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Original Research

The effects of mebudipine on myocardial arrhythmia induced by ischemia-reperfusion injury in isolated rat heart

R. Ghiasi[1,2], M. Mohammadi[1], M. Majidinia[3], B. Yousefi[4], A. Ghiasi[5], R. Badalzadeh[1,2,6*]

[1] Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[2] Department of Physiology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[3] Department of Clinical Biochemistry, Faculty of Medicine, Urmia University Medical Sciences, Urmia, Iran
[4] Molecular Targeting Therapy Research Group, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[5] Department of Health Care Management, School of Public Health, Zabol University of Medical Sciences, Zabol, Iran
[6] Cardiovascular Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

Abstract: Reperfusion of the heart after an ischemic insult may lead to potentially lethal arrhythmias and cardimyocyte cell death via apoptosis and necrosis. In addition, previous studies showed that calcium channel blockers may have a protective role in the myocardium against arrhythmia and irreversible tissue injury. Therefore, this study was aimed to investigate the effects of mebudipine on myocardial arrhythmias and tissue injury induced by ischemia/reperfusion injury in isolated rat hearts. Male Wistar rats (250-300g) were randomly divided to Sham group (without ischemia), control group (ischemia without drug), drug group (ischemia with mebudipine 0.1nM) and vehicle group (ischemia with ethanol 0.01%). The hearts of anaesthetized rats were removed and mounted on Langendorff apparatus and perfused by Krebs-Henseleit solution under constant pressure of 75 mmHg at 37°C. Impulsive heart rate was monitored with bipolar golden electrodes. The electrocardiographs were recorded throughout the experiment and interpreted using the Lambeth convention. LDH and CPK activities in coronary effluent were analyzed spectrophotometrically. Hematoxilin & Eosin staining was performed for evaluation of microscopic architecture of the myocardium and tissue injury. Pretreatment with mebudipine significantly decreased the number of ventricular premature beats (VPB) as compared with control group. The similar findings were seen in the number of ventricular tachycardia (VT) and fibrillation (VF) among groups. In addition, mebudipine significantly reduced the severity of arrhythmias in comparison with control hearts. Moreover, the drug group demonstrated marked improvement in edema and infiltration of inflammatory cells especially with regard to the degree of myonecrosis and cell lysis.Mebudipine diminished the number and the incidence of myocardial arrhythmias induced by reperfusion injury and the severity of tissue injury.

Key words: Reperfusion, myocardial ischemia, arrhythmia, mebudipine.

Introduction

Ischemic heart disease (IHD) is the leading cause of death and disability and its occurrence is continuously increasing world-wide (1).The effects of IHD are usually attributable to the detrimental effects of myocardial ischemia/reperfusion injury (2). Myocardial ischemia develops when coronary blood supply to the myocardium is decreased, either in terms of absolute flow rate (low-flow or no-flow ischemia) or relative to increased tissue demand (demand ischemia) (3). An important feature of ischemia is that the oxygen supply to the mitochondria is inadequate to support oxidative phosphorylation. The ischemia may be followed by reperfusion, that is, the readmission of oxygen and metabolic substrates and washout of ischemic metabolites(4). The process of reperfusion is associated with further structural, biochemical, and functional changes in myocardium, including "myocardial stunning", microvascular injury and ''no-reflow'' phenomenon, as well as inflammatory responses and thus, the reperfusion may have a pivotal role in cell survival and death (5,6).

In addition, during ischemia and reperfusion, arrhythmias may develop ranging in severity from isolated ventricular premature beats, through runs of ventricular tachycardia, to ventricular fibrillation. Ventricular tachycardia and ventricular fibrillation remain the most important causes of sudden death following spontaneous restoration of blood flow (7-9). Growing evidence from both animal experiments and clinical observations indicates that myocardial infarction after ischemia and reperfusion is caused by necrosis and apoptosis (10, 11).

It has been long recognized that ischemia and reperfusion cause intracellular calcium overloading in cardiac cells and the administration of Ca^{2+} antagonists before ischemia to cadioplegic solutions has produced protective effects against ischemia/reperfusion injury (12,13). Ca^{2+} overload induces contracture (i.e. a sustained shortening and stiffening of myocardium), and contracture development cause tissue necrosis and cell death (14). Previous studies have demonstrated the efficacy of the Ca^{2+} channel blockers in protecting the myocardium against irreversible tissue injury (9,15,16). Mebudipine [(f)-t-butyl, methyl-1,4- dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate] is a new Ca^{2+} channel blocker with dihydropyridine structure that has comparable pharmacological effect while offering some advantages such as longer biological half time to reach

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*** Corresponding author:** Dr. Reza Badalzadeh, Department of Physiology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran. Email: rafighehg@gmail.com

peak effect, tissue selectivity as well as potent vasodilating activity and weak cardiodepressant action (17,18). Therefore, the present study was scheduled to evaluate the effects of mebudipine on myocardial arrhythmia and tissue injury induced by ischemia/reperfusion injury in isolated rat hearts.

Materials and Methods

Animals

Forty male Wistar rats (250-300 g) were obtained from laboratory animal house of Tabriz University of Medical Sciences. They were housed in an animal room at 22–24 ºC and given free access to commercial rat chow and tap water. All the experimental procedures employed, as well as rat care and handlings were in accordance with guidelines provided by the Animal Care Committee of the Tabriz University of Medical Sciences.

Isolated Heart Protocol

All animals were anesthetized with sodium pentobarbital (60 mg/kg i.p) and heparinized with sodium heparin (300IU i.p). After opening the chest cavity, the hearts were quickly excised and immersed in ice-cold krebs-Henseliet (K-H) solution. Then, the aorta was connulated and the hearts were retrogradely perfused via the aortic cannula in a Longendorff apparatus with K-H solution (pH=7.4) containing: 118 mM Nacl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.0 mM KH_2PO_4, 27.2 mM $NaHCO_3$, 10 mM glucose and 1.25 mM $CaCl_2$. The perfusate was bubbled with a mixture of 95% $O2$, and 5% CO_2. Perfusate and bath temperatures were maintained at 37°C by thermostatically controlled water circulator (Satchwell Sunvic LTD). The heart was perfused at constant mean pressure of 75-80 mmHg.

Experimental protocol

The animals were randomly divided in four groups (n=10):

(1) Sham group: in which the isolated hearts of animals were not subjected to ischemia, and continuously perfused with a normal K-H solution.

(2) Control group: in which after the surgical preparation and stabilization periods, the isolated hearts of animals were subjected to ischemia and reperfusion with a normal K-H solution.

(3) Drug group: in which the condition was similar to control group except that the hearts were perfused with a K-H solution containing mebudipine 0.1nM for 20 min before ischemia.

(4) Vehicle group: in which the condition was similar to control group except that the hearts were perfused with a K-H solution containing ethanol 0.01% (as a solvent of mebudipine) for 20 min before ischemia.

I/R injury protocol

We performed the I/R injury protocol as described previously with slight modifications previously (19). Briefly,the isolated hearts were allowed to equilibrate for 20 min prior to each study. For ischemic-control group, the hearts were perfused with K-H solution for 20 min, and then global ischemia was conducted by interrupting the aortic flow for 30 min followed by reperfusion with K-H solution up to 120 min. In the drug and vehicle groups, before ischemia the hearts were perfused with mebudipine (0.1nm) or ethanol (0.01%)-enriched solution for 25min, respectively, and the remaining of the experiment was similar to control group.

Ventricular arrhythmias and the Lambeth convention guidelines

Impulsive heart rate was monitored with bipolar golden electrodes that was described previously[20] which were placed close to the apex of the right atrium (RA), on the base (RV_1) and on the apex (RV_2) of the right ventricle. Electocardiograms were recorded throughout the experiment. Criteria for classification of ventricular arrhythmias were based on the Lambeth conventions. Accordingly, arrhythmias were categorized as a premature ventricular complex (PVC), ventricular salvos (VS), ventricular bigeminy (VB), which all these three items were named as ventricular premature beats (VPB) and were not analyzed separately, ventricular tachycardia (VT, run of 4 or more consecutive VPBs with corresponding effective left ventricular pressure), and ventricular fibrillation (VF, ventricular electrograms of irregular morphology without corresponding effective left ventricular pressure). During the first 30 minutes of reperfusion phase, the electrocardiograms were analyzed for (1) the number of ventricular premature complexes; (2) the number, timing, and incidence of ventricular tachycardia and ventricular fibrillation; and (3) the severity of arrhythmias. The scoring of arrhythmias was based on a 5-grade evaluation system, as follows: grade 0 for no arrhythmia, grade 1 for VPB, grade 2 for VB or VS, grade 3 for VT, and grade 4 for VF. If there was more than one type of arrhythmia in one sample, the highest grade of arrhythmia was reported.

CPK and LDH release measurement

For evaluation of myocardial cellular damage, lactate dehydrogenase (LDH) and cereatin phospho kinase (CPK) were measured in coronary effluent collected during the reperfusion period. CPK and LDH activities were evaluated spectrophotometrically with a commercially available assay kits (Roche Diagnostics, Germany. The absorbance of the solutions for CPK and LDH were detected at 340 nm and 492 nm, respectively. The results were reported in U/L.

Tissue injury studies

At the end of the experiment, myocardial tissue was immediately fixed in 10% buffered neutral formalin solution. The tissues were carefully embedded in molten paraffin, and kept under freezing plates to allow the paraffin to solidify. Cross sections (4 μm thick) of the fixed myocardial tissues were cut. These sections were stained with Hematoxylin and Eosin (H&E) and visualized under light microscope to study the light microscopic architecture of the myocardium. The degree of necrosis was graded and scored as follows: (-): Absence of any inflammation, edema and necrosis (healthy tissue); (+): Edema and myocardial cell intervals more than normal; (++): Edema and inflammation; (+++): Edema and inflammation and relative destruction of cells; (++++): Edema, inflammation and cell lysis.

Statistical analysis

All quantitative data was reported as mean ± SEM. Differences between incidences of arrhythmias was analyzed by *Fisher exact* test. The nonparametric *Kruskal–Wallis* test was used to analyze the data for count and duration of arrhythmias between groups. The parametric data were analyzed using *one-way ANOVA* followed by *Tukey* post hoc test. A level of $P<0.05$ was accepted as statistically significant.

Results

The effect of mebudipine on Ventricular arrhythmias

There were no significant differences in any baseline values between all groups as well as in any values of reperfusion phase between control and vehicle groups. According to the Lambeth convention, PVC, bigeminy and salvos were counted and all presented as ventricular premature beats (VPB). Preadministration of mebudipine significantly decreased the number of VPB as compared with control group (figure 1). In addition, the numbers of VT and VF in mebudipine-treated hearts were significantly lower than other groups (figure 2).

Furthermore, the durations of VT and VF in drug group were significantly lesser than those of control group (figure 3). The incidence of VT and VF were also reduced with mebudipine preadministration, but only the difference of VF incidence was statistically significant between groups (figure 4). Although ischemia reduced the heart rates from pre-ischemic values, there was no significant difference in heart rates between all groups (data not shown).

The effect of mebudipine on LDH and CPK activities

The activities of LDH and CPK released into the coronary effluent during the reperfusion phase in experimental groups are shown in Fig. 6. In the drug group, the hearts released less LDH and CPK than the control hearts ($p<0.05$). In other words, when compared to the control group, mebudipine significantly reduced the LDH and CPK levels in reperfusion injury.

The effect of mebudipine on tissue injury

Histological findings revealed that myocardial tissues receiving no ischemia (the sham group) were characterized by an organized pattern and showed normal architecture of the myocardial cells (figure 7A). Conversely, rat hearts subjected to I-R injury but without administration of drug (control and vehicle groups) demonstrated marked edema, confluent areas of myonecrosis, myofibrillary loss and sever inflammation as compared to those in the sham group (figure 7B, C). In myocardial

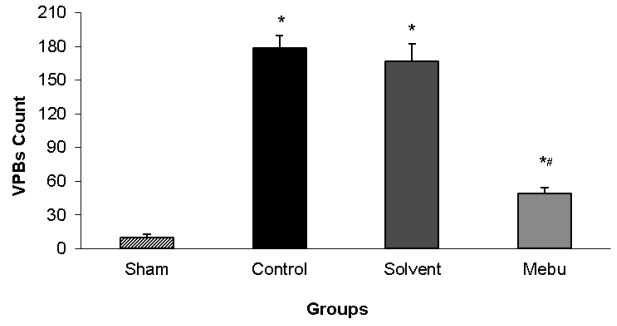

Figure 1. Effect of mebudipine on the number of ventricular premature beats (VPBs) during the reperfusion periods in experimental groups. Data are shown as the mean ± S.E.M. *p<0.05 as compared with sham group; and #p<0.05 as compared with control group. Mebu: mebudipine.

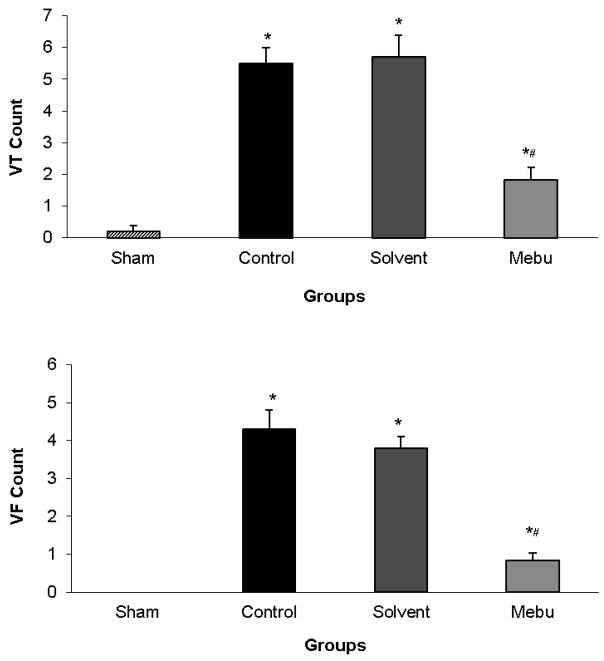

Figure 2. Effect of mebudipine on the number of ventricular tachycardia (VT) and number of ventricular fibrillation during reperfusion periods in experimental groups. Data are shown as the mean ± S.E.M. *p<0.05 as compared with sham group; and #p<0.05 as compared with control group. Mebu: mebudipine.

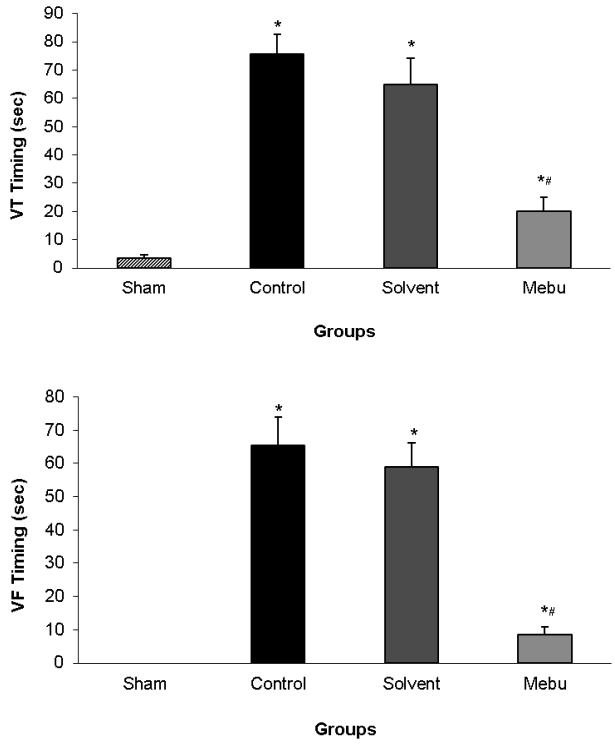

Figure 3. Effect of mebudipine on the timing of ventricular tachycardia (VT) during reperfusion periods in experimental groups. Data are shown as the mean ± S.E.M. *p<0.05 as compared with sham group; and #p<0.05 as compared with control group. Mebu: mebudipine.

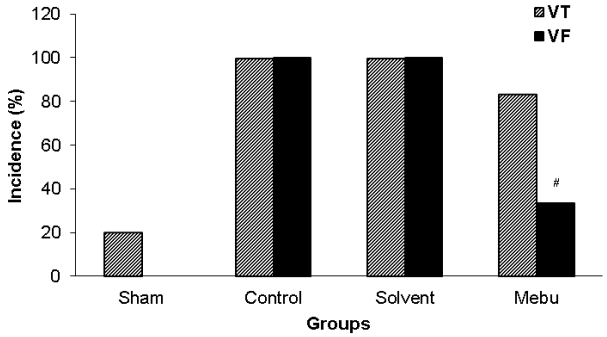

Figure 4. ect of mebudipine on the incidence of ventricular tachycardia (VT) and incidence of ventricular fibrillation (VF) during reperfusion periods in experimental groups. Data are shown as the mean ± S.E.M. #p<0.05 as compared with control group. Mebu: mebudipine.

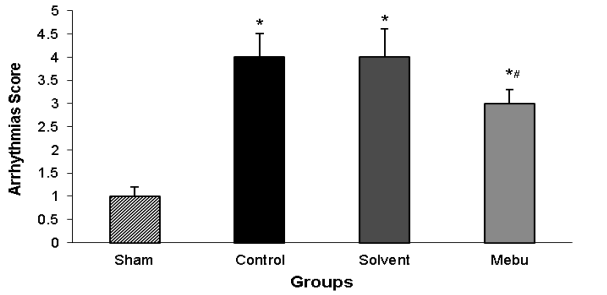

Figure 5. Effect of mebudipine on the severity of arrhythmias during reperfusion periods in experimental groups. Data are shown as the mean ± S.E.M. *p<0.05 as compared with sham group; and #p<0.05 as compared with control group. Mebu: mebudipine.

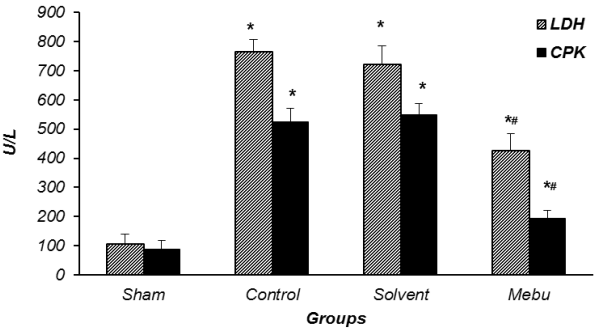

Figure 6. Effect of mebudipine on LDH and CPK levels during reperfusion periods in experimental groups. Data are shown as the mean ± S.E.M. *p<0.05 as compared with sham group; and #p<0.05 as compared with control group. Mebu: mebudipine.

cells of I-R hearts that received mebudipine displayed edema and inflammation, but demonstrated marked improvement in tissue injury especially with regard to the degree of myonecrosis and cell lysis, as compared to the control and vehicle groups (figure 7D).

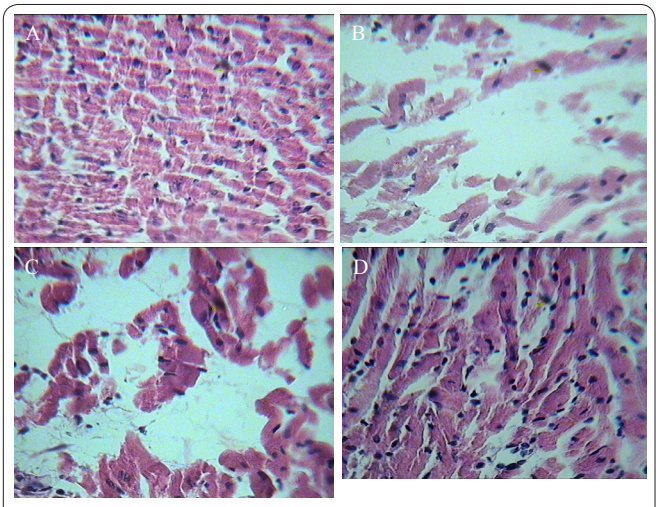

Figure 7. Effect of mebudipine on tissue injury. (A) Normal aspect of myocardial tissue in control hearts. (B, C) Acute, extensive myofibrillary degeneration, related to the inflammation and interstitial edema (marked myocardial injury) in myocardial tissue in control and vehicle groups. (D) Focal myocyte damage or small multifocal degeneration with slight degree of inflammatory process (mild myocardial injury) in myocardial tissue in mebudipine group (H&E, 660×).

Discussion

The present study indicated that mebudipine reduced the incidence of myocardial arrhythmias and tissue injury during ischemia/reperfusion injury in isolated rat hearts.

During ischemia, the cells become dependent on anaerobic glycolysis for their ATP supply (6,21). This causes to an accumulation of lactate, protons, and NAD+ and, therefore, leads a drop in cytosolic pH. For reestablishing normal pH, the cell extrudes H+ ions in exchange for Na+ via the plasmalemmal Na+/H+ exchanger (NHE). The Na+ ions are, in turn, exchanged for Ca2+ by the plasmalemmal Na^+/Ca^{2+} exchange (22). This increase in cytosolic Ca^{2+} is exacerbated upon reperfusion, where removal of extracellular H+ ions further increases the proton gradient across the plasmalemma, thereby accelerating NHE exchanger function. Additionally, the endoplasmic/sarcoplasmic reticulum Ca^{2+} store is also affected during ischemia/reperfusion injury. In particular, Ca^{2+} reuptake into the ER/SR by the SERCA ATPase is impaired by ischemia/reperfusion, whereas Ca^{2+} release through the ryanodine receptor is enhanced, both of which further exacerbate the lethal elevations in intracellular Ca^{2+} (23,24). This cellular Ca^{2+} overload during myocardial ischemia/reperfusion injury might induce arrhythmias.

Generally, the main cause of ischemia-induced arrhythmias is aberrations in impulse generation or a defect in impulse conduction. However, the main cause of reperfusion-induced arrhythmias remains poorly studied. A delay after depolarization-induced activity and an increase in Ca^{2+} concentration are thought to play a role (25). On the other hand, blocking the Ca^{2+} channel has been shown to be effective in preventing reperfusion arrhythmias in rats. In a study by Farkas *et al*. it was reported that the administration of verapamil and mibferadial before the main ischemia have elicited anti-arrhythmic effects in isolated rat hearts (26). Other studies have revealed that anti-arrhythmic influence of Ca^{2+} antagonists is attributable to the inhibition of slow inward ionic current (27,28); the site of action of these agents is likely the ischemic zone. Therefore, the Ca^{2+} antagonists maybe useful in treating the ischemia/reperfusion induced arrhythmias, for better efficacy, one should consider the tissue selectivity and voltage dependence of drug, which mebudipine has these pro-

perties. Ca^{2+} antagonists including mebudipine have higher affinity to affect the calcium with higher rising membrane potential. Therefore, the potency of Ca^{2+} antagonists in blocking Ca^{2+} channels is increased by any condition with increased membrane potential, since the potential of ischemic zone is greater than the other areas. In our study, pre-administration of mebudipine showed anti-arrhytthmic effects and it may imply the better effect of mebudipine in controlling ischemia/reperfusion-induced arrhythmias.

Previous studies have revealed that reactive oxygen species (ROS) are responsible in electrophysiological alterations and arrhythmias induced by reperfusion (29-31) and the endogenous or exogenouse agents scavenging or reducing ROS production, have decreased the reperfusion induced arrhythmias. In a previous study, we showed that mebudipine has anti-oxidative effects and it can increase the antioxidant enzymes levels, calatase and superoxide dismutase (31). Therefore, this may partly explain the reduced arrhythmias induced by mebudipine.

Mebudipine pretreatment also reduced the level of tissue injury indicated by lower CPK and LDH activity in drug group. Abnormal Ca^{2+} homeostasis and increased its cytosolic level during reperfusion cause cellular alteration leading to tissue injury and necrosis. In addition, increased intracellular Ca^{2+} concentration over-activates the hydrolytic enzymes which disturb the energy generation mechanism. These alterations may result in disruption of cell membrane integrity and finally lead to tissue necrosis. It has been reported also that the Ca^{2+} overload is associated with apoptotic cell death. Therefore, reduced tissue injury in mebudipine treated hearts is likely linked to its direct effect on reducing intracellular Ca^{2+} content.

It can be concluded that by decreasing the incidence of myocardial arrhythmias in ischemia and reperfusion injury, and the severity of tissue injury, mebudipine may has important protective effects against ischemia/reperfusion injuries in rat heart.

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