The effects of diazepam in cardio depressant concentration on the function of isolated rat heart in ischemia-reperfusion

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ABSTRACT

الأهداف: فحص آثار تركيز مخفضات الجهاز القلبي لعقار ديازيبام على وظيفة القلب المعزول للجرذ في حالة نقص – إعادة التروية.

الطريقة: أجريت هذه الدراسة بمركز الأبحاث الأحيائية الطبية – كيرمانشاه – إيران، خلال الفترة ما بين نوفمبر 2006م وحتى مارس 2007م. تم إخضاع قلوب الجرذان المعزولة لمدة 40 دقيقة من نقص التروية و45 دقيقة من إعادة التروية. تمت إضافة عقار ديازيبام بمقدار(100µM) في محلول التسريب لمدة 10 دقائق قبل نقص التروية في مجموعة الاختبار. كما تم قياس متغيرات القلب المختلفة بما فيها ضغط البطين الأيسر (LVDP)، ومعدل النبض (HR)، وتيار الدم في الشريان التاجي (CF). تم حساب منتج ضغط النبض (RPP)، وحُدد التقلص خلال فترة نقص التروية حتى لحظة تقلص نقص التروية والتقلص الأقصى. في إعادة التروية تم قياس تدفق خمائر اللبن المنزوعة الهيدروجين المفرزة (LDH) تم تحديد الشفاء الوظيفي القلبي.

النتائج: تبين أن عقار ديازيبام يخفض بنجاح معدل إنتاج الضغط (RPP)، ويزيد من تيار الدم في التاجي (CF) قبل نقص التروية. في المجموعة التي تلقت عقار ديازيبام (عدد= 10)، أثناء نقص التروية، كان التقلص الأقصى ملحوظاً بشكل أقل من مجموعة التحكم (عدد=14). كما زاد عقار ديازيبام بشكل ملحوظ من الشفاء الوظيفي وتيار الدم في التاجي (CF) عند إعادة التروية.

خاتمة: يخفض عقار ديازيبام (100µM) التقلص الأقصى خلال نقص التروية، ويحسن من عملية الشفاء لوظيفة عضلة القلب وتيار الدم في الشريان التاجي (CF) في حالة إعادة التروية. تظهر النتائج أن تركيز مخفضات الجهاز القلبي لدى عقار ديازيبام آمن، ويحمي بشكل نسبي في حالة القلب المعزول للجرذ المصاب الذي أجريت له نقص التروية – إعادة التروية. قد تتوسط هذه الآثار بواسطة تثبيت الكالسيوم الحالي في خلايا عضلة القلب.

Objective: To investigate the effects of cardio depressant concentration of diazepam on the function of isolated rat heart in ischemia-reperfusion.

Methods: This study was performed at the Medical Biology Research Center, Kermanshah, Iran from November 2006 to March 2007. Isolated, perfused rat hearts were subjected to 40 minutes normothermic global ischemia and 45 minutes reperfusion. Diazepam (100µM) was added to the perfusion solution for 10 minutes before ischemia in the test group. Different cardiac variables including left ventricular developed pressure, heart rate, and coronary flow (CF) were measured. Rate pressure product (RPP) was calculated, during the ischemic period time until onset of ischemic contracture and maximum contracture were determined. In reperfusion, released lactate dehydrogenase enzyme in effluent was measured and cardiac functional recovery was determined.

Results: It was found that diazepam significantly decreased RPP and increased CF before ischemia. In the diazepam group (n=10), during ischemia, maximum contracture was significantly lower than the control group (n=14). Also, diazepam significantly increased functional recovery and coronary flow in reperfusion.

Conclusion: Diazepam (100 µM) significantly decreased maximum contracture during ischemia, improved the recovery of myocardial function and CF in reperfusion. The results show that the cardio depressant concentration of diazepam is safe and relatively protective in the ischemia-reperfused isolated rat heart. These effects may be mediated by inhibition of calcium current in cardiomyocytes.

Saudi Med J 2008; Vol. 29 (6): 847-853

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Received 15th January 2008. Accepted 7th May 2008.

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Diazepam, a benzodiazepine derivative, is commonly used as a tranquilizer, a muscle relaxant, and an anticonvulsant agent in clinical medicine.[1] The benzodiazepine receptors are classified as central and peripheral types.[2,3] Peripheral-type benzodiazepine receptors (PBRs), also named 18 kDa Translocator Protein,[2,4] are abundant in the cardiovascular system.[3,5] The role of these receptors on the cardiac responses, which are induced by diazepam has been examined in different previous studies. Diazepam is a benzodiazepine with moderate affinity to mitochondrial benzodiazepine receptors,[6] and as a ligand, induces mitochondrial permeability transition and ultrastructural alterations in isolated cardiac mitochondria as well as in myocardiocytes.[7] The opening of the mitochondrial permeability transition pore allows free passage of any molecule of <1.5 kDa,[8] and can cause the dissipation of inner mitochondrial transmembrane potential, disrupting mitochondrial structure. It was shown that during reperfusion, the recovery of the heart depends on subsequent pore closure.[7] Regarding the effects of PBR ligands on ischemia, the irreversible PBR antagonist, SSR180575, greatly reduces the contractile dysfunction associated with ischemia reperfusion of the heart.[9,10] Some data indicate that PBR ligands including diazepam produce mitochondrial swelling and ultrastructural alterations in cardiomyocytes.[7] Therefore, with regard to diazepam effect on PBR, it could exacerbate the ischemic damage of cardiomyocytes. Alternatively, previous studies have suggested that diazepam could also affect cardiac contractility.[1,11,12] It has been shown that diazepam produces a concentration dependent negative inotropic effect that is independent on benzodiazepine receptors, and is probably mediated through inhibition of calcium current [I Ca^{++}] in guinea-pig heart preparations.[1] The mechanisms underlying the myocardial depressant effects of the benzodiazepines are not fully understood.[13-16] However, some direct and indirect evidence indicate that inhibition of I Ca^{++} could play an important role in direct myocardial depression caused by diazepam. In adult rat ventricular myocytes, and in cultured fetal mouse cardiac myocytes it has been reported that the cardio depressant effect of diazepam appears to be mediated by a decrease in L-type Ca^{++} channel activity.[14,17] It is well known that the cytoplasmic calcium overload and Ca^{++} accumulation in the mitochondria during ischemia-reperfusion leads to cellular dysfunction through multiple pathways.[18] Also it has been reported that calcium channel blockers can result in mitochondrial protection.[19] On the basis of these data, it might be concluded that diazepam as a calcium channel blocker can protect the heart during ischemia reperfusion. However, there is some evidence on the exacerbation of ischemic damage through the diazepam effect on PBR. Actually, consistent with this controversy, different studies indicate that the PBR classical ligand (PK 11195) may exacerbate or prevent damage caused by ischemia, or have no effect.[3] Regarding this controversy, there are not enough data on the effect of diazepam, especially in cardio depressant concentration on myocardial function in ischemia- reperfusion. So. the goal of this study is to investigate the effect of diazepam, in cardio depressant concentration, on the function of isolated rat heart in ischemia-reperfusion.

Methods. This study was performed at the Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran, from November 2006 to March 2007. The Ethics Committee of Kermanshah University of Medical Sciences approved all experiments, and all animals used in the present study received humane care in compliance with institutional animal care guidelines. Male Wistar rats (300-400 gr) were anesthetized by intraperitoneal administration of 60 mg/kg pentobarbital sodium (Sigma, Steinheim, Germany). Hearts were excised and immediately arrested in ice-cold Krebs solution. The hearts were rapidly cannulated and retrogradely perfused through the aorta in a non-circulating Langendorff apparatus with Krebs solution (containing in mmol/l: sodium chloride 118, sodium hydrogen carbonate 25, potassium chloride 4.8, potassium dihydrogen phosphate 1.2, magnesium sulphate 1.2, glucose 11, and calcium chloride 1.2), at pH 7.4. The buffer was bubbled with 95% O_2 and 5% CO_2 at 37°C and perfusion was performed under a constant hydrostatic pressure[20] of 65 mm Hg. Following removal of the left atrial appendage, a deflated water filled latex balloon was inserted through the mitral valve into the left ventricle. This balloon was connected via a rigid polyethylene tube to a pressure transducer (MLT 844; AD Instruments), which in turn was connected via a power lab (model ML825; AD Instruments) to a computer for continuous monitoring of cardiac performance. At the beginning of the experiment the balloon volume was adjusted to achieve a stable end diastolic pressure of 5-10 mm Hg. This volume was then kept constant for the duration of the study. The index of myocardial function was left ventricular developed pressure (LVDP in mm Hg), which was defined as peak systolic pressure minus end diastolic pressure and heart rate (HR; beat per minute-BPM). Rate pressure product (RPP) was calculated as: RPP = LVDP × HR. Coronary flow (CF) was measured by timed collections of the coronary effluent. Baseline data were recorded after a

Disclosure: This study was supported by the Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran.

30 minute stabilization and equilibration period. The samples with stable baseline values were used for the next steps and data collection. Global normothermic ischemia was induced by clamping the aortic cannula. The temperature was maintained by immersing the heart in perfusion medium at 37ºC. Hearts in the control group (n=14) were subjected to global ischemia for 40 minutes followed by reperfusion for 45 minutes. In addition to this protocol, hearts in the test group (n=10) were perfused for 10 minutes before ischemia with Krebs solution containing 100 micromole per litre (μM) diazepam (Chemi Darou Pharmaceuticals Co. Ltd. Tehran, Iran). The level of ischemia-reperfusion injury was assessed based on the functional recovery, time until onset of ischemic contracture, maximum contracture during ischemia and the release of lactate dehydrogenase (LDH). Time until onset of ischemic contracture is the moment at which the end diastolic pressure starts to increase during ischemia (in minutes) as described previously.[21] The extent of reperfusion injury was determined on the basis of the release of a marker intracellular enzyme into the effluent. To measure the LDH, coronary effluent was collected at the first minute of reperfusion. The samples were measured using a Cell Cytotoxicity kit (LDH) (Roche, Mannheim, Germany) using known quantities of LDH (Sigma, Steinheim, Germany) as a standard.

Results are expressed as mean ± SEM. Comparisons between data sets were made using paired or unpaired t-test as appropriate or ANOVA with a Tukey post test as offered by GraphPad Instat, version 3.05, GraphPad software. Differences were considered to be statistically significant when $p<0.05$.

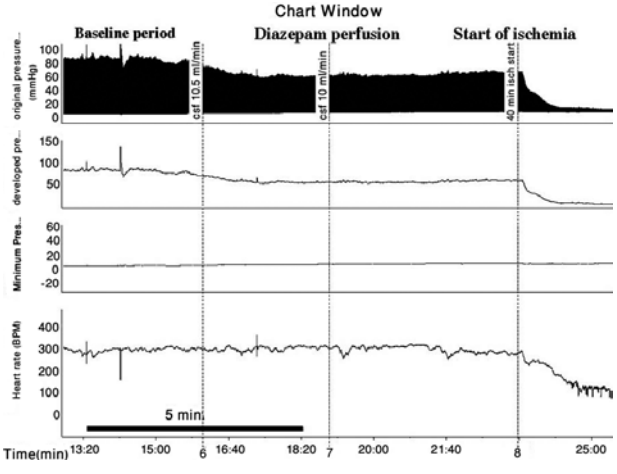

Figure 1 - This powerlab recorded graph shows the typical effect of diazepam (100 μM) on the left ventricular developed pressure of isolated Langendorff rat heart. Diazepam produced a depressant effect on myocardial function.

Results. A typical effect of diazepam on the left ventricular developed pressure of an isolated rat Langendorff heart is shown in **Figure 1**. Diazepam (100 μM) reduced cardiac contraction force and RPP, and this reduction was continued to the end of diazepam perfusion. Heart rate, LVDP, CF, and RPP in different periods of experiment are summarized in **Table 1**. There were no significant differences between the groups at baseline. The RPP average in the control group did not significantly vary throughout the time of experiment before ischemia. Conversely, in the test group the mean of RPP after diazepam perfusion significantly reduced to 77.14 ± 1.45% of the baseline. During the reperfusion period, the recovery of RPP on the forty-fifth minute

Table 1 - The effect of diazepam on myocardial function, before and after exposure to 40-minute global normothermic ischemia in the isolated langendorff rat heart. The columns represent cardiac parameters in the baseline period (after stabilization), after 10 minutes Krebs or Diazepam perfusion (100 μM) in control and test groups and 45th minute of reperfusion.

Parameters and periods	Control group	Test group
Baseline values		
LVDP	84.64±4.8	86.3±5.0
HR	264.9±7.3	264.5±10.7
CF	14.1±0.74	12.25±0.95
RPP	22383±1333	22697±1374
Kreb's perfusion (control group) *Diazepam perfusion (test group)*		
LVDP	85.57±4.51	78.3±5.2
HR	266.71±8.3	227.1±13.5*
CF	14.09±0.78	14.45±1.68
RPP	22770±1277	17449±1026‡
45th min of reperfusion		
LVDP	47.07±2.5	60.30±3.0‡
HR	216.4±9.5	238±12.3
CF	7.37±0.69	8.1±0.94
RPP	10229±716	14368±1125†

LVDP - left ventricular developed pressure (in mm Hg), HR - heart rate (in beats per minute), CF - coronary flow (in ml/min), RPP - rate pressure product (LVDP x HR). Data are means±SEM of control (n=14) and test (n=10). *p=0.024, †p=0.0074, ‡p=0.0039, and, p=0.0036 versus control.

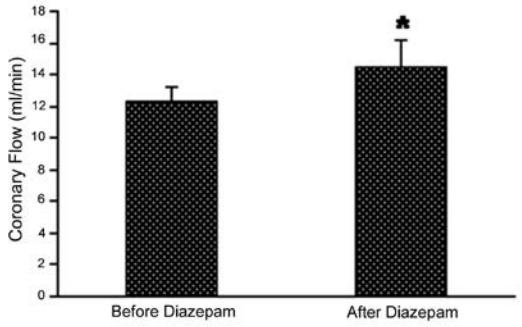

Figure 2 - Coronary flow of the hearts in test group, before and after 10 minutes perfusion with diazepam. Data shown are mean ± SEM, *p=0.0256.

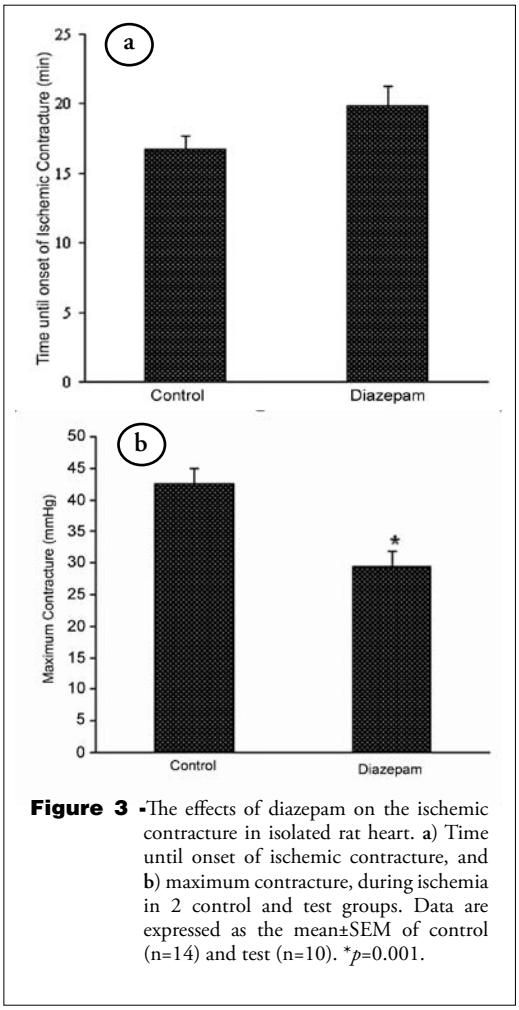

Figure 3 - The effects of diazepam on the ischemic contracture in isolated rat heart. **a**) Time until onset of ischemic contracture, and **b**) maximum contracture, during ischemia in 2 control and test groups. Data are expressed as the mean±SEM of control (n=14) and test (n=10). *p=0.001.

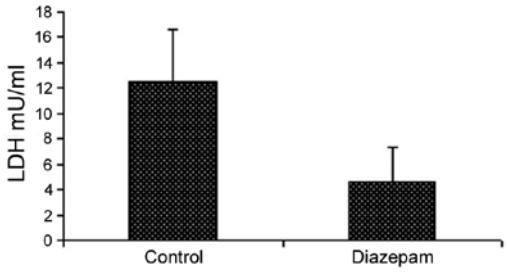

Figure 4 - The concentration of lactate dehydrogenase (LDH) enzyme (in mU/ml) that was released during the first minute of reperfusion following 40-minute global normothermic ischemia in 2 control and test groups. Data shown are mean±SEM of control (n=14) and test (n=10).

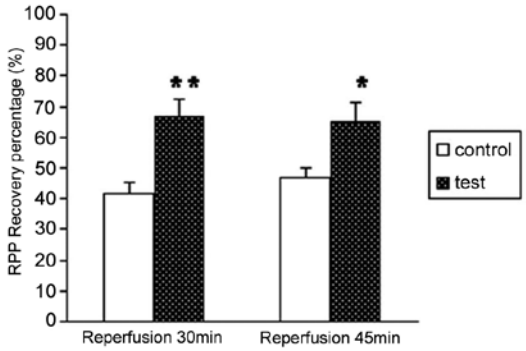

Figure 5 - Recovery percentage (in comparison to baseline) of the rate pressure product (RPP) at 30 and 45-minutes reperfusion following the 40 minutes global normothermic ischemia in 2 diazepam and control groups. Data are expressed as the mean± SEM of control (n=14) and test (n=10). *p=0.0176 **p=0.0027 versus control.

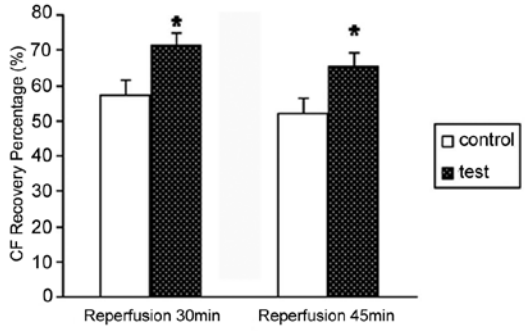

Figure 6 - Recovery percentage (in comparison to baseline) of coronary flow (CF) on the 30th and 45th minutes of reperfusion following 40 minutes global normothermic ischemia in 2 control and test groups. Data shown are mean±SEM of control (n=14) and test (n=10). *p=0.0147, p=0.0353 versus control.

of reperfusion was significantly better in the test group. After diazepam perfusion, CF significantly increased (15.67±4.99%) from 12.25±0.96 to 14.45±1.69 ml/min (p=0.0256) (**Figure 2**). During ischemia, the time until onset of ischemic contracture was determined and there was no significant difference between the test and control groups (19.9±1.39 versus 16.71±0.99 minutes) (p=0.0789) (**Figure 3a**). However, the maximum contracture during ischemia was significantly lower in the diazepam group (29.5±2.396 versus 42.571±2.467 mm Hg, p=0.001) (**Figure 3b**). **Figure 4** shows the concentration of lactate dehydrogenase released during the first minute of reperfusion from the hearts. The amount of released enzyme was not significantly different in the hearts treated with diazepam in comparison with the control (4.63±2.72 versus 12.54±4.14 mU/ml). The recovery percentages of RPP in the diazepam group at thirtieth and forty-fifth minutes of reperfusion (66.7 ± 5.9 and 65.26 ± 6.09% of baseline) were significantly (p=0.0027, and p=0.0176) greater than the control (41.91 ± 3.61 and 46.64 ± 3.29% of baseline) in the post ischemic period (**Figure 5**). Also in the diazepam group, the recovery percentages of CF on the thirtieth and forty-fifth minutes of reperfusion (71.49 ± 3.42 and 65.24 ± 3.98%) were significantly greater than the control [57.34 ± 4.08% (p=0.0147) and 52.22 ± 4.21% (p=0.0353)] (**Figure 6**).

Discussion. The main findings of the present study include: 1) the perfusion of diazepam (100 μM) significantly depressed the myocardial function (RPP) and increased CF before ischemia; 2) it significantly reduced the maximum contracture during ischemia; and 3) it significantly increased the recovery of cardiac function and CF in reperfusion. These results show the cardio depressant and relatively cardioprotective effect of diazepam in the isolated Langendorff rat heart. It has been shown in previous studies that diazepam elicits a biphasic inotropic response in isolated rat heart preparations,[11,12,15] produces reduction of chronotropism in isolated rat atria,[16] and inhibits cardiac contractility at 100 μM in the isolated guinea pig heart.[1] In this study, consistent with other reports, diazepam (100 μM) significantly depressed cardiac function (**Figure 1 and Table 1**). It was reported that the diazepam-induced negative inotropic effect in cultured fetal mouse cardiac myocytes may not be related to the known benzodiazepine receptors.[17] Also, in isolated guinea pig heart, it has been reported that neither the central type, nor the peripheral type of benzodiazepine receptor antagonists influences the negative inotropic effects of diazepam.[1] However, there is some direct and indirect evidence indicating that the inhibition of L-type calcium channel current (I Ca^{2+}) could play an important role in direct myocardial depression caused by diazepam.[14] The L-type calcium channel is considered the most significant calcium channel in the human heart.[19] In ventricular cardiomyocyte, the L-type calcium channel is the major trigger for calcium release from the sarcoplasmic reticulum.[18] In another study, the calcium current [I Ca^{2+}] suppression was reported by using 100 μM diazepam.[1] The myocardial depressant effect of diazepam in this investigation is consistent with previous studies, and according to those reports it could be explained by the reduction of calcium channel activity. Also, in this experiment, diazepam increased the CF rate (**Figure 2**). The same result has been reported in a previous study,[22] and it can be explained by the mentioned action of diazepam as a calcium channel blocker. It has been shown that the calcium channel blockers cause the relaxation of the vascular smooth muscle and coronary vasodilatation.[23] Consistent with this explanation, the maximum contracture during ischemia in the diazepam group significantly decreased in comparison with the control (**Figure 3**). The gradual increase in myocardial resting tension during ischemia has been attributed to an increase in the cytoplasmic calcium concentration.[18] It has been reported that the calcium channel blockers reduce the contracture and diastolic dysfunction.[19] Therefore, the cardio depressant concentration of diazepam could reduce the ischemic contracture, possibly through its action as a calcium blocker, as mentioned in previous reports. It has been shown that PBR ligands including diazepam induce mitochondrial permeability transition and ultrastructural alterations in isolated cardiac mitochondria as well as cardiomyocytes.[7] However, in the present study, there was no significant difference in LDH released during reperfusion in 2 diazepam and control groups (**Figure 4**). Indeed, released LDH is a marker of myocardial cell damage and shows the level of reperfusion injury.[24] In contrast to the reported effect of PBR ligands on the cardiomyocytes, the present study indicates that diazepam (100 μM) does not exacerbate the ischemia-reperfusion injury in the isolated rat heart. It is a new finding and represents the safety of diazepam (100 μM) in myocardial ischemia.

Diazepam (100 μM) significantly increased the cardiac functional recovery after ischemia (**Figure 5**) and recovery of CF (**Figure 6**) in comparison with the control. These new findings are not consistent with some previous studies. It has been shown that approximately 100 μM diazepam depressed functional and metabolic recovery during reperfusion.[25] Also it has been reported that diazepam as a benzodiazepine ligand induces mitochondrial permeability transition in myocardiocytes.[7] This transition causes mitochondria to become uncoupled and capable of hydrolyzing rather than synthesizing adenosine triphosphate (ATP). Such a condition causes the rapid decline of intracellular ATP concentration and leads to the disruption of ionic and metabolic homeostasis and results in cellular damage.[8] Furthermore, it contributes to the reperfusion damage by depolarizing the mitochondrial inner membrane potential.[26] There is evidence that mitochondrial permeability transition pore opening is critical in the transition from reversible to irreversible reperfusion injury.[27,28] In addition, it has been reported that the functional recovery of the Langendorff-perfused heart from ischemia inversely correlates with the extent of pore opening, and inhibition of the mitochondrial permeability transition pore in cardiomyocyte provides protection against reperfusion injury.[8,29] It has been demonstrated that PBR antagonist reduces the ischemic damage,[3] and the cardioprotection at the level of PBR is linked to the limitation of mitochondrial membrane permeabilization.[10] Hence, the improved recovery of the myocardial function in this study can not be explained through the action of diazepam on the peripheral benzodiazepine receptor. It was reported that both central and peripheral type benzodiazepine receptors do not play an important role on the depressant effect of diazepam on the isolated hearts. However, diazepam with a concentration of 100 μM significantly decreases the calcium current in cardiomyocytes.[1,30] Although we did not measure the calcium current in this investigation (as a limitation of the study), it seems as if the improved

functional recovery after using a cardio depressant concentration of diazepam might be explained by the reduction of calcium current in cardiomyocytes. In spite of the previous reports, it seems that the role of each mechanism (PBRs and calcium channels) in different concentration of diazepam still needs to be elucidated in future studies. Also, the present study with regard to LDH result shows that the cardio depressant concentration of diazepam is safe in ischemia-reperfusion; and with regard to other findings in the present study, including lower maximum contracture, and improved recovery of myocardial function and CF, diazepam shows some cardioprotective properties, probably due to its calcium current effects, as reported in previous studies.

In conclusion, diazepam (100 μM) before ischemia significantly depressed the myocardial function and increased CF. During ischemia, it significantly decreased maximum contracture. It did not significantly change the LDH release after ischemia and significantly improved the recovery of myocardial function and CF in reperfusion. The results show that the cardio depressant concentration of diazepam is safe and relatively protective in the ischemia-reperfused isolated rat heart. These effects may be mediated by inhibition of calcium current in cardiomyocytes.

Acknowledgment. *We would like to thank Miss Zahra Mirabbassi for expert technical assistance and Mr Bahman Mehraban for grammatical checking of the manuscript.*

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