginning, but significant when the concentration is more than 200 μ M. (**Fig. 1**).

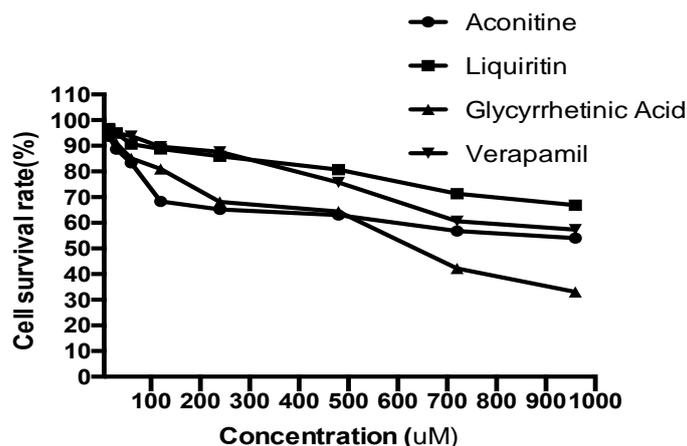


Figure 1: Myocardial cell viability is detected by MTT with gradient concentrations of Aconitine, Liquiritin, Glycyrrhetic Acid and Verapamil (5, 15, 30, 60, 120, 240, 480, 720, 960 µM). Dynamic curve is drawn with concentrations (µM) as the horizontal coordinate and Myocardial cell survival rate (%) as the longitudinal coordinate. With n=3.

We select the concentration of Aconitine, Liquiritin, Glycyrrhetic Acid and Verapamil based the damage on cultured myocardial cells, as the highest concentration used for experimental grouping by orthogonal experimental design. We select the concentration of Aconitine as 120 µM, with which myocardial cell survival rate is relatively low (68.37±12.71%). Other groups choose the highest concentration of survival rate at about 85%: Liquiritin is 240 µM (cell survival rate is 85.97±11.02%), Glycyrrhetic Acid is 60 µM (cell survival rate is 85.13±16.05%), and Verapamil is 240 µM (cell survival rate is 87.69±14.57%).

Experiments and Treatment Groups Designed by Orthogonal Experimental Design

(3)- (11) orthogonal combination groups: the concentration of Aconitine, Liquiritin and Glycyrrhetic Acid was drawn from the cell survival rate test (Table1-2).

Table 1: Compatibility of main active substances with different doses (µM)

| Levels | Factors | | | |
|--------|----------------|-----------------|------------------------|-----------------|
| | A Aconitine | B Liquiritin | C Glycyrrhetic Acid | D Error Term |
| 1 | 30 | 60 | 15 | 1 |
| 2 | 60 | 120 | 30 | 2 |
| 3 | 120 | 240 | 60 | 3 |

Table 2: Nine combination experiment groups designed by orthogonal design using table L₉(3⁴)

| Group Number | Factors/dose (uM) | | | |
|---------------|-------------------|-----------------|------------------------|-----------------|
| | A Aconitine | B Liquiritin | C Glycyrrhetic Acid | D Error Term |
| Combination 1 | 30 | 60 | 15 | 1 |
| Combination 2 | 30 | 120 | 30 | 2 |
| Combination 3 | 30 | 240 | 60 | 3 |
| Combination 4 | 60 | 60 | 30 | 3 |
| Combination 5 | 60 | 120 | 360 | 1 |
| Combination 6 | 60 | 240 | 15 | 2 |
| Combination 7 | 120 | 60 | 60 | 2 |
| Combination 8 | 120 | 120 | 15 | 3 |
| Combination 9 | 120 | 240 | 30 | 1 |

Combination of Aconitine with Liquiritin and Glycyrrhetic Acid Prevents Myocardial Cells from Damage on Account of Toxicity of Aconitine

In the visual field of inverted microscope, cells had spindle shape, adhering to the wall, and had regular shape, beating strongly and rhythmically with the frequency of 50~70 times/min. After affected 4 h by Aconitine, myocardial cells pseudopodia became shorted, turned round, and there were vacuolation and granule degeneration in a few cells. Cells spontaneous rhythmic beats decreased, had irregular rhythm, and irregular fibrillation and shrinkage, even death of advanced cells.

Compared with the Aconitine group, the cells in the groups which affected by nine combination groups for 4 h had improvement in the aspects of morphology and shrinkage, such as the shrinkage of pseudopodia, cytoplasm cavitation and granule matters were decreased. The improvement in the combination group 5 (which with 60uM Aconitine, 60uM Liquiritin and 60uM Glycyrrhetic Acid) was the most obviously. (Fig. 2)

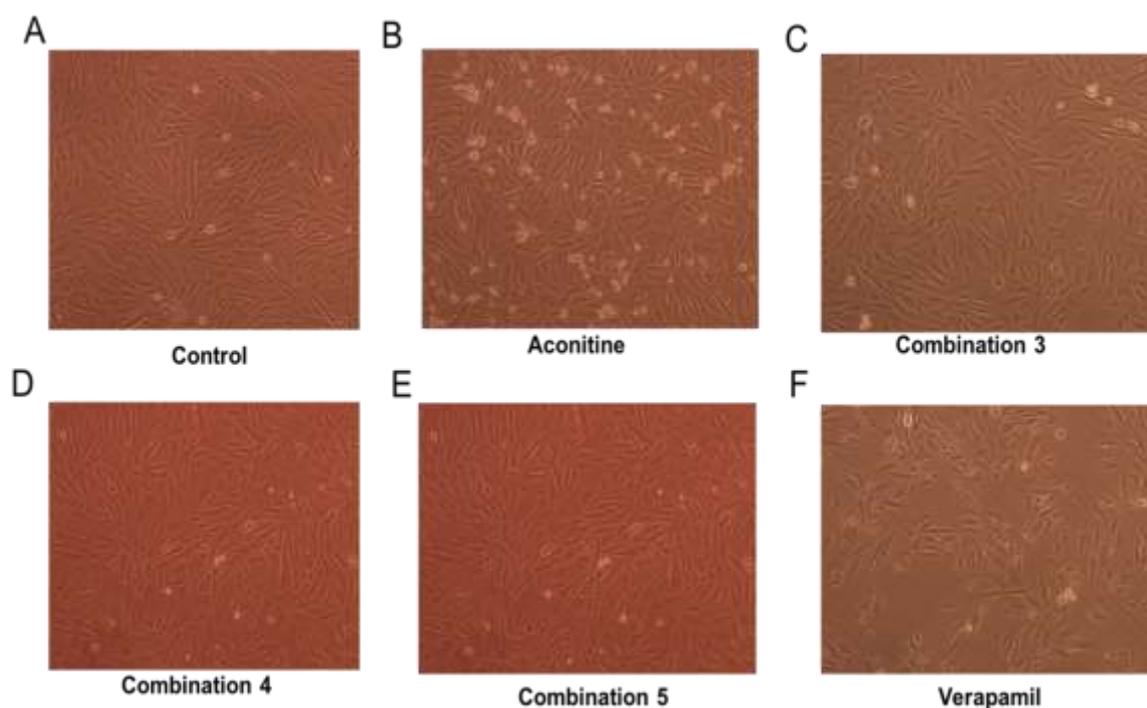


Figure 2: The growth conditions and morphological characteristics of each group myocardial cells were observed by invert microscope at 4 h separately. Morphological changes of myocardial cells ($\times 100$): A. Control group; B. Aconitine group; C. Combination 3; D. Combination 4; E. Combination 5; F. Verapamil group.

Combination of Aconitine with Liquiritin and Glycyrrhetic Acid Improve Cells Viability

The survival rate of myocardial cells in Aconitine group at the concentration of 120uM was decreased obviously ($P < 0.01$), while nine combination groups improved it with different degrees (extremely significant difference with $P < 0.01$, significant difference with $P < 0.05$). (Fig. 3)

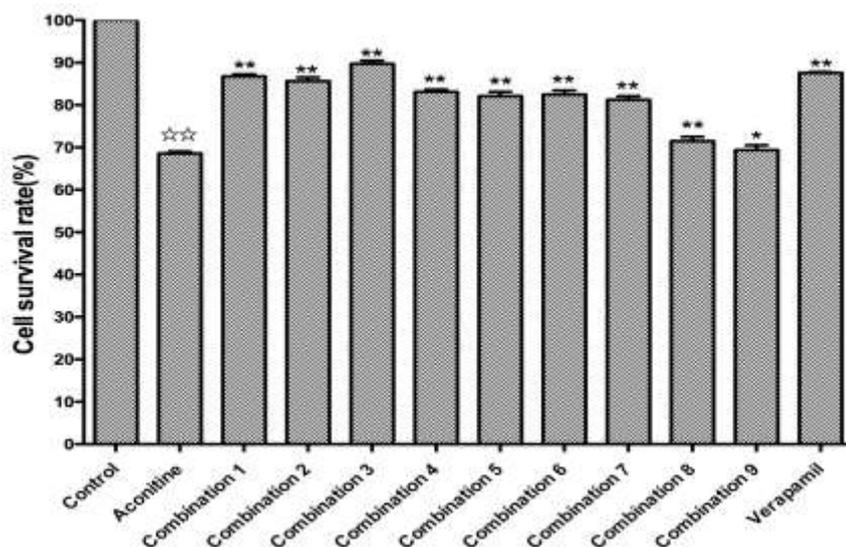
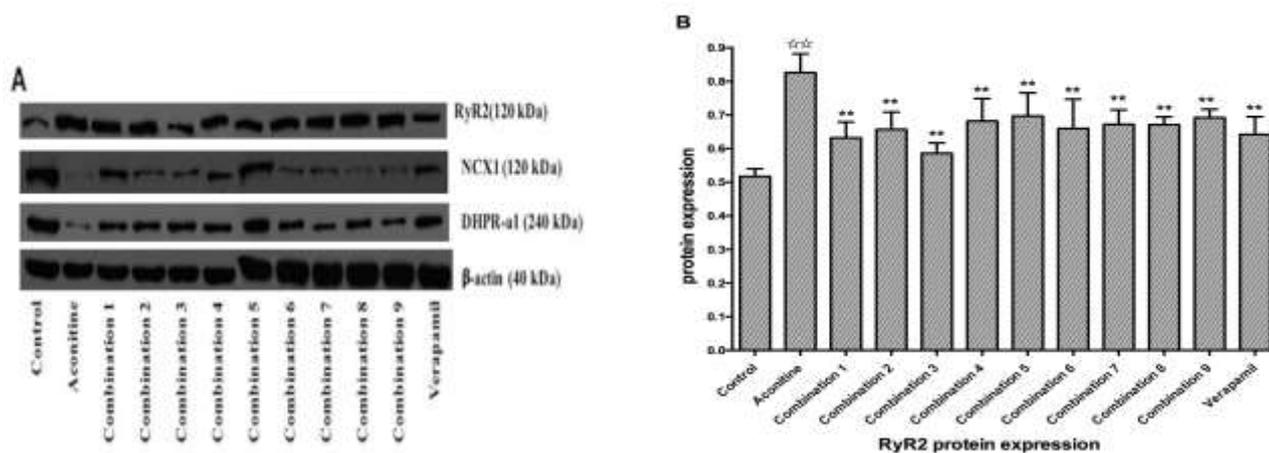


Figure 3: The survival rate of myocardial cells in combination of Aconitine with Liquiritin and Glycyrrhetic Acid. Data were expressed as mean+s.e.m., ** $P < 0.01$ vs control group; * $P < 0.05$, ** $P < 0.01$ vs Aconitine group. $n = 6$.

Combination of Aconitine with Liquiritin and Glycyrrhetic Acid Regulate Calcium Regulatory Proteins (RyR2, NCX1, DHPR-a1)

The expression level of RyR2 protein was obviously higher, while the expression levels of NCX1 and DHPR-a1 were significantly lower in Aconitine group compared with the control group. Compared with Aconitine group, RyR2 proteins of Combination 1-9 were all decreased significantly, while NCX1 and DHPR-a1 proteins were increased obviously ($P < 0.01$). Different combinations of Aconitine with Liquiritin and Glycyrrhetic Acid had different effects, particularly that the expression level of RyR2 protein in Combination 3 group was lowest, and the expression levels of NCX1 and DHPR-a1 protein in Combination 5 group were highest. (Fig.4 A, B, C, D).



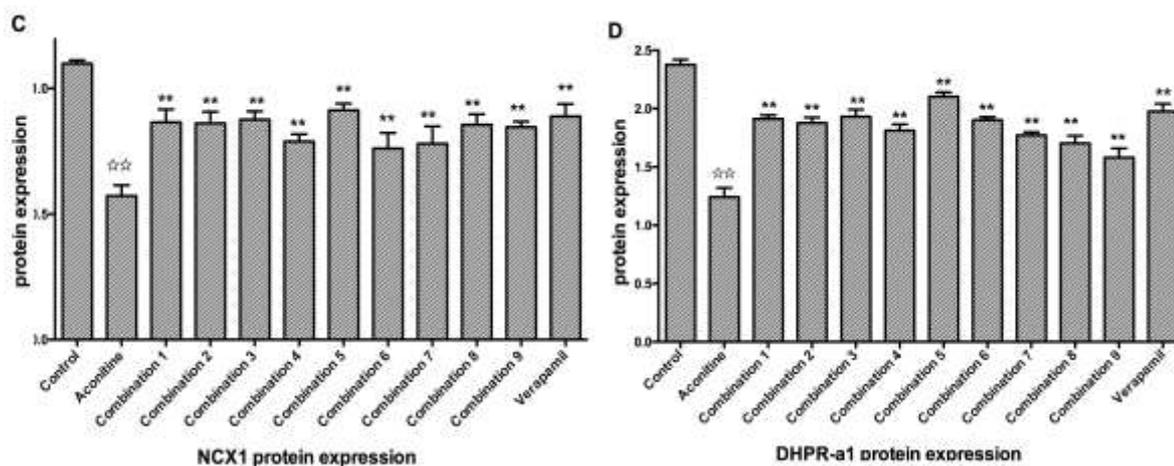


Figure 4: The expression of calcium regulated proteins (NCX1, RyR2, DHPR-a1) with western blotting. There were 12 groups: 1: Control group; 2: Aconitine group; 3-11: Combination 1-9 (compatibility groups of Aconitine, Liquiritin and Glycyrrhetic Acid); 12: Verapamil group. n=6. The protein expressions of myocardial cells of RyR2 (B), NCX1 (C), and DHPR-a1 (D) were expressed as mean+s.e.m., ☆☆ $P < 0.01$ vs control group; ** $P < 0.01$ vs Aconitine group.

Discussion

There are three main processes of calcium circulation in myocardial cells, named Sarcoplasmic reticulum calcium release, calcium taken back and calcium stored. The depolarization of myocardial-fibrosa activated L-form Voltage-gated calcium channel, the channel of membranes dihydropyridine receptor opened, which induced RyR2 (ryanodine receptor type2) on sarcoplasmic reticulum (SR) calcium release channel to open, plenty of calcium were released from SR, that led to calcium-induced calcium release (CICR) (Hideki et al, 2012; Jayasinghe1 et al, 2012; Eugenio et al, 2007). Then the intracytoplasmic calcium was pumped into SR quickly by SR calcium pump or discharged cells by NCX when cardiac muscle fibers systole and diastole. Finally, the cycle of systole and diastole was finished (Igal et al, 2011).

Intracellular calcium homeostasis of the myocardial cell is maintained by transmembrane transport of calcium or regulated by related calcium regulatory proteins like intracellular calcium pump to make a dynamic balance in myocardial cells (Ayaz and Howlett, 2015). There are several kinds of calcium regulatory proteins, including NCX1, RyR2, DHPR and SERCA2a in SR, which are all play a role in regulating the intracellular calcium homeostasis (Luisa et al, 2009). Therefore, calcium regulatory proteins play a key role in the process of calcium cycle.

RyR2, the calcium release channel in myocardial cell, mainly takes part in the process of mechanical systole and plays an important part in excitation-contraction coupling of myocardial cell (Xander et al, 2003; Ching et al, 2000). NCX1 involves not only in the process of myocardial contraction, but also in diastole, which mainly maintaining intracellular calcium homeostasis by transporting a part of cytoplasm calcium out of cells. Under the pathology condition, NCX1 is one of the essential ways that leading calcium overloads in cytoplasm. Calcium channel of L-form is the important regulator of calcium concentration in myocardial cells (Petros et al, 2015). Voltage-gated calcium channels of DHPR/L-form induce internal flowing of calcium, which stimulates more ensuing external flowing of calcium in sarcoplasmic reticulum (Pochaev et al, 2013). Once the dysfunction of calcium regulatory proteins mentioned above happens, or one of the processes of calcium cycle in myocardial cells is affected by external factors. Calcium stability in myocardial cells would be affected and causes calcium overloads. Thus, the function of the myocardial cell is affected.

Disruption of the intracellular Ca^{2+} homeostasis is a crucial mechanism of arrhythmic toxicity of Aconitine (Wright, 2002), Aconitine causes unbalance of calcium homeostasis in myocardial cell though affects the expression of

calcium regulatory proteins in myocardial cell excitation-contraction coupling system. The early studies showed that the biophysical characteristics of the sodium channel could be changed by Aconitine (Fu et al, 2007) and blocked the inactivation of the channel, which caused the sodium channel opening continuously (Sawanobori et al, 1987), thus persistent depolarization of myocardial cells stimulated NCX, and internal flowing of calcium increased. Secondly, Aconitine could increase the expression level of RyR2 gene and protein on myocardial cell sarcoplasmic reticulum calcium release channels, it could increase the calcium release of sarcoplasmic reticulum and increased the concentration of intracellular calcium (Hashimoto et al, 2007). The dynamic equilibrium of intracellular calcium was broken and caused arrhythmia. Aconitine may cause dysfunction of the central nervous system, heart and muscle system by affecting voltage-gated sodium channel or sodium-calcium exchange system (Fu et al, 2007).

In our study, we found the damage and effects of calcium-regulatory proteins (RyR2, NCX1, DHPR-a1) on myocardial cells of Aconitine. The results showed that 120 μ mol/L Aconitine damaged the myocardial cell, increased the expression of RyR2 and reduced the expression of NCX1, DHPR-a1 obviously. The underlying mechanism may be that after myocardial cell affected by Aconitine, it induced the high expression of RyR2 and increased calcium concentration in cytoplasm, which released from sarcoplasmic reticulum, and thus calcium overloads happened. At the same time, Aconitine decreased calcium excretion by inhibiting the expression of NCX1, and led the persistency of calcium overloads in cytoplasm eventually. Due to the high concentration of intracellular calcium, decreasing the expression of L-form voltage-gated calcium channel DHPR protein by negative feedback system to reduce calcium internal reduce, and then decline the range of calcium-induced calcium release and calcium transient, then reduce the systolic function of myocardial cell.

Also, we found that there had different protective effects of different doses compatibility of Aconitine with Liquiritin and Glycyrrhetic Acid on the survival rate and the expression level of calcium regulatory proteins (RyR2, NCX1, DHPR-a1) of the myocardial cell. The effect of the third combination group (30 μ M Aconitine, 60 μ M Liquiritin and 240 μ M Glycyrrhetic Acid) was extremely significant on the aspect of RyR2 expression, and the effect of the fifth combination group (60 μ M Aconitine, 60 μ M Liquiritin and 60 μ M Glycyrrhetic Acid) was extremely significant ($P < 0.01$) on the aspects of NCX1 and DHPR-a1 expression. The underlying mechanism may be that the compatibility of Aconitine, Liquiritin and Glycyrrhetic Acid inhibits the high expression of RyR2 induced by Aconitine, prevents the calcium overloads caused by calcium releasing, and maintains the intracellular calcium homeostasis. It also induces the expression of NCX1, increases excretion calcium by increasing the forward transportation of NCX1, decreases the intracellular calcium concentration and the intracellular calcium overload, and then reduces the happening of delayed depolarization. The compatibility of drugs regulates the expression of L-form voltage-gated calcium channel (DHPR) protein to reach the homeostasis by L-form internal flow of calcium when excitation-contraction coupling of myocardial cell happens, and finally enhances the contraction function of the myocardial cell.

Based on the analysis above, the orthogonal combination of Aconitine with Liquiritin and Glycyrrhetic Acid may regulate the expression of calcium regulatory proteins to inhibit the cytotoxicity of myocardial induced by Aconitine, enhance the calcium regulatory adaption of myocardial cell, maintain the function of heart diastole and systole, and whence achieve the effect of toxicity reducing and efficacy enhancing.

Conflict of interest: The authors declare no conflict of interests.

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