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# Myocardial Function of Hyperthyroid Rats in Presence of Diazepam in Langendorff Setup

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ABSTRACT

**Background:** Modulation of Ischemia-Reperfusion (I/R) injury is highly important in medicine, especially in hyperthyroidism condition. The effect of diazepam as a benzodiazepine on cardiac I/R injury is also clinically important. The chronic effect of diazepam in this case has been reported previously.

**Objectives:** This study aimed to investigate the effect of acute diazepam perfusion on isolated heart of hyperthyroid rats during I/R.

**Materials and Methods:** Male rats (n = 32, weighing 250 - 300 gr) were randomly divided into 4 groups: control, hyperthyroid, control diazepam perfused, and hyperthyroid diazepam perfused. Isolated hearts were perfused through Langendorff method. Four sets of data were collected at baseline (20 minutes), diazepam perfusion (10 minutes, 100 µmol/L), ischemia (40 minutes), and reperfusion (45 minutes) periods. Cardiac parameters, including Left Ventricular Developed pressure (LVDP; mmHg) and Rate Pressure Product (RPP; mmHg × beats/min) were measured, as well. Lactate dehydrogenase (LDH; mU/mL) was also assessed for evaluation of I/R injury. The data were analyzed using ANOVA and Student t-test and $P < 0.05$ was considered as statistically significant.

**Results:** RPP significantly declined in the hyperthyroid group after diazepam perfusion compared to the baseline (24046 ± 1381 versus 18269 ± 711, P = 0.012, 95% CI: 1724 - 9828). Besides, RPP and LVDP significantly increased in the hyperthyroid diazepam perfused group compared to the hyperthyroid group at the end of the reperfusion period (12469 ± 1422 versus 4007 ± 258, $P < 0.001$, CI: 5066 - 11856 and 47 ± 2.8 versus 23 ± 2.8, $P < 0.001$, CI: 11-35, respectively). These findings were confirmed by LDH levels (19.08 ± 1.06 versus 41.07 ± 8.14, P = 0.002, CI: -34.4 - -9.4-35).

**Conclusions:** The results showed that acute diazepam perfusion to isolated hyperthyroid rats' hearts could significantly improve cardiac function following ischemia and protect the heart against I/R injury.

►*Implication for health policy/practice/research/medical education:*
Regarding the critical role of modulation of Ischemia-Reperfusion (I/R) injury in medicine, this study performed to clarify the effect of diazepam on myocardial function of hyperthyroid rats' hearts. We showed diazepam perfusion could decline the exacerbated I/R injury of isolated hearts in hyperthyroidism.

## 1. Background

Heart is one of the main target organs for action of thyroid hormones and any changes in thyroid hormones affect the heart function (1). Thyroid hormones change the cardiac parameters through acting on some molecular pathways in the heart (1). For example, T3 directly affects L-type calcium channels and enhances calcium entry into cardiomyocytes (2-4). Calcium influx has some important physiological roles in cell contraction, gene transcription, protein phosphorylation, and secretion (5). On the other hand, it has been shown that hyperthyroidism increases

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the vulnerability of the heart against Ischemia-Reperfusion (I/R) injury (6-9). Mitochondrial respiratory dysfunction and excessive production of reactive oxygen species are some of the mechanisms which are responsible for increase of I/R injury in hyperthyroid hearts (7). Furthermore, combination of oxidative stress and increase in $Ca^{2+}$ concentration that occurs in myocardial cells during I/R could decline mitochondrial respiration (1). Therefore, calcium enhancement in these conditions can be considered as a factor in exacerbation of I/R injury.

It has been reported that diazepam could also affect cardiac contractility (10-12). Diazepam is a benzodiazepine derivative which is commonly used in medicine (13). Benzodiazepines have different effects on the heart (14). For example, they can exacerbate cardiac I/R injury by different mechanisms, including regulation of permeability of mitochondrial membrane transition pore, cell necrosis (15, 16), apoptosis, respiratory chain, and ion channel activity (17, 18). It has been reported that chronic diazepam (5 mg/kg) administration increases the vulnerability of the heart against I/R injury (13). Regarding to the fact that hyperthyroid patients are more prone to cardiac dysfunction, the interaction of diazepam and thyroid hormones on the heart is clinically important. Our previous study showed that chronic diazepam administration in hyperthyroid conditions had no negative effects on ischemic heart (19). Based on other studies, acute and chronic diazepam administration triggered different mechanisms in isolated heart (13, 19, 20). Now, the question is that how acute dose of diazepam could affect hyperthyroid rats' hearts. To the authors' knowledge, no document is available regarding the effect of acute diazepam perfusion on hyperthyroid condition.

## 2. Objectives

It is clear that the results of the current study will reveal the safety of diazepam in hyperthyroid conditions.

## 3. Materials and Methods

All the chemicals were purchased from Merck (Darmstadt, Germany) in the highest grades available. Lactate dehydrogenase (LDH) concentration was measured using a commercial cytotoxicity detection kit (Roche, Mannheim Germany). L-Thyroxine (T4) was purchased from Sigma Aldrich chemicals (Steinheim, Germany) and diazepam was provided by Chemi Daru Company (Iran, Tehran).

### *3.1. Animals and Their Treatments*

This study was carried out at Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran. It was also approved by the Ethics Committee of the University. All the animals used in the current study received humane care in compliance with the institutional animal care guidelines. Male Wistar rats weighing 250 - 300 gr were obtained from the animal care center at Kermanshah University of Medical Sciences and were housed in the animal room the Medical Biology Research Center until the end of the treatment. They were maintained under standard laboratory conditions (controlled temperature of 21°C, 12-h light/dark cycle). The animals were divided into four groups of eight according to the similar studies (13, 20).

Animal Groups:

1: Control group (n = 8): The animals were fed with normal rat pellet and normal water.

2: Hyperthyroid group (n = 8): Hyperthyroidism was induced by adding T4 (Sigma, catalog number T-2376; 12 mg/L) to the drinking water for 21 days (21).

3: Control diazepam perfused group (n = 8): The animals received the same treatment as group 1, but were perfused for 10 min before ischemia with Krebs buffer containing diazepam (100 µmol/L) (20, 22).

4: Hyperthyroid diazepam perfused group (n = 8): The animals received the same treatment as group 2, but were perfused for 10 min before ischemia with Krebs buffer containing diazepam (100 µmol/L).

### *3.2. Thyroid Hormone Assessment*

At first, the animals were selected randomly. Then, blood samples were collected from abdominal aorta and were immediately centrifuged at 1500 revolutions per minute (rpm) for 5 min. Radioimmunoassay method was applied for estimation of serum thyroid hormone concentration (23).

### *3.3. Isolated Heart Preparation*

Isolated rats' hearts were perfused at constant hydrostatic pressure (65 mmHg) according to Langendorff technique (24, 25). The rats were anaesthetized with an intraperitoneal administration of 60 mg/kg pentobarbital sodium (Sigma, Steinheim, Germany). The hearts were then excised and were immediately arrested in ice-cold Krebs buffer (4°C), quickly cannulated, and retrogradely perfused through the aorta in noncirculating Langendorff apparatus (Harvard Apparatus Ltd., Edenbridge, UK) with Krebs buffer 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 of 7.4 (26). The Krebs buffer was filtered through the Whatman paper No. 1002 - 125 and then bubbled with 95% oxygen and 5% carbon dioxide at 37°C. Following the removal of the left atrial appendage, a water-filled latex balloon was inserted into the left ventricle. This balloon was connected to a pressure transducer (MLT 844; AD Instruments, New South Wales, Australia) which in turn was connected via ML110 BP Amp (AD Instruments), a ML825 Power Lab 2.25 (AD Instruments) system, to a computer for continuous monitoring of heart functions. At first, the balloon volume was adjusted to achieve a stable end diastolic pressure of 5 - 10 mmHg. This volume was then kept constant during the study. Different hemodynamic parameters were measured, including Left Ventricular Developed Pressure (LVDP; mmHg), which was defined as the peak systolic pressure minus the end diastolic pressure, and Heart Rate (HR; beats/min). The Rate Pressure Product (RPP; LVDP × HR) was also calculated. Coronary Flow (CF; mL/min) was measured by collecting the coronary effluent. After a 20-min stabilization period, baseline parameters were recorded.

In addition to this protocol, the hearts in groups 3 and 4 were perfused for 10 minutes before ischemia with Krebs solution containing 100 µmol/L diazepam. This dose has been shown previously to have cardio depressant effects on the heart (10, 13). Global normothermic ischemia was induced by halting perfusion and immersing the heart in Krebs buffer at 37°C. The hearts were subjected to global ischemia (40 min) followed by reperfusion (45 min).

*3.4. Lactate Dehydrogenase (LDH) Assessment*

LDH is an intracellular enzyme whose increase is an indicator of tissue damage with the consequent release into the coronary effluent. Therefore, LDH concentration in coronary effluent collected in the first min of reperfusion was evaluated to determine the myocardial injury. The samples were measured using a cell cytotoxicity detection kit (LDH (Roche, Mannheim, Germany)) as well as known quantities of standard LDH (Sigma, Steinheim, Germany).

*3.5. Statistical Analysis*

The data were expressed as mean ± Standard Error of Mean (SEM). The data were analyzed using Unpaired Student's t-test, Analysis of Variance (ANOVA), and Tukey post hoc test as appropriated. All the analyses were performed using the SPSS software, version 20 (SPSS Inc, Chicago, IL, USA) and $P < 0.05$ was considered as statistically significant.

## 4. Results

*4.1. Thyroid State*

According to the results of unpaired t-test, hyperthyroidism induced by L-thyroxine (12 mg/L for 21 days) led to a significant increase ($P = 0.002$, 95% CI: -0.35 - -0.11) in T3 level (nmol/L) in the hyperthyroid group ($N = 5$, $0.92 \pm 0.033$) compared to the control group ($N = 5$, $0.68 \pm 0.04$).

*4.2. Hemodynamic Function*

The four groups' values of cardiac functional parameters, including CF, HR, LVDP, and RPP, have been presented in Table 1. At base line, the values of CF (mL/min) and HR (beats/min) were higher in the hyperthyroid group compared to the controls. Moreover, comparison of HR and RPP (mmHg × beats/min) before and after acute diazepam perfusion at baseline revealed a significant decrease in these two parameters in the control diazepam perfused group after diazepam perfusion (HR: 240 ± 19.4 to 172 ± 22, $P = 0.013$, CI: 19 - 117; RPP: 20674 ± 1474 to 15596 ± 1604, $P = 0.007$, CI: 1908 - 8246). Similarly, HR and RPP parameters reduced significantly in the hyperthyroid diazepam perfused group after diazepam perfusion compared to baseline (HR: 296 ± 16 to 221 ± 9, $P = 0.002$, CI: 37 - 111; RPP: 24046 ± 1381 to 18269 ± 711, $P = 0.012$, CI: 1724 - 9828). Diazepam improved RPP recovery at the 45th min of reperfusion in the hyperthyroid diazepam perfused group (12469 ± 1422) in comparison to the hyperthyroid group (4007 ± 252) ($P < 0.001$, CI: 5066 - 11856).

*4.3. Lactate Dehydrogenase Release*

The extent of reperfusion injury was determined in the 4 groups based on the released LDH. The results showed that the concentration of the released LDH during the first min of reperfusion in the hyperthyroid group (41 ± 8.14 mU/mL) was significantly ($P < 0.001$, CI: 15.9 - 43.3) higher than that of the control group (11.45 ± 6.25 mU/mL). However, no significant difference was found between hyperthyroid diazepam perfused (19.08 ± 1.69 mU/mL) and control diazepam perfused (19.88 ± 1.35 mU/mL) and control groups (11.45 ± 6.25 mU/mL). Nonetheless, there was a significant difference between hyperthyroid diazepam perfused (19.08 ± 1.69 mU/mL) and hyperthyroid groups (41 ± 8.14 mU/mL) ($P = 0.002$, CI: -34.4 - -9.4).

## 5. Discussion

The results of the current study clearly revealed that acute diazepam perfusion to isolated hyperthyroid rats' hearts before ischemia significantly improved heart recovery in the reperfusion period. Our findings showed that thyroxine administration (12 mg/kg) for 21 days in rats induced hyperthyroidism and changed the cardiac parameters. At baseline, HR (beats/min) and CF (mL/min) significantly increased in the hyperthyroid group compared to the controls (Table 1). Increase in HR in this group was consistent with the findings of the previous studies. Other studies have explained several mechanisms for hyperthyroid induced tachycardia, including increased L-type calcium channels (2, 3), increased $Ca^{+2}$ ATPase activity (27), increased alpha-myosin heavy chain gene expression (2), and decreased phospholamban (28). On the other hand, as illustrated in Table 1, recovery of cardiac function after ischemia significantly reduced compared to the controls. This was also confirmed by the results of LDH (mU/mL) assessment (Figure 1). The released LDH concentration in the first minute of reperfusion after ischemia increased

**Table 1.** Cardiac Parameters before and after Exposure to Global Normothermic Ischemia for 40 Min in the Study Groups

| Groups | Baseline (10th min) | | | | Reperfusion (45th min) | | | |
|---|---|---|---|---|---|---|---|---|
| | HR | LVDP | CF | RPP | HR | LVDP | CF | RPP |
| **Control (N = 8)** | 251 ± 8 | 83.6 ± 3.3 | 10 ± 0.5 | 21208 ± 741 | 249 ± 8.3 | 35.2 ± 2.6 | 5.4 ± 0.5 | 8578 ± 453 |
| **Hyperthyroid (N = 8)** | 294 ± 11 [a] | 77.5 ± 3.9 [a] | 12.3 ± 0.3 | 22674 ± 1084 | 220 ± 37 | 23.9 ± 4.1 [b] | 4.6 ± 0.3 [b] | 4007 ± 258 [a, b] |
| **Control diazepam Perfused (N = 8)** | 240 ± 19.4 | 83.9 ± 6.1 | 10.8 ± 0.8 | 20674 ± 1474 | 252 ± 23 | 29.2 ± 5.2 | 6.3 ± 0.4 | 7845 ± 1788 |
| **Hyperthyroid diazepam perfused (N = 8)** | 296 ± 16 [c] | 81.8 ± 4.8 [c] | 13 ± 0.5 | 24045 ± 1381 | 257 ± 18 | 48.8 ± 4 [a, c] | 8.1 ± 0.5 [c, d] | 12469 ± 1422 [c] |

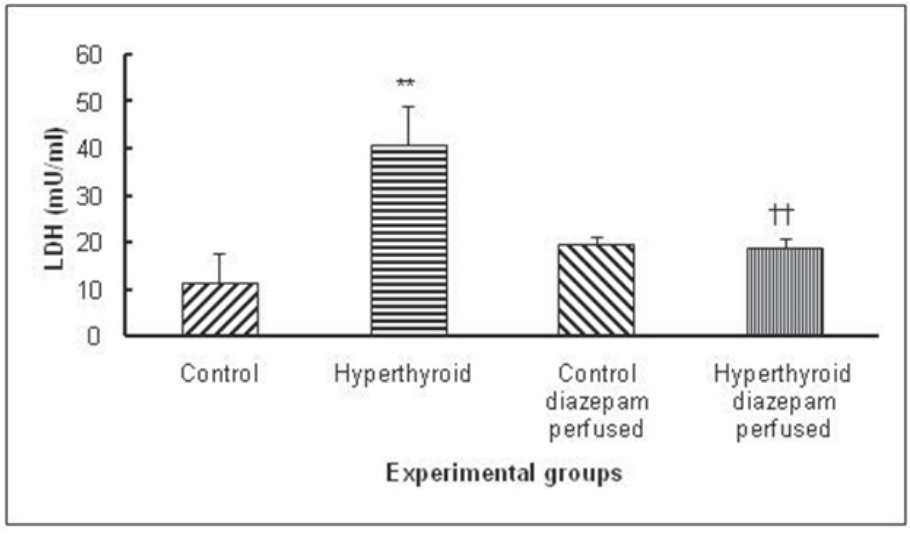
Abbreviations: LVDP, Left ventricular developed pressure (mmHg); HR, Heart rate (beats/min); CF, Coronary flow (mL/min); RPP, Rate pressure product (LVDP × HR).

Data sets were analyzed by analysis of variance (ANOVA) and expressed as mean ± SEM.

[a] or [b] or [c], Significant at $P < 0.05$, [d] or [e] or [f], Significant at $P < 0.01$, [g] or [h] or [i], Significant at $P < 0.001$

[a] Significant from control, [b] Significant from control diazepam perfused, [c] Significant from hyperthyroid diazepam perfused

**Figure 1.** The Concentration of Lactate Dehydrogenase (LDH; mU/mL) in the Four Experimental Groups.




Data Sets Were Analyzed by Analysis of Variance (ANOVA) and Expressed as Mean ± SEM and 95% Confidence Interval (CI). ** P < 0.001, CI: 15.9 - 43.3, versus the controls and †† P = 0.002, CI:-34.4 - 9.4 versus the hyperthyroid group

in the hyperthyroid hearts compared to the control group, indicating further damage of hyperthyroid rats' hearts. This observation was consistent with the previous reports indicating higher susceptibility of hyperthyroid rats' hearts to I/R (6, 7, 29). It has been stated that mitochondrial function was impaired in hyperthyroidism due to excessive production of reactive oxygen species and dysfunction of mitochondrial state 3 respiration (9). Oxidative damage to heart mitochondria was also exacerbated by increased H2O2 production (7). These factors led to mitochondrial dysfunction and reduced cardiac functional recovery in the reperfusion period (7).

According to Table 1, acute diazepam perfusion to hyperthyroid hearts significantly increased RPP (mmHg × beats/min), LVDP (mmHg), and CF (mL/min) recovery in the reperfusion period. In other word, the hyperthyroid hearts were more resistant to I/R injury in the presence of diazepam. These findings were supported by LDH results (Figure 1). In fact, decreased release of LDH in the first minute of reperfusion caused lower cardiac I/R injury of the hyperthyroid hearts in the presence of diazepam compared to its related control group (hyperthyroid group).

Exacerbation of cardiac I/R injury in hyperthyroid condition has been reported previously (6). Our previous study showed that intraperitoneal injection of diazepam (1 mg/kg) for 5 days modified the exacerbated cardiac I/R injury, in a way that the significant difference between the hyperthyroid rats treated with diazepam and the control group disappeared after chronic diazepam administration (19). In our study, it was determined for the first time that presence of diazepam during heart perfusion significantly improved cardiac function of the hyperthyroid rats compared to the controls. This positive effect was stronger than chronic usage of diazepam. Other studies have reported different mechanisms for the effect of acute and chronic benzodiazepine administration (13, 19, 20). In long-term diazepam administration, changes in benzodiazepine receptor density and changes in cardiomyocytes vulnerability against I/R were reported (14). However, different mechanisms are involved in acute usage of benzodiazepines. For instance, it has been demonstrated that diazepam could act on cardiomyocytes as a calcium channel blocker agent (10, 30). The beneficial effect of calcium channel blockage on reduction of acute ischemic myocardial injury is well known (31). Furthermore, calcium channel blocker agents are clinically important and can improve cardiac I/R injury (32-35). Enhancement of L-type calcium channels which are located in sarcolema occurs in hyperthyroid myocytes (2, 36). These channels play an important role in cardiac contraction because they are of great importance in calcium intake during excitation (5). Increased intracellular calcium is a factor that exacerbates I/R injury (37). It has been shown that diazepam could reduce $Ca^{2+}$ influx to myocytes by inhibiting L-type calcium channels (30). Therefore, reduced $Ca^{2+}$ influx to myocytes could probably decrease I/R injury in the hyperthyroid cardiomyocytes in the presence of diazepam. However, as a limitation of this study, intracellular calcium levels were not measured and the exact role of this channel is required to be investigated in future studies. Yet, according to the previous reports, it can be concluded that diazepam might modify the heart through this pathway.

Negative inotropic and chronotropic effects of diazepam have been reported previously (10, 20). As mentioned in the results, HR significantly decreased after diazepam perfusion. It is believed that L-type $Ca^{2+}$ channel blockage plays an important role in this regard (38). The important role of calcium channels have been reported, as well (10, 20). The results of the present study, including the negative chronotropic effects of diazepam and decreased I/R injury induced by diazepam, could also be explained by this pathway.

In conclusion, the study results showed that presence of diazepam during perfusion of isolated hyperthyroid rats' hearts significantly improved cardiac function following ischemia and protected the hearts against I/R injury. Regarding the important role of calcium channels in myocardial I/R injury, it seems that the beneficial effects of diazepam in the current study could be explained by

blockage of these channels.

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**Authors' Contribution**

Study concept and design: Dareuosh Shackebaei; Acquisition of data: Atefeh Asadmobini, Mahvash Hesari, and Maryam Vaezi; Analysis and interpretation of data: Atefeh Asadmobini, Mahvash Hesari, and Dareuosh Shackebaei; Drafting of the manuscript: Atefeh Asadmobini, Mahvash Hesari, and Dareuosh Shackebaei; Critical revision of the manuscript for important intellectual content: Mahvash Hesari and Atefeh Asadmobini; Statistical analysis: Maryam Vaezi; Administrative, technical, and material support: Atefeh Asadmobini, Dareuosh Shackebaei, and Mahvash Hesari; Study supervision: Dareuosh Shackebaei.

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