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Cardioprotective effect of diazepam on ischemia-reperfused isolated hyperthyroid rat heart

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Abstract: Hyperthyroidism increases the vulnerability of the heart to ischemia-reperfusion (I/R) injury. Regarding the fact that diazepam also affects cardiac I/R injury, the current study was conducted to investigate the effect of diazepam on ischemia-reperfused isolated hyperthyroid rat heart. The animals were divided into 6 groups: the control, hyperthyroid, control diazepam (1 mg/kg), hyperthyroid diazepam (1 mg/kg), control diazepam (5 mg/kg), and hyperthyroid diazepam (5 mg/kg) groups. Langendorff-perfused rat hearts underwent 40 min of global ischemia and 45 min of reperfusion. Different cardiac parameters, including the left ventricular developed pressure, heart rate, coronary flow, and rate pressure product (RPP), were measured. Lactate dehydrogenase (LDH) release was determined in reperfusion to evaluate the severity of the myocardial injury. The results showed that the RPP recovery percentage significantly decreased in the hyperthyroid group compared with control group, whereas comparison of the hyperthyroid diazepam (1 mg/kg) with the control and control diazepam (1 mg/kg) groups did not reveal a significant difference. These results were confirmed by LDH test, demonstrating that administration of diazepam (1 mg/kg) protected the heart against I/R injury in the hyperthyroid group and significantly decreased the I/R injury, which was probably due to the function of diazepam as a phosphodiesterase 4 inhibitor.

Key words: Diazepam, hyperthyroidism, isolated heart, ischemia-reperfusion, cardioprotective

Introduction

Normal heart structure and function are dependent on normal thyroid hormone levels (1). Changes in thyroid hormone levels alter the vulnerability of the heart against ischemia-reperfusion (I/R) injury (2). For instance, it has been found that in hyperthyroidism, the susceptibility of the myocardium to I/R injury increases due to mitochondrial respiration dysfunction (2), H_2O_2 production, and higher oxidative damage (3). Furthermore, thyroid hormone can affect peripheral benzodiazepine receptor (PBR) density (4).

The PBR is an 18-kDa protein, consisting of 169 amino acids and located in the outer mitochondrial membrane (5). It has been suggested that PBRs are involved in several processes, including the regulation of the opening of the mitochondrial permeability transition pore (MPTP), cell necrosis (6,7), apoptosis, respiratory chain, and ion channel activity (8,9). Meanwhile, cellular resistance to oxidative stress has been shown to be related to the expression of these receptors in cells (10).

It has been shown that chronic diazepam administration regulates PBR density in the peripheral

organs (11). For example, it was demonstrated that a 14-day diazepam administration produced upregulation of heart PBR density (12). Therefore, cardiac resistance to I/R injury might be affected by chronic diazepam administration. The protective effects of diazepam have also been reported (13). For example, it was indicated that diazepam, as a selective type-4 phosphodiesterase inhibitor, leads to cyclic adenosine monophosphate (cAMP) enhancement in cardiac tissue (14). In vitro and in vivo studies have demonstrated that an increase of cAMP inhibits apoptosis in cardiomyocytes and reduces mortality in acute myocardial infarction (13). Moreover, studies have shown that diazepam is able to affect cells by paths related (8) or unrelated (15) to PBR.

According to the dual effects of diazepam on cells, it is not clear how it can affect the hyperthyroid heart during I/R. This is a point that has not been reported in previous studies. Therefore, regarding the positive and negative effects of diazepam on I/R injury and the common clinical use of benzodiazepines, the current study was conducted to investigate effects of diazepam on ischemia-reperfused isolated hyperthyroid rat heart.

Materials and methods

Materials

All of the chemicals (Merck, Darmstadt, Germany) were of the highest grades available. Lactate dehydrogenase (LDH) was assessed using a cell cytotoxicity kit (Roche, Mannheim, Germany). L-thyroxine (T_4) was purchased from Sigma (Steinheim, Germany) and the diazepam was from the Chemi Daru Company (Iran, Tehran).

Animals and their treatments

This investigation was approved by the ethics committee of the Kermanshah University of Medical Sciences. All of the animals used in the present study received humane care in compliance with the institutional animal care guidelines. Male Wistar rats (250-300 g) were obtained from the animal care center at the Kermanshah University of Medical Sciences. They were maintained under standard laboratory conditions (controlled temperature of 21 °C, 12-h light/dark cycle).

The animals were divided into 6 groups, as follows.

1. General control group (n = 8): This group was fed normal rat pellet and normal water, and had an intraperitoneal (IP) injection of normal saline (sodium chloride) for 5 days.
2. Hyperthyroidism group (n = 8): In this group, hyperthyroidism was induced by adding T_4 (Sigma, catalog number T-2376; 12 mg/L) to the drinking water for 21 days (16), and the animals also had an IP injection of normal saline during the last 5 days of the treatment.
3. Control diazepam (1 mg/kg)-administered group (n = 8): This group received the same treatment as group 1, but received a daily IP injection of 1 mg/kg diazepam for 5 days.
4. Hyperthyroidism diazepam (1 mg/kg)-administered group (n = 8): This group received the same treatment as group 2, but received a daily IP injection of 1 mg/kg diazepam during the last 5 days of treatment.
5. Control diazepam (5 mg/kg)-administered group (n = 9): This group received the same treatment as group 1, but received a daily IP injection of 5 mg/kg diazepam for 5 days.
6. Hyperthyroidism diazepam (1 mg/kg)-administered group (n = 10): This group received the same treatment as group 2, but received a daily IP injection of 5 mg/kg diazepam during the last 5 days of treatment.

Thyroid hormone concentration

The animals were randomly selected, and after anesthesia, blood samples were collected from the abdominal aorta, placed in tubes, and immediately centrifuged at 1500 rpm for 5 min. The serum thyroid hormone concentration was then estimated using the radioimmunoassay method (17).

Isolated heart preparation

Isolated rat hearts were perfused at a constant hydrostatic pressure according to the Langendorff technique (18,19). The rats were anesthetized with an IP administration of 60 mg/kg pentobarbital sodium (Sigma). The hearts were rapidly excised, placed in ice-cold Krebs buffer (4 °C), rapidly cannulated and retrogradely perfused through the aorta in a noncirculating Langendorff apparatus (Harvard

Apparatus Ltd., Edenbridge, UK) with Krebs solution [containing sodium chloride (118 mmol/L), 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 Krebs solution was filtered through Whatman paper No. 1002-125 and then bubbled with 95% oxygen and 5% carbon dioxide at 37 °C, and perfusion was performed under a constant hydrostatic pressure of 65 mmHg. 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 an ML110 BP amp (AD Instruments), an ML825 Power Lab 2.25 (AD Instruments) system, and Chart 5 software (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 mmHg. This volume was then kept constant for the duration of the study. Different indices of myocardial function were assessed, including left ventricular developed pressure (LVDP, in mmHg), which was defined as the peak systolic pressure minus the end diastolic pressure, and heart rate (HR, in beats per minute). The rate pressure product (RPP) was calculated as: RPP = LVDP × HR. Coronary flow (CF) was measured by timed volumetric collection of the coronary effluent. The baseline data were recorded after a 20-min 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 40 min, followed by reperfusion for 45 min.

LDH assessment

When cells die, they release their intercellular LDH. Measuring this activity in the resulting supernatant reveals the extent of cell death. Therefore, the level of I/R injury was assessed based on the functional recovery and the release of LDH 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) and known quantities of LDH (Sigma) as a standard.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) and were compared using the unpaired Student's t-test as appropriate or ANOVA and Tukey's posttest, as offered by GraphPad InStat version 3.0 (GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was considered significant.

Results

Thyroid state

The thyroid state of different groups of animals was characterized by the data reported in Table 1. The values indicate a significant rise in T_3 after oral L-thyroxine administration ($P = 0.004$).

Hemodynamic function

The data for HR, LVDP, CF, and RPP at different periods of the experiment are summarized in Table 2. HR, CF, and RPP during the baseline period were higher ($P < 0.05$) in the hyperthyroid and hyperthyroid diazepam (5 mg/kg)-administered groups compared with their related controls. The LVDP and RPP at minute 45 of reperfusion in the hyperthyroid and hyperthyroid diazepam (5 mg/kg)-administered groups were lower than in the related control groups ($P < 0.05$).

Cardiac functional recovery percentage

The recovery percentage at minute 45 of reperfusion in the hyperthyroid group (18 ± 1.5%) was significantly lower ($P < 0.01$) than in the control group (43 ± 3.1%), and there was also a significant

Table 1. Comparison of the thyroid function test. Value was expressed as mean ± SEM.

Groups	T_3 (nmol/L)
Control (n = 6)	0.72 ± 0.048
Hyperthyroid (n = 6)	0.93 ± 0.029*

*P = 0.004 versus control (unpaired t-test).

Table 2. Cardiac parameters before and after exposure to 40 min of global normothermic ischemia in the groups.

Groups	Baseline values				45 min of reperfusion			
	LVDP (mmHg)	HR (beats/min)	CF (mL/min)	RPP (mmHg × beats/min)	LVDP (mmHg)	HR (beats/min)	CF (mL/min)	RPP (mmHg × beats/min)
Control	80.20 ± 3	247 ± 6	10.3 ± 0.58	19666 ± 640	34.88 ± 2.7	247 ± 6	5.65 ± 0.57	8503 ± 475
Hyperthyroid	78.8 ± 4	275.75 ± 8*	11.75 ± 0.28*	22148 ± 960*	23.95 ± 4.1*	220 ± 37	4.68 ± 0.33	4007 ± 258*
Control diazepam (1 mg/kg)	84.35 ± 5	263 ± 16	12.12 ± 0.50	21913 ± 1102	35.22 ± 7.13	252 ± 14	6.45 ± 0.47	9862 ± 1337
Hyperthyroid diazepam (1 mg/kg)	80.41 ± 5	267 ± 15	12.43 ± 0.53	22386 ± 1194	32.15 ± 4.7	285 ± 16	7.17 ± 0.61	8877 ± 1117
Control diazepam (5 mg/kg)	89.9 ± 4.7	208 ± 15	9.3 ± 0.61	18295 ± 1102	36 ± 5.1	199 ± 39	5.7 ± 0.6	6047 ± 829
Hyperthyroid diazepam (5 mg/kg)	81.56 ± 3	254 ± 6#	13.15 ± 0.86#	21639 ± 826#	17.31 ± 2.77#	217 ± 21	7.64 ± 1.2	3684 ± 658#

LVDP: left ventricular developed pressure (mmHg), HR: heart rate (beats/min), CF: coronary flow (mL/min), RPP: rate pressure product (LVDP × HR). Data were analyzed using unpaired t-test and expressed as mean ± SEM between the test and related control groups. The groups include: control (n = 8), hyperthyroid (n = 8), control diazepam (1 mg/kg)-administered (n = 8), hyperthyroid diazepam (1 mg/kg)-administered (n = 8), control diazepam (5 mg/kg)-administered (n = 9), and hyperthyroid diazepam (5 mg/kg)-administered (n = 10). *$P < 0.05$ versus the control group and #$P < 0.05$ versus the control diazepam (5 mg/kg)-administered group (unpaired t-test).

difference between the control diazepam (5 mg/kg)-administered group (33 ± 4.2%) and the hyperthyroid diazepam (5 mg/kg)-administered group (17 ± 3%) ($P < 0.05$). However, there was no significant difference between the hyperthyroid diazepam (1 mg/kg)-administered group (40 ± 5.3%) and either the related control group (45.3 ± 5.5%) or the general control group (43 ± 3.1%). The recovery percentage in the hyperthyroid diazepam (1 mg/kg)-administered group (40 ± 5.3%) was significantly higher ($P < 0.01$) than in the hyperthyroid group (18 ± 1.5%). Moreover, there was a significant difference between the control and hyperthyroid diazepam (5 mg/kg)-administered groups ($P < 0.001$) (Figure 1).

Lactate dehydrogenase release

The extent of reperfusion injury in the 6 groups was determined based on 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 hyperthyroid group (41 ± 8.14 mU/mL) was significantly higher than that of the control group (11.45 ± 6.25 mU/mL) ($P < 0.01$); there was no significant difference between the hyperthyroid diazepam (1 mg/kg)-administered group (20.28 ± 4.16 mU/mL) and either the related control group (12.86 ± 0.32 mU/mL) or the general control group (11.45 ± 6.25 mU/mL). There was a significant difference between the control diazepam (5 mg/kg)-administered group (37.2 ± 1.02 mU/mL) and the hyperthyroid diazepam (5 mg/kg)-administered group (57.95 ± 1.55 mU/mL) ($P < 0.05$). The LDH in the hyperthyroid diazepam (1 mg/kg)-administered group (20.28 ± 4.16 mU/mL) was significantly lower than that in the hyperthyroid group ($P < 0.05$). Levels of LDH in the hyperthyroid diazepam (5 mg/kg)-administered group (57 ± 1.55 mU/mL) were significantly higher than those of the hyperthyroid diazepam (1 mg/kg)-administered group ($P < 0.001$).

Discussion

The results of the current study demonstrate that diazepam modulates hyperthyroidism effects. These new findings are indicative of the effects of benzodiazepine on changes of heart activity caused by hyperthyroidism. As illustrated in Table 2, chronic diazepam (1 mg/kg) administration to hyperthyroid rats led to protection against I/R injury in the heart. Moreover, the exacerbated I/R injury that occurred in the hyperthyroid group was not seen in the hyperthyroid diazepam (1 mg/kg) group (Figure 1). This finding was also confirmed by LDH results (Figure 2). In fact, compared with the hyperthyroid group, the significant reduction of released LDH in the hyperthyroid animals that received diazepam (1 mg/kg) represents decreased I/R injury in this group. As illustrated in Figure 1, in the hyperthyroid group, cardiac functional recovery following I/R was significantly lower than that of the control group. This finding was confirmed by the LDH results, which are presented in Figure 2. The increase in concentration of released LDH in the first minutes of reperfusion in the hyperthyroid group versus the control indicates further damage to the heart during the I/R period. This observation confirms previous reports indicating higher susceptibility of the hyperthyroid rat heart to I/R (2,20,21). Previous studies have revealed that mitochondrial function is impaired in hyperthyroidism due to the excessive production of reactive oxygen species, dysfunction of mitochondrial respiration at stage 3 (3), and increased H_2O_2 production (2). Moreover, it was reported that chronic thyroxine administration gives rise to rat PBR density (4). Enhancement of PBR density leads

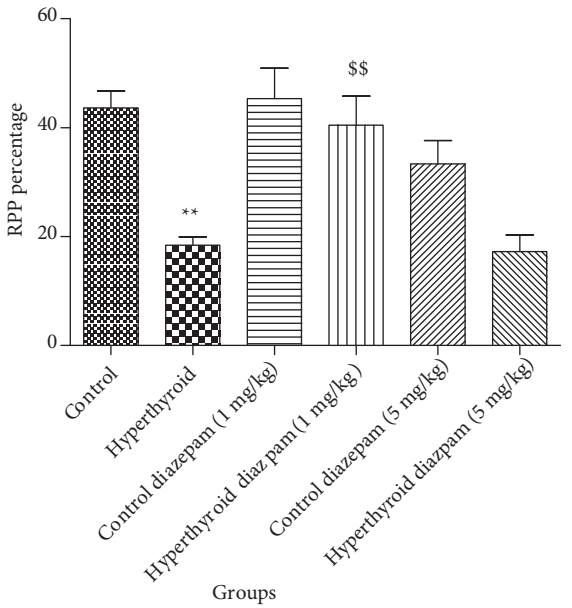

Figure 1. Recovery percentage (in comparison with the baseline) of the RPP at 45 min of reperfusion following 40 min of global normothermic ischemia in the control group (n = 8), hyperthyroid group (n = 8), control diazepam (1 mg/kg)-administered group (n = 8), hyperthyroid diazepam (1 mg/kg)-administered group (n = 8), control diazepam (5 mg/kg)-administered group (n = 9), and hyperthyroid diazepam (5 mg/kg)-administered group (n = 10). Data are expressed as mean ± SEM with ANOVA. **P < 0.01 versus the hyperthyroid diazepam (1 mg/kg)-administered group. $$P < 0.01 versus the hyperthyroid diazepam (5 mg/kg)-administered group.

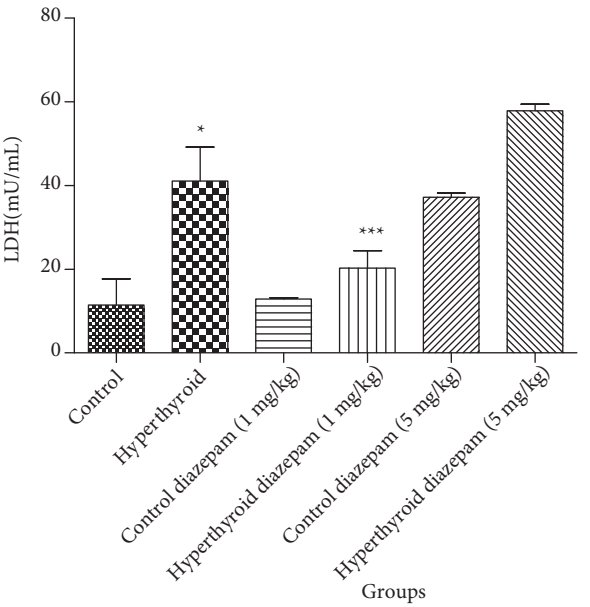

Figure 2. The concentration of LDH enzyme (mU/mL) that was released during the first minute of reperfusion following 40 min of global normothermic ischemia in the control group (n = 8), hyperthyroid group (n = 8), control diazepam (1 mg/kg)-administered group (n= 8), hyperthyroid diazepam (1 mg/kg)-administered group (n = 8), control diazepam (5 mg/kg)-administered group (n = 9), and hyperthyroid diazepam (5 mg/kg)-administered group (n = 10). Data are expressed as mean ± SEM with ANOVA. *P < 0.05 versus the hyperthyroid diazepam (1 mg/kg)-administered group. ***P < 0.001 versus the hyperthyroid diazepam (5 mg/kg)-administered group.

to an increase in the intramitochondrial calcium of cardiomyocytes, which in turn increases MPTPs (22), mitochondrial dysfunction, and, consequently, cell death (23). Therefore, the exacerbated I/R injury in the hyperthyroid group can be justified by these mechanisms. In addition, the findings of the present study indicate cardiac protection in the hyperthyroid diazepam (1 mg/kg) group. Other studies have demonstrated that chronic diazepam administration can alter the vulnerability of cardiac I/R injury by the enhancement of PBR density (24). The enhancement of PBR density also occurs in hyperthyroidism (4). Therefore, it is expected that diazepam would lead to negative effects in the hyperthyroid heart during I/R. However, the findings of the current study suggest that diazepam not only improved heart function, but also had a nonnegative effect and protected the heart against I/R injury. Previous studies have reported that diazepam acts as an inhibitor of phosphodiesterase 4 (25). Inhibition of this isoenzyme results in increased cAMP levels (25), and thereby suppresses tumor necrosis factor-alpha (TNF-α) (26). Furthermore, the inhibition of phosphodiesterase results in the blockage of TNF-α gene transcription and, consequently, its protein production (27). The reduction in TNF-α production could be regarded as an important mechanism that protects the heart against I/R injury (27). TNF-α is one of the cytokines that may contribute to cell death by apoptosis, which occurs during I/R injury (28). Moreover, it has been observed that after chronic thyroxine administration, TNF-α will increase (29). Thus, the exacerbation of I/R injury may have been due to increased TNF-α in the hyperthyroid group. It seems that in the current study, diazepam (1 mg/kg) was able to affect heart function through the inhibition of phosphodiesterase type 4, the enhancement of cAMP, and, subsequently, the reduction of TNF-α. Therefore, the protective effect of diazepam in the hyperthyroid heart may be explained by this mechanism. However, the exact role of this mechanism remains to be elucidated in future studies.

Another valuable finding of the current study was the modulation of heart function in the hyperthyroid group after diazepam (1 mg/kg) administration.

As illustrated in Table 2, there was a significant difference between the baseline heart rate in the hyperthyroid group and its related control group. This difference was not seen, however, after diazepam (1 mg/kg) administration. It has been reported that hyperthyroidism increases the heart rate via several mechanisms, such as increased L-type calcium channels (30,31), activity of Ca^{+2} ATPase (32), alpha-myosin heavy chain gene expression (31), and decreased phospholamban (33). The chronotropic effect of hyperthyroidism was eliminated after diazepam (1 mg/kg) administration, which represents the modulatory effects of diazepam (1 mg/kg) on heart function. Further studies are required to reveal the cellular mechanism of diazepam on the heart rate of hyperthyroid rats.

The cardioprotective effect of diazepam (1 mg/kg) was not seen after the administration of diazepam (5 mg/kg) in the hyperthyroid group. Moreover, the heart rate in the hyperthyroid diazepam (5 mg/kg) group was significantly higher than that of its related control group (Table 2).

Exacerbated I/R injury was seen in the hyperthyroid diazepam (5 mg/kg) group compared with the related control groups, as was the case for the hyperthyroid group compared with its related control groups. In addition, the enhancement of released LDH in these groups confirmed I/R injury (Figure 2). In fact, diazepam (5 mg/kg) cannot protect from exacerbated I/R injury. The negative effects of diazepam (5 mg/kg) were shown in a previous study (11). Dose-dependent effects of benzodiazepines have also been reported (34). For example, previous studies demonstrated that PBR ligands could induce MPTP opening in a dose-dependent manner (34). Therefore, the protective effect of diazepam is limited to a dosage of 1 mg/kg. Overall, the findings of the current study reveal that diazepam modulates hyperthyroid cardiac function; these effects are dose-dependent and are seen at 1 mg/kg.

The current study showed that administration of diazepam (1 mg/kg) significantly reduced the exacerbated I/R injury in the hyperthyroid group. This effect was probably due to the effect of diazepam as an inhibitor of phosphodiesterase 4.

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