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Importance of Timing of Magnesium Administration in the Isolated Ischemic-Reperfused Rat Heart: Role of K_{ATP} Channels

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Summary

There is a growing interest for the beneficial effect of magnesium (Mg) in cardiovascular disorders. A number of cardiovascular disorders including myocardial infarction, arrhythmias and congestive heart failure have been associated with low extra-cellular or intracellular concentrations of Mg. The efficiency of the preconditioning effect of Mg on cardiac function and infarct size in the globally ischemic-reperfused isolated rat heart was studied together with the role of ATP-sensitive potassium (K_{ATP}) channels in protection induced by Mg. Rat hearts were Langendorff perfused, subjected to 30 min of global ischemia and 90 min of reperfusion, including treatment groups which focused on different times of Mg (8 mmol/l) use. Infarct size was measured by triphenyltetrazolium chloride (TTC) method. The left ventricular function was assessed by left ventricular developed pressure (LVDP), heart rate (HR) and coronary flow (CF). The administration of Mg before ischemia had an anti-infarct effect in rat hearts and improved cardiac function. The protective effects of magnesium was abolished by the blocking of K_{ATP} channels and suggests that K-ATP channel has an important role in the heart protection effect of Mg as a preconditioning agent.

Key words

K_{ATP} channel • Preconditioning • Reperfusion • Magnesium • Diazoxide • Glibenclamide

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Introduction

A considerable number of experimental, epidemiological and clinical studies point to an important role of magnesium in the etiology of cardiovascular pathology. A number of cardiovascular disorders including myocardial infarction, arrhythmias, sudden cardiac death, ventricular complications in diabetes mellitus and congestive heart failure have been associated with low extracellular or intracellular concentrations of Mg or abnormalities in Mg metabolism (Fox *et al.* 1997, Schechter *et al.* 2000, Chakraborti *et al.* 2002, Fuentes *et al.* 2006).

Magnesium plays an essential role in a wide range of fundamental cellular reactions in patients with ischemic heart disease. It also controls cardiac excitability, neuromuscular transmission, vasomotor tone, and blood pressure, among other functions such as maintaining myocardial electrical stability in hemodialysis patients (Yokoyama and Kawamura 2005).

There is growing interest for beneficial effect of Mg on the cardiovascular disorders. From an epidemiological view point, chronic Mg deficiency due to its low intake could play a role in the onset of ischemic heart disease, vasospasm of coronary artery and exacerbate several coronary risk factors such as hypertension, diabetes mellitus, and mental stress. Blood concentration of Mg in patients with ischemic heart disease, especially acute coronary syndrome, was lower than that of healthy subjects and may result in serious cardiac ischemia (Ueshima 2005).

The clinical application of Mg therapy in acute myocardial infarction still remains controversial (Stuhlinger *et al.* 2002). According to the large-scale

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clinical trials, the efficacy of Mg administrated to patients with acute myocardial infarction has not been established, but supplementary Mg may keep adequate blood Mg levels and protect from cardiac injury or cardiac ischemia (Ueshima 2005).

Despite these conflicting results, several experimental myocardial infarction studies have suggested that Mg treatment is potentially effective in reducing infarct size in different animal species. Furthermore, some studies have suggested that Mg supplementation should be considered as a preventive measure in atherosclerosis and ischemic heart disease and the intake of magnesium may have a modest inverse association with the risk of congestive heart diseases among men (Al-Delaimy *et al.* 2004, Ueshima 2005). Thus the optimum time and efficacy for administering Mg remain to be determined. The better understanding of mechanisms by which Mg acts as a cardioprotective agent will provide the ability of choosing the correct time for reasonable use of Mg.

The aim of this study was to compare protective effects of magnesium as a pharmacological preconditioning agent (Mg-Pre) and Mg administration during reperfusion (Mg-Rep) in the isolated globally ischemic-reperfused rat heart. Mg was administered shortly before ischemia for 5 min to see its preconditioning effect and during reperfusion time for 90 min to see its protection after ischemia. We also assessed whether heart protection against ischemia-reperfusion injury induced by Mg is mediated by K_{ATP} channels or not. To accomplish these goals, a K_{ATP} channel opener, diazoxide, and a channel blocker, glibenclamide, was used (Miura and Miki 2003). It has been suggested that classic sulfonylureas such as tolbutamide and glibenclamide (also known as glyburide) may have adverse effects on the cardiovascular system mainly because they also close mitochondrial K_{ATP} channels, which play a central role in ischemic preconditioning (IPC) protection (Li *et al.* 2000). Glibenclamide has been widely assumed to block both sarcolemmal and mitochondrial K_{ATP} channels, while Garlid *et al*. (1997) reported that diazoxide opened mitochondrial K_{ATP} channels with a $k_{1/2}$ of 0.8 mmol/l and 800 mmol/l was required to open the sarcolemmal K_{ATP} channels.

In this study, infarct size was used as the end point of injury because this measure is a robust indicator of preconditioning-induced cardioprotection.

Materials and Methods

Animals

A total of 77 male Sprague-Dawley rats (200-250 g) were used. Animals were kept in the normal animal room under standard laboratory conditions. All experiments were conducted in accordance with the institutional guidelines of Tehran University of Medical Sciences (Tehran, Iran) and the National Institutes of Health guidelines for the care and use of laboratory animals. The animals were randomly assigned to 11 treatment groups (n=7) and anesthetized by pentobarbital sodium (60 mg/kg b.w., intraperitonally).

Chemicals

Diazoxide, glibenclamid, $MgSO_4$ and TTC were obtained from Sigma-Aldrich (Deisinhofen, Germany) and general laboratory chemicals were purchased from Merck (Darmstadt, Germany). Stock solutions of diazoxide and glibenclamide were prepared separately and then diluted to appropriate concentrations in Krebs-Henseleit bicarbonate (KHB) buffer and equilibrated with 95 % O_2 - 5 % CO_2 (pH 7.4 at 37 ºC).

Isolated heart perfusion

Rats were anesthetized with pentobarbital sodium (60 mg/kg, ip) 30 min after treatment with heparin sodium (500 IU). Hearts were cannulated *in situ* after the induction of anesthesia and mounted on a non-recirculating, constant-pressure (80-100 mm Hg) Langendorff perfusion system and were perfused with an oxygenated (95 % O_2 - 5 % CO_2) normothermic (37 ºC) KHB buffer which had the following composition (in mmol/l): $NaHCO_3$ 25; KCl 4.7; NaCl 118.5; $MgSO_4$ 1.2; KH_2PO_4 1.2, glucose 11, $CaCl_2$ 2.5 (pH 7.4). The perfusion apparatus was water-jacketed to maintain a constant perfusion temperature of 37 ºC and during prolonged global ischemic periods hearts were immersed in KHB buffer at 37 ºC. Hearts were allowed to beat spontaneously throughout the experiments. A latex, fluid-filled, isovolumic balloon was introduced into the left ventricle through the left atrial appendage and inflated to give a preload of 8 to 10 mm Hg and connected to a pressure transducer (Harvard).

Hemodynamic data was monitored with a homemade program (Ossilo Graph Monitor, Medicore). Left ventricular developed pressure, heart rate, and coronary flow were registered at regular intervals. Ischemia was achieved by clamping the aortic perfusion

catheter in such a way that coronary flow was reduced to zero.

The rats were assigned into 11 experimental groups as: 1) controls (Con): hearts were perfused for 160 min; 2) ischemia-reperfusion (IR): in this group 30 min of stabilization were followed by 30 mins of global ischemia and 90 min reperfusion; 3) ischemic preconditioning (IPC): in this group, after stabilization, hearts were subjected to 5 min of ischemia and 5 mins reperfusion before global ischemia; 4) magnesium preconditioned group (Mg-Pre): after a stabilization, hearts were perfused with Mg (8 mmol/l) (Ebrahimi *et al*. 2004) for 5 min before global ischemia; 5) magnesium reperfused group (Mg-Rep): protocol for this group was as for the IR group except that hearts were perfused with Mg (8 mmol/l) during 90 min of reperfusion; 6) diazoxide-treated group (Dia): after stabilization, hearts were perfused with Dia (30 μmol/l) (Hicks *et al.* 1999) for 5 min before global ischemia; 7) glibenclamide-treated group (Gli): after stabilization, hearts were perfused with Gli (10 μmol/l) (Hicks *et al.* 1999) for 5 min before global ischemia; 8) magnesium preconditioned and diazoxide-treated group (Mg-Pre + Dia): after stabilization, hearts were perfused with 8 mmol/l Mg + 30 μmol/l Dia for 5 min before global ischemia; 9) magnesium preconditioned and glibenclamide-treated group (Mg-Pre + Gli): after stabilization, hearts were perfused with Mg (8 mmol/l) + Gli (10 μmol/l) for 5 min before global ischemia; 10) magnesium reperfused and diazoxide-treated group (Mg-Rep + Dia) was like Dia group, except that hearts were perfused with Mg (8 mmol/l) during 90 min reperfusion, and 11) magnesium reperfused and glibenclamide-treated group (Mg-Rep + Gli), was like Gli group, except that hearts were perfused with Mg (8 mmol/l) during 90 min reperfusion. Perfusions of drugs for 5 min in all experimental groups were started 10 min before global ischemia.

Hearts were perfused for 30 min to establish hemodynamic equilibrium. Equilibration was established when HR and LVDP were maintained at the same level for three continuous measurement periods timed 5 min apart. Baseline measurements were recorded at the end of this time.

Administration of drugs for 5 min were performed *via* the second arm of perfusate cannula which was connected to the main perfusion cannula and the experimental conditions were kept constant throughout the experiment.

Infarct size measurement

At the end of the reperfusion period, hearts were frozen and kept in a –20 ºC freezer to facilitate slicing of 2 mm transverse sections across the long axis. All hearts had approximately the same size (1.2 cm; atria and great vessels excluded). Slices were incubated in 1 % TTC in a phosphate buffer (pH 7.4) for 30 min at 37 ºC. After staining, slices were immersed in 10 % formaline for 24 hours to enhance the contrast between stained and unstained tissues. Tissues that were stained brick red were taken as viable, whereas pale or white tissues were taken as necrotic. The areas of the left ventricle and infarcted tissues were measured by way of a planimetry from the scanned hearts by using Photoshop program. Volumes were obtained by multiplying the area by the thickness of the slices. Infarct size was expressed as a percentage of left ventricular volume for each heart.

Statistical analyses

A statistical analysis was performed using SPSS (version 11.5 for Windows, SPSS, Chicago, IL, USA). Data are expressed as the mean ± S.E.M. To account for inter-animal variability, the functional indices were measured during treatment periods and at the end of 90 min reperfusion period and expressed as a percentage of the control value recorded for each heart before any test intervention was introduced. Groups were compared by one-way ANOVA. If a significant *F*-value was obtained, the Tukey test was used to identify individual group differences. Differences were considered statistically significant at $P< 0.05$.

Results

Because heart rate (HR) and left ventricular developed pressure (LVDP) may recover to different degrees, rate pressure product (RPP) was calculated by multiplying heart rate with LVDP and presented as reliable left ventricular function parameter for the isolated heart.

The infarct size was used as the end point of injury because this measure is a robust indicator of preconditioning-induced protection. The infarct sizes were determined by using computer-aided planimetry. RPP is expressed as a percentage of an individual baseline and infarct size is expressed as a percentage of left ventricular volume.

No differences were obtained between the experimental groups for RPP at the end of 30 min of

Table 1. Baseline hemodynamic characteristics.

Group	n	LVDP (mm Hg)	CF (ml/min)	HR (bpm)
Con	7	105.28± 4.43	10.2 ± 1.20	220 ± 12
IR	7	110.42 ± 2.32	10.15 ± 1.71	198 ± 16
IPC	7	108.17 ± 4.48	11.02 ± 0.98	203 ± 15
Dia	7	111.56 ± 3.42	10.76 ± 1.5	230 ± 14
Gli	7	112.32 ± 3.10	10.54 ± 1.48	223 ± 13
Mg-Pre	7	107.58 ± 3.76	11.08 ± 1.1	216 ± 10
Mg-Pre + Dia	7	109.52 ± 3.48	12.08 ± 1.3	210 ± 16
Mg-Pre + Gli	7	108.36 ± 5.88	10.89 ± 1.02	227 ± 10
Mg-Rep	7	107.32 ± 6.05	10.11 ± 2.02	213 ± 12
Mg-Rep + Dia	7	110.54 ± 5.28	11.00 ± 3.05	209 ± 21
Mg-Rep + Gli	7	105.09 ± 4.32	10.01 ± 1.19	221 ± 17

Values are mean ± S.E.M. n, number of hearts in each group; LVDP, left ventricular developed pressure; CF, coronary flow; HR, heart rate; Con, control; IR, ischemia-reperfusion; IPC, ischemic preconditioning; Dia, diazoxide; Gli, glibenclamide; Mg, magnesium; Pre, preconditioning; Rep, reperfusion.

adaptation before starting treatments and global ischemia (Table 1). During 30 min of global ischemia there was a reduction in RPP to zero which started to recover gradually by continuation of the reperfusion.

Diazoxide (30 μmol/l), as a mitochondrial K_{ATP} channel opener, increased the recovery of the RPP in Dia group (69 % basal value) compared with the IR group (38 % basal value, $P<0.05$), and significantly decreased infarct size in Dia group (10.2±1.27 %) compared with IR group (44.47±3.14 %, $P<0.001$). There was no significant difference in the Dia group compared with the control group for RPP (90 % basal value) and infarct size (1.0± 0.03 %).

In the IPC and Mg-Pre groups, RPP ultimately recovered to 80 % and 77 % basal value vs IR group ($P<0.001$) at the end of 90 min reperfusion.

The protection was associated with magnesium in Mg-Pre (9.79±0.76 %) and with ischemia in IPC (8.69 ±1.28 %) groups in decreasing infarct size compared with IR group ($P<0.001$). There were no significant differences in the RPP and infarct size in both IPC and Mg-Pre groups compared with control and Dia groups. Thus using Mg before the ischemia showed the same protection as a mitochondrial K_{ATP} channel opener (Diazoxide) and classical IPC.

The protection was associated with magnesium in Mg-Rep group in decreasing infarct size (29.25±3.41 %) which was significantly different from IR group ($P<0.01$). The infarct size and recovery of the RPP (58 % of basal value) in the Mg-Rep group were significantly different from the Mg-Pre group ($P<0.05$).

When Mg (both as Pre and Rep) was used with diazoxide, there were no significant differences in the recovery of RPP between Mg-Pre and Mg-Pre + Dia (70 % of basal value) or between Mg-Rep and Mg-Rep + Dia (58 % of basal value) groups at the end of 90 min reperfusion and also co-administration of Dia with Mg-Pre (11.26±1.4 %) did not show any further protection in decreasing infarct size in comparison with Dia and Mg-Pre alone.

Consequently, they did not show any useful effect of using two protective agents simultaneously, which may be a signal that Mg may induce its protection *via* mitochondrial K_{ATP} channels. However, the co-administration of Dia with Mg-Rep (20.08±3 % for Mg-Rep + Dia) decreased infarct size in so that there was a significant difference from IR group ($P<0.001$).

Glibenclamide was not able to increase the recovery of the RPP (52 % of basal value) and to decrease the infarct size (33.08±1.4 %) in the Gli group. There were significant differences between the Gli and Control groups ($P<0.001$) in the recovery of RPP and infarct size.

When glibenclamide was used in combination with Mg-Pre (52 % of basal value for Mg-Pre + Gli), recovery of the RPP was significantly different from Mg-Pre ($P<0.05$) and the control group ($P<0.001$).

Glibenclamide also abolished the protective effect of Mg-Pre group (29.1±2.17 % in Mg-Pre + Gli vs. 9.76±0.76 % in Mg-Pre, $P<0.001$) in decreasing infarct

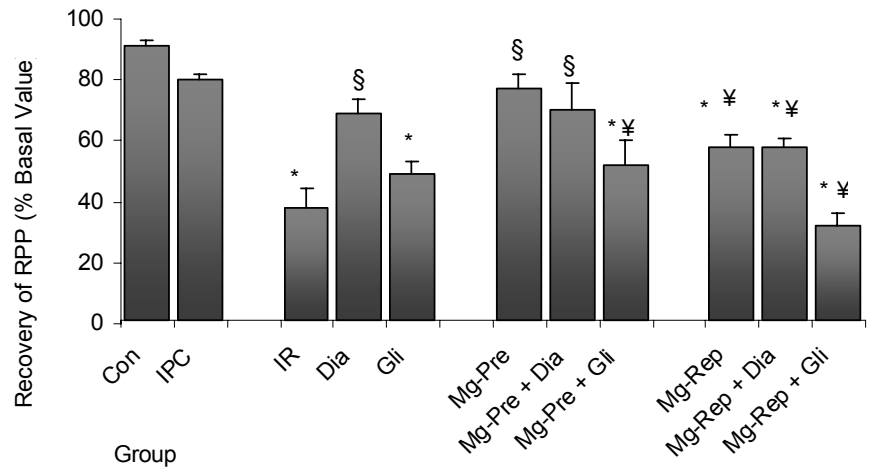

Fig. 1. Recovery of RPP (Basal Value %) in Con, IPC, IR, Dia, Gli, Mg-Pre, with Dia and Gli, and Mg-Rep, with Dia and Gli, groups. (n=7), data are presented as Mean ± S.E.M. Con, control; IR, ischemia- reperfusion; IPC, ischemic preconditioning; Dia, diazoxide; Gli, glibenclamide; Mg, magnesium; Pre, preconditioning; Rep, reperfusion. * Significant difference with Con group (P<0.001). § Significant difference with IR group (P<0.05). ¥ Significant difference with Mg-Pre group (P<0.05).

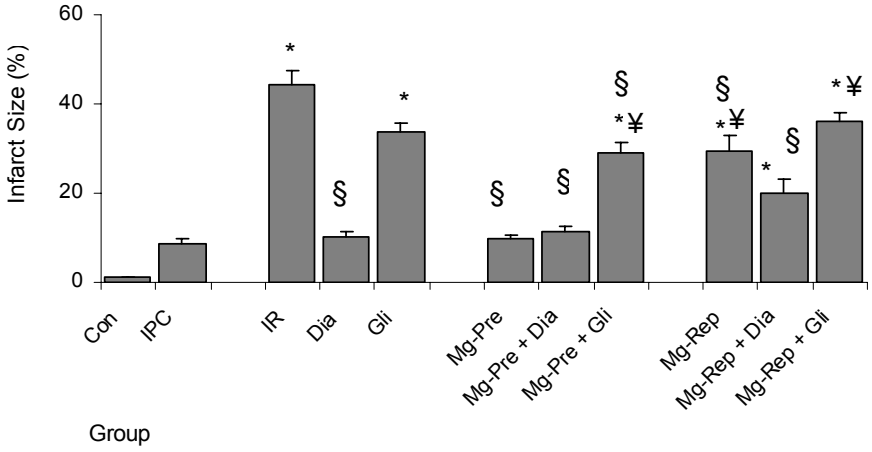

Fig. 2. Infarct Size (% of left ventricular) in Con, IPC, IR, Dia, Gli, Mg-Pre, with Dia and Gli, and Mg-Rep, with Dia and Gli, groups. (n=7), data are presented as Mean ± S.E.M. Con, control; IR, ischemia- reperfusion; IPC, ischemic preconditioning; Dia, diazoxide; Gli, glibenclamide; Mg, magnesium; Pre, preconditioning; Rep, reperfusion. * Significant difference with Con group (P<0.001). § Significant difference with IR group (P<0.01). ¥ Significant difference with Mg-Pre group (P<0.001).

size, whereas it had no effect on the Mg-Rep group (Figs 1 and 2).

Glibenclamide abolished Mg-Pre induced protection in recovery of the RPP and decreasing of infarct size, which suggests the role of K_{ATP} channels in this protection.

Discussion

Ischemic preconditioning (IPC) is still a laboratory-based phenomenon that has not been documented conclusively in patients (Robert and Engler 1996). Although there is no drug known that completely prevents myocyte necrosis, some agents can slow the rate of cell death. The administration of Mg has been reported to protect the myocardium against ischemia and reduce reperfusion injury (Naik *et al.* 1999). It has been reported that Mg therapy starting early after reperfusion is effective in reducing infarct size in a swine model (Jynge and Falk 1995). In contrast, Mg therapy reduced infarct size when it was administered before, but not after, reperfusion in a rat model (Maulik *et al.* 1999). Although many studies have shown the involvement of Mg in cardioprotection by enhancing post-ischemic functional recovery and/or myocardial infarct size, there is some controversy regarding the results of these studies (Sameshima *et al.* 1998, Maulik *et al.* 1999, Abbott *et al.* 2003, Shirey 2004, Ueshima 2005). In fact, some clinical studies have shown that chronic supplementation of oral Mg is well tolerated and could improve endothelial function in symptomatic heart failure patients and is a preventive element in atherosclerosis and ischemic heart disease. Al-Delaimy *et al.* (2004) suggest that the intake of magnesium may lower the risk of congestive heart disease (Ueshima 2005, Fuentes *et al.* 2006).

Strong evidence has supported the role of K_{ATP} channels as a mediator in IPC-induced cardio-protection against ischemia-reperfusion injury (Arena and Kass 1989, Wilde *et al.* 1989). Similarly, many studies have demonstrated that opening of K_{ATP} channels by pharmacological tools such as diazoxide, a selective mitochondrial K_{ATP} channels opener in cardiac myocyte, reduces myocardial injury, while blockade of these channels by some agents like glibenclamide eliminates

the protection resulting from diazoxide and IPC (Hamada *et al.* 1990, Wilde *et al.* 1990). It is generally accepted that the mitochondrial K_{ATP} is intimately involved in the cardioprotection induced by IPC. Garlid *et al.* (1997) provided the first direct evidence to support the role of mitochondrial K_{ATP} channels in cardioprotection. They found that the cardioprotective effect of diazoxide was abolished by the K_{ATP} channel antagonist 5-HD and glibenclamide, suggesting that mitochondrial K_{ATP} channels were responsible for this action (Garlid *et al.* 1997). The sacolemmal K_{ATP} channels may indeed act as a trigger for the opening of the mitochondrial K_{ATP}, or confer its own cardioprotection *via* a protein kinase C-dependent mechanism, which ultimately leads to a reduction in Ca overload during ischemia. Moreover, kir6.2-deficient mice are insensitive to IPC. Thus it appears that both sarcolemmal and mitochondrial K_{ATP} channels have complementary roles in the cardioprotection afforded by IPC; indeed they may act in concert (Peart and Gross 2002).

In the present study, we assessed the preconditioning effect of Mg in the protection of the heart with respect to both post-ischemic functional recovery and infarct size in comparison with the effect of Mg which was used during reperfusion time.

So far Mg has not been used as a preconditioning agent in isolated rat heart and only one study from our lab has demonstrated its protective effect when it was used as bolus injection (Ebrahimi *et al.* 2004). Mg was perfused 10 min before global ischemia for 5 min. The recovery of post-ischemic hemodynamic functions and the reduction of infarct size induced by Mg were significant and similar to those of IPC. When Mg was used after ischemia, it was not able to improve cardiac function, but decreased infarct size which was different from the control and IPC (Figs 1 and 2).

Experiments in animal models of myocardial infarction have provided the evidence that early Mg overload during or after ischemia (Ichikawa 1998) and adversely affect of mitochondria to generate ATP, which may be partly mediated by an increase in calcium overload in cytosol and/or mitochondria, leading to necrotic or apoptotic cell death in the ischemia-reperfused heart (Millane *et al.* 1994).

In this study it was clear that mitochondrial K_{ATP} channels play a role in the protection afforded by Mg used before ischemia. Matsusaka and Jin (2002) were the first to report on the mechanism of the infarct size-limiting effect of Mg in acute myocardial infarction. Recently, it was reported that matrix Mg regulates mitochondrial K_{ATP} channels in myocardium (Bednarczyk *et al.* 2005). In order to determine whether preconditioning effect of Mg is mediated *via* these channels, Mg was tested in the presence of a K_{ATP} channel opener, diazoxide, and a potassium channel blocker, glibenclamide. Our results demonstrated that administration of glibenclamide abolished the protective effect of Mg.

Therefore mitochondrial K_{ATP} channels may be the target for the preconditioning effect of magnesium. Elam and Lansman (1995) also demonstrated that Mg has been a contributing factor in the gating of K^+ channels and binds to the closed channels during hyperpolarization and prevents its opening until it is occupied by potassium. We did not study any other possible mechanism for Mg preconditioning, but blockade of this effect by glibenclamide, a K_{ATP} channels blocker, was clear in our experiment. In addition, the co-administration of diazoxide with magnesium in Mg-Pre + Dia group did not show any further protection in comparison with Dia and Mg-Pre groups, which may be a signal that Mg induces its protection almost exclusively *via* mitochondrial K_{ATP} channels.

Diazoxide, K_{ATP} channel opener, had the same cardioprotective effect as Mg-Pre and classic ischemic preconditioning and the concentration of diazoxide (30 μmol/l) is able to open mitochondrial subtype of K_{ATP} channels only. Therefore the opening of mitochondrial K_{ATP} channels is an important mechanism for induced cardioprotection when Mg is used as a pharmacological preconditioning agent (Garlid *et al.* 1997).

Continuous magnesium infusion effectively reduces the rate of arrhythmias following cardiopulmonary bypass surgery for congenital heart disease and causes quick recovery without increasing the incidence of dysrhythmia in pediatric patients, and should, therefore, be routinely used (Dittrich *et al.* 2003, Hoshino *et al.* 2003, Jian *et al.* 2003). Its antiarrhythmic effect may be related to its pharmacological properties but not to normalization of the circulating magnesium concentration (Kiziltepe *et al.* 2003).

Hhypomagnesemia appears to have an adverse pathophysiological effect and Mg deficit and other electrolyte abnormalities are frequent disorders in patients with congestive heart failure (Cohen *et al.* 2003). The therapeutic value of Mg in the management of coronary risk factors and ischemic heart disease has been clarified and the administration of magnesium can be

considered as clinically valuable.

While the beneficial effects of Mg are apparent, further research is needed for the incorporation of these findings on the myocardial protective role of Mg in order to reduce morbidity and mortality of patients suffering from a variety of cardiac diseases.

Conflict of Interest

There is no conflict of interest.

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