Effects of *N*-*n*-butyl haloperidol iodide on rat myocardial ischemia and reperfusion injury and L-type calcium current[1]

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KEY WORDS haloperidol; ischemia-reperfusion injury; myocardium; L-type calcium channels; electron microscopy; patch-clamp techniques

ABSTRACT

AIM: To study the effects of *N*-*n*-butyl haloperidol iodide (F_2) on rat heart ischemia/reperfusion (I/R) injury and L-type calcium current (I_{Ca}) in rat ventricular myocytes. **METHODS**: Rat heart I/R injury was induced by occluding the left anterior descending coronary artery for 30 min and restoring perfusion for 30 min. F_2 (1, 2, and 4 mg/kg) were iv injected before ischemia. Plasma creatine kinase (CK), creatine kinase isoenzyme MB (CK-MB), lactate dehydrogenase (LDH), α-hydroxybutyrate dehydrogenase (HBDH), glutamic-oxaloacetic transaminase (GOT), malondialdehyde (MDA) concentrations, and superoxide dismutase (SOD) activity were measured. The pathologic changes of I/R myocardium were assessed by the transmission electron microscopy. Single rat ventricular myocyte was obtained by enzymatic dissociation method. The currents were recorded with the whole-cell configuration of the patch-clamp technique. **RESULTS:** F_2 reduced the release of CK, CK-MB, LDH, HBDH and GOT, preserved the activity of SOD, and decreased the MDA contents dose-dependently. For morphology, F_2 mollified the pathologic changes of myocardium induced by I/R injury. F_2 1 μmol/L decreased I_{Ca} from (1775±360) pA to (464±129) pA (n=8, P<0.01) and shifted the current-voltage of I_{Ca} upward, without affecting the voltage-dependent properties of I_{Ca}. **CONCLUSION:** F_2 played a protective role against rat heart I/R injury in a dose-dependent manner, and inhibited I_{Ca} in rat ventricular myocytes. The cardioprotective and vasodilatory mechanisms of F_2 may be related to its inhibitory effect on L-type calcium channel.

INTRODUCTION

Haloperidol (Hal) is a typical antipsychotic agent and clinically used for the treatment of psychological disorders such as schizophrenia and mania. Since 1993, our past research had shown that Hal had effects on vasodilation and anti-myocardial ischemia[1-4]. But its side effects on the extrapyramidal system limited large sample observation and further study. Therefore, in order to eliminate the side effects of Hal on the central nervous system (CNS), the chemical structure of Hal needs to be modified to decrease its passage through the blood-brain barrier. Using the piperidine group, we designed and synthesized a series of quaternary ammonium salt derivatives of Hal. F_2 is one of these compounds. It was named as *N*-*n*-butyl haloperidol iodide

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(Fig 1). Because of the high polarity and the low lipid solubility of the quaternary ammonium salt, it would be impossible for F_2 to pass through the blood-brain barrier. So the extrapyramidal side effects would be minimized. But the cardiac and vascular effects would hopefully be preserved. The following research confirmed our proposition. Rats treated with Hal deve-loped the Parkinson-like syndrome, such as increased muscle tone and tremors, oculogyric response and ataxia. However, F_2 did not result in any CNS reactions[5]. We further found that F_2 antagonized the reduction of coronary flow induced by pituitrin on guinea pig isolated heart[5], blocked the porcine coronary artery strip contraction induced by KCl[6]. F_2 was also shown to decrease the intracellular calcium fluorescence intensity[7]. In the present study, the *in vivo* animal model of heart I/R injury was used to examine the global effect of F_2 on myocardial ischemia. Furthermore, we investigated the effect of F_2 on I_{Ca} with single enzymatically-dissociated ventricular myocyte by the whole-cell configuration of the patch-clamp technique to elucidate the cardioprotective and vasodilatory mechanisms of F_2.

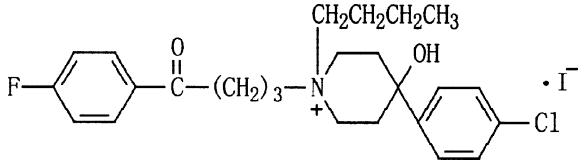

Fig 1. Chemical structure of *N*-*n*-butyl haloperidol iodide (F_2)

MATERIALS AND METHODS

Treatment of myocardial ischemia and reperfusion injury The model of heart I/R injury was processed on anesthetized Sprague-Dawley rats (234 g±18 g, *n*=60), provided by Experimental Animal Center of Shantou University Medical College, by the method similar to that previously described[8]. The 60 rats were randomly assigned into 6 groups: Group 1 (sham group, *n*=10). The coronary artery was surrounded by a silk thread but not ligated. Group 2 (I/R group, *n*=10). This group consisted of rats undergoing ischemia 30 min and reperfusion 30 min. Group 3 (Ver group, *n*=10). The operation of this group was the same as I/R group, but the rats received iv verapamil (2 mg/kg, Knoll AG, Germany) before induction of ischemia. Group 4, 5, 6 (*n*=10, for each group). The rats were given iv F_2 1, 2, and 4 mg/kg, respectively before induction of ischemia.

Determination of myocardial damage Creatine kinase (CK), creatine kinase isoenzyme MB (CK-MB), lactate dehydrogenase (LDH), α-hydroxybutyrate dehydrogenase (HBDH), and glutamic-oxaloacetic transaminase (GOT) were used as the markers of myocardial damage. The serum concentrations of these enzymes were measured by Automatic Analyzer (MODEL 7060, HITACHI, Japan). The kits were purchased from Randox Laboratories LTD (United Kingdom).

Determination of antioxidant enzyme and lipid superoxide level Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were used as indices of oxygen free radical and lipid superoxide level. They were measured using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China) with a spectrophotometer (UV-120-02, SHIMADZU, Japan).

Pathomorphological examination of myocardium After collection of blood, small pieces of myocardium at ischemic areas were collected and were cut into fragments (diameter=1 mm). Then they were fixed in 2.5 % glutaraldehyde, post-fixed with 2 % osmium tetroxide, dehydrated with the graded series of ethanol, passed through propyleneoxide, and then embedded in PDAP. Ultra-thin sections were stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (H-300, HITACHI, Japan) and photographed .

Isolation of rat ventricular myocytes Ventricular myocytes were isolated from Sprague-Dawley rats (235 g±15 g, *n*=15) by a collagenase enzymatic method similar to that previously described[9]. In brief, the heart was suspended in a constant flow Langendorff system. The heart was then perfused via the coronary artery with some modified Tyrode's solution in the following sequence: Ca^{2+}-free Tyrode's solution (mmol/L, pH 7.4): NaCl 135, KCl 5.4, $MgCl_2$ 1.0, NaH_2PO_4 0.33, HEPES10, glucose 10 for 6 min; enzymatic solution (mmol/L, pH 7.4): collagenase P 0.12 g/L (Roche Diagnostics, Boehringer Mannheim, Germany); taurine 20, $CaCl_2$ 0.075, NaCl 125, the other components the same as the Tyrode's solution for nearly 20 min. All of the solutions were saturated with 95 % O_2 and 5 % CO_2 at 37 °C ±0.5 °C. The retrograde perfusion pressure of Langendorff apparatus was 70 cm H_2O. Upon sufficient digestion of the tissue, the ventricle was cut into small pieces and gently agitated in the Kraft-Brühe (KB) solution (mmol/L, pH 7.2): KOH 85, *L*-glutamic acid 50, KCl 30, taurine 20, $MgCl_2$ 1.0, KH_2PO_4 30, HEPES 10, Glucose 10, egtazic acid 0.5. The solution was filtered,

then the cells were stored in KB solution at 4 °C.

Electrophysiologic measurement Cell preparations were perfused (2 mL/min) with modified Tyrode's solution containing (mmol/L, pH 7.4) $CaCl_2$ 1.8, tetraethylammonium-Cl (TEA) 0.01, the other components the same as the Tyrode's solution in a chamber (1 mL) on an inverted microscope (Olympus IX 71, Japan). Only rod-shaped cells with a clear margin and striation were used. The tight-seal whole cell recording techniques were used[10]. The heated-polished electrode had a resistance of 2-5 MΩ, when filled with the pipette solution containing (mmol/L) KCl 150, $MgCl_2$ 1.0, HEPES 5.0, egtazic acid 5.0, ATP-K_2 3.0, 4-amino-pyridine (4-AP) 5.0 (pH 7.2). Transmembrane currents were recorded with a patch-clamp amplifier (Axopatch 200B, Axon Instruments, USA). The current signal was filtered at 2 Hz and via a data acquisition system on a computer equipped with an AD converter (Digtal 1200, Axon Instruments, USA). The sampling and data analysis were obtained using the pClamp 8.1 software (Axon Instruments, USA).

Analysis of statistics Data were presented as mean±SD. The significance of group differences was determined by the Student's *t*- test. The effects of F_2 on currents were evaluated using the Student's paired *t*-test.

RESULTS

Biochemical studies F_2 reduced the release of CK, CK-MB, LDH, HBDH, and GOT from ischemic and reperfused myocardium, preserved the activity of SOD and decreased the MDA contents dose-dependently (Tab 1).

Morphological studies The sham-operated rat hearts showed normal ultrastructures (Fig 2A). In the myocardium from the ischemic-reperfused hearts, ultrastructures were damaged. They consisted of intracellular edema, myofibrillar derangements and rupture, swollen and damaged mitochondria, marginated and concentrated nucleus (Fig 2B). Similar to the Ver group (Fig 2C), F_2 obviously mollified these kinds of injuries (Fig 2D, E, F).

Effects of F_2 on L-type calcium currents Voltage pulses were applied every 5 s at holding potential of -40 mV, depolarizing potential of -40 mV to +60 mV, with 10-mV increment. I_{Ca} was elicited by depolarization from the depolarizing potential of -30 mV to +50 mV (Fig 3). When cells were exposed to Ver (1 μmol/L), the elicited current was almost completely blocked, indicating the characteristic of calcium current[11] (Fig 4). F_2 1 μmol/L reduced I_{Ca} by 73.9 % [from (1775±360) pA to (464±129) pA] (n=8, P<0.01). After washout of F_2 with modified Tyrode's solution for 5 min, calcium currents partially recovered (Fig 5). In addition, F_2 shifted the current-voltage curve of I_{Ca} upward, without affecting the voltage-dependent properties of I_{Ca} (Fig 6).

"Rundown" phenomenon of L-type calcium current It was possible that the so-called "rundown" phenomenon of I_{Ca} occurred[12]. Thus, the change in amplitude of I_{Ca} with time using the same experimental condition specimen was observed (n=8). In the control group ("rundown" group), after 5, 10, 15, and 20 min, the peak current was reduced by

Tab 1. Effects of *N-n*-butyl haloperidol iodide (F_2) on serum CK, CK-MB, LDH, HBDH, GOT concentrations, SOD activity, and MDA contents in I/R hearts of rats. n=10. Mean±SD. ^aP>0.05, ^bP<0.05 , ^cP<0.01 *vs* I/R group; ^dP>0.05, ^fP<0.01 *vs* F_2 1 mg/kg group; ^gP>0.05, ^iP<0.01 *vs* F_2 2 mg/kg group; ^jP>0.05, ^kP<0.05 *vs* verapamil (Ver) group.

Group	CK/U·L^{-1}	CK-MB/U·L^{-1}	LDH/U·L^{-1}	HBDH/U·L^{-1}	GOT/U·L^{-1}	SOD/kU·L^{-1}	MDA/mmol·L^{-1}
Sham	629±98^c	900±95^c	228±25^c	183±33^c	97±16^c	149±17^c	3.9±0.61^c
I/R	1825±310	2720±442	506±71	364±42	218±28	78± 9	9.6±1.2
Ver	979±144^c	1312±245^c	309±83^c	230±78^c	181±36^c	138±9^c	5.4±0.7^c
F_2 1 mg/kg	1591±202^a	2253±201^c	452±47^b	332±26^b	214±17^a	85±6^a	8.9±0.9
F_2 2 mg/kg	1178±131^{cf}	1538±114^{cf}	378±56^{cf}	298±45^{cd}	186±21^{cf}	127±8^{cf}	6.2±0.5^f
F_2 4 mg/kg	946±107^{cfij}	1116±140^{cfik}	305±31^{cfij}	228±37^{cfij}	167±21^{cfij}	134±7^{fgj}	5.0±0.8^{cfij}

CK: creatine kinase; CK-MB: creatine kinase isoenzyme MB; LDH: lactate dehydrogenase; HBDH: α-hydroxybutyrate dehydrogenase; GOT: glutamic-oxaloacetic transaminase; SOD: superoxide dismutase; and MDA: malondialdehyde.

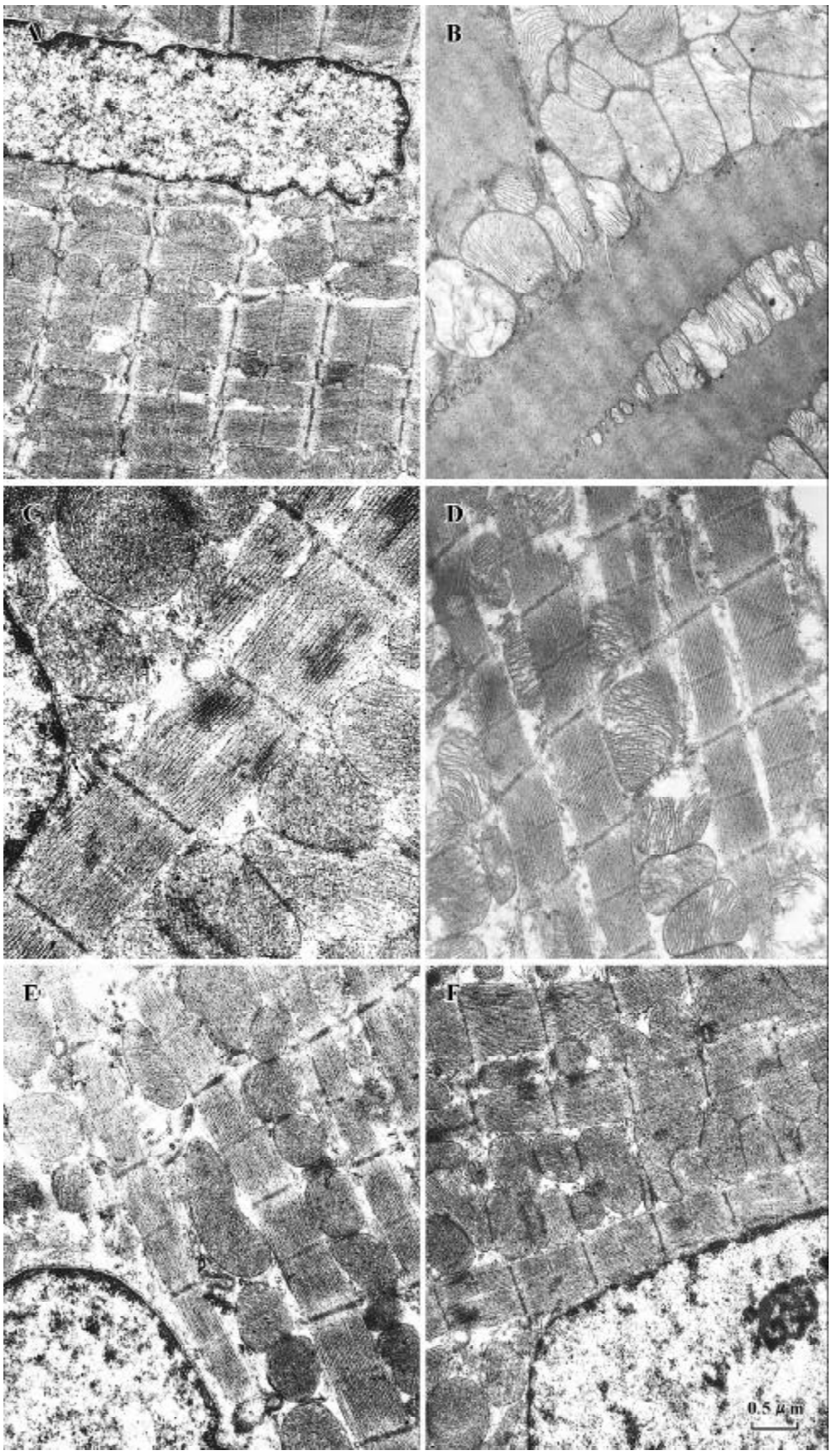

Fig 2. Transmission electron microscopy of rat myocardium. ×15 000. A: Sham group. B: Ischemia/reperfusion group. C: Ver group. D: F_2 1 mg/kg group. E: F_2 2 mg/kg group. F: F_2 4 mg/kg group.

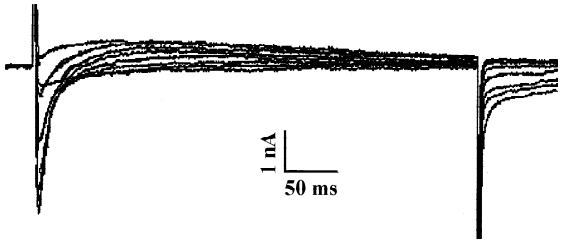

Fig 3. L-type calcium current. Voltage pulses were applied every 5 s at holding potential of -40 mV, depolarizing potential of -40 mV to +60 mV, with 10-mV increment. I_{Ca} were elicited by depolarization from the depolarizing potential of -30 mV to +50 mV. The peak I_{Ca} was elicited at the potential of 0 mV.

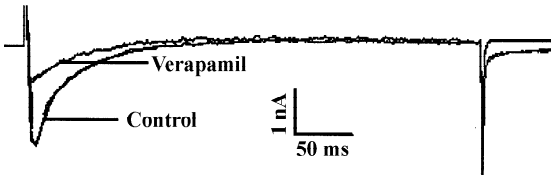

Fig 4. Effect of verapamil on L-type calcium current. The amplitude of peak I_{Ca} was decreased by verapamil (1 μmol/L).

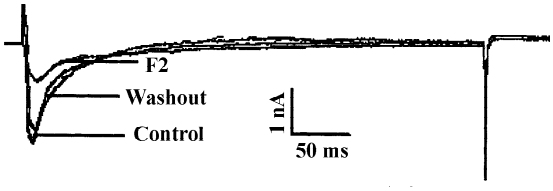

Fig 5. Effect of F_2 on L-type calcium current. The amplitude of peak I_{Ca} was decreased by F_2. After washout of out F_2, I_{Ca} partially recovered.

0.03 % (P>0.05 *vs* 0 min), 8.6 % (P>0.05 *vs* 0 min), 21.3 % (P<0.05 *vs* 0 min), and 38.3 % (P<0.01 *vs* 0 min).

DISCUSSION

Myocardial enzymes may be released from the injuried myocytes induced by ischemia and (or) reperfusion. So enzyme analysis has proved considerably valuable in the diagnosis of myocardial infarction. Meanwhile, myocardial enzymes such as GOT, LDH, HBDH, CK and CK-MB were often used as the markers of myocyte damage[13]. According to the present study, we found that F_2 similar to Ver[14], apparently decreased the serum concentrations of those enzymes. In addition, pathomorphological studies showed modifications of

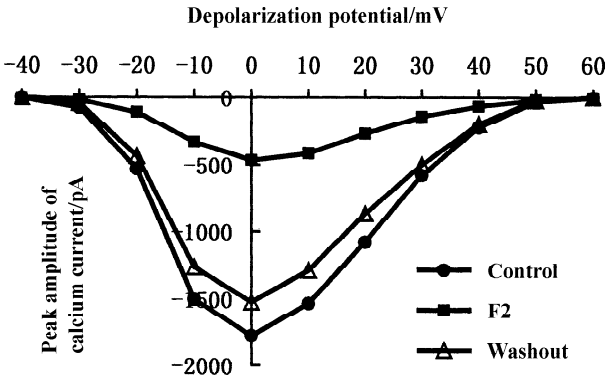

Fig 6. Effect of F_2 on *I-V* relation of I_{Ca} in ventricular myocytes. The current-voltage curve of I_{Ca} was shifted upward with the voltage-dependent properties of I_{Ca} not being affected.

myocardial damage induced by I/R injury in animals treated with F_2. All of these suggested that F_2 exerted a beneficial effect on ischemic and reperfused rat hearts.

Our previous research had shown that F_2 decreased the intracellular calcium concentration. In order to elucidate its cardioprotective and vasodilatory mechanisms, the effect of F_2 on L-type calcium channel of ventricular myocytes was investigated.

It is well known that there are two types (L and T) calcium channels in cardiac myocytes[15]. Under the condition of individual cell depolarization from holding potential of -40 mV, the L-type calcium channel was activated, while T-type Ca^{2+} channel and Na^+ channel were inactivated[16,17]. Moreover, TEA, a non-specificity K^+ channel blocker, was administered in the extracellular solution. The pipette solution was also filled with 4-AP (a K^+ channel blocker). So the outward K^+ currents were completely blocked. Furthermore, the recorded current could be completely inhibited by Ver, a typical L-type Ca^{2+} channel antagonist. Therefore, the inward current we recorded under these conditions was L-type Ca^{2+} current.

In this study, F_2 obviously suppressed the cardiac L-type calcium current. *I-V* relationship of I_{Ca} showed that the peak I_{Ca} was decreased by F_2 at all depolarizing potentials. But the activated potential, peak amplitude potential, and reversal potential of I_{Ca} were not changed. This indicated that the blocking effect of F_2 on calcium channels was voltage-independent. The so-called "run-down" phenomenon of I_{Ca} was also observed. The results showed that the currents were stable within the initial 10 min, with the administration of F_2 within the first 3 min. Five minutes after washing out F_2, I_{Ca}

recovered, indicating that the L-type calcium channel blocked by F_2 could be reactivated. These suggested that the blocking effects of F_2 on I_{Ca} were not the consequence of the "rundown" phenomenon. Based on the above, we could draw a conclusion that F_2 was a calcium channel blocker. So it not only gave support to the idea that F_2 decreased the intracellular calcium concentration[7], but also explained its vasodilatory effect due to calcium channel blockers.

As we all know, calcium overload and oxygen free radical have been postulated as the main underlying mechanisms for myocardial I/R injury. Calcium antagonists played a beneficial role on ischemic and reperfused myocardium[18]. Since F_2 could block the L-type calcium channel in myocytes, the intracellular calcium concentration is reduced because of the decrease of calcium influx. Thus, F_2, through attenuating "calcium overload", maintained the integrity of myofibrillar membrane and mitochondria, restored ATPase activity, and minimized ATP depletion. Besides, it induced coronary artery vasodilation, decreased heart rate and cardiac contractility, reduced myocardial oxygen consumption and ATP utilization. Therefore, F_2 exerted a protective effect against I/R injury.

Our present study showed that F_2 strongly preserved the activity of SOD and decreased the production of MDA, the lipid peroxidation metabolite. The result indicated that the protective effect of F_2 on myocardial I/R injury could be related to the antioxidation. However, we could not confirm F_2 as a direct oxygen radical scavenger or whether it influenced oxidation by acting as a calcium antagonist. It needs to be further investigated.

Based on the results of our present studies, we can conclude that F_2 exert a significant cardioprotective effect against I/R injury and block the L-type calcium channel. Our past research showed its effects of vasodilation and anti-myocardial ischemia. Moreover, with a structure different to the current cardiovascular agents, such as vasodilators, calcium channel blockers, potassium channel openers, it is worthwhile to further study other effects of F_2. For these reasons, we have obtained the Chinese national invention patent (No ZL96119098.1). We hope to develop it to be a novel drug to treat ischemic heart disease. However, other pharmacodynamics, pharmacokinetics, and toxicology of F_2 need to be further studied.

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