The effect of repeated diazepam administration on myocardial function in the ischemia-reperfused isolated rat heart

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ABSTRACT

الأهداف: لتقييم ما إذا كان إعادة تلقي عقار ديازبام يؤثر على القلب في إعادة التسريبم/نقص التروية.

الطريقة: أجريت هذه الدراسة بمركز الأبحاث الحيوية الطبية – خيرمانشاه – إيران، خلال الفترة مابين مارس2008م وحتى سبتمبر 2008م. تلقت أربعة مجموعات من الجرذان يومياً عقار ديازيبام بالحقن بمقدار (0.5mg/kg) لمدة (21 يوم)، (2.5mg/kg و 5) لمدة (5 أيام)، (IP) والمحلول الملحي (21 يوماً) في مجموعات الاختبار 1، 2، 3 ومجموعة التحكم، على التوالي. تلقت الجرذان المعزولة التسريب لمدة 40 دقيقة من نقص التروية الشامل و45 دقيقة من إعادة التسريب. تم قياس الضغط الحاصل في البطين الأيسر (LVDP) وضربات القلب وتيار الدم في الشريان التاجي. كما تم حساب ناتج ضغط المعدل (RPP)، وفي إعادة التسريب، تم قياس تحرير أنزيم ديهادروجينيس لاكتيت (LDH).

النتائج: لقد تبين أن شفاء (RPP) و (LVDP) في حالة إعادة التسريب منخفض بشكل ملحوظ في مجموعة الاختبار الثالثة (عدد=9) مقارنتاً مع مجموعة التحكم (عدد=8). خلال فترة إعادة التسريب، ازداد (LDH) المحرر بشكل ملحوظ في المجموعة الثانية (عدد=8) والمجموعة الثالثة مقارنتاً مع مجموعة التحكم.

خاتمة: تُظهر النتائج أن إعادة تلقي عقار ديازيبام (5mg/kg) لمدة (خمسة أيام) يخفض من أداء القلب في إعادة التسريب ويشتد بشكل ملحوظ في إصابة نقص التروية/إعادة التسريب، ومن الأرجح أن ذلك يتوسط بواسطة تغير استعداد القلب في نقص التروية نتيجة لإعادة تلقي عقار ديازيبام.

Objectives: To evaluate whether repeated diazepam administration affects the heart in ischemia-reperfusion.

Methods: This study was performed at the Medical Biology Research Center, Kermanshah, Iran, from March to September 2008. Four groups of rats were subjected to a daily injection of diazepam (group 1 [0.5 mg/kg for 21 days], group II [2.5 mg/kg for 5 days], and group III [5 mg/kg for 5 days] intraperitoneally), and saline solution (21 days) in the control groups. Isolated, perfused hearts were subjected to 40 minutes global ischemia, and 45 minutes reperfusion. The left ventricular developed pressure (LVDP), heart rate, and coronary flow were measured. Rate pressure product (RPP) was calculated. In reperfusion, released lactate dehydrogenase (LDH) enzyme in effluent was measured.

Results: It was observed that the recovery of the RPP and LVDP in reperfusion significantly decreased in the test group III (n=9) in comparison to the control (n=8). During the reperfusion period, the released LDH significantly increased in test group II (n=8) and group III in comparison with the control.

Conclusion: The results show that repeated administration of diazepam (5 mg/kg for 5 days) reduced the cardiac performance in reperfusion, and significantly exacerbated the ischemia-reperfusion injury. It is probably mediated by the changing of cardiac susceptibility in ischemia due to repeated administration of diazepam.

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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] Benzodiazepines produce their pharmacological effects through binding to specific receptors. These receptors are classified as central and peripheral types.[2,3] Peripheral-type benzodiazepine receptors (PBRs), also known as, 18 kDa translocator proteins,[4] are abundant in the cardiovascular system.[3] The peripheral benzodiazepine receptor is a 169-amino acid protein with 5 transmembrane domains, associated with the mitochondrial outer membrane.[5] It was suggested that PBRs might be involved in the control of several mitochondrial functions, including respiratory chain and ion channel activities, and in the regulation of apoptosis, which occurs during cardiac injury.[6-8] Also, it is involved in the regulation of mitochondrial permeability transition, and plays an important role in the processes of cell apoptosis and necrosis.[8] It was shown that during reperfusion, a significant pore opening does occur, and recovery of the heart depends on subsequent pore closure.[9] The peripheral benzodiazepine receptor plays a major role in the regulation of cardiac ischemia-reperfusion (I/R) injury,[7] and it was demonstrated that the level of PBR expression is correlated with the resistance of the cell to oxidative stress.[10] On the other hand, it has been reported that chronic benzodiazepine exposure regulates peripheral benzodiazepine receptors in peripheral organs. For example, it has been demonstrated that a 14-day administration of diazepam produces an up-regulation of heart peripheral benzodiazepine receptors.[11] Also, it has been shown that the chronic (21 days) diazepam treatment (0.5 mg/kg, intraperitoneal [IP]) results in a significant increase (18%) in the density of peripheral benzodiazepine binding sites in the heart.[12] Therefore, the cardiac resistance to I/R injury might be affected by chronic diazepam exposure in the organism. The chronic usage of benzodiazepines with different doses and duration is common in medicine, however, enough data on its possible effect on the cardiac vulnerability in ischemia are not available. The present investigation is designed to evaluate whether repeated diazepam administration affects myocardial function in I/R isolated rat heart.

Disclosure. This study was supported by the Medical Biology Research Centre in Kermanshah University of Medical Sciences, Kermanshah, Iran. The authors declare that there is no conflict of interest relevant to the content of this study.

Methods. This study was performed at the Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran, from March 2008 to September 2008. All experiments were approved by the Ethics Committee of Kermanshah University of Medical Sciences, and all animals used in the present study received humane care, in compliance with the institutional animal care guidelines. Male Wistar rats were randomly divided into control and test groups I-III. In the test group I (n=7), the animals were subjected to a daily injection of diazepam (Chemi Darou Pharmaceuticals Co. Ltd. Tehran, Iran) (0.5 mg/kg, IP) for 21 days, and in the control group (n=8), animals were subjected to a daily injection of the saline solution with the same time and volume. The animals received a daily injection of diazepam (2.5 mg/kg, IP for group II [n=8], and 5 mg/kg, IP for group III [n=9]) for 5 days. The experiments were performed on the day after the last injection in each group. Male Wistar rats (250-300 g) were anesthetized by IP administration of 60 mg/kg pentobarbital sodium (Sigma, Steinheim, Germany). The hearts were excised and immediately arrested in ice-cold Krebs solution (Merck, Darmstadt, Germany). The hearts were rapidly cannulated and retrogradely perfused through the aorta in non-circulating Langendorff apparatus (Harvard Apparatus Ltd., Edenbridge, United Kingdom) (**Figure 1**) with Krebs solution (containing sodium chloride [118 mmol/l],

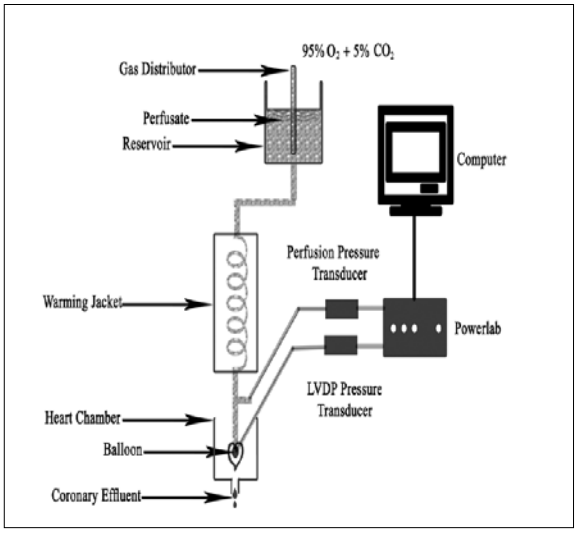

Figure 1 - Schematic of our Langendorff (constant pressure) heart perfusion system. All parts of the apparatus are water-jacketed with warm circulating water to maintain the temperature of the heart at 37°C. Coronary flow is measured by timed collection of the coronary effluent. Intraventricular pressure is measured by an indwelling balloon. This balloon is connected to a pressure transducer, which in turn is connected via a power lab to a computer, for continuous monitoring of cardiac performance. LVDP - left ventricular developed pressure

sodium bicarbonate [25 mmol/l], potassium chloride [4.8 mmol/l], potassium dihydrogen phosphate [1.2 mmol/l], magnesium sulfate [1.2 mmol/l], glucose [11 mmol/l], and calcium chloride [1.2 mmol/l]) at pH 7.4. The buffer was bubbled with 95% oxygen/5% carbon dioxide at 37ºC, and perfusion was performed under a constant hydrostatic pressure of 65 mm Hg. Following the 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, New South Wales, Australia), which in turn was connected via a power lab (model ML825; AD Instruments, New South Wales, Australia) 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.[13] The baseline data were recorded after a 30-minute stabilization and equilibration period. Global normothermic ischemia was induced by clamping the aortic cannula. The temperature was maintained by immersing the heart in perfusion medium at 37ºC. The hearts were subjected to global ischemia for 40 minutes followed by reperfusion for 45 minutes. The level of I/R injury was assessed based on the functional recovery and the release of lactate dehydrogenase (LDH). The extent of reperfusion injury was determined from the release of a marker intracellular enzyme into the effluent. In order to measure the LDH, coronary effluent was collected at the first minute of reperfusion. The samples were measured using a cell cytotoxicity detection kit (LDH [Roche, Mannheim, Germany]) using known quantities of LDH (Sigma, Steinheim, Germany) as a standard.[14]

Results are expressed as mean ± standard error of the mean (SEM). Comparisons between data sets were made using paired or unpaired t-test as appropriate or analysis of variance (ANOVA) with Tukey post test as offered by Instat. Differences were considered to be statistically significant when $p<0.05$.

Results. ***Hemodynamic function.*** The HR, LVDP, CF, and RPP in different periods of the experiment are summarized in **Table 1**. There were no significant differences between groups at baseline. The average of RPP did not significantly vary throughout the time of experiment before ischemia. There are no significant differences in the RPP of test groups I and II versus the control in the recovery period. Conversely, the recovery of the RPP and LVDP in the forty-fifth minute of reperfusion was significantly lower in test group III, in comparison to the control group and group I. Also, there is a significant difference between the recoveries of LVDP in the test groups I and II at the forty-fifth minute of reperfusion (**Table 1** & **Figure 2**).

Functional recovery percentage. The recovery percentage of RPP at the forty-fifth minute of reperfusion in test group III (29.98 ± 4.1% of baseline) was significantly lower in comparison to the control (53.88 ± 6.07%) (p=0.004, p=0.01), and group I (48.67 ± 4.93%) (**Figure 3**).

Lactate dehydrogenase release. The extent of reperfusion injury in the 4 groups was determined from the release of a marker intracellular enzyme into the effluent. The concentration of released LDH during the first minute of reperfusion from the hearts in the test group II (26.78 ± 2.09) mU/ml, and group III (20.15 ± 2.81) (p=0.0001, p=0.041) were significantly higher than the control group (12.76 ± 1.53) (**Figure 4**).

Table 1 - Cardiac parameters before and after exposure to a 40-minute global normothermic ischemia in the control and diazepam treated groups.

Groups	Baseline values				Forty-fifth minute reperfusion			
	LVDP	HR	CF	RPP	LVDP	HR	CF	RPP
Control	83.1 ± 2.6	254 ± 11	11.8 ± 0.6	21047 ± 1033	53.7 ± 4.4	208.8 ± 15.5	6.4 ± 0.4	11258 ± 1377
Group I (0.5 mg/kg), 21days	99.3 ± 6.1	250 ± 14.6	10.7 ± 0.5	24427 ± 1629	60.8 ± 2.6	192 ± 17	6.8 ± 0.6	11572 ± 927
Group II (2.5 mg/kg), 5 days	85 ± 3.3	276.7 ± 11.5	11 ± 0.5	23520 ± 1362	46.5 ± 5¶	202.5 ± 20.8	6.4 ± 0.3	9469 ± 1490
Group III (5 mg/kg), 5 days	89.9 ± 3.6	230.5 ± 14	10.5 ± 0.5	20453 ± 1143	36 ± 5.1*†	199.5 ± 39.7	5.7 ± 0.6	6047 ± 829‡§

Left ventricular function of Langendorff perfused hearts in different groups of the experiment. LVDP - left ventricular developed pressure (mm Hg), HR - heart rate (beat/minute), CF - coronary flow (ml/minute), RPP - rate pressure product (LVDP×HR). Data are mean ± SEM of control (n=8), group I (n=7), group II (n=8), and group III (n=9). *p=0.044 versus control, †p=0.004 versus group I, ‡p=0.017 versus control, and §p=0.015 versus group I (analysis of variance), ¶p=0.0323 versus group I (unpaired t-test).

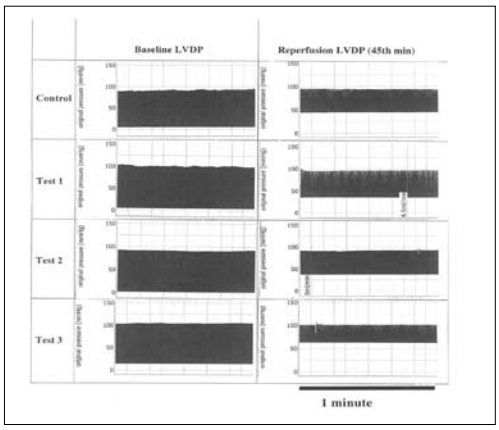

Figure 2 - These power lab recorded graphs show the typical left ventricular developed pressure (LVDP mm Hg), before and after exposure to 40 minute global normothermic ischemia in control and test groups.

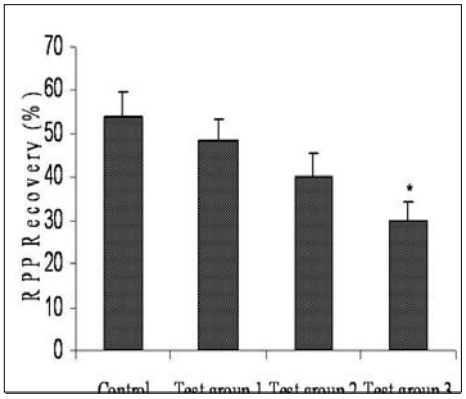

Figure 3 - Recovery percentage (in comparison to the baseline) of the rate pressure product (RPP) at 45th minute of reperfusion following the 40 minute global normothermic ischemia in the control (n=8) and group I (n=7) (0.5 mg/kg diazepam, intraperitoneally), group II (n=8) (2.5 mg/kg), group III (n=9) (5 mg/kg). Data are expressed as Mean ± SEM. *p=0.004, versus control, and *p=0.01 versus group I.

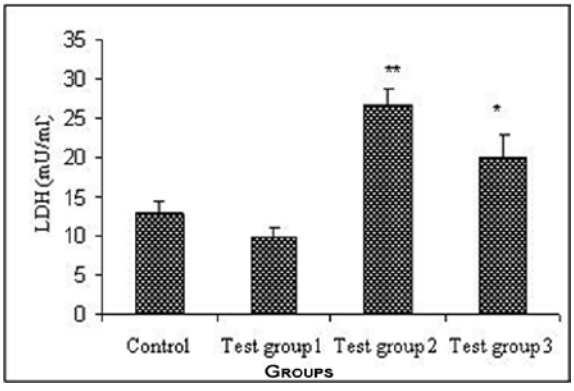

Figure 4 - The concentration of lactate dehydrogenase (LDH) enzyme (mU/ml) that was released during first minute of reperfusion following 40 minute global normothermic ischemia in the control (n=8) and test group I (n=7) (0.5 mg/kg Diazepam, intraperitoneally, 21 days), group II (n=8), and group III (n=9) (2.5 and 5mg/kg, 5 days). Data shown are Mean ± SEM. *p=0.041 and **p=0.0001, versus control.

Discussion. The results of the current study provide evidence of marked increase in cardiac I/R injury as a result of repeated exposure to diazepam. In addition, the results demonstrate significant differences between the effects of different doses of diazepam on myocardial function in I/R. We previously demonstrated that the cardiodepressant concentration of diazepam is safe, and relatively protective in the ischemia-reperfused isolated rat heart.[13] However, the data of the present study shows that repeated administration of diazepam (2.5 and 5 mg/kg for 5 days) reduced the cardiac performance during reperfusion. With the low dose (2.5 mg/kg), it somehow reduced the recovery of cardiac function (LVDP), and by using the higher dose (5 mg/kg), it significantly exacerbated the myocardial dysfunction in reperfusion. Also, the exacerbated reperfusion injury was confirmed by increased LDH release in these groups. Indeed, released LDH is a marker of myocardial cell damage and shows the level of reperfusion injury.[15] In other studies, it was reported that repeated (21 days) diazepam treatment (0.5 mg/kg, IP) significantly increased the density of peripheral benzodiazepine binding sites in the heart (18%),[12] and a 14-day administration of diazepam produced an up-regulation of peripheral benzodiazepine receptors in rat heart.[11] The PBRs, are primarily located on the outer mitochondrial membrane and associated with the voltage-dependent anion channel. It is one of the proteins which might regulate the mitochondrial permeability transition pore.[16] The opening of the mitochondrial permeability transition (MPT) pore can cause the dissipation of inner mitochondrial transmembrane potential, disrupting mitochondrial structure and leading to the release of proapoptotic intermembrane proteins from the mitochondrion.[9] Indeed, prolonged permeability transition pore opening is known to induce massive swelling of the mitochondria, leading to membrane rupture. Through mitochondrial membrane permeabilization, I/R induces the release of cell death effecters, and ultimately the loss of mitochondrial functions, which are fundamental for cell survival.[17] It was reported that during reperfusion a significant opening of the MPT pore does occur, and the recovery of the heart depends on subsequent pore closure.[9] In addition, it was shown that a rise of PBR levels inevitably cause an increase in the calcium concentration, necessary to induce MPT opening on heart isolated mitochondria.[7,18] Although we did not measure the heart PBR density in this study (as a limitation of the study), the present findings can be explained by the changing of PBR density in the heart due to repeated administration of diazepam, as reported in the mentioned studies.

It was shown that the levels of PBR might be affected by the duration of stress, some disorders, and their treatments.[3,19] On the basis of this fact, the different time duration of diazepam administration was used in the present study. The results demonstrate that the repeated administration of diazepam with a high dose in a short period (group III) significantly decreased cardiac recovery in comparison to the group that received diazepam with a low dose in a longer period (group I). It shows that the dose of diazepam is probably more important than the time duration of its administration in myocardial I/R injury. However, the effects of dose and duration of diazepam administration on the PBR density remains to be elucidated in future studies. Indeed, the exacerbated I/R injury were shown in the present study by the application of diazepam in supra-clinical dose. However, there are some common factors which induce the up regulation of PBR density, including some types of stress.[19,20] In these cases, the exacerbated I/R injury probably can be induced by a lower dose of diazepam. Relating to these facts, the safe dose of diazepam in chronic administration remains to be clarified in future studies.

In conclusion, the results of the present study show that repeated administration of diazepam (5 mg/kg for 5 days) reduced the cardiac performance in reperfusion and significantly exacerbated the I/R injury. It is probably mediated by the changing of cardiac susceptibility and PBR density due to repeated administration of diazepam.

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