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The Effect of Diazepam on the Function of Hypertrophied Rats' Hearts in Ischemia-Reperfusion Conditions

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ABSTRACT

***Background*:** Hypertrophied hearts are susceptible to ischemic injury. Besides, cardiac vulnerability could be changed in the presence of diazepam.
***Objectives*:** The current study aimed to investigate the effect of diazepam on hypertrophied rats' hearts in ischemia-reperfusion conditions.
***Materials and Methods*:** Male Wistar rats (body weight 210 - 270 gr) were administered with isoproterenol (4 mg/kg body weight, intraperitoneally for 7 days) alone or along with diazepam (1 and 5 mg/kg body weight, intraperitoneally for 5 days). The control rats received normal saline intraperitoneally. The animal s' hearts were isolated according to langendorff setup and were passed through baseline, ischemia, and reperfusion stages. Then, cardiac mass index (ratio of heart weight to body weight) was measured. Cardiac functional parameters, including left ventricular developed pressure and rate pressure product, were also assessed at baseline and following ischemia. The data were analyzed using ANOVA and $P < 0.05$ was considered to be statistically significant.
***Results*:** Isoproterenol-induced cardiac hypertrophy was significantly reduced by both doses of diazepam compared to the group only treated with isoproterenol ($P < 0.05$) although it did not reach the control level. However, diazepam administration (1 and 5 mg/kg) did not change isoproterenol-induced exacerbated ischemia-reperfusion injury compared to the control group ($P = 0.001$ and $P = 0.013$, respectively).
***Conclusions*:** Diazepam relatively prevented the isoproterenol–induced cardiac hypertrophy in the animal model. This effect could be probably explained by the modification of oxidative stress and preservation of intracellular calcium concentration. Considering the common clinical usage of diazepam, as a peripheral benzodiazepine ligand, antihypertrophic effects of diazepam are recommended to be investigated in clinical trials.

►*Implication for health policy/practice/research/medical education:*
Administration of diazepam could partially reduce myocardial hypertrophy induced by isoproterenol, but it could not prevent myocardial ischemia-reperfusion injury of hypertrophied hearts following ischemia.

1. Background

Hypertrophy of the heart is a common physiological, pathological, and clinical state in humans (1). Cardiac hypertrophy occurs as a physiological adaptation to increased hemodynamic load; however, sustained hypertrophy can lead to maladaptation and has been established as an independent risk factor for cardiac morbidity and mortality (2, 3). Hypertrophied hearts exhibit an increased sensitivity to ischemic injury and their function is weaker compared to normal hearts at this condition (4-6). Ischemia of the heart is one of the most important factors resulting in human mortality (1, 7). There are several potential mechanisms for myocardial hypertrophy, including pregnancy, valvular disorders, exercise, myocardial disorders, high blood pressure, and familial and genetic backgrounds (1, 4, 6, 8). Several reports have shown that reversible perfusion

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abnormalities are common in patients with hypertrophied hearts, because hypertrophied myocardium needs more oxygen compared to normal hearts (1, 4, 6, 8-12). It has also been revealed that myocardial ischemia occurs in patients with hypertrophic cardiomyopathy (12, 13). Some cellular factors could affect cardiac hypertrophy and its related complications, as well.

Peripheral Benzodiazepine Receptors (PBRs), also known as 18kDa translocator proteins, are expressed in various tissues, including the heart, and play an important role in regulation of cardiac hypertrophy and Ischemia–Reperfusion (I/R) injury (3, 14-17). Hence, they represent a novel therapeutic target in cardiovascular diseases (18).

Diazepam, a benzodiazepine derivative, is clinically used as a tranquilizer, a muscle relaxant, and an anti-convulsant agent (16). Repeated administration of diazepam can change the density of PBRs in peripheral tissues and organs (14, 15, 17). Its administration can also affect myocardial susceptibility to ischemic injury (7, 18). Furthermore, the crucial role of PBRs in modulation of cardiac hypertrophy has been reported previously (3).

2. Objectives

Considering the role of PBR ligands in myocardial function, cardiac hypertrophy, and its related complications, the present study aims to investigate the effect of diazepam on hypertrophied rats' hearts. Obviously, the outcomes of the current study would be useful for clinical consideration of benzodiazepines in this regard.

3. Materials and Methods

This experimental study was performed at the Medical Biology Research Center, Kermanshah University of Medical Sciences (KUMS), Kermanshah, Iran. It was approved by the Ethics Committee of KUMS. Also, all the animals used in the 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).

3.1. Materials

All the chemicals used in the current study were obtained from Merck (Darmstadt, Germany) in the highest grade available. Besides, isoproterenol and sodium pentobarbital were purchased from Sigma-Aldrich Co., USA and diazepam was provided by Chemi Daru Company, Tehran, Iran.

3.2. Experimental Scheme

Isoproterenol (4mg/kg body weight) was injected intraperitoneally once daily for 7 days (2) to induce cardiac hypertrophy. Also, 2 doses of diazepam, (1 and 5 mg/kg body weight) were injected intraperitoneally for 5 days (7). The animals weighing 210 - 270 g were randomly allocated to 6 groups. The treatment scheme for different groups (n = 7 - 10) was as follows:

Control: normal saline (vehicle, 1ml/kg body weight) intraperitoneally,

Iso: isoproterenol 4mg/kg body weight,

Diaz 1: diazepam 1 mg/kg body weight,

Diaz 5: diazepam 5 mg/kg body weight,

Iso + Diaz 1: isoproterenol 4 mg/kg body weight + diazepam 1 mg/kg body weight, and

Iso + Diaz 5: isoproterenol 4 mg/kg body weight + diazepam 5 mg/kg body weight.

The rats were anesthetized by intraperitoneal administration of 60 mg/kg sodium pentobarbital and weighed before heart isolation. Hypertrophy was characterized and calculated for each group by the ratio of heart weight to body weight (mg/gr), which was defined as Cardiac Mass Index (CMI) (3). The ratio of CMI changes in different groups in comparison to the control group was assessed and defined as CMI%. The percentage of change in cardiac hypertrophy was also calculated on the basis of CMI% in different groups compared to the control group according to the following formula:

$$\frac{(\text{HW/BW test} - \text{HW/BW control})}{(\text{HW/BW control})} \times 100$$

3.3. Isolated Heart Preparation

After anesthesia, 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, NaHCO3 25, KCl 4.8, KH2PO4 1.2, MgSO4 1.2, glucose 11, and CaCl2 1.2) at the pH of 7.4 (19).

The buffer was bubbled with 95% O2 and 5% CO2 at 37ºC 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 into the left ventricle through the mitral valve. This balloon was connected via a rigid polyethylene tube to a pressure transducer (MLT 844; AD Instruments, New South Wales, Australia). The pressure transducer was connected via a power lab (model ML825; AD Instruments) to a computer for 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 during 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/minute). Rate Pressure Product (RPP) was also calculated as: LVDP×HR. Additionally, Coronary Flow (CF) was measured by collections of the coronary effluent per minute. The baseline data were recorded after a 15-minute stabilization and equilibration period. Global normothermic ischemia was induced by clamping the aortic cannula for 45 minutes. The temperature was maintained by immersing the heart in perfusion medium at 37ºC. After ischemia, the hearts were subjected to global reperfusion for 45 minutes. Hereby, the level of I/R injury was assessed by comparing the cardiac parameters before and after ischemia. The recovery percentage was also defined by the ratio of RPP at the 45th minute of reperfusion to that at the 15th minute of baseline in each group.

3.4. Statistical Analysis

The results have been expressed as mean ± Standard Error of Mean (SEM). Comparisons between the data sets were made by ANOVA and Tukey's post hoc test using the SPSS statistical software, version 16 (SPSS Inc., Chicago, IL, USA). Besides, $P < 0.05$ was considered to be statistically significant.

4. Results

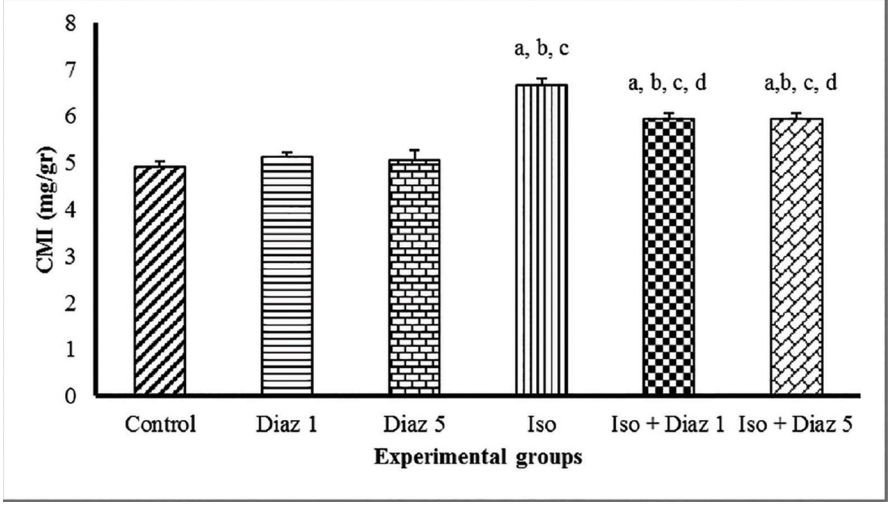
The mean body weight of animals was 262 ± 18 (gr) in the control group, 236 ± 13 (gr) in the Iso group, 258 ± 13 (gr) in the Diaz 1 group, 232 ± 12 (gr) in the Diaz 5 group, 218 ± 13 (gr) in the Iso + Diaz 1 group, and 218 ± 7 (gr) in the Iso + Diaz 5 group. However, the differences were not statistically significant.

According to Figure 1, CMI was significantly higher in the Iso-treated groups compared to the control, Diaz 1, and Diaz 5 groups ($P < 0.001$). Additionally, both doses of diazepam (1 and 5 mg/kg) significantly prevented the Iso-induced increase in CMI compared to the only Iso-treated group ($P = 0.008$ and $P = 0.013$, respectively). However, this index was significantly higher in comparison to the other groups (control, Diaz 1, and Diaz 5). In addition, the CMI% of the Iso-treated groups, including Iso, Iso + Diaz 1, and Iso + Diaz 5, were 35.44%, 20.87%, and 20.7%, respectively.

4.1. Hemodynamic Function

The means of the cardiac parameters, including HR, LVDP, CF, and RPP, have been demonstrated in Table 1. Accordingly, the hearts showed normal performance at baseline and there were no significant differences among the study groups in this period. However, RPP was significantly

Figure 1. The Effect of Diazepam on Cardiac Mass Index (CMI) of the Experimental Groups



The data have been presented as mean ± SEM in Diaz 1, diazepam 1 mg/kg; Diaz 5, diazepam 5 mg/kg; Iso, isoproterenol-treated group; Iso + Diaz 1, isoproterenol co-administered with diazepam 1 mg/kg; Iso + Diaz 5, isoproterenol co-administered with diazepam 5 mg/kg. [a] Significant compared to the control group, [b] significant compared to the Diaz 1 group, [c] significant compared to the Diaz 5 group, and [d] significant compared to the ISO group. $P < 0.05$ is significant.

Table 1. The Effect of Diazepam on Cardiac Parameters in Isoproterenol-Induced Cardiac Hypertrophy at Baseline and 45th Minute of Reperfusion Following Ischemia

Periods and Parameters	Baseline				The 45th Min Reperfusion			
Groups	**LVDP**	**HR**	**CF**	**RPP**	**LVDP**	**HR**	**CF**	**RPP**
Control n = 7	92.8 ± 3.8	252 ± 15	14 ± 1.2	23393 ± 1553	53.4 ± 5.2	202 ± 19	8.36 ± 1.57	10601 ± 1060
Diaz 1 n = 8	89.2 ± 10.4	270 ± 23	14.4 ± 1.1	22603 ± 1559	50.6 ± 7.4	213 ± 19	7.94 ± 0.68	10429 ± 1387
Diaz 5 n = 8	91.4 ± 6.7	277 ± 17	12 ± 0.48	24668 ± 916	31.6 ± 7.5	231 ± 47	5.19± 0.47	5268 ± 755 [a, b]
Iso n = 8	92.3 ± 7.8	265 ± 22	12.8 ± 0.6	23452 ± 867	34.9 ± 10.1	212 ± 54	7.06 ± 0.84	5045 ± 818 [a, b]
Iso + Diaz 1 n = 10	87.3 ± 2.3	250 ± 7	11 ± 0.82	21911 ± 586	28.2 ± 3.9	178 ± 19	5.55 ± 0.42	4801 ± 575 [a, b]
Iso + Diaz 5 n = 8	77.7 ± 3.3	278 ± 13	12.1 ± 0.6	21369 ± 381	30.6 ± 5.4	193 ± 9	7 ± 0.77	5734 ± 988 [a, b]

Abbreviations: LVDP, left ventricular developed pressure (mmHg); HR, heart rate (beats/minute); CF, coronary flow (mL/minute); RPP, rate pressure product (LVDP×HR)

Diaz 1, diazepam 1 mg/kg; Diaz 5, diazepam 5 mg/kg; Iso, isoproterenol-treated group; Iso + Diaz 1, isoproterenol co-administered with diazepam 1 mg/kg; Iso + Diaz 5, isoproterenol co-administered with diazepam 5 mg/kg. Values have been presented as mean ± SEM.

[a] Significant compared to the control group, and [b] significant compared to the Diaz1 group. $P < 0.05$ is significant.

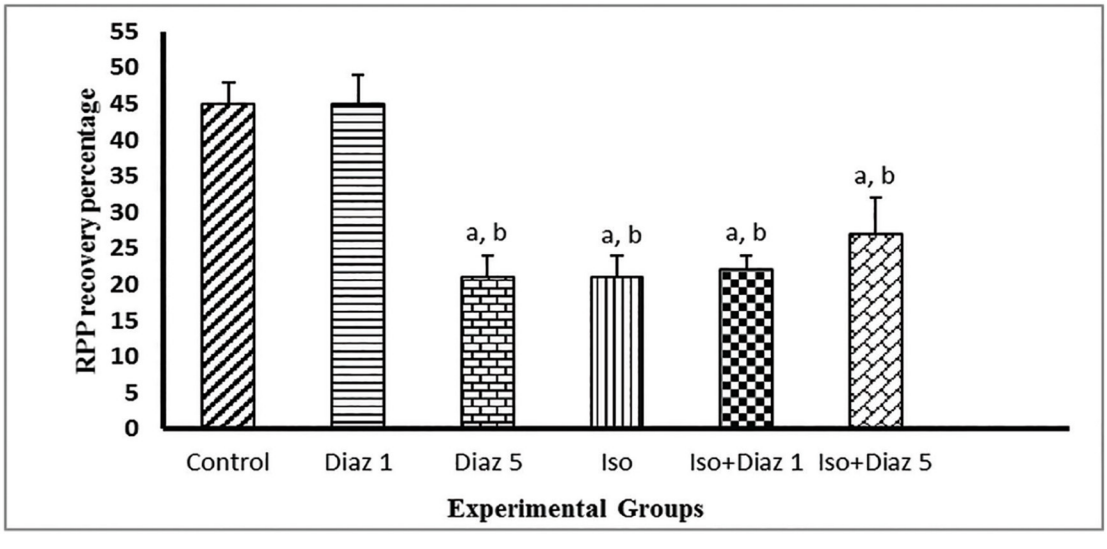
reduced in Diaz 5, Iso, Iso + Diaz 1, and Iso + Diaz 5 groups compared to the control and Diaz 1 groups at the 45th minute of reperfusion (p-values have been shown in Table 1). As illustrated in Figure 2, the RPP recovery percentage was significantly reduced in the isoproterenol-treated groups, including Iso, Iso + Diaz 1, Iso + Diaz 5, and Diaz 5 groups compared to the control group (21.4 ± 3.4%, 21.85 ± 2.6%, 26.75 ± 4.4%, 21.4 ± 3.1%, and 45.33 ± 3.2%, respectively).

5. Discussion

The results of the current study revealed that CMI was significantly increased in the isoproterenol-treated groups, indicating cardiac hypertrophy following administration of isoproterenol. Previous studies also showed that administration of isoproterenol could lead to cardiac hypertrophy (2, 20). In fact, chronic stimulation of myocardial β-receptors could induce the process of hypertrophy (21, 22). Our findings also showed that administration of diazepam 1 and 5 mg/kg significantly decreased the CMI of the isoproterenol-treated groups, which revealed the preventive role of diazepam against isoprpterenol-induced cardiac hypertrophy. However, CMI was still higher compared to the control group. This implies that diazepam could not completely prevent this process and hypertrophy relatively remained. These findings were confirmed by the results of CMI% that, compared to the control group, was about 35% higher in the isoproterenol-treated group and changed to about 20% in the Iso-Diaz groups. This showed the relative reduction of CMI% in the presence of diazepam. Consistently, several studies have shown that the presence of PBR ligands, including Ro5-4864 and SSR180575, could reduce oxidative stress, thereby preventing cardiac hypertrophy (3, 23, 24). Oxidative stress is implicated in development and progression of cardiac hypertrophy (25). It has been reported that the PBR ligands are involved in protection of cardiac cells against isoproterenol-induced hypertrophy due to their modulatory effects on oxygen radical damage (3). Similarly, our results showed for the first time that diazepam, as a common clinical drug, could prevent isoproterenol-induced cardiac hypertrophy, which could probably be explained by the above-mentioned modulatory effect of this drug as a PBR ligand. On the other hand, several studies have shown that diazepam was able to affect cells by mechanisms related or unrelated to PBRs (24, 26). For example, diazepam decreased the Ca^{2+} transient and the L-type Ca^{2+} channel, which are both important in the negative inotropism and chronotropism caused by this drug (27-29). In the same line, another study on guinea pigs' hearts indicated that the negative inotropic effect of diazepam referred to its calcium channel blocking effect (15). Ca^{2+}, as a second messenger and a regulatory factor, plays a pivotal role in progression of cardiac hypertrophy (30). Regarding the important role of Ca^{2+} in the hypertrophy process (31, 32), the results of the current study might be also explained by the calcium blocking effect of diazepam. However, intracellular calcium levels and oxidative stress were not measured, which is a limitation to this study. Thus, it is not clear which of these two mechanisms is involved in the preventive role of diazepam against isoproterenol-induced cardiac hypertrophy, and it needs to be elucidated in future studies.

In addition, the results of the current study showed that following ischemia, the RPP of the isoproterenol-treated groups was significantly lower than that of the control group, indicating exacerbation of I/R injury in the hypertrophied hearts. In fact, the lower cardiac recovery percentage shows higher susceptibility of the isoproterenol-treated groups to I/R injury. In the previous studies, several

Figure 2. The Recovery Percentage of the Rate Pressure Product (RPP) in the Isolated Hearts of the Study Groups after Ischemia-Reperfusion



The data have been presented as mean ± SEM in Diaz 1, diazepam 1 mg/kg; Diaz 5, diazepam 5 mg/kg; Iso, isoproterenol-treated group; Iso + Diaz 1, isoproterenol co-administered with diazepam 1 mg/kg; Iso + Diaz 5, isoproterenol co-administered with diazepam 5 mg/kg. [a] Significant compared to the control group, and [b] significant compared to the Diaz 1 group. $P < 0.05$ is significant.

mechanisms were reported for the exacerbated I/R injury of the hypertrophied hearts, including imbalance between myocardial oxygen supply and demand (1). This imbalance is caused by inadequate capillary density in relation to the increased myocardial mass and limitation of blood flow despite high oxygen demand of the myocardium (9, 33-35). Therefore, it could exacerbate the myocardial injury after ischemia (5, 21, 22). As it was expected, our findings showed the exacerbation of I/R injury in the isoproterenol-induced hypertrophied hearts. However, exacerbation of I/R injury in the hypertrophied hearts was not affected by the presence of diazepam. This means that diazepam administration could not prevent the exacerbated I/R injury in the isoproterenol-treated groups probably due to the fact that cardiac hypertrophy was still remained in these groups, and exacerbation of I/R injury occurred along with remaining of cardiac hypertrophy. The exacerbated I/R injury was also detected in the Diaz 5 group in the current study. The side effects of high doses of diazepam, including reduced recovery percentage and exacerbated cardiac I/R injury, have been reported previously (7). In addition, this negative effect of high dose of diazepam did not affect the exacerbated I/R injury of the hypertrophied hearts in a synergistic manner, as shown in the Iso + Diaz 5 group in the current study. The non-synergistic effects of diazepam on this process are probably due to the complex effects of PBR ligands on cardiomyocytes, including modification of oxidative stress in hypertrophied hearts (3). In agreement with the other reports, the unpredictable effects of PBR ligands were shown in the presence of diazepam in the current study.

In conclusion, administration of diazepam relatively reduced the isoproterenol–induced cardiac hypertrophy in animal model. This effect could probably be explained by the modification of oxidative stress and preservation of intracellular calcium concentration. In addition, presence of diazepam could not affect the exacerbated myocardial I/R injury of the hypertrophied hearts. Considering the common clinical usage of diazepam, as a peripheral benzodiazepine ligand, antihypertrophic effects of diazepam are recommended to be investigated in clinical trials.

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Authors' Contribution

Study concept and design: Dareuosh Shackebaei, Mahvash Hesari; Acquisition of data: Farid Feizollahi, Mahvash Hesari; Analysis and interpretation of data: Farid Feizollahi, Mahvash Hesari, Dareuosh Shackebaei; Drafting of the manuscript: Dareuosh Shackebaei, Mahvash Hesari, Farid Feizollahi; Critical revision of the manuscript for important intellectual content: Dareuosh Shackebaei, Mahvash Hesari; Statistical analysis: Farid Feizollahi; Administrative, technical, and material support: Dareuosh Shackebaei, Mahvash Hesari, Gholamreza Bahrami; Study supervision: Dareuosh Shackebaei, Gholamreza Bahrami, Mahvash Hesari

Financial disclosure

The sponsor had no role in the design and conduct of the study, collection, management, and analysis of the data, or preparation, review, and approval of the manuscript.

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