



## Cardioprotective effect of fasting on ischemia reperfused rat heart after diazepam administration

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### ABSTRACT

**Background:** Fasting and calorie restriction have some cardioprotective effects. In view of the effect of fasting on peripheral benzodiazepine receptors and widespread administration of benzodiazepines in medicine, the present study was designed to evaluate whether fasting may affect myocardial vulnerability to cardiac ischemia–reperfusion (I/R) following repeated diazepam administration.

**Methods:** Rats were divided into six groups of 8 or 10 animals. Groups I and II were controls which received intra peritoneal injection of normal saline solution for 5 days. Also, Control II underwent fasting on 5th day of experiment. Four test groups received intra peritoneal injection of diazepam for 5 days (groups I and II 1mg/kg; groups III and IV 5 mg/kg). Also, test groups II and IV fasted on 5th day of experiment. The Langendorff isolated hearts were subjected to 25 minutes ischemia and 25 minutes reperfusion. Cardiac parameters including left ventricular developed pressure and rate pressure product were determined. Infarct size was measured by Triphenyltetrazolium staining.

**Results:** Recovery of the left ventricular developed pressure in diazepam groups were significantly lower than control I and II ( $P=0.049$  and  $P=0.046$  respectively). But there was no significant difference among the controls and test group II, which fasted following diazepam administration. This showed the preservation of the cardiac performance in the fasting animals following administration of diazepam (1 mg/kg).

**Conclusion:** The results obtained showed the exacerbation of ischemia reperfusion injury in the presence of diazepam and demonstrated the protective effect of fasting which is probably due to modulation of the mitochondrial permeability transition pore.

#### ► Implication for health policy/practice/research/medical education:

The cardio protective effects of fasting on diazepam induced ischemia reperfusion injury.

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## 1. Introduction

Diazepam, a benzodiazepine derivative, is used as a tranquilizer, a muscle relaxant and an anti-convulsant agent in clinical medicine (1). Benzodiazepines exert their pharmacological effects through binding to specific receptors. These receptors are classified as central and

peripheral types. The peripheral-type benzodiazepine receptors (PBRs), also known as 18kDa translocator proteins (2-4), are abundant in the cardiovascular system (4). PBR is a 169-amino acid protein with 5 trans-membrane domains, associated with the mitochondrial outer membrane (5). It is suggested that, PBRs might be involved in the control of several mitochondrial functions, including respiratory chain, ion channel activities and regulation of apoptosis, which occurs during cardiac injury (6,7). Also, the PBRs play a major role in the regulation of

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cardiac ischemia – reperfusion (I/R) injury (8), and it was demonstrated that the level of PBR expression is correlated with the resistance of the cell to oxidative stress (9). On the other hand, it has been reported that chronic benzodiazepine exposure, changes PBRs density in peripheral organs. For example, it has been shown that, repeated diazepam administration results in a significant increase in the density of peripheral benzodiazepine binding sites in the heart (10). Also it reduces the cardiac performance in reperfusion, and significantly exacerbated I/R injury that is probably mediated by the changing of cardiac vulnerability to ischemia (11,12). Furthermore, PBRs density may be affected by stress (13). It has been demonstrated that fasting stress decreases PBRs density in peripheral organs including heart (14). Some cardiovascular protective effect has been reported by fasting and calorie restriction. Fasting protects the heart against ischemia reperfusion injury (15) and the reduction of oxidative stress and free radicals are probably responsible for this protection (16,17). Therefore it seems that fasting is a good candidate for preservation of the myocardium following repeated diazepam administration.

In regard to the common usage of benzodiazepines in medicine, the present investigation is conducted to evaluate whether fasting may affect myocardial vulnerability to I/R following repeated diazepam administration.

## 2. Materials and Methods

This study was performed at the Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, IR, Iran. It was approved by the Ethics Committee of Kermanshah University of Medical Sciences and performed on male Wistar rats (250 to 300 g). All animals used in the current study received human care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). Animals were randomly divided into two control groups (I, II) and four experimental groups (I-IV). In the control group I (n= 9), the animals were subjected

to intraperitoneal (IP) injection of normal saline solution for 5 days. The control group II (n=10), in addition, fasted for 24 hrs on 5th day of experiment with free access to water. Test group I (n=8), was subjected to daily injection of diazepam, 1 mg/kg, IP (Chemi Darou Pharmaceuticals Co. Ltd. Tehran, IR, Iran) for 5 days, before heart isolation. Test group II (n=9), in addition to the above- mentioned protocol for the test group I, were fasted for 24h on the 5th day of experiment with free access to water. Experimental protocol for test groups III and IV (n=9), was the same as the test groups I and II, but with different dose of diazepam (5 mg/kg, IP). Male Wistar rats (250-300gr) were anesthetized by IP administration of 60 mg/kg sodium pentobarbital (Sigma, Steinheim, Germany). The hearts were excised and immediately arrested in ice cold Krebs solution. The hearts were quickly cannulated and retrogradely perfused through the aorta using non circulating Langendorff apparatus (Harvard Apparatus Ltd., Eden bridge , United Kingdom) with Krebs buffer (containing in mmol/l: NaCl 118 , NaHCO<sub>3</sub> 25 , KCl 4.8, KH<sub>2</sub>PO<sub>4</sub> 1.2 , MgSO<sub>4</sub> 1.2, glucose 11 and CaCl<sub>2</sub> 1.2) at pH 7.4 (11).

The buffer was bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 37°C and perfusion was performed under, a constant hydrostatic pressure of 60 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) 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 kept constant for the duration of the study. The indices of myocardial function were Left ventricular developed pressure (LVDP in mm Hg), which was defined as peak systolic pressure minus end diastolic pressure, and heart rate (HR, beats 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.

**Table 1.** Cardiac parameters before and after exposure to a 25 minutes global normothermic ischemia in the control and diazepam treated groups

Groups	Baseline values				25th min of Reperfusion			
	LVDP	HR	CF	RPP	LVDP	HR	CF	RPP
Control I	72.9±3.9	277.9±12	12.9±0.5	20192±1472	42.1±3.4	221.9±9.3	8.1±0.4	9386.7±588.01
Control II (24 hrs fasting)	79.7±4.9	256.2±12	13.3±0.7	20124±1163	44.9±5.5	238.4±21.4	9.6±0.8	10313±1338
Group I (diaz 1 mg/kg)	69.3±7.8	268±7.6	12.2±0.9	18457±2082	21.3±2.9 ‡‡‡ §§§	218.9±16.2	8.3±0.8	4767±806 ††† **
Group II (diaz 1mg/kg and fasting)	78.6±3.3	254.6±13.2	13.7±0.5	19902±1125	27.1±4.09	202.8±16.7	9.2±0.6	5642±936 †
Group III (diaz 5mg/kg)	66.7±3.4	258.9±15.5	11.0±0.9	17139±1173	27.3±7.3 §‡	183.6±28.5	7.5±1.1	5211±1659 ***
Group IV (diaz 5mg/kg and fasting)	76.4±4.6	247.6±15.7	12.0±0.6	18806±1511	26.5±5.2 §§‡‡	194.2±12.6	8.3±0.5	5383±1210 * ††

Left ventricular function of Langendorff perfused hearts in different groups of the experiment. LVDP- left ventricular developed pressure (mm Hg), HR- heart rate (beats/minute), CF- coronary flow (ml/minute) and RPP- rate pressure product (LVDP×HR). Data are mean ± SEM of control I (n=9), control II (n=10), group I (n=8), group II (n=9), group III (n=9) and group IV (n=8). †P= 0.019, ‡P= 0.014, ‡‡P= 0.004, §P= 0.049, §§P= 0.036 and §§§P= 0.003 versus control I; \*P= 0.021, \*\*P= 0.012, \*\*\*P= 0.01, ‡P= 0.046, ‡‡P= 0.034 and ‡‡‡P= 0.004 versus control II (Kruskal-Wallis test).

The baseline data were recorded after a 30 minutes 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 37°C. The hearts were subjected to global ischemia for 25 minutes followed by reperfusion for 25 minutes. Hereby, the level of I/R injury was assessed by comparing the cardiac parameters before and after ischemia.

### 2.1. Assessment of infarct size

The infarct size was assessed based on triphenyltetrazolium staining. This technique relies on the ability of dehydrogenase enzymes and cofactors in the tissue to react with tetrazolium salts (Sigma, Steinheim, Germany) to form a formazan pigment, so the surviving tissue should turn a deep red color and ischemic dead cells should turn pale gray (18, 19).

### 2.2. Statistical Analysis

Results are expressed as mean  $\pm$  standard error of the mean (SEM). Comparison between data sets was made by Kruskal-Wallis test using SPSS version 20 software. Differences were considered to be statistically significant when  $P < 0.05$ .

## 3. Results

### 3.1. Hemodynamic function

The averages of the cardiac parameters including HR, LVDP, CF and RPP in different periods of the experiment are demonstrated in Table 1. The hearts showed normal performance at baseline and there were no significant difference between groups in these periods. The significant decline of different cardiac parameters (compared with

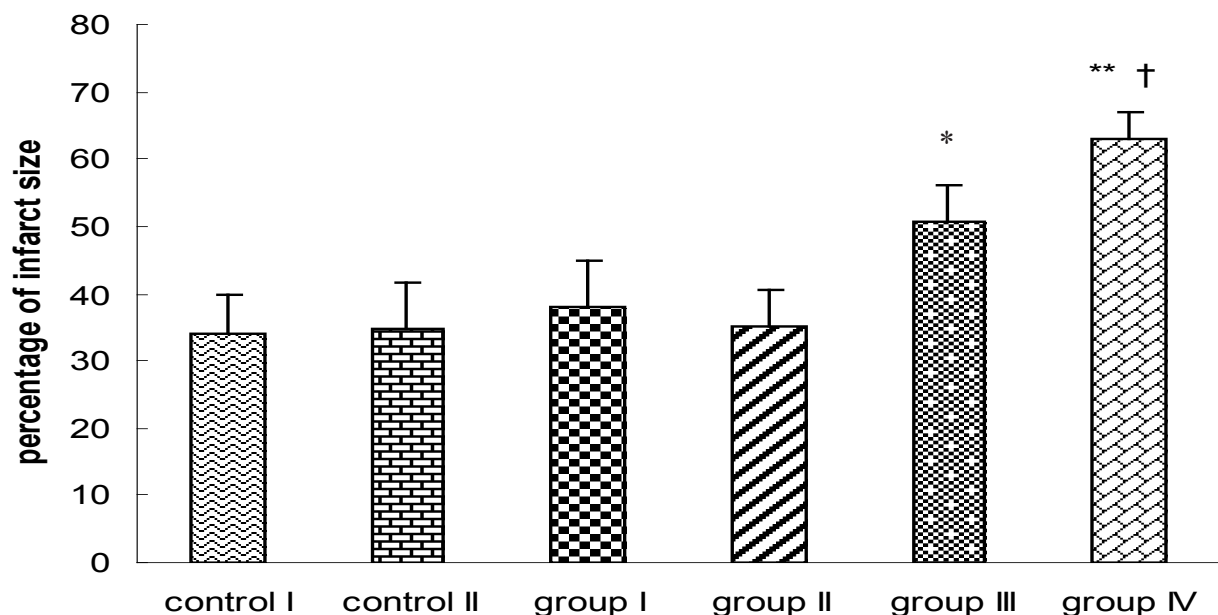
baseline) at 25th minute of reperfusion following 25 minute of normothermic global ischemia, showed the ischemia-reperfusion injury in all groups. In addition, the LVDP averages at 25th minute of reperfusion showed significant differences between groups which received diazepam 1mg/kg (group I), 5mg/kg (group III), 5mg/kg and fasting (group IV) versus control groups I and II (Table 1). In other words, compared with controls I and II, there was significant decline in the average of LVDP following ischemia reperfusion in these three groups which showed exacerbated ischemia reperfusion injury. However there was not any significant difference between group II and controls which showed the protective effect of fasting in the presence of diazepam. Also similarly, RPP was significantly decreased in the test groups I, III and IV versus control II. However, there was no significant difference between group II and control II.

### 3.2. Myocardial Infarct size

The percentage of cardiac infarct size following ischemia reperfusion in different groups is shown in the Figure 1. The statistical analysis of data sets by Kruskal-Wallis test showed that there were not significant differences between test groups which received diazepam 1 mg/kg ( groups I and II) compared with controls, meanwhile the infarct sizes in the test groups which received diazepam 5 mg/kg ( groups III & IV) were significantly greater than controls.

## 4. Discussion

The results of the current study revealed significant increase in cardiac I/R injury following repeated administration of diazepam. Previous studies have shown that, the cardio depressant concentration of diazepam in perfusion solution did not exacerbate I/R injury (20). On



**Figure 1.** The percentages of myocardial infarct size in experimental groups following 25 minutes ischemia and 25 minutes reperfusion. data are mean  $\pm$  SEM of control I (n=9), control II (24 hrs fasting, n=9), group I (diazepam 1 mg/kg, n=8), group II (diazepam 1mg/kg and fasting, n=9), group III (diazepam 5 mg/kg, n=9) and group IV (diazepam 5mg/kg and fasting, n=8), for statistical analysis Kruskal-Wallis test was applied (\* $P < 0.04$  and † $P = 0.001$  versus control I; \*\* $P = 0.004$  versus control II)

the other hand, it has been shown that the repeated exposure of rats to diazepam for 5 days significantly decreased the cardiac performance of their ischemia reperfused isolated hearts (11). This finding is confirmed by the present study and the results show deterioration of cardiac I/R injury due to repeated administration of diazepam for 5 days. Another finding of the current study showed the preservation of the cardiac performance of the fasted animals which received the diazepam 1 mg/kg under the same protocols (group II). This is a novel finding of the present study.

The cardioprotective effects of the fasting have been reported in the numerous studies. The several mechanisms have been proposed for these protective effects including the restriction of calorie intake and the reduction of free radicals which decreases the oxidative stress in ischemia reperfused hearts (16,17). Consistent with these reports, the present study showed the protective effect of the fasting on I/R injury. Also, in the same way with the myocardial functions (Table 1), the result of the present study showed that the repeated administration of diazepam (5mg/kg), significantly increased infarct size in I/R isolated hearts compared with controls (Figure. 1). Meanwhile fasting could not affect this process which confirms the negative and harmful effects of the repeated administration of diazepam on ischemia reperfused hearts. Therefore fasting does not show the protective effect in this case, which can be probably due to the huge myocardial injury of animal's heart which treated with the high doses of diazepam. Overall present study showed that the fasting can relatively protect ischemic heart against negative effect of the repeated diazepam administration in a dose-dependent manner.

Previous studies have shown that the repeated administration of diazepam increases the PBR density in the heart (10,21). Regarding to the important role of this receptors in I/R injury and oxidative stress (8,9), it seems that the exacerbated I/R injury can be explained by changing the PBR density in the heart (11). The PBRs are located on the mitochondrial membranes and associated with the voltage-dependent anion channels. It is one of the proteins which might regulate permeability of the mitochondrial permeability transition pore (MPT) (22). The opening of MPT can cause dissipation of inner mitochondrial transmembrane potential, disrupting mitochondrial structure and leading to release of proapoptotic intermembrane proteins from the mitochondria (23).

In addition, it was shown that a rise of PBR levels inevitably causes an increase in the calcium concentration, necessary to induce MPT opening in the myocyte isolated mitochondria (8,24). So, the opening of the MPT possibly have an important role in the exacerbated I/R injury following repeated diazepam administration. On the other hand, it has been reported that the fasting can protect the ischemia reperfused heart, probably through reducing MPT (25). Therefore, considering the key roles of MPT in I/R injury, it seems that reduction of MPT by fasting could protect the heart in the presence of diazepam. However, the limitation of this study was that we actually did not measure the PBR and MPT densities and the exact role of the PBR and MPT remains to be elucidated in the future.

In conclusion, to our knowledge this study showed for the first time the cardioprotective effect of fasting on diazepam- induced exacerbated I/R injury, which might be explained by the impact of MPT and PBR density.

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## Financial Disclosure

The authors declare that they have no conflicts of interest.

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