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Calcium antagonistic effects of *Bambusa Rigida* investigated by ^{45}Ca and its protection on myocardial ischemia of rats

LIN Rushan YE Ling YANG Yuanyou* LIAO Jiali MO Shangwu LIU Ning

Key Laboratory of Radiation Physics and Technology (Sichuan University), Ministry of Education, Institute of Nuclear Science and Technology, Sichuan University, Chengdu 610064, China

Abstract An investigation was conducted using ^{45}Ca as a radioactive tracer to evaluate calcium antagonistic effects of several extracts from *Bambusa Rigida* in living rats. The relationship between the flavonoid and saccharide contents of *Bambusa Rigida* and calcium antagonistic effects were also analyzed. The protective effects of the alkali extracts of *Bambusa Rigida* on myocardial ischemia were investigated in living rats. The results indicated that the alkali extracts of *Bambusa Rigida* had a prominent influence on Ca^{2+} influx and efflux in the isolated rat aorta and heart, as they could obviously block ^{45}Ca entering into cells and stimulate efflux of intracellular Ca^{2+}. Moreover, the alkali extracts of *Bambusa Rigida* had favorable protective effects on myocardial ischemia induced either by isoproterenol injection (ISO) or by the ligation of coronary artery. These results implied that the *Bambusa Rigida* had attractive potential for the treatment of heart, cerebrovascular and other diseases. However, the conclusion that whether the flavonoid or saccharide in *Bambusa Rigida* affected the calcium antagonistic effects and Ca^{2+} channels or not was hard to make within the results of the investigation.

Key words Influx, Efflux, ^{45}Ca, *Bambusa Rigida* extracts, Myocardial ischemia

CLC numbers TL99, Q4, R817.1

1 Introduction

Ca^{2+}, as an important and abundant metal cation and the second cell messenger in the human body, possesses several physiological and biochemical functions. It regulates the normal physiological actions of some key visceral organs. And it is involved in many physiological actions and life activities[1-5]. The metabolism of calcium in organisms and the dynamic changes of plasma calcium are regulated by vitamin D, parathyroid, calcium opsonin and other factors to maintain its relative equilibrium. Any breakage of the equilibrium of calcium in organisms would cause abnormal physiological states or diseases. For example, disturbance to Ca^{2+} stable state in cells may well be an early and important factor to cause cell injury, while overloading of Ca^{2+} in synapse cells may cause senile disturbance of memory[6]. Moreover, coronary and other diseases in cardiac or brain blood vessels are due to excessive influx of Ca^{2+} into cytoplasm[7-9].

Usually, there exist three calcium channels in the cell membranes: (1) the leak Ca^{2+} channels in normal physiological status; (2) the potential-dependent Ca^{2+} channel (PDC), which has six subsets (T, L, N, P, Q, R) and opens when the membrane is depolarized; (3) the receptor-operated Ca^{2+} channels (ROC) controlled by receptor activation. The diseases mentioned above might therefore be cured or controlled if Ca^{2+} channels in cell membranes are blocked or the superfluous Ca^{2+} in intracellular fluid is lured out from cytoplasm by calcium antagonist[10-13]. Many calcium antagonists have been developed for treating coronary and other diseases[7,14,15]. However, overuse of the chemical calcium antagonists may cause side effects[16,17].

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* Corresponding author. *E-mail address:* yangyyou@163.com

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To minimize the side effects, substantial interest has been focused on Chinese crude drugs with calcium antagonistic effects. In thousands of years, traditional Chinese medicine has been efficaciously used to treat coronary and other diseases in cardiac or brain blood vessels. Among the enormous number of Chinese crude drugs, some bamboos that contain cellulose, hemicellulose, amino acid, flavones, polysaccharose and minerals possess many advantageous biological functions. Flavonoids and saccharides are ubiquitous compounds in plants and are biologically active at nontoxic concentrations in organisms. Structure diversity of flavonoids and saccharides allow them to exhibit activities against cancers and other diseases[18-20]. Herbal remedies containing flavonoids and saccharides are in folk medicine of many countries, especially China[21]. However, efforts shall be made to clarify whether the bamboos have calcium antagonistic effects, and whether flavonoids and saccharides are related to the calcium antagonistic effects or Ca^{2+} channels. In this paper, the influence of *Bambusa Rigida*, an abundant kind of bamboo in southwest China, on Ca^{2+} influx and efflux in the isolated rat aortas and hearts was investigated using ^{45}Ca as a radioactive tracer, and calcium antagonistic effects of the flavonoid/sacchsrides contents were evaluated. Additionally, the protective effects of alkali extract from *Bambusa Rigida* on myocardial ischemia were evaluated in living rats.

2 Materials and methods

2.1 Materials

$^{45}CaCl_2$, in specific radioactivity of 1.57×10^{10} $Bq\cdot g^{-1}$, was from Amershan (UK). Nifedipine (Nif) was from No.1 South-west China Pharmaceuticals (Chongqing, China). Isoproterenol (ISO) was from Hefeng Pharmaceuticals (Shanghai, China). Urethane was from No.1 Chemical Reagents Manufacturing (Tianjin, China). Other chemical reagents were of analytical or chromatographic grade and were used without further purification.

Bambusa Rigida was gathered from Wangjiang Park in Chengdu. It was pretreated as follows. After 1 h extraction at reflux temperatures with H_2O, 70% ethanol, hydrochloric acid (1 $mol\cdot L^{-1}$) and saturated lime water (pH 9~10), respectively, the extractive solutions were filtrated. The filtrates were vacuum-dried, and the residues were dissolved in warm alcohol and centrifuged. The supernatants were vacuum-dried to obtain the extracts, which were stored in a refrigerator for further experiments.

The flavonoid contents in *Bambusa Rigida* were analyzed in a spectrophotometer at 510 nm, with rutin as the reference compound[22], while the saccharide contents were analyzed at 490 nm using glucose as the reference compound[23].

Wistar rats of both sexes weighing 180~240 g were from Sichuan Industrial Institute of Antibiotics (Chengdu, China). The animals were stunned and sacrificed. Their thoracic aortas and hearts were promptly removed and placed in physiological saline solution (PSS, pH 7.4, 37℃) containing (in $mmol\cdot L^{-1}$) NaCl (137), $CaCl_2$ (1.5), $MgCl_2$ (1.0), KCl (4.6), 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethansulfonsaure (HEPES, 20) and glucose (10). Fat and connective tissues were removed and the isolated aortas were cut into rings of 4~5 mm while the hearts were cut into fractions of 8~9 mg for further experiments.

2.2 Ca^{2+} influx and efflux determination

Ca^{2+} influx in isolated aorta rings and heart fractions of the rats were analyzed with the ^{45}Ca radioactive tracer method in Refs.[9, 12] with some modifications (Fig.1). The aorta rings and heart fractions were equilibrated in PSS and O_2 at 37℃ for 60 min before they were incubated at 37℃ in turn with the following solutions: 2 mL $^{45}Ca^{2+}$ solution (3.7×10^4 $Bq\cdot mL^{-1}$, in PSS) containing the extracts from *Bambusa Rigida* for 3 min and a K^+-depolarizing solution (pH=7.4) containing (in $mmol\cdot L^{-1}$) NaCl (1.5), $MgCl_2$(1.0), KCl (100), HEPES (20), glucose(10) and $^{45}Ca^{2+}$(3.7×10^4 $Bq\cdot mL^{-1}$) for 5 min. Usually, the rings and fractions were incubated for 40 min in the $^{45}Ca^{2+}$ solution containing the extracts from *Bambusa Rigida*, and washed for 60 min at 0~2℃ in a solution (pH=7.4) containing (in $mmol\cdot L^{-1}$) ethylene glycol-bis-(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, 10), NaCl(137), $MgCl_2$(1.0), KCl(4.6), HEPES(10) and glucose (10). Subsequently, every ring or fraction was blotted dry with filter paper, weighed, and dissolved in a mixture of 70% perchloric acid (25 μL)

and 30% H_2O_2 (50 μL) at 75℃ for 20 min. The solution was allowed to cool, before 5 mL scintillation solution was added. The radioactivity was analyzed by a liquid scintillation counter using ESCR (external standard channel ratio method) as quench correction.

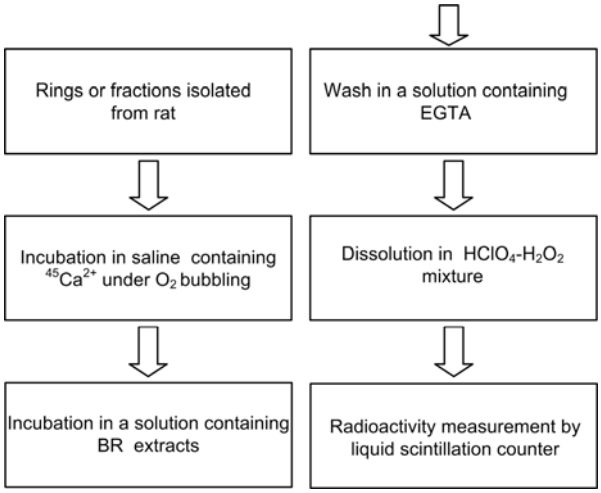

Fig.1 Diagram for the influx measurement procedure.

The Ca^{2+} efflux was determined in a procedure (Fig.2) that is somewhat different from the Ca^{2+} influx experiment. In order to let $^{45}Ca^{2+}$ enter tissue cells of each visceral organ first, the rings or fractions were incubated in a K^+-depolarizing solution (pH=7.4) containing (in mmol·L^{-1}) NaCl (1.5), $MgCl_2$(1.0), KCl (100), HEPES (20), glucose(10) and 3.7×10^4Bq·ml^{-1} $^{45}Ca^{2+}$ at 37℃ for 10 min before the Ca^{2+} efflux experiment. They were incubated with the extracts from *Bambusa Rigida* to promote ^{45}Ca efflux outward into the PSS solution.

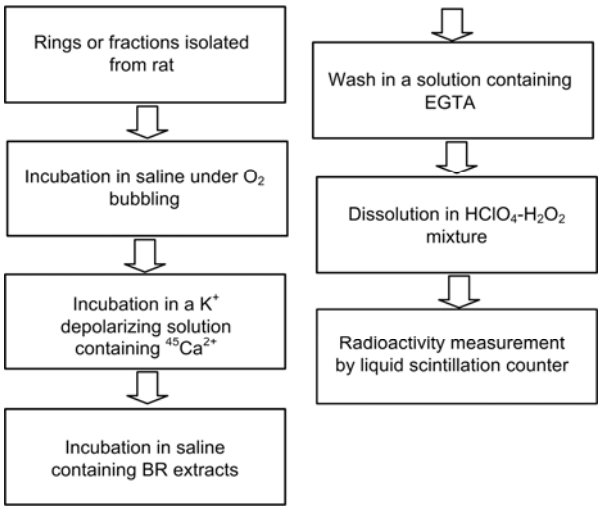

Fig.2 Diagram for the efflux measurement procedure.

The result of each aorta ring or heart fraction was converted to the apparent tissue content of Ca^{2+} by

$$Ca^{2+}(\mu mol\cdot kg^{-1}) = \frac{\text{Dpm of } ^{45}Ca \text{ in aorta}}{\text{aorta wet weight / kg}} \times \frac{\mu mol\ Ca/mL\ solution}{\text{Dpm/mL solution of } ^{45}Ca}$$

The data are expressed as ($\overline{X} \pm SD$). The statistical analysis was made with Student's *t*-test, and *P* values smaller than 0.05 were considered to be significant.

2.3 Influences of the alkali extracts of *Bambusa Rigida* on myocardial ischemia injury

2.3.1 Effects of the alkali extracts on myocardial ischemia injury induced by ISO injection

Male rats fed with nothing but water for 12 h before the experiment were divided at random in accordance with body weight into three groups, i.e. the blank control group, positive control group (Nif) (15 mg·kg^{-1}), and alkali extracts of *Bambusa Rigida* group (150 mg·kg^{-1}). Nif and the alkali extracts of *Bambusa Rigida* for each group were given by pouring into the stomach in 7 days at a dose of 4 mL·kg^{-1}; equivalent physiological saline was given to blank control groups at a dose of 4 mL·kg^{-1}. Exactly one hour after pouring on the 7th day, the rats were anaesthetized by i.p. administration with 20% urethane (4 mL·kg^{-1}), and were connected to a physiological meter (RM-6000, Photoelectricity Co., Japan) to determine Lead II electrocardiogram (ECG). The anode and cathode of RM-6000 were linked to the left lower and right upper limbs, respectively. ISO (4 mg·kg^{-1}) was injected in the tail vein and the electrocardiogram was recorded in 5 min after the i.v. injection. If one of the following indices appeared, it was considered as the positive myocardial ischemia: J-spot increased over 1.5 mV; T wave was low (decreasing primary T wave over 50%), diphasic and inverted; level in ST section decreased to 0.5 mV; arrhythmia appeared. The statistical analysis was made by Student's *t*-test, and *P* value smaller than 0.05 was considered to be significant.

2.3.2 Effects of the alkali extracts on myocardial ischemia injury induced by ligation of coronary artery

Male rats were divided according to body weight at random into the control, the positive control (Nif) and the alkali extracts of *Bambusa Rigida* (50 mg·mL^{-1}) groups, and were fed with nothing but water before the experiment. The rats were fixed by dorsal position,

anaesthetized by i.p. administration with 20% urethane (4 mL·kg^{-1}), and connected to the biological system BL-40 (Taimeng Technologies, Chengdu, China) to record Lead II ECG for 15 s. The drug for each group was given by i.v. injection in the tail with a dose of 4mg·kg^{-1}, and equivalent physiological saline was given to the control group. After 5 min, the rat chest was opened to ligate the left coronary artery and record Lead II ECG at 5，10，15，20，30min after ligature. The average drift value at J-spot ($\overline{X} \pm SD$) were considered as index for myocardial ischemia and evaluation of the drug efficacy, and the statistical analysis was made by Student's *t*-test.

3 Results

The Ca^{2+} influx and efflux in the isolated aortas and hearts of the rats in the presence of the *Bambusa Rigida* (BR) extracts are shown in Tables 1 and 2. The contents of flavonoid and saccharide were summed up to explore if the contents are consistent with the results about the Ca^{2+} influx and efflux. As shown in Tables 1 and 2, not all extracts obtained by different methods could inhibit Ca^{2+} influx or promote Ca^{2+} efflux. Among the four kinds of extracts, the alkali extracts have the most significant effect on calcium channels, even the alkali extract was 2 or 4 times diluted (Table 3). However, no statistically significant effect of the ethanol, hydrochloric acid or water extracts on the Ca^{2+} influx and efflux was observed (Tables 1 and 2). Additionally, there was no significant relationship between the content of flavonoid or saccharide and the calcium antagonistic effects. For example, although the flavonoid or saccharide contents of 70% ethanol extracts (43.1 mg·L^{-1}) are higher than other three kinds of extracts (≦21.1 mg·L^{-1}), its effects on Ca^{2+} influx and efflux were lower than the alkali extracts.

Table 1 Ca^{2+} influx in isolated rat aorta and heart in the presence of *Bambusa Rigida* extracts (n=6, $\overline{X} \pm SD$)

Extracts		Saccharide / mg·L^{-1}	Flavonoid / mg·L^{-1}	Artery / CPM·mg^{-1}	Heart / CPM·mg^{-1}
BR leaves	Alkali extract	30.4	34.5	109.8±39.6***	471.4±76.6***
	Water extract	21.6	43.1	157.4±41.0**	710.3±120.0
	Hydrochloric acid extract	22.9	33.8	237.3±46.7	666.1±88.5*
	70% ethanol extract	38.2	63.4	184.7±31.5*	738.9±158.8
BR culms	Alkali extract	39.2	6.3	108.4±15.2***	515.4±88.9***
	Water extract	9.6	3.2	275.0±39.7	969.5±254.9
	Hydrochloric acid extract	7.9	10.8	211.5±27.5	769.0±228.8
	70% ethanol extract	13.9	17.1	185.2±35.9*	854.7±168.2

$^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 compared with the blank control group, which had Ca^{2+} influx in artery and heart of 235.5±24.4 and 769.9±52.1, respectively.

Table 2 Ca^{2+} efflux in isolated rat aorta and heart in the presence of *Bambusa Rigida* extracts (n=6, $\overline{X} \pm SD$)

Extracts		Saccharide / mg·L^{-1}	Flavonoid / mg·L^{-1}	Artery / CPM·mg^{-1}	Heart / CPM·mg^{-1}
BR leaves	Alkali extract	34.5	30.4	50.1±5.9***	154.9±34.1**
	Water extract	43.1	21.6	65.7±9.6*	196.8±39.1*
	Hydrochloric acid extract	33.8	22.9	69.5±13.6	244.2±43.9
	70% ethanol extract	63.4	38.2	93.8±8.2	301.3±68.1
BR culms	Alkali extract	39.2	6.3	49.2±8.1***	125.1±19.7***
	Water extract	9.6	3.2	102.3±25.2	363.8±38.6*
	Hydrochloric acid extract	7.6	10.8	79.3±9.6	311.6±60.8
	70% ethanol extract	13.9	17.1	108.8±27.2	352.1±71.5

$^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 compared with the blank control group, which had Ca^{2+} efflux in artery and heart of 82.5±10.5 and 280.7±59.4, respectively.

Table 3 Ca^{2+} influx and efflux ($CPM \cdot mg^{-1}$) in isolated rat aorta and heart in the presence of alkali extract of *Bambusa Rigida* culms (n=6, $\overline{X} \pm SD$)

Concentrationa / $g \cdot mL^{-1}$	Influx		Efflux	
	Artery	Heart	Artery	Heart
0.25	157.5±31.3***	621.7±139.7**	59.1±12.5*	228.3±45.6*
0.5	103.8±21.4***	496.7±98.3***	52.3±11.4**	215.7±60.3*
1.0	76.9±15.1***	357.8±67.1***	38.7±6.7***	155.8±34.2***

[a]Concentrations for *Bambusa Rigida* were calculated with the weight before extraction. *P<0.05, **P<0.01, ***P<0.001 compared with the blank control, which had Ca^{2+} influx in artery and heart of 391.8±82.8 and 1223.3±224.3, and Ca^{2+} efflux in artery and heart of 93.8±20.6 and 302.9±49.6, respectively.

Also, no significant differences were observed between the leaves and culms of *Bambusa Rigida* on Ca^{2+} influx and efflux.

Effects of the alkali extracts on myocardial ischemia induced by ISO are shown in Table 4. It can be seen that oral medication of the alkali extracts with 150 $mg \cdot kg^{-1}$ reduced J-spot drift of electrocardiogram with myocardial ischemia evoked by ISO, especially at 10 min post administration. Compared with the blank control group, the values for J-spot drift in the alkali extracts of *Bambusa Rigida* group were obviously different, but no statistically obvious difference was found in comparison with positive drug (Nif).

Table 5 shows that the influence of the alkali extracts on myocardial ischemia injury was induced by the ligation of coronary artery. We note that the alkali extracts decreased J-spot drift of electrocardiogram with myocardial ischemia induced by the ligation of the coronary artery compared with the control group, similar to the ISO results, but no statistically obvious difference was observed in comparison with positive drug (Nif).

Table 4 J-spot shift (mV) of Lead II ECG induced by ISO in rats (n=6, $\overline{X} \pm SD$)

Groups	5 min	10 min	20 min	30 min	60 min
Blank control	0.050±0.075	0.098±0.052	0.072±0.062	0.061±0.063	0.044±0.059
Positive control	0.011±0.013	0.003±0.007**	0.005±0.009*	0.005±0.009 *	0.007±0.006 *
Alkali extracts	0.009±0.012	0.001±0.006**	0.004±0.007*	0.005±0.006 *	0.004±0.003*

*P<0.05, **P<0.01 compared with the blank control.

Table 5 J-spot shift (mV) of Lead II ECG induced by coronary artery-ligated rats (n=6, $\overline{X} \pm SD$)

Groups	5 min	10 min	15 min	20 min	30 min
Blank control	0.51±0.25	0.45±0.20	0.31±0.19	0.24±0.11	0.19±0.12
Positive group	0.05±0.03**	0.03±0.05**	0.04±0.02 *	0.05±0.03**	0.04±0.03*
Alkali extracts	0.03±0.04**	0.05±0.06*	0.05±0.04*	0.04±0.03**	0.04±0.02*

*P<0.05, **P<0.01 compared with the control group.

4 Discussions

The Ca^{2+} influx or efflux in isolated aorta rings or heart fractions of the rats were investigated by using the radioactive tracer of ^{45}Ca, which has a half-life of 163.5 days and emits low energy β rays. Adding the ^{45}Ca, however, increases Ca^{2+} content of the solution by $[Ca^{2+}]=C/40A$ ($mol \cdot L^{-1}$), where C is radioactivity concentration ($MBq \cdot L^{-1}$) of ^{45}Ca in physiological solution, A is the specific radioactivity ($MBq \cdot g^{-1}$) of ^{45}Ca provided by the producer with decay correction, and 40 is the atomic weight of Ca. Unfortunately, the influence of this fact was not taken into account previously. In this study, we kept the Ca^{2+} concentration in physiological solution constant by reducing the concentration of $CaCl_2$.

It is very important to keep the influxed ^{45}Ca in the cytoplasm while the ^{45}Ca on the surfaces of cells

should be entirely removed. In this experimental procedure, a solution containing EGTA of 10 mmol·L^{-1} was effectively used to wash the ^{45}Ca on the surface of cells. This is different from the procedure reported by others[12].

Some kinds of bamboo are used in Chinese traditional drugs. However, it is of interest whether *Bambusa Rigida* has calcium antagonistic effects as chemical calcium antagonists. The results showed that the Ca^{2+} influx and efflux in isolated aortas or hearts were significantly inhibited in a concentration-dependent manner by the alkali extracts of *Bambusa Rigida*. The effects are similar to chemical calcium antagonists, such as Verapamil, being less potent, though, than Verapamil in some way, duo to less effective components in the *Bambusa Rigida* extracts.

The alkali extracts do have protective effect on myocardial ischemia induced by isoproterenol or by the ligation of coronary artery. This is encouraging and consistent with the results about the Ca^{2+} influx and efflux by isotope tracer technique. However, it is very difficult to conclude that whether the flavonoid or saccharide in *Bambusa Rigida* is the primary chemical compound to influence the calcium channels or not, just based on the results up to now. This is probably because the components of *Bambusa Rigida* are very complicate and no unique compound is involved in the calcium antagonistic effects and Ca^{2+} channels.

5 Conclusion

An efficient and sensitive way was developed to measure the Ca^{2+} influx and efflux in the isolated rat aortas or hearts. The calcium antagonistic effects of *Bambusa Rigida* extracts was evaluated. It implied that some *Bambusa Rigida* extracts are able to block the Ca^{2+} influx into cell or lure the Ca^{2+} out of cell as chemical calcium antagonists. The alkali extracts, even 4 times diluted, have significant effects on calcium channels in visceral organs. Moreover, the alkali extracts have favorable protective effect on myocardial ischemia induced by drugs or by the ligation of the coronary artery. The results indicated that the *Bambusa Rigida* has attractive potentials in treatments of heart, cerebrovascular and other diseases. These should be further explored, and involvement of the flavonoid or saccharide in *Bambusa Rigida* in the calcium antagonistic effects and Ca^{2+} channels shall be further studied, too.

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