The role of K –ATP channel in the preconditioning effect of magnesium in the rat isolated heart

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ABSTRACT

There is growing interest for beneficial effect of Mg in the cardiovascular disorders. A number of cardiovascular disorders including myocardial infarction, arrhythmias and congestive heart failure have been associated with low extracellular or intracellular concentrations of Mg. The aim of present study was to investigate the preconditioning effects of magnesium (Mg) on cardiac function and infarct size in the globally ischemic-reperfusion in isolated rat heart. Rat hearts were Langendorff-perfused, subjected to 30 minutes of global ischemia and 90 minutes of reperfusion, and assigned to one of the following treatment groups with 7 hearts in each group: (1) control, (2) ischemic-reperfusion, (IR), (3) ischemic preconditioning, (IPC) of 5 minutes of global ischemia - reperfusion before lethal ischemia; or pretreatment with (4) 30 μmol/L of Diazoxide (Dia), (5) 8 mmol/L magnesium, (6) 10 μmol/L glibenclamid (Gli), (7) magnesium and Dia and (8) magnesium and Gli. Infarct size was measured by the triphenyltetrazolium chloride method. Left ventricular function was assessed by left ventricular developed pressure (LVDP), heart rate and coronary flow (CF). Mg limited infarct size (9.76 % vs 44.47% in IR, $P< 0.001$) as did Dia (10.2 % vs 44.4 % in IR $P< 0.001$) and IPC (8.69 % vs 44.47% in IR, $P< 0.001$). The protective effect of magnesium was abolished by Gli. Administration of Mg had an anti-infarct effect in ischemic-reperfusion isolated rat hearts and improved cardiac function. Blockade of K-ATP channel abolished the protective effects of magnesium and suggest that K-ATP channel has an important role in this effects.

Key words: K-ATP channel, infarction, ischaemia, magnesium, Diazoxide, Glibenclami

INTRODUCTION

There is growing interest for the beneficial effects of Mg in the cardiovascular disorders. A number of cardiovascular disorders including myocardial infarction, arrhythmias and congestive heart failure have been associated with low extracellular or intracellular concentrations of Mg (1-3).

While elevation of the extracellular Mg by the use of cardioplegic solutions has a variety of clinical uses (4) and implication of Mg imbalances in cardiovascular pathophysiologies (5), the clinical application of Mg therapy in acute myocardial infarction remains controversial (6). It has been reported that Mg therapy reduced the mortality rate in acute myocardial infarction in the Leicester Intravenous Magnesium Intervention Trial 2 (LIMIT-2). However, a significant effect on mortality rate for Mg could not be demonstrated in the International Study of Infarct Survival 4 (ISIS-4)(7).

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(8). However, the mechanism for the efficacy of Mg is not fully understood.

The aim of this study was to compare protective effects of magnesium, IPC and one of the preconditioning mimetic agents, diazoxide, which is a K-ATP channel opener (9). We also assessed whether the mechanism by which magnesium protects the rat heart against ischemic-reperfusion injury, is mediated by ATP-sensitive potassium (K-ATP) channel or not. To accomplish these goals, the effect of the coadministration of Mg, with diazoxid, an ATP-sensitive potassium (K-ATP) channel opener, and glibenclamid, a potassium channel (K-ATP) blocker was investigated. Glibenclamide is one of the sulfonylurea drugs, which induces membrane depolarization and results in an influx of calcium in pancreatic β-cells. Calcium acts as a second messenger for the release of insulin to the bloodstream(9). However, it has been suggested that classical 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-

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ATP channels which play a central role in ischemic preconditioning (IP) protection(10).

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

MATERIALS AND METHODS

Animals

A total of 56 male Sprague-Dawley rats (200-250 g) were used. Animals were kept in the normal animal room under standard conditions. Animals were randomly assigned to 1 of 8 treatment groups (n=7), anaesthetized by pentobarbital sodium (60 mg/kg bodyweight, intraperitonally.).

Chemicals

Diazoxide, Glibenclamid, MgSO4 and TTC were obtained from Sigma-Aldrich (Deisinhofen, Germany). Chemicals were from Merck (Darmstadt, Germany). Stock solutions of diazoxide and Glibenclamid were prepared separately and then diluted to appropriate concentrations in KHB and equilibrated with 95% O_2–5% CO_2 (pH 7.4 at 37 ºC).

Isolated Heart Perfusion

Rats were anesthetized with sodium pentobarbital (60 mg/kg, ip) 30 minutes after treatment with heparin sodium (500 IU). Hearts were cannulated in situ after induction of anesthesia and mounted on a nonrecirculating, constant-pressure (80-100 mm Hg) Langendorff perfusion system and were perfused with oxygenated (95% O 2–5% CO2) normothermic (37ºC) Krebs'–Henseleit bicarbonate (KHB) buffer which had the following composition (mmol/L):

NaHCO3 25; KCl 4.7; NaCl 118.5; MgSO4 1.2; KH2PO4 1.2; glucose 11;CaCl2 2.5 (pH 7.4). The perfusion apparatus was water-jacketed to maintain a constant perfusion temperature of 37 º C and, during prolonged global ischemic periods, hearts were immersed in KHB buffer at 37 º C. 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 transduser (Harvard). Homodynamic data were 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 experimental groups were : 1- control (Con), hearts were perfused for 160 minute; 2- ischemic-reperfusion (IR), in this group 30 minute of stabilization followed by 30 minute of global ischemia and 90 minute reperfusion; 3-ischemic preconditioning (IPC), in this group after stabilization, hearts were subjected to 5 minutes of ischemia and 5 minute perfusion before global ischemia; 4-diazoxide treated group(Dia), after stabilization, hearts were perfused with 30 µmol/L Dia for 5 minute before global ischemia; 5-magnesium treated group(Mg), after stabilization, hearts were perfused with 8 mmol/L Mg for 5 minute before global ischemia; 6-glibenclamid treated group(Gli), after stabilization, hearts were perfused with 10 µmol/L Gli for 5 minute before global ischemia; 7-Magnesium and Dia treated group (Mg + Dia), after stabilization, hearts were perfused with 8 mmol/L Mg+30 µmol/L Dia for 5 minute before global ischemia and 8- Magnesium and Gli treated group (Mg+Gli), after stabilization, hearts were perfused with 8 mmol/L Mg + 30 µmol/L Dia for 5 minute before global ischemia. All groups had a 90 minute reperfusion after global ischemia. Control hearts were perfused for 165 minute without any ischemia but all the other groups were subjected to 30 minutes of global ischemia.

Hearts were perfused for 30 minute to establish equilibrium haemodynamics which was established when HR, LVDP and CF were maintained at the same level for three continuous measurement periods of 5 minute apart. Baseline measurements were recorded at the end of this time.

Magnesium was dissolved in the Krebs buffer in the concentration of 8 mMol/L. Diazoxide and Glibenclamide were prepared in high concentration as stock solutions and were diluted in the Krebs buffer just before the use.

The Mg group was perfused for 5 min with Mg sulphate (8 mMol/L)(11) in Krebs buffer followed by 5 minutes washout 10 min before global ischemia. The diazoxide group (Dia) and glibenclamid (Gli) group were treated with 30µmol/L of diazoxside and with 10µmol/L of glibenclamid (12) similar to magnesium group. In group 7 and 8 treatment protocols consisted of co-administration of magnesium with diazoxide for the group 7 and with glibenclamide for the group 8.

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

Infarct size measurement

At the end of the reperfusion period, hearts were frozen and kept at –20º C 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% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) for 30 min at 37º C. After staining, slices were immersed in 10% formalin 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 infracted tissues were measured by planimetry from the scanned hearts by using Photoshop. Volumes were obtained by multiplying the area by the thickness of the slice. Infarct size was expressed as a percentage of left ventricular volume for each heart.

Statistical analyses

Statistical analysis was performed using SPSS (version 11.5 for Windows; SPSS, Chicago, IL, USA). Data are expressed as the mean ± SEM. To account for interanimal variability, the functional indices were measured during treatment and 120 min reperfusion periods and expressed as a percentage of the control value recorded for each heart before any test intervention was made. 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

A- Hemodynamic Data

1-Heart Rate (HR)

No differences were noted between the experimental groups for basal heart rate at the end of 30 minute adaptation (table 1).During 30 minute global ischemia heart rate reduced to zero and returned gradually to the baseline after continuation of the reperfusion. There was no significant differences in the heart rate of IPC, Mg, Dia and Mg +Dia groups at the end of 90 minute reperfusion with control group, (Figure 1). At the end of experiment, recovery of heart rate for IPC was 93% vs 90% for control, for Dia was 75 % vs 90% for control, for Mg was 88% vs 90% for control and for Mg +Dia was 70% vs 90% for control. Data are expressed as a percentage of individual baselines.

The heart rate by the simultaneous use of Mg+Dia (70% for Mg+Dia Vs 88% for Mg group) was a little lower than those by the use of individual agents which can be a signal that Mg may induce its protection almost via K-ATP channels.

Heart rate was not reached to baseline in IR, Gli and Gli + Mg groups at the end of 90 minute reperfusion and showed significant differences in comparison with control group ($P < 0.001$, Figure 2). Recovery of heart rate in IR group was 26% vs 90% for control, $P < 0.001$, and for Gli group was 35% vs 90% for control, $P < 0.001$ at the end of 90 minute reperfusion which showed significant differences in comparison to the control group (Figures 1 and 2).

When glibenclamide was used in combination with magnesium, it abolished the protection induced by magnesium (40 % for Mg + Gli vs 26 % for IR, $P < 0.001$).This data was not significantly different from Gli group (40 % for Mg + Gli vs 35 % for Gli) and interestingly was significantly different from Mg group (40 % for Mg + Gli vs 88 % for Mg , $P < 0.001$),which may suggests a function of K-ATP channels in magnesium induced recovery of heart rate (Figure 2).

2-Rate Pressure Product (RPP)

Because heart rate (HR) and Left Ventricular Developed Pressure (LVDP) may recover to different degrees, RPP was calculated via multiplying heart rate by LVDP and presented as a reliable homodynamic data for isolated heart.

No differences were obtained between the experimental groups for RPP at the end of 30 minute adaptation, before starting treatments and global ischemia (table 1).During 30 minute global ischemia there was a reduction in RPP to zero which started to recover gradually by continuation of the reperfusion. Data are expressed as a percentage of individual baselines. In IPC (80 % basal value), Mg (77 % basal value), Dia (69 % basal value) and Mg +Dia (70 % basal value) groups at the end of 90 minute reperfusion the recovery of RPP were not significantly different from control group (90% basal value, Figure 3).

Recovery of RPP in Mg + Dia group (70% for Mg + Dia vs 77 % for Mg) at the end of 90 minutes reperfusion did not show any useful effect of using two protective agents simultaneously which may be a signal that magnesium induces its protection almost via K-ATP channels.

Recovery of RPP was not reached to baseline in IR (38 % vs 90% for control, $p < 0.001$) and Gli (48.9 % vs 90% for control, $p <0.001$) groups at the end of 90 minute reperfusion and showed significant differences in comparison with control group (Figure 3).

When glibenclamide was used in combination with magnesium, it abolished the protection induced by magnesium (52 % for Mg + Gli vs 77 % for Mg) which was significantly different from Gli (52 % for Mg + Gli vs 48.9 % for Gli) group

Table 1. Baseline haemodynamic Characteristics Values are Mean ± SEM

Group	n	LVDP(mmHg)	CF(mL/min)	HR(b.p.min)
Con	7	105.28± 4.43	10.2 ± 1.2	220 ± 12
IR	7	110.42 ± 2.32	10.15 ± 1.71	198 ± 16
IPC + IR	7	108.17 ± 4.48	11.02 ± 0.98	203 ± 15
Dia + IR	7	111.56 ± 3.42	10.76 ± 1.5	230 ± 14
Mg + IR	7	107.58 ± 3.76	11.08 ± 1.1	216 ± 10
Gli + IR	7	112.32 ± 3.1	10.54 ± 1.48	223 ± 13
Mg + Dia + IR	7	109.52 ± 3.48	12 .08 ± 1.3	210 ± 16
Mg + Gli + IR	7	108.36 ± 5.88	10.89 ± 1.02	227 ± 10

n, number of hearts in each group; LVDP, left ventricular developed pressure; CF, coronary flow; HR, heart rate; Con, control; IR, ischemic- reperfusion; IPC, ischemic preconditioning; Dia, diazoxide; Mg, magnesium; Gli, glibenclamid.
There were no significant differences between groups after 30 minute of stabilization

as well. Recovery of RPP in Mg group was significantly different from IR group (77 % for Mg vs 38 % for IR, P < 0.01), but when magnesium was used with glibenclamid, there was no significant differences in the recovery of RPP between Mg + Gli and IR groups (52 % for Mg + Gli vs 38 % for IR) at the end of 90 minute reperfusion, which suggests the role of K-ATP channels in magnesium induced recovery of RPP (Figure 4).

B- Infarct Size

The infarct sizes were determined by using computer-aided planimetry. In control group, infarct size was 1 ± 0.03% of the heart .The protection associated with K-ATP channel opener (Dia), Mg and IPC groups, in decreasing infarct size were 10.2 ± 1.27%, 9.76 ± 0.76 % and 8.69 ± 1.28 % respectively. In the IR and Gli groups infarct size were 44.47 ± 3.14% and 33.08 ± 1.4 % respectively.

Table 2. Infarct size, expressed as percentage of left ventricular volume. Values are mean ± SEM

Group	Mean of Infarct Size
Con	1.00±.03
IR	44.47 ± 3.14
IPC + IR	8.69 ± 1.28
Dia + IR	10.2 ± 1.27
Mg + IR	9.76 ± 0.76
Gli + IR	33.08 ± 1.4
Dia + Mg + IR	11.26 ± 1.4
Gli + Mg + IR	29.1 ± 2.17

Con, control; IR, ischemic- reperfusion; IPC, ischemic preconditioning; Dia, diazoxide; Mg, magnesium; Gli, glibenclamid.

The infarct size of Mg + Gli group (29.1 ± 2.17 %) was significantly different from IPC group (8.96 ± 1.28 %) and from Mg group (9.76 ± 0.76 %) with P < 0.001.

Co-administration of Dia + Mg (11.26 ± 1.4 %) did not show any further protection in comparison with Dia (10.2 ± 1.27 %) and Mg (9.76 ± 0.76 %) alone (Table 2).

As expected, IPC significantly reduced the amount of infarcted tissue in comparison with IR hearts (8.69 ± 1.28 % for IPC vs 44.47 ± 3.14% for IR, P<0.001; Figures 4) as did Mg (9.76 ± 0.76 % for Mg vs 44.47 ± 3.14% for IR, P<0.001) and Dia (10.2 ± 1.27 % for Dia vs 44.47 ± 3.14% for IR, P<0.001).Glibenclamide abolished the protective effect of Magnesium (29.1 ± 2.17 % in Gli+Mg vs 9.76 ± 0.76 % in Mg, P<0.001), whereas diazoxide did not increase protective effect of Mg (11.26 ± 1.4 % in Dia + Mg vs 9.76 ± 0.76 % in Mg; Figure 5).

DISCUSSION

Ischemic preconditioning (IPC) is still a laboratory-based phenomenon that has not been documented conclusively in patients(13). Although drug is known to prevent myocyte necrosis completely, some agents can slow the rate of cell death. Administration of Mg has been reported to protects the myocardium against ischemia and reduces reperfusion injury(14). It has been reported that Mg therapy starting early after reperfusion is effective in reducing infarct size in a swine model(15).In contrast, Mg therapy has been reported to reduce infarct size when it is administered before, but not after reperfusion in a rat model(16).Although many studies have shown involvement of Mg in cardio protection by post ischemic functional recovery and/or myocardial infarct size, there are some controversy regarding the results of these studies (8, 16-19). This may be due to differences in species, end-point, experimental models or intervention which have been used. Thus the optimum time for administration of Mg remains to be determined and understanding the mechanisms by which Mg

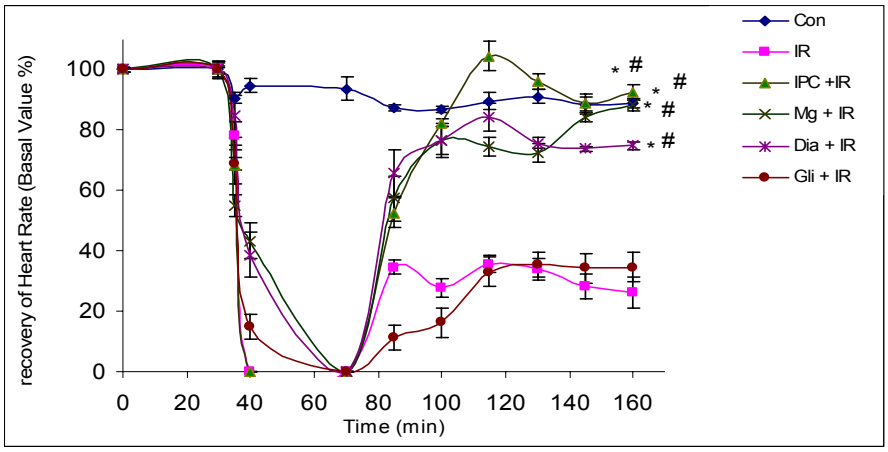

Figure 1. Recovery of heart rate (Basal Value %) in Con, IR, IPC, Mg, Gli and Dia groups. (n=7)
Con, control; IR, ischemic- reperfusion; IPC, ischemic preconditioning; Dia, diazoxide; Mg, magnesium; Gli, glibenclamid.
* Significant difference with IR group ($P < 0.001$), # Significant difference with Gli group ($P < 0.001$)

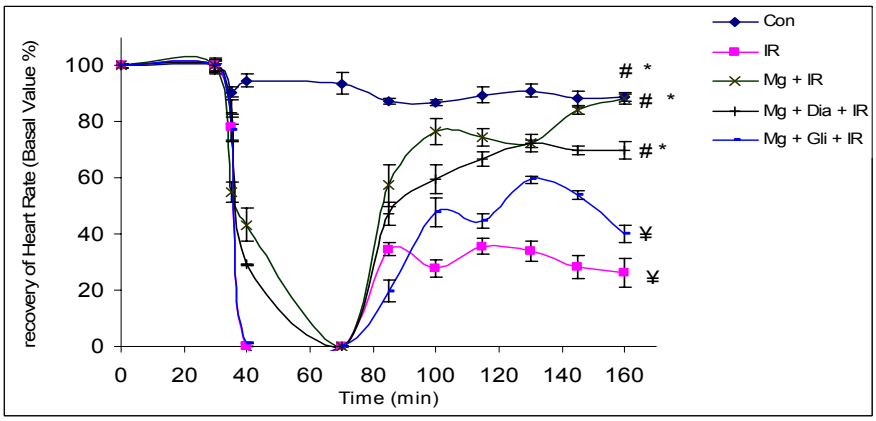

Figure 2. Recovery of heart rate (Basal Value %) in Con, Mg, IR, Mg + Dia and Mg + Gli groups. (n=7)
Con, control; IR, ischemic- reperfusion; IPC, ischemic preconditioning; Dia, diazoxide; Mg, magnesium; Gli, glibenclamid.
* significant difference with IR group ($P < 0.001$), # significant difference with Mg + Gli group ($P < 0.001$)
¥ significant difference with Mg group ($P < 0.001$)

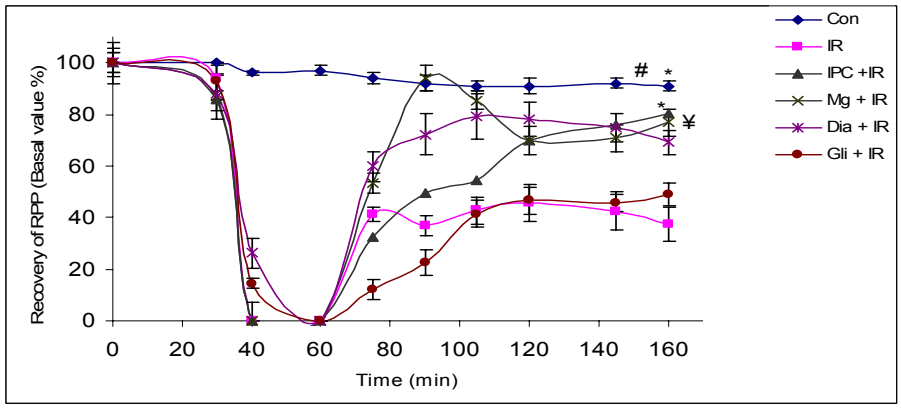

Figure 3. Recovery of RPP (Basal Value %) in Con, IR, IPC, Mg, Gli and Dia groups. (n=7)
Con, control; IR, ischemic- reperfusion; IPC, ischemic preconditioning; Dia, diazoxide; Mg, magnesium; Gli, glibenclamid.
* significant difference with IR group ($P < 0.001$), ¥ significant difference with IR group ($P < 0.01$)
# significant difference with Gli group ($P < 0.001$)

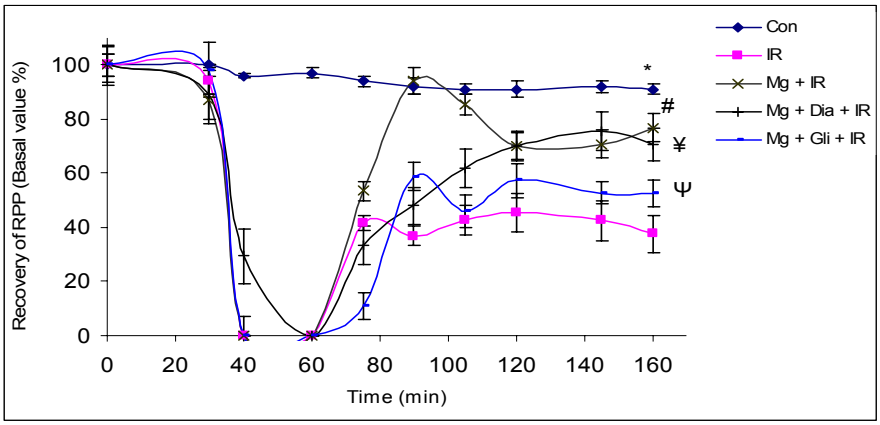

Figure 4. Recovery of RPP (Basal Value %) in Con, Mg, , IR, Mg + Dia and Mg + Gli groups. (n=7)
Con, control; IR, ischemic- reperfusion; IPC, ischemic preconditioning; Dia, diazoxide; Mg, magnesium; Gli, glibenclamid.
* significant difference with IR group ($P < 0.001$), # significant difference with IR group ($P < 0.01$)
¥ significant difference with IR group ($P < 0.05$), Ψ significant difference with Con group ($P < 0.001$)

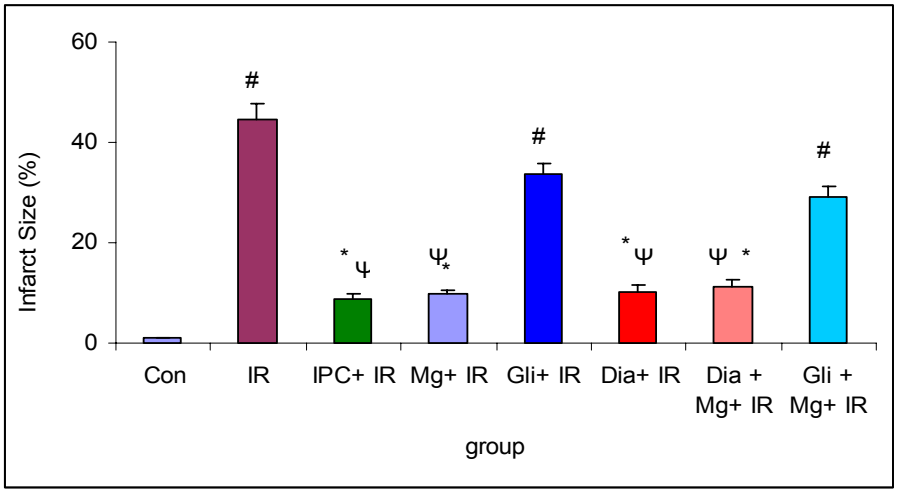

Figure 5. Infarct Size (%) in Con, IR, IPC, Mg, Dia, IPC, Gli, Mg + Dia and Mg + Gli groups. (n=7)
Con, control; IR, ischemic- reperfusion; IPC, ischemic preconditioning; Dia, diazoxide; Mg, magnesium; Gli, glibenclamid.
* significant difference with IR group ($P < 0.001$), # significant difference with Mg group ($P < 0.001$)
Ψ significant difference with Gli group ($P < 0.001$)

acts as cardio protective agent will provides opportunity for the correct time and reasonable uses of Mg.

Strong evidences have supported the role of K-ATP channels as mediators in IPC and cardio protection against ischemia and reperfusion injury(20, 21), and many studies have demonstrated that opening of K-ATP channels by pharmacological agents such as diazoxide, a selective m K-ATP channel opener in cardiac myocytes, reduces myocardial injury and blockade of these channels by some agent like glibenclamide, eliminates protective effects of diazoxide and IPC (22, 23).

In the present study, the preconditioning effect of Mg in the protection of the heart with respect to both post ischemic functional recovery and infarct size was determined and compared with those of IPC and diazoxide, a K-ATP channel opener.

Mg has not been used as a preconditioning agent in isolated rat heart and only one study has demonstrated its protective effect when it was used as bolus injection (11). In the present study Mg was perfused 10 minutes before global ischemia for 5 minutes and the recovery of post ischemic homodynamic functions and the reduction of infarct size induced by Mg were significant and similar to those of IPC and Dia (Figures 1, 2 and 3).

The other aim of this study was to clarify the role of K-ATP channel in the protection afforded by Mg. Matsusaka et al, for the first time reported the mechanism of the infarct size-limiting effect of Mg in acute myocardial infarction (24). Recently it was reported that matrix Mg regulates the m K-ATP channels in myocardium (25). In the present study in order to determine whether preconditioning effect of Mg is expressed via

these channels, Mg was tested in the presence of diazoxide as a K-ATP channel opener, and glibenclamid as a potassium channel blocker. Our results demonstrated that administration of glibenclamid completely abolished the protective effects of Mg. Therefore K-ATP channel may be the target for preconditioning effects of Mg. Experiments in animal models of myocardial infarction have provided evidences that early Mg infusion may limits infarct size by prevention of calcium overload during or after ischemia (26) and defects in ability of mitochondria to generate ATP, may at least in part, be mediated by an increases in calcium overload in cytosol and/or mitochondria, leading to necrotic or apoptotic cell death in the ischemic-reperfused heart (27). Lansman has also demonstrated that Mg has contribution in gating of K channels and it binds to the closed channel during hyperpolarization and prevents its opening until it is occupied by potassium (28). While other possible mechanisms for Mg preconditioning has been determined, in our experiment blockade of this effect by glibenclamid, a K-ATP channel blocker, was clear. Therefore opening of K–ATP channel might be an important mechanism of Mg protection as a pharmacological preconditioning agent in myocard.

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