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Importance of timing of magnesium administration in the isolated ischemic-reperfused rat heart: role of K-ATP channels.

Short Title: Importance of timing of magnesium administration

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Abstract

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. Also the role of ATP-sensitive potassium (K-ATP) channels in protection induced by Mg was studied. Rat hearts were Langendorff perfused, subjected to 30 minutes of global ischemia and 90 minutes of reperfusion, including treatment groups which focused on different times of Mg (8 mM/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, Glibenclamid

Introduction

A considerable number of experimental, epidemiological and clinical studies are now available which point to an important role of Mg 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 (Burrows et al. 1997; Merz et al. 2000; Chakraborti 2002; Fuentes et al. 2006).

Mg 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 2005).

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

While the elevation of extracellular Mg along with the use of cardioplegic solutions, has a variety of clinical uses (Hanafusa et al. 1997) and implication involved in Mg imbalances in cardiovascular pathophysiologies is clear (Agus 2001). The clinical application of Mg therapy in acute myocardial infarction still remains controversial (Stuhlinger 2002). According to the large-scale clinical trials, the efficacy of Mg administrated to patients with acute myocardial

infarction has not been established, however, supplementary Mg may keep blood Mg levels adequately in check 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 element 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. Also the understanding of mechanisms by which Mg acts as a cardio protective agent will provide the ability of choosing correct time and 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 administrated shortly before ischemia for 5 minutes to see its preconditioning effect and during reperfusion time for 90 minutes to see its protection after ischemia. We also assessed whether heart protection against ischemic-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

reported that diazoxide opened mitochondrial K-ATP channels with a k 1/2 of 0.8 mM/L and 800 mM/L was required to open the sarcolemmal K-ATP channels (Garlid et al 1997).

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 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. In addition animals were randomly assigned to 1 of 11 treatment groups (n=7), anesthetized by pentobarbital sodium (60 mg/kg bodyweight, intraperitonally).

Chemicals

Diazoxide, Glibenclamid, $MgSO_4$ and TTC were obtained from Sigma-Aldrich (Deisinhofen, Germany) and general laboratory chemicals were acquired from Merck (Darmstadt, Germany). Stock solutions of diazoxide and Glibenclamid 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 37º C).

Isolated Heart Perfusion

Rats were anesthetized with pentobarbital sodium (60 mg/kg, ip) 30 minutes after treatment with heparin sodium (500 IU). Hearts were cannulated in situ after the induction of anesthesia and mounted on a nonrecirculating, constant-pressure (80-100 mm Hg) Langendorff perfusion system and were perfused with an oxygenated (95% O 2–5% CO2) normothermic (37º C) KHB buffer which had the following composition (in 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 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 experimental groups were assigned into 11 groups as: 1- control (Con): hearts were perfused for 160 minutes; 2- ischemic- reperfusion (IR): in this group 30 minutes of stabilization followed by 30 minutes of global ischemia and 90 minutes reperfusion; 3-ischemic preconditioning (IPC): in this group, after stabilization, hearts were subjected to 5 minutes of ischemia and 5 minutes reperfusion before global ischemia; 4-magnesium preconditioned group(Mg-Pre): after stabilization, hearts were perfused with Mg (8 mM/L) (Ebrahimi et al, 2004) for 5 minutes 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 mM/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 minutes before global ischemia; 7-glibenclamid treated group(Gli): after stabilization, hearts were perfused with Gli (10 µmol/L) (Hicks et al. 1999) for 5 minutes before global ischemia; 8-magnesium preconditioned and Dia treated group (Mg-Pre + Dia): after stabilization, hearts were perfused with 8 mmol/L Mg + 30 µmol/L Dia for 5 minutes before global ischemia; 9- magnesium preconditioned and Gli treated group (Mg-Pre +Gli): after stabilization, hearts were perfused with Mg (8 mmol/L) + Gli (10 µmol/L) for 5 minutes before global ischemia; 10-magnesium reperfused and Dia treated group(Mg-Rep + Dia) was like Dia group, except that hearts were perfused with Mg (8 mM/L) during 90 min reperfusion and 11-magnesium reperfused and Gli treated group(Mg-Rep + Gli), was like Gli group, except that hearts were perfused with Mg (8 mM/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 minutes to establish hemodynamics equilibrium and Equilibration was established when HR and LVDP were maintained at the same level for three continuous measurement periods timed 5 minutes apart. Baseline measurements were recorded at the end of this time.

Addminstration of drugs for 5 minutes were performed via the second arm of perfusate cannula which was connected to the 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 in a –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% TTC in a phosphate buffer (pH 7.4) for 30 min at 37º C. After staining, slices were immersed in 10% formalin 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 infracted 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 ± SEM. To account for interanimal 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 via multiplying heart rate by LVDP and presented as reliable left ventricular function parameter for the isolated heart.

Also 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 minutes adaptation before starting treatments and global ischemia (table 1). During 30 minutes 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± 0.03 %).

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 (0<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.

So using Mg before the ischemia showed protection same 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% 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 with Mg-Pre + Dia (70% basal value) or between Mg-Rep with Mg-Rep + Dia (58% 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 induces its protection almost via mitochondrial K-ATP channels. But co-administration of Dia with Mg-Rep (20.08 ± 3% for Mg-Rep + Dia) decreased infarct size in a way that there was significant difference with IR group ($P<0.001$).

Glibenclamide was not able to increase the recovery of the RPP (52% 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% basal value for Mg-Pre + Gli), recovery of the RPP was significantly different from Mg-Pre ($p<0.05$) and the control group ($P < 0.001$).

Also glibenclamide 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 size, whereas it had no effect on the Mg-Rep group (Fig 1,2).

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(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 1999). It has been reported that Mg therapy starting early after reperfusion is effective in reducing infarct size in a swine model(Jyage 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 involvement of Mg in cardio protection by 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 there are 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 and suggests that the intake of magnesium may have a modest inverse

association with risk of congestive heart disease among men (Al-Delaimy et al. 2004; Ueshima 2005; Fuentes et al. 2006).

Strong evidence has supported the role of K-ATP channels as a mediator in IPC and cardioprotection against ischemia and reperfusion injury (Arena and Kass 1989; Wilde et al. 1989). Similarly many studies have demonstrated that opening of K-ATP channels by pharmacological factors such as diazoxide, a selective mitochondrial K-ATP channels opener in cardiac myocyte, reduces myocardial injury and blockage of these channels by some agents like glibenclamide, eliminates 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 provided the first direct evidence to support a role for 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). Also 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 complimentary 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 those of Mg which was used during reperfusion time.

Mg has not been used as a preconditioning agent in isolated rat heart and only one study in our lab has demonstrated its protective effect when it was used as bolus injection (Ebrahimi et al. 2004). Mg was perfused 10 minutes before global ischemia for 5 minutes. 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 (Fig 1 and 2).

Experiments in animal models of myocardial infarction have provided evidences that early Mg infusion may limit infarct size by the preventing calcium overload during or after ischemia (Ichikawa 1998) and defect the ability of mitochondria to generate ATP, which may, at least in part, mediate by an increase in calcium overload in cytosol and/or mitochondria, leading to necrotic or apoptotic cell death in the ischemic-reperfused heart (Millane et al. 1994).

In this study it was clear that mitochondrial K-ATP channels played a role in the protection afforded by Mg usage before ischemia. Matsusaka et al, for the first time reported the mechanism of the infarct size-limiting effect of Mg in acute myocardial infarction (Mutsusaka 2002). 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 expressed via these channels, Mg was tested in the presence of a K-ATP channel opener, diazoxide, and a potassium channel blocker, glibenclamid. Our results demonstrated that administration of glibenclamid abolished the protective effect of Mg.

Therefore mitochondrial K-ATP channels may be the target for the preconditioning effect of Mg. Lansman 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 (Lansman 1995). We did not study any other possible mechanism for Mg preconditioning but in our experiment blockage of this effect by glibenclamid, a K-ATP channels blocker, was clear. Also 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 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 (Garlid et al. 1996). Therefore opening of mitochondrial K –ATP channels is the important mechanism of Mg protection as a pharmacological preconditioning agent in myocardium.

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; Su et al. 2003). Its antiarrhythmic effect may relate to its pharmacological properties and unrelate to normalization of the circulating magnesium concentration (Kiziltepe et al. 2003).

Reportedly, hypomagnesemia 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 administration of magnesium can consider clinically valuable.

While the beneficial effects of Mg are apparent, further research is needed for the incorporation of these findings toward the development of novel myocardial protective role of Mg to reduce

morbidity and mortality of patients suffering from a variety of cardiac diseases. Thus the optimum time for administration of Mg remains to be determined and also the understanding of mechanisms by which Mg acts as cardioprotective agent will provide the ability of choosing correct time and reasonable use of Mg.

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Group	n	LVDP(mmHg)	CF(mL/min)	HR(b.p.min)
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

Table 1: Baseline hemodynamic Characteristics.
Values are Mean ± SEM.
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; Gli, glibenclamid; Mg, magnesium; Pre, preconditioning; Rep, reperfusion.
There were no significant differences between groups after 30 minutes of stabilization.

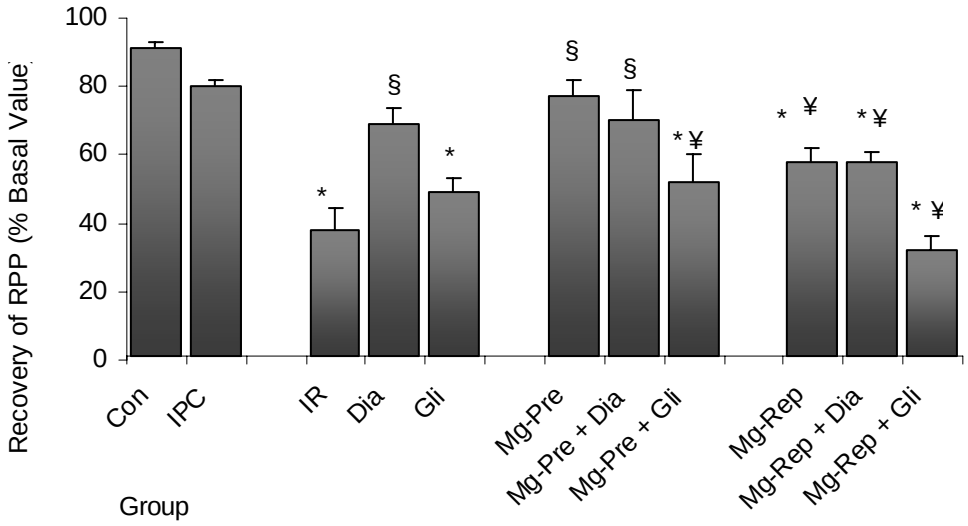

Figure 1: Recovery of RPP (Basal Value %) in Con, IPC, IR, Dia, Gli, Mg-Pre, with Dia and Gli, and Mg-Rep, with Dia and Gli, groups. (n=7),
Group data are presented as Mean ± SEM.

Con, control; IR, ischemic- reperfusion; IPC, ischemic preconditioning; Dia, diazoxide; Gli, glibenclamid; 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 ($P < 0.05$)

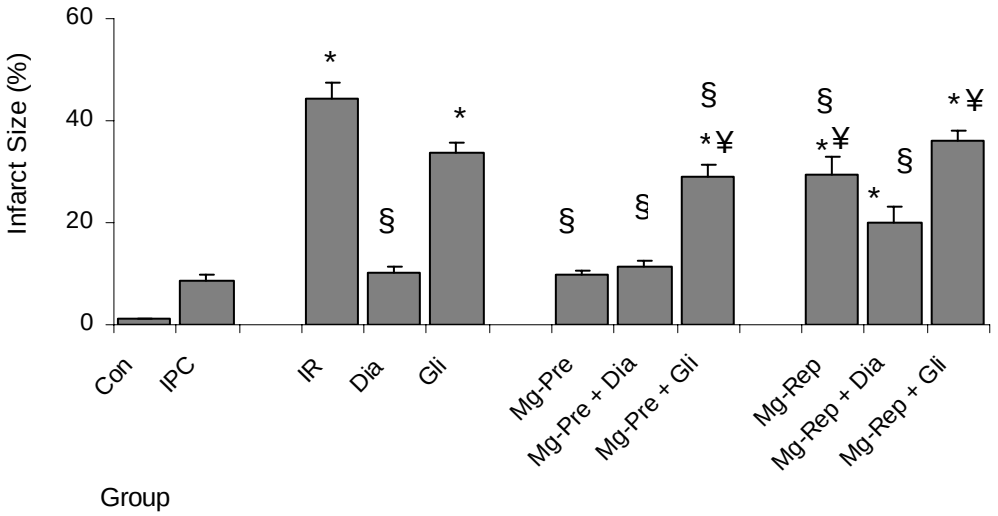

Figure 2: Infarct Size (% of left ventricular) in Con, IPC, IR, Dia, Gli, Mg-Pre, with Dia and Gli, and Mg-Rep, with Dia and Gli, groups. (n=7),
Group data are presented as Mean ± SEM.

Con, control; IR, ischemic- reperfusion; IPC, ischemic preconditioning; Dia, diazoxide; Gli, glibenclamid; 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 ($P < 0.001$)