Protective effects of strychnos alkaloids on the xanthine and xanthine oxidase-induced damage to cultured cardiomyocytes

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(Received May 8, 1995. Accepted July 13, 1995.)

Abstract

Nine strychnos alkaloids were investigated for their effects on the xanthine and xanthine oxidase (X-XOD) -induced damage to cultured cardiomyocytes by means of electron microscopy. In the presence of isobrucine (**7**), isobrucine *N*-oxide (**9**) or 2-hydroxy-3-methoxystrychnine (**5**), the damage to fine organized structures in cardiomyocytes was appreciably suppressed. The other alkaloids examined also suppressed the damage in the cells though their effects were not so evident as the above three compounds.

Since brucine (**2**), brucine *N*-oxide (**4**), isobrucine (**7**), isobrucine *N*-oxide (**9**), 2-hydroxy-3-methoxystrychnine (**5**) *etc.* remarkably inhibited XOD and has SOD-like activities in both systems of X-XOD and phenazine methosulfate-NADH, the suppression of the X-XOD-induced damage in cardiomyocytes may be due to the inhibition of the generation of superoxide anions and to the scavenging of reactive oxygen species by these alkaloids.

Key words brucine, brucine *N*-oxide, isobrucine, isobrucine *N*-oxide, cardiomyocyte, *Strychnos nux-vomica*, superoxide anion, xanthine oxidase.

Abbreviations BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetra-acetate; FBS, fetal bovine serum; IC_{50}, a concentration that is required to 50 % inhibition; NADH, a reduced form of nicotinamide adenine dinucleotide; NBT, nitroblue tetrazolium; SOD, superoxide dismutase; X, xanthine; XOD, xanthine oxidase.

Introduction

The tissue damage caused by reactive oxygen species is more or less associated with diverse disorders in the body, such as rheumatism, nephritis, carcinogenesis, inflammatory diseases and heart dysfunctions.[1] A variety of pathological models induced by reactive oxygen species have been established to explore the free radical mechanism of diseases. Injection of xanthine and xanthine oxidase (X-XOD) to rats was shown to induce free radical-mediated damages to the myocardium.[2]

In the course of our studies on the processing of nux vomica,[3-6] we investigated the effects of strychnos alkaloids on fine organized structures in cardiomyocytes exposed to reactive oxygen species. In the present experiment, cultured cardiomyocytes prepared from the rat ventricle were exposed to X-XOD in the presence of various strychnos alkaloids

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isolated from the processed seeds of *Strychnos nux-vomica* L.(Loganiaceae) and the resulting damage in the cells was observed by electron microscopy. Furthermore, we investigated the effects of these alkaloids on the generation of superoxide anions in the X-XOD system and in the phenazine methosulfate-NADH system, as well as the effects on XOD.

Materials and Methods

Chemicals : Xanthine oxidase from butter milk (1.16 units/mg protein; EC 1.2.3.2) was purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan) ; Superoxide dismutase (Cu, Zn type of SOD, 3500 units/mg protein; EC 1.15.1.1) from bovine erythrocyte, nitroblue tetrazolium (NBT), xanthine, phenazine methosulfate and quercetin were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan) ; bovine serum albumin (BSA) from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) ; a reduced form of nicotinamide adenine dinucleotide (NADH) from Boehringer Mannheim Gmbh (Mannheim, Germany) ; Dulbecco's modified Eagle's medium (DMEM) from Life Technologies Inc. (Gaithersburg, Md., U.S.A.) ; fetal bovine serum (FBS) from Nanjing Medical University (Nanjing, China) ; trypsin from Shanghai Biochemical Pharmaceutical Factory (Shanghai, China).

For experiments of free radical scavenging activity and xanthine oxidase inhibitory activity, ultra pure water was used in all procedures. All other chemicals were of reagent grade.

Strychnine (**1**), brucine (**2**), strychnine *N*-oxide (**3**), brucine *N*-oxide (**4**), 2-hydroxy-3-methoxystrychnine (**5**), isostrychnine (**6**), isobrucine (**7**), isostrychnine *N*-oxide (**8**) and isobrucine *N*-oxide (**9**) were isolated from the processed seeds of *S. nux-vomica*.[3] These compounds were identified by spectroscopic means and the purity was more than 96 % judging from thin layer chromatography-densitometry. Each alkaloid was dissolved in a small amount of EtOH and diluted with water to give a solution of 50 mM, just before addition to cardiomyocytes in culture. The final concentration of EtOH was less than 0.1 %. The solutions were adjusted to pH 7.2-7.4 with 0.5 M H_3PO_4 or 0.5 M NaOH and sterilized with membrane filter.

Apparatus : A transmission electron microscope Hitachi H-600 (Tokyo, Japan) and a Shimadzu UV-2200 digital double beam spectrophotometer (Shimadzu Co., Kyoto, Japan) were used in this experiment.

Preparation of cardiomyocytes : According to the method of Su,[7] the whole ventricle was taken from neonatal Wistar rats 24 to 48 hours after birth and cut into pieces about 1 mm^3 in size, which were then dispersed in 0.1 % trypsin with mechanical stirring. The suspension of cardiomyocytes was diluted with 80 % DMEM supplemented with 20 % FBS in a polystyrene culture vessel and incubated at 37°C in an atmosphere of 5 % carbon dioxide and 95 % air.

Exposure of superoxide anions to cardiomyocytes in the presence or absence of strychnos alkaloids : Cardiomyocytes ($1-3 \times 10^5$ cells/ml) were suspended in a 1 ml of 80 % DMEM-20 % FBS containing 50 μM alkaloid (**1-9**), and 0.42 mM xanthine and 0.64×10^{-3} unit/ml XOD were added. The mixture was incubated for 4 days at 37°C in an atmosphere of 5 % carbon dioxide and 95 % air. The cardiomyocytes were detached, centrifuged at 1600 rpm for 10 min, fixated with 2.5 % glutaraldehyde at room temperature for 70 min, cut into about 1 mm^3 lumps with a microtome. The lumps were then washed three times with 0.1 M phosphate buffer (pH 7.4) for 2 hours, followed by fixation with 1 % osmium tetra-oxide for 1.5 hours at 4°C. As a control, cardiomyocytes were cultured in 80 % DMEM-20 % FBS containing no strychnos alkaloid under the same conditions and treated as described above.

Electron microscopic observation : The specimens were dehydrated with 50 %, 70 %, 90 % and 100 % acetone in this order for 13 min each and permeated with a 1 : 1 mixture of acetone and an embedding medium (6 ml of epon 618, 4 ml of maleic anhydride, 0.3 ml of dibutyl phthalate and 0.1 ml of diethylaniline) for 1.5 hours at 37°C. Each specimen was transferred to the bottom of a capsule and the embedding medium was added. The mixture was kept at 37°C for 8 hours and further at 68°C for 48 hours to give a solid block of plastic and the block was cut into thin sections (40 to 70 nm in thickness) with a fine glass knife attached to a microtome. Each section was placed on a small circular copper grid (200 th type of sieve), which was put on a wax plate followed by addition of

an uranate solution (2 g of uranyl acetate in 100 ml of 50 % EtOH). The resulting section was electronically stained for 20 min and washed with bidistilled water.

The section with a copper grid was then stained with a lead solution (1.33 g of lead nitrate and 1.76 g of sodium citrate in 30 ml of water) for 10 min, washed with bidistilled water and dried on a filter paper. The specimens were observed with a transmission type electron microscope.

Scavenging activity of strychnos alkaloids for superoxide anions generated by xanthine - xanthine oxidase : According to the method of Imanari *et al.*,[8] SOD and SOD - like activities were determined. A mixture containing 1.1 ml of 50 mM carbonate buffer (pH 10.2), 0.05 ml of 3 mM xanthine, 0.05 ml of 3 mM disodium ethylenediaminetetra-acetate (EDTA-2Na), 0.05 ml of 1.5 mg/ml BSA, 0.05 ml of 0.75 mM NBT and various concentrations of strychnos alkaloids and quercetin was preincubated for 10 min at 25°C and 0.05 ml of XOD (0.053 unit / ml) was added. The mixture was incubated for 20 min at 25°C with gentle shaking and the reaction was stopped by adding 0.05 ml of 6 mM $CuCl_2$. The SOD-like activity was determined by measuring the absorbance at 560 nm.

Percentage of inhibition was calculated as follows : Inhibition (%) = 100 × [absorbance of control − (absorance of sample − absorbance of blank)]/absorbance of control. The results were expressed as the means and standard errors (S.E.) of four tests.

Scavenging activity of strychnos alkaloids for superoxide anions generated by phenazine methosulfate-NADH : According to the methods of Nishikimi *et al.*[9] and Robak and Gryglewski,[10] a mixture containing 20 μM phenazine methosulfate, 130 μM NBT and 175 μM NADH with or without various concentrations of strychnos alkaloids was incubated for 5 min at 25°C. The absorbance was measured at 560 nm.

Inhibitory activity of strychnos alkaloids against XOD : XOD activity was measured spectrophotometrically by a modified method of Robak and Gryglewki[10] and Noro *et al.*[11] using xanthine as a substrate. A mixture contained various concentrations of strychnos alkaloids (or quercetin), and 0.5 ml of XOD (0.04 unit/ml) in 0.1 mM phosphate buffer (pH 7.5). After preincubation of the mixture for 15 min at 25°C, the reaction was initiated by adding 17 μl of 3 mM xanthine in 0.05 mM carbonate buffer (pH 10.2) and 0.5 ml of 0.1 mM phosphate buffer (pH 7.5). The mixture was incubated for 30 min at 25°C. The reaction was stopped by adding 0.22 ml of 1 N HCl, and the absorbance of the mixture was measured at 295 nm.

Results

Morphological changes in the fine organized structures of the cardiomyocytes exposed with superoxide anions in the presence or absence of strychnos alkaloids

Fig. 1 shows typical electron micrographs of the fine organized structures within cultured cardiomyocytes. In a control group (Fig. 1A) where the cells had been cultured under the ordinary conditions, mitochondria were distributed even around the nucleus and innumerable myofilaments were integrated. Z lines, sarcomeres and mild contraction bands and sarcoplasmic reticula were visible. Nuclei were essentially normal.

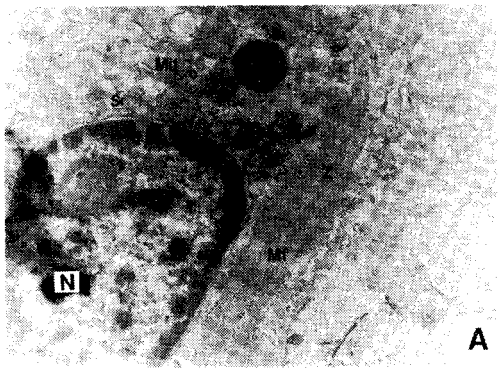
On the other hand, in a group that was treated with X-XOD in the absence of strychnos alkaloids (Fig. 1B), repeated structures of myofilaments were completely broken and myofibrils were dissolved. Swollen, dissolved, broken organelles were observed around the nucleus. These findings showed that the fine structures in the cell were severely damaged by X-XOD treatment.

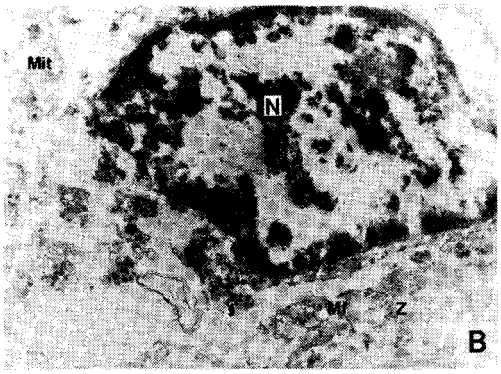
However, in the presence of the strychnos alkaloids, the X-XOD-induced damage to the fine structures in the cells appreciably suppressed and the electron micrograms were classified into three categories on the basis of their morphological changes. The extent of suppression was reduced in the order of the first category to the third one.

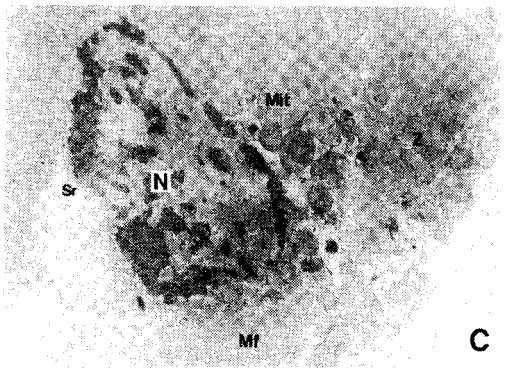
In the first category (Fig. 1C), many mitochondria were present, their cristae well defined and myofilaments parallel. Sarcomeres and Z lines were well defined, and small inclusion bodies and endoplasmic reticula were visible. Furthermore, nuclei were normal and the most of organelles were similar in shape to those of control shown in Fig. 1A. The first category included the groups that had been treated with X-XOD in the presence of isobrucine (**7**), isobrucine *N*-oxide (**9**) or 2-hydroxy-3-methoxystrychnine (**5**) (Fig. 2).

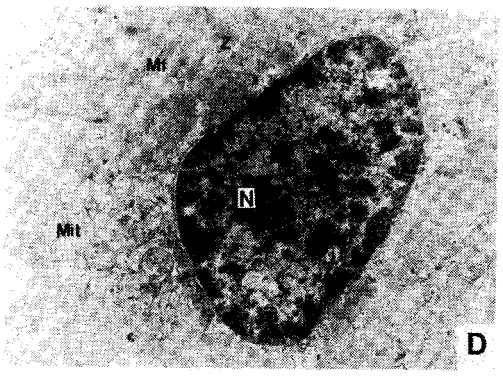
In the second category (Fig. 1D), mitochondria were a little swollen and some matrix granules were deposited. The repeated structures of myofilaments were not clear but contraction bands of myofilaments were clear. Myofibrils were rather dissolved and sarcoplasmic reticula were distended. The groups that had been treated with X-XOD in the presence of brucine *N*-oxide (**4**), isostrychnine (**6**) or isostrychnine *N*-oxide (**8**) belonged to this category.

In the third category (Fig. 1E), where the groups









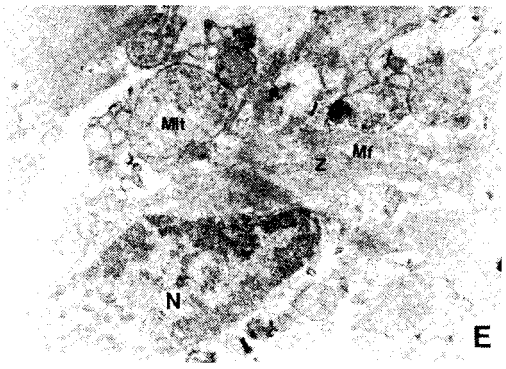
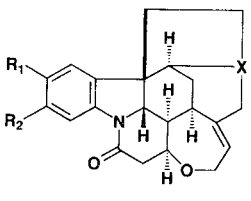
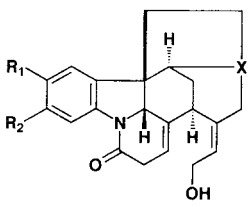


Fig. 1 Transmission type electron micrographs (×15,000) showing morphological changes in the fine structures of cultured cardiomyocytes which treated with X-XOD in the presence or absence of strychnos alkaloids.
A, control; B, treated with X XOD; C, treated with X XOD and isobrucine *N* oxide (**9**); D, treated with X XOD and brucine *N* oxide (**4**); E, treated with X-XOD and strychnine (**1**). N, nucleus; Mf, myofilament; Mit, mitochondria; Z, Z lines; SR, sarcoplasmic reticulum.





1 : R_1-R_2-H, X N
2 : R_1-R_2-OMe, X-N
3 : R_1 R_2 H, X=N→O
4 : R_1 R_2-OMe, X N-→O
5 : R_1 OH, R_2-OMe, X-N
6 : R_1 R_2-H, X-N
7 : R_1-R_2-OMe, X N
8 : R_1-R_2 H, X N →O
9 : R_1 R_2-OMe, X N→O

Fig. 2

that had been treated with X-XOD in the presence of brucine (**2**), strychnine (**1**) or strychnine *N*-oxide (**3**) were included, mitochondria were obviously swollen with some small granules. Some myofilaments were broken and myofibrils were randomly oriented.

Superoxide anions scavenging activity of strychnos alkaloids

Superoxide anion scavenging activity of strychnos alkaloids was examined using the enzymatic and non-enzymatic systems, X-XOD and phenazine methosulphate-NADH. Of these alkaloids, 2-hydroxy-3-methoxystrychnine (**5**) showed the most potent SOD-like activity when superoxide anions were determined by the NBT method (Tables I and II). The IC_{50} of **5** (28.3±0.5 μM) in the X-XOD system was almost equivalent to that of quercetin (31.9±2.5 μM) known as a naturally occurring antioxidant (Table I).[10] Brucine (**2**) and its derivatives (**4, 7** and **9**) showed moderate SOD-like activity with IC_{50} of 55-100 μM in the X-XOD system and with IC_{50} of 9-13 μM in the phenazine methosulphate-NADH system. Brucine (**2**) and its derivatives showed more potent SOD-like activity compared to strychnine (**1**) and its derivatives (**3, 6** and **8**).

Table I Effects of various strychnos alkaloids and quercetin on the superoxide anion generation in the X-XOD system.

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
Strychnine and its derivatives		*Brucine and its derivatives*	
Strychnine (**1**)	130.7±1.3	Brucine (**2**)	97.3±1.2
Strychnine *N*-oxide (**3**)	108.9±3.1	Brucine *N*-oxide (**4**)	70.6±2.1
Isostrychnine (**6**)	115.1±10.3	Isobrucine (**7**)	55.4±1.7
Isostrychnine *N*-oxide (**8**)	116.9±10.6	Isobrucine *N*-oxide (**9**)	86.5±4.1
Others			
2-Hydroxy-3-methoxystrychnine (**5**)	28.3±0.5		
Quercetin	31.9±2.4		

Values are means±S.E. (n=4).

Table II Effects of various strychnos alkaloids on the superoxide anion generation in the phenazine methosulphate and NADH system.

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
Strychnine and its derivatives		*Brucine and its derivatives*	
Strychnine (**1**)	35.2±1.7	Brucine (**2**)	10.7±1.2
Strychnine *N*-oxide (**3**)	22.4±2.1	Brucine *N*-oxide (**4**)	13.0±1.1
Isostrychnine (**6**)	29.9±5.9	Isobrucine (**7**)	10.0±1.4
Isostrychnine *N*-oxide (**8**)	29.5±0.7	Isobrucine *N*-oxide (**9**)	8.9±1.2
Others			
2-Hydroxy-3-methoxystrychnine (**5**)	5.9±0.8		

Values are means±S.E. (n=4).

Inhibitory activity of strychnos alkaloids against xanthine oxidase

Since the generation of superoxide anions is inhibited by XOD inhibitors such as allopurinol in the X-XOD system, we examined the effects of strychnos alkaloids on XOD (Table III). Contrary to the case of SOD-like activity, isobrucine *N*-oxide (**9**) showed the most potent inhibition to XOD with an IC_{50} of 13.3±6.0 μM but 2-hydroxy-3-methoxystrychnine (**5**) showed moderate inhibition (IC_{50}= 33.7 ± 1.2 μM). Similar to the case of SOD-activity, brucine (**2**) and its derivatives (**4, 7** and **9**) were stronger in their inhibitory potency than the corresponding strychnine (**1**) and its derivatives (**3, 6** and **8**), IC_{50} values of the

Table III Inhibitory effects of various strychnos alkaloids and quercetin on XOD.

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
Strychnine and its derivatives		*Brucine and its derivatives*	
Strychnine (**1**)	48.6±1.1	Brucine (**2**)	35.0±1.8
Strychnine *N*-oxide (**3**)	39.9±2.0	Brucine *N*-oxide (**4**)	21.0±5.0
Isostrychnine (**6**)	39.3±4.6	Isobrucine (**7**)	23.5±1.5
Isostrychnine *N*-oxide (**8**)	35.8±3.8	Isobrucine *N* oxide (**9**)	13.3±6.0
Others			
2 Hydroxy-3-methoxystrychnine (**5**)	33.7±1.2		
Quercetin	18.6±6.0		

Values are means±S.E. (n=4).

former group being in a range of 13-35 μM and those of the latter group in a range of 36-49 μM.

Discussion

For the purpose of evaluating the processing of nux vomica, we have previously compared chemical constituents before and after processing of the seeds of *S. nux-vomica* and found that ring-opened and *N*-oxidized compounds were produced from major alkaloids, strychnine (**1**) and brucine (**2**), by heating in sand bath or in oil bath.[3,4] Furthermore, we compared alkaloid contents, their composition and acute toxicity among the seeds treated by seven different processing methods.[5] The ring-opened and *N*-oxidized alkaloids, isostrychnine *N*-oxide (**8**) and isobrucine *N* oxide (**9**), were also shown to be potent cytotoxic against HeLa, chronic myelomatosis and laryngocarcinoma cells.[6]

In the present paper, we demonstrated that strychnos alkaloids (**1**-**9**) inhibited free radical-mediated damage to the cardimyocytes in culture. These alkaloids also inhibited the generation of superoxide anions in the enzymatic X-XOD system and in the non-enzymatic phenazine methosulfate-NADH system. The most potent inhibitory activity was observed in both systems for 2-hydroxy-3-methoxystrychnine (**5**) which was newly formed from brucine (**2**) through the processing of nux vamica with the use of a hot sand bath or a hot oil bath. The inhibitory activities of brucine (**2**) and is derivaties (**4**, **7** and **9**) were generally stronger than those of strychnine (**1**) and its derivatives (**3**, **6** and **8**), indicating that two methoxyl substituents attached to the aromatic ring in **2**, **4**, **7** and **9** enhance radical scavenging potency. However, the most of strychnos alkaloids (**1**-**9**) also inhibited XOD at lower concentrations when measured by the formation of uric acid from xanthine. The major protective action of strychnos alkaloids (**1**-**9**) against the cellular damage may be due to the inhibition of XOD, which reduces the generation of superoxide anions, although it still remains a possibility that these alkaloids take part in reduction of reactive oxygen species as free radical scavengers.

The X-XOD system is frequently used as a generator of superoxide anions.[12] The native form of xanthine oxidase and xanthine dehydrogenase exists in three types: xanthine dehydrogenase (D type), xanthine oxidase (O type) and xanthine dehydrogenase/xanthine oxidase (D/O type).[13] The O type is an enzyme that generates superoxide anions. In normal heart tissue, about 8 % are present as the O type and more than 90 % as the D type.[14] The D type has no ability of producing superoxide anions but can be converted to the O type under such conditions as in cardiac ischemia-reperfusion injury,[15] which subsequently leads to damage to cardiomyocytes by reactive oxygen species generated in the X-XOD system.

Since strychnos alkaloids (**1**-**9**) suppress the free radical mediated-damage to cultured cardiomyocytes, it is suggested that these alkaloids may be applicable towards the prevention and treatment of various diseases caused by abnormal production of reactive oxygen species.

和文抄録

Xanthine-xanthine oxidase 系で発生する superoxide anion による培養心筋細胞の傷害に対する9種の馬銭子アルカロイドの影響を電子顕微鏡を用いて観察した結果，isobrucine, isobrucine *N*-oxide, 2-hydroxy-3-methoxystrychnine は細胞内の微細構造の破壊を顕著に抑制することを見い出した。他のアルカロイドも同様な作用を示すが，上記化合物ほど明らかではなかった。Brucine, brucine *N*-oxide, isobrucine, isobrucine *N*-oxide, 2 hydroxy-3-methoxystrychnine は顕著に xanthine oxidase を阻害し，しかも酵素的 (xanthine-xanthine oxidase) および非酵素的 (phenazine methosulphate-NADH) superoxide anion 生成系での実験から superoxide dismutase 様活性を示した。これらの事実から，馬銭子アルカロイドは，xanthine-xanthine oxidase 系での superoxide anion の生成阻害や活性酸素捕獲作用により心筋細胞傷害を抑制するものと思われる。

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