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Adiazoninol-Steroid Derivative With Biological Activity Against Ischemia-Reperfusion Injury.

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ABSTRACT

Several drugs have used to myocardial ischemia/reperfusion injury; however the molecular mechanism involved in their biological activity is very confusing. Therefore the aim of study was to evaluate the biological activity ofdiazoninol-steroidderivativeon myocardial ischemia/reperfusion injury model. To characterize the molecular mechanism involved in their effect some pharmacological tools such as mifepristone, flutamide, tamoxifen, nifedipine, milrinone, ouabaine, levosimedan, adenosine, WB-4101, metoprolol, isoxsuprine and butaxaminewere used.The results showed that the effect exerted by the diazoninol-steroidderivativewas similar toeffect produced by isoxsuprine. Additionally, the biological activity induced by the diazoninol-steroid-derivativewas inhibited in the presence of butaxamine. Other data showed that the biological activity of the diazoninol-steroid-derivativeincreased the cAMP levels in a time-dependent manner. In conclusion, the effect induced by the diazoninol-steroid-derivativeproduce a cardioprotective effect against myocardial ischemia-reperfusion injury translated as a decrease in the area of infarction. This phenomenon was viaβ_2-receptor adrenergic activation whichconsequently brings changes in cAMP levels.
Keywords: Ischemia, reperfusion, cAMP, diazoninol, β_2-receptor.

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INTRODUCTION

There are several reports which indicate that cardiovascular diseases is one of the leading causes of death in the world [1-3]; this clinical pathologies may be due to the cardiac myocyte cell death caused by prolonged myocardial ischemia that can be translated as myocardial infarction [4]; this phenomenon results in some changes in the cardiac work of both ventricles [5]. In addition, there is a study which suggests that restoration of blood flow in some case can decrease the cardiac necrosiscaused by prolonged myocardial ischemia[6]; nevertheless, other studies indicate that the reperfusion may increase the tissue injury [7]. These studies have led to the search for therapeutic alternatives for treatment of ischemia-reperfusion injury; for example, the cyclosporin-Awhich showed biological activity against ischemia-reperfusion injury in an isolated heart model [8].In addition, a study showed that rapamycin which can producebenefic effectson myocardial infarction in isolated mouse heart via mitochondrial potassium channel activation[9]. Other report suggests that a sulfonylurea(glibenclamide) can lower myocardial damage in ischemia/reperfusionmodel through potassium channel activation [10].Also, a study indicates that a benzenamine-derivative can lower the reperfusion injury using an"in vitro" model via Na^+/Ca^{2+} exchanger inhibition [11].All these results indicate that severaldrugs mayinduce effects on reperfusion injury; however, the molecular mechanism involved in its biological activity is very confusing, perhaps this phenomenon is due to differences in protocols used. The aim of this study was designed to evaluate the biological activity exerted by adiazoninol-steroid derivativeagainst ischemia-reperfusion injury using an isolated rat heart model.

MATERIAL AND METHODS

General

The diazoninol-steroid derivative ((1Z,4E,7aR,9aS)-10-(E)-1-((2-aminoethyl) imino)ethyl)-7a,9a-dimethyl-2,3,6,7,7a,7b,8,9,9a,10,11,12,12a,12b,13,14-hexadecahydrocyclopenta- [5,6]-naphto[2,1-e][1,4]diazonin-5-ol) was prepared using previously method reported [12].The others drugs used in this work were purchased from Sigma-Aldrich Co. Ltd.

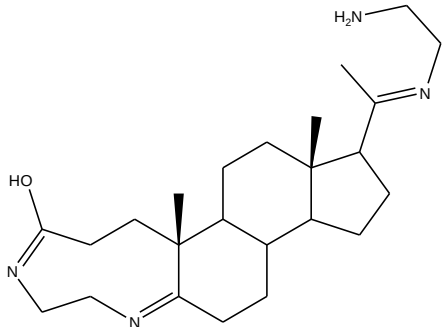

Figure 1: Structure chemical of diazoninol-derivative

Biological activity

The experimental techniques carried out in this study used in this research were reviewed and approved by the animal care and use of the Autonomous University of Campeche with No. PI-420/12using the animal management guides [13]. Male Wistar rats; weighing 200-250 g were obtained from Laboratory of Pharmacochemistry from University Autonomous of Campeche (Faculty of Chemical-Biological Sciences).

Reagents

It noteworthy that drugs involved in this study were dissolved in methanol and from this solution all dilutions were obtained using a Krebs-Henseleit solution (v/v).

Experimental design I

Animals were anesthetized with pentobarbital (50 mg/Kg body weight) via intraperitoneal administration. After the animal was opened by a thoracic abdominal laparotomy and the heart was perfused via retrogradewith the Krebs-Henseleit solution through a non-circulating perfusion system with a constant flow rate [14].

*Krebs-Henseleit solution (pH = 7.4, 37°C) composed by following system; 117.8 NaCl; 6 KCl; 1.75 $CaCl_2$; 1.2 NaH_2PO_4; 1.2 $MgSO_4$; 24.2 $NaHCO_3$; 5 glucose and 5 sodium pyruvate (mmol). The solution was then bubbled with a mixture of O_2/CO_2 (95:5/5 %). The coronary flow (10 ml / min) was adjusted with a peristaltic pump and a period of equilibration was carried out for 15 min.

Ischemia-Reperfusion model

It is important to mention that after the equilibrium time, the hearts underwent a period of ischemia for 40 minutes due to closure of the perfusion system such as indicate some reports[15] in absence (control) or presence of each drug involved in this study (see design experimental). Following, the system was restarted and the hearts were reperfused by other 40 minutes with Krebs-Henseleit solution.

Histological Analysis

For histological evaluation, the technique modifies reported by Engelhardtand col. [16] was used. Cross sections from the heart were fixed with 10% paraformaldehyde for 8 h. Then, the samples are placed in a histocasette (Leica Mod. TP1020 SN: 042231418), for 12 h to be processed. After, the sections of heart were placed in a Paraffin Embedding Center (Leica EG1160 model) to form paraffin blocks which are cut into 2 µm slices using an aparatus Leica 50138178 model and following were introduced to bath water (Riossa-Rocha B7 SN: 070909 model). Then, the samples were placed on a slide, which was dried at 60 ^{o}C for 30 minutes in a Binder-ED23 apparatus. After, a solution of ethanol:xylol(1: 1) was added to the slides for cell clearance; after 10 minutes, was wash with distilled water.To observe the tissue morphology,hematoxylin is added to the sample (for 1 minute), after which time it is washed again. Then eosin is added for 1 minute. Finally, ethanol/xylol (1:1) is added to the sample and the tissue is observed under the microscope. For morphometrical analysis, photographs of 20 ventricular sections were taken at 3320 magnifications (ZeissIM-35).

Biological evaluation

Step I

Effects inducedbydiazoninol-steroidderivativeagainst infarct area. The ischemia-reperfusion injury was evaluated in absence or presence of diazoninol-steroid derivativeat dose of 0.001-100 nM. It's important to mention that after of each experiment the hearts were cut into two sections at right angles to the vertical axis. It is noteworthy that the areas of the normal left ventricle non-risk region, area at risk, and infarct region were evaluated using a previously reported method [17].

Step II

Effects induced by the diazoninol-steroid derivative against ischemia-reperfusion injury via hormone-receptors activation. The diazoninol-steroidderivative was perfused(1 nM/min) in the hearts and infarct area was evaluated; then this experiment was repeated in presence of mifepristone(1 nM/min) or flutamide (1nM/min) or tamoxifen(1nM/min).

Step III

Effect produced by the diazoninol-steroidderivative on ischemia-reperfusion injury via calcium channels activation. The diazoninol-steroid derivative was perfused(1 nM/min) in the hearts and infarct area was evaluated; after, this experiment was repeated in presence of nifedipine (10 nM/min)or levosimedan(300 nM/min).

Step IV

Biological activity induced by the diazoninol-steroidderivative on ischemia-reperfusion injury via ATP-ase inhibition. The diazoninol-steroidderivative was perfused(1 nM/min) in the hearts and infarct area was evaluated; then, this experiment was repeated in presence of ouabaine (10µM/min).

Step V

Biological activity induced by the diazoninol-steroidderivative on ischemia-reperfusion injury via phosphodiesterase-3 inhibition. The diazoninol-steroidderivative was perfused(1 nM/min) in the hearts and infarct area was evaluated; then, this experiment was repeated in presence of milrinone(50nM/min).

Step VI

Biological activity induced by the diazoninol-streroidderivativeand adenosine on ischemia-reperfusion injury. The diazoninol-steroid derivative was perfused(1 nM/min) in the hearts and infarct area was evaluated; then, this experiment was repeated in presence of adenosine(140 µg/min).

Step VII

Biological activity exerted by the diazoninol-steroidderivativeagainst infarct area via adrenergic-receptors activation. The diazoninol-steroidderivative was perfused(1 nM/min) in the hearts and infarct area was evaluated; then this experiment was repeated in presence of metoprolol (1 nM/min) or WB-4101(10 nM/min), isoxaprine (0.18 mg/min)

Biological activity exerted by the diazoninol-steroid derivative against infarct area via β_2-receptor activation. The diazoninol-steroid derivative was perfused (1 nM/min) in absence or presence of butaxamine (50 pg/min) and in the hearts and infarct area was evaluated;

Step VIII

Effect exerted of the diazoninol-steroidderivative on cAMPconcentration. Isoproterenol (100 nM/min) was perfused on the heart for 3, 6, 9, 12 or 18 minutes and its effect on cAMP levels was determined. After, the results obtained were compared with the biological activity exerted by the diazoninol-steroidderivative(1 nM/min) and control on the concentration of cAMP. Then, atrial tissue was removed, and the ventricles were immediately frozen with liquid nitrogen and stored at 270 ^{o}Cuntil assayed. To mixture was addedtrichloroacetic acid (6%) and stirringby centrifugation at 2000 rpm at 4°C for 15 minutes. After thesupernatant wasseparated and washed with the water:diethyl ether (5:1 v/v) system. The sample was separated and thecAMPconcentration was determinate using a standard ^{125}I radioimmunoassay kit supplied by Amersham International [18].

It is important to mention that dose administered of drugs involved in this study such as mifepristole [19, 20]; metoprolol [21]; flutamide [21]; tamoxifen [22]; Levosidan [23] and nifedipine [24]; digoxine [25] milrinone [26]; WB-4101 [27], adenosine [28]; isoxaprine[29] and butaxamine [30]have been previously reported in other type of experiments.

Statistical analysis

The results were expressed as average ± SE, using each heart (n = 9) as its own control. In addition, the results were put under Analysis of Variance (ANOVA) with the Bonferroni correction factor [31] using the SPSS 12.0 program. The differences were considered significant when p was equal or smaller than 0.05.

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RESULTS

Biological activity

Biological evaluation

Effects induced bythe diazoninol-steroidderivativeagainst ischemia-reperfusion injury.The effect exerted by the diazoninol-steroid derivativeand control conditions on infarct area was evaluated using an ischemia-reperfusion injury model (Figure 2). The results showed that the diazoninol-steroid derivative [0.001 to 100 nM] decreased the infarct area in a dose-dependent manner compared with conditions control.

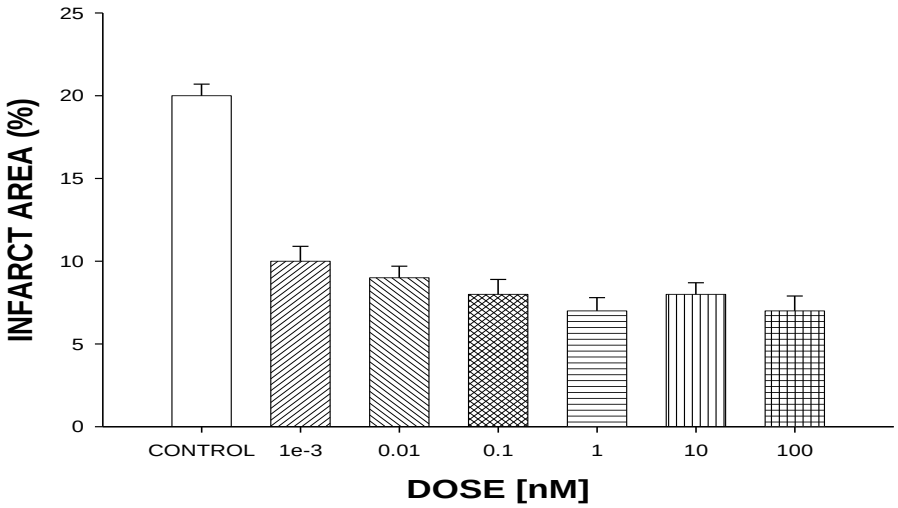

Figure 2: Biological activity exerted by the diazoninol-steroid derivative(0.001 to 100 nM) against ischemia-reperfusion injury translated as infarct size. The experimental results showed that effect exerted by the diazoninol-derivative decrease the infarct area in a dose-dependent manner compared with conditions control. Each bar represents the mean ± S.E. of 9 experiments.

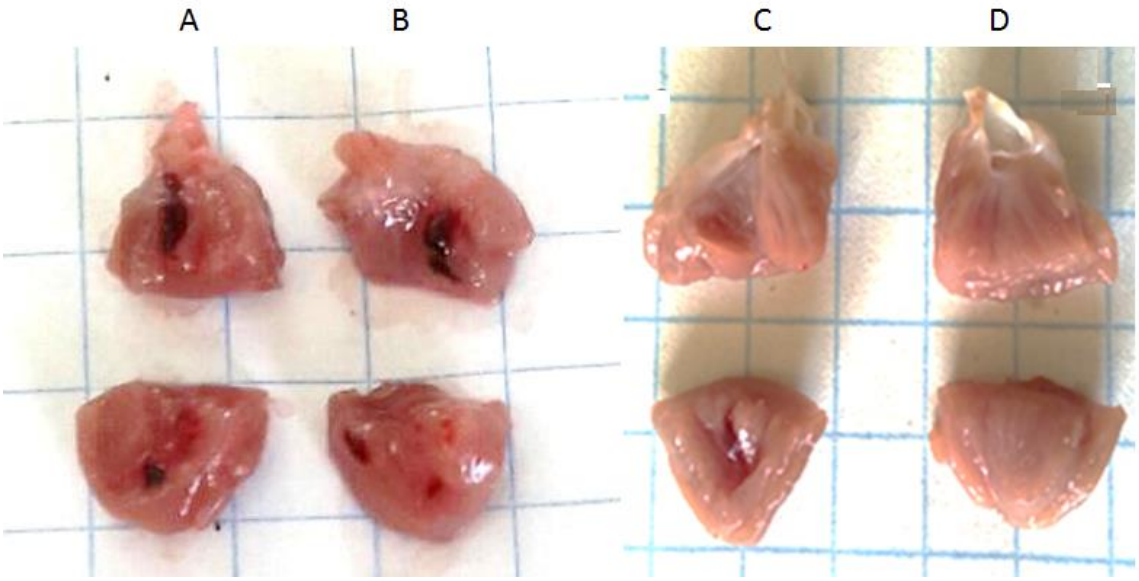

Figure 3: The scheme shown the myocardial tissue infarction is higher in vehicle (A, B) treated rats subjected to occlusion (for 40 min and reperfusion for 40 min); however, in presence of the diazoninol-steroid derivative (C, D; 0.001 nM) this phenomenon was lower.

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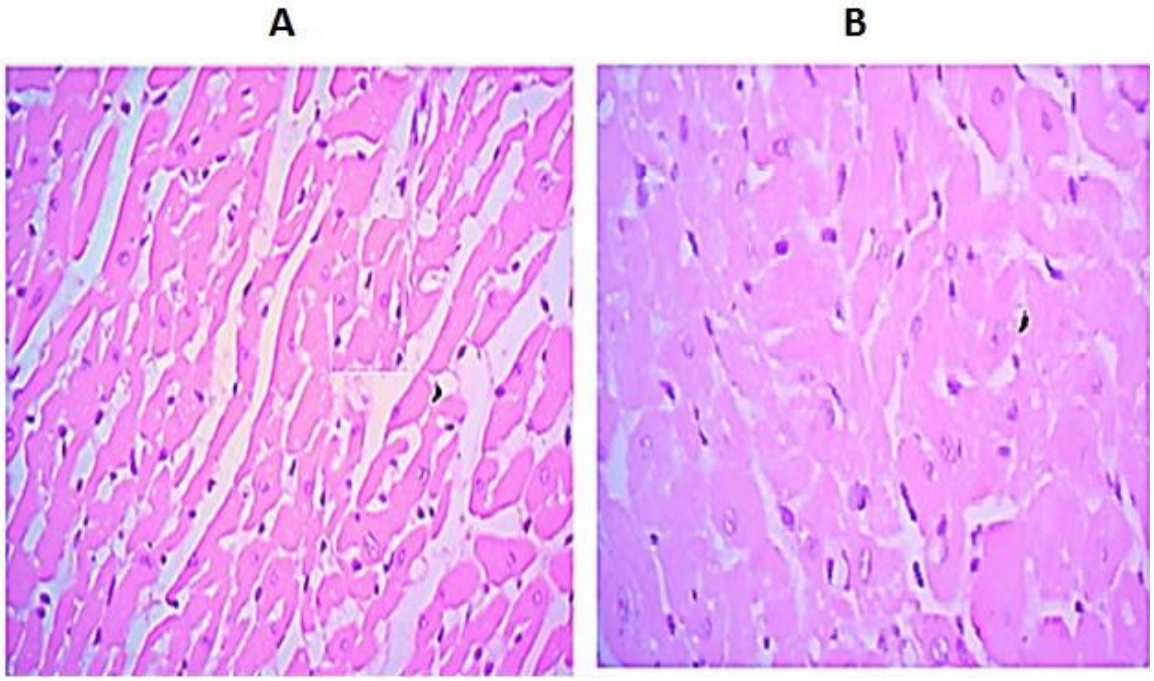

Figure 4: Histological evaluation of effect exerted by the diazoninol-steroid derivativeon ischemia-reperfusion using the technique modifies reported by Engelhardt [28]. The scheme A(control) showed a marked alteration of the structure of the myocardium section characterized by the appearance of extensive necrosis: In addition, several bands of contraction and thinning of myofibrils were observed. The scheme B show a decreased of myocardial necrosis by presence of diazoninol-steroid derivative (0.001 nM).

Histological Analysis

In the Figures 3 and 4 are shown a marked disruption of the myocardial structure characterized by appearance of extensive necrosis in conditions control; however this was lower in presence of the diazoninol-steroid derivative.

Effects induced by the diazoninol-steroid derivative on ischemia-reperfusion injury via sex hormones. The biological activity induced by the diazoninol-steroid derivative (0.001 nM/min)on infarct area was evaluated in absence or presence of mifepristone or flutamide or tamoxifen at dose of 1 nM/min (Figure 5).The results shown that diazoninol-steroid derivative[0.001 nM/min] decreased infarct size (**p** = 0.05) and this effect was no inhibited in presence ofmifepristone or flutamide or tamoxifen.

Step V

Effect produced by the diazoninol-steroidderivativeon ischemia-reperfusion injury via calcium channels.The biological activity induced by the diazoninol-steroidderivative(0.001 nM/min)on infarct area was evaluated in absence or presence of levosimedanor nifedipine at a dose of 1 nM/min (Figure 6). The results showed that diazoninol-steroid derivative[0.001 nM/min] decreased infarct size (**p** = 0.05) and this effect was no inhibited bylevosimedanor nifedipine.

Biological activity induced by the diazocinol-steroid derivative or milrinone or digoxine or adenosine against ischemia injury.The experimental data (Figure **7**) showed that effect induced by the diazoninol-steroid derivative[0.001 nM/min] was lower (**p** = 0.05)compared with milrinone (50 nM/min), digoxine (10 µM/min) and adenosine (140 µg/min).

Effect induced by the diazoninol-steroid derivative against ischemia-reperfusion injury via adrenergic system activation. The results showed in the Figure **8** indicated that infarct area was lower (**p** = 0.05) in presence of diazoninol-steroid derivative[0.001 nM/min] compared with metoprolol (1 nM/min) or WB-4101(10 nM/min)

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orisoxaprine (0.1 µmol/min). Other data found shown that butaxamine (50 pg/min) exert a similar effect that diazoninol-steroid derivative.

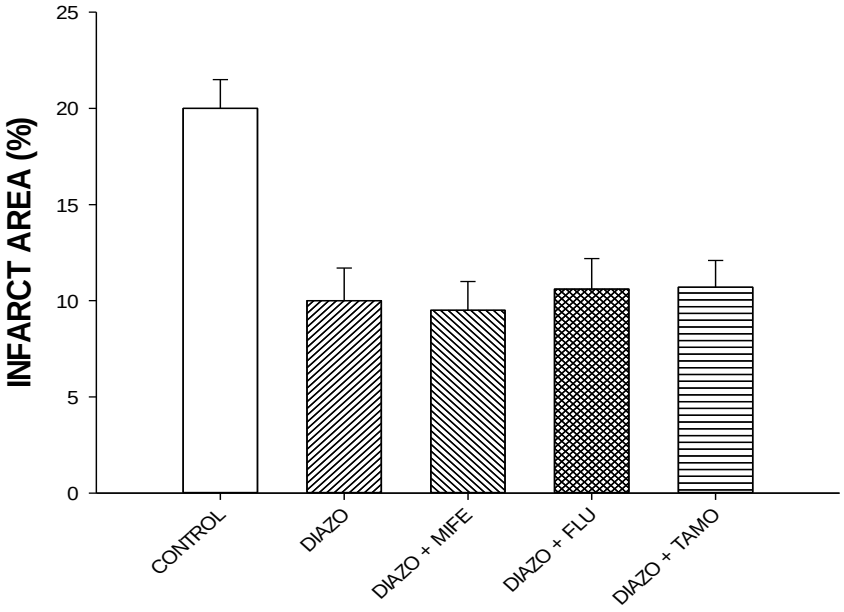

Figure 5: Effect induced by the diazoninol-steroid derivative (DIAZO) in absence or presence of mifepristone (MIFE) or flutamide (FLU) or tamoxifen (TAMO) on ischemia-reperfusion injury translated as infarct size. The experimental data showed that effect exerted by DIAZO (0.001 nM/min;p = 0.05) was lower compared with the control; however, this effect was not inhibited by MIFE [1nM/min], FLUTA [1 nM/min] and TAMO [1 nM/min]. Each bar represents the mean ± S.E. of 9 experiments.

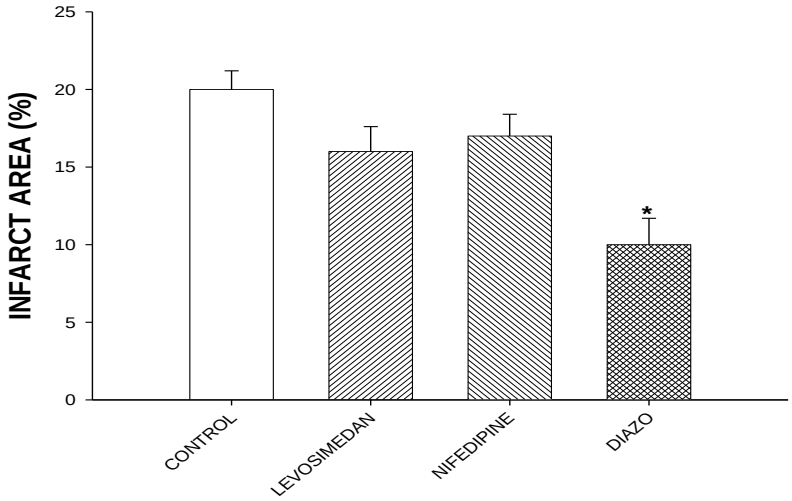

Figure 6: Biological activity exerted by the diazoninol-steroid derivative(DIAZO) or levosimedan or nifedipine against ischemia-reperfusion injury translated as infarct size. The results indicated that effect exerted by DAIZO (p = 0.05) was lower compared with levosimedan [30 nM/min] or nifedipine [1nM/min] and the control. Each bar represents the mean ± S.E. of 9 experiments.

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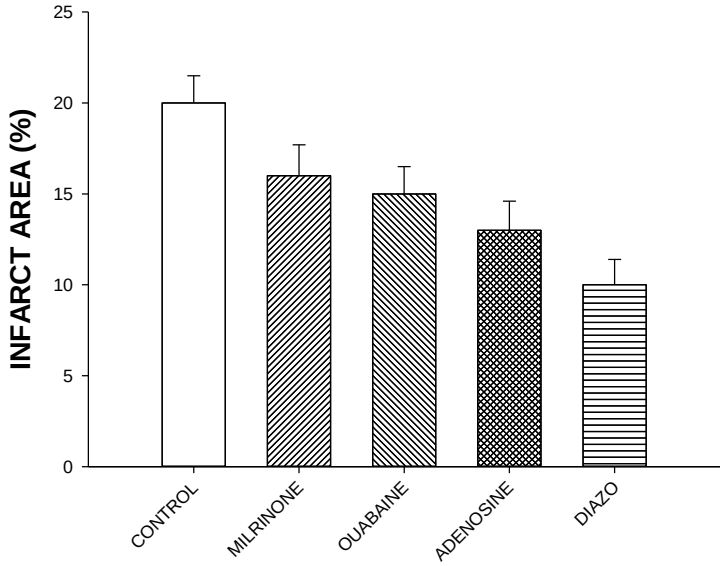

Figure 7: Effect induced by the diazoninol-steroid derivative (DIAZO) or milrinone oroubaineor adenosine against ischemia-reperfusion injury translated as infarct size. The scheme shows that the DIAZO [0.001 nM/min] significantly decreased (p = 0.05) the area of infarction compared to milrinone[50 nM/min] or oubaine [10 µM/min] or adenosine [140 µg/min] and control.

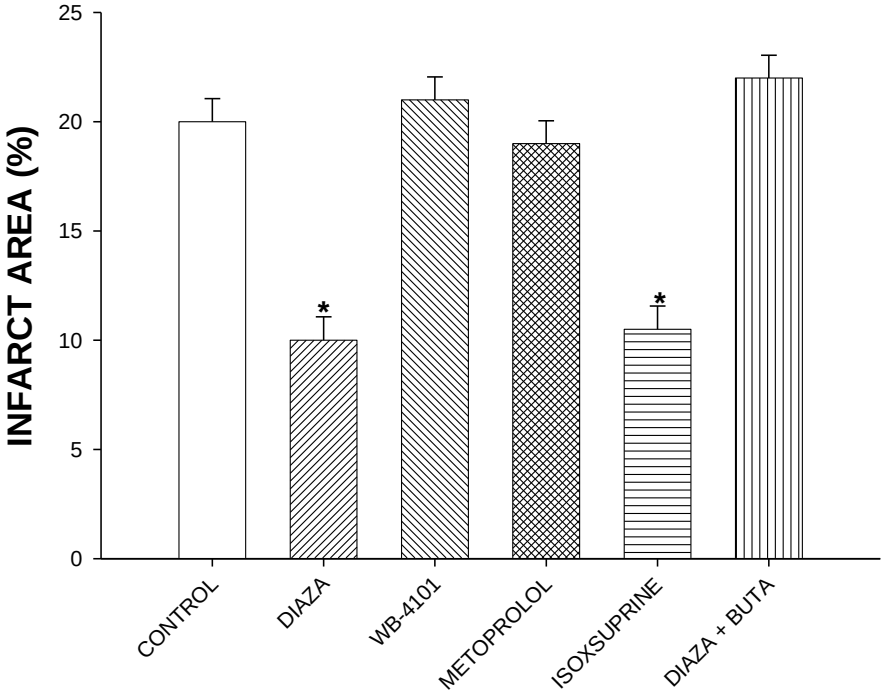

Figure 8: Biological activity exerted by the diazoninol-steroid derivative via adrenergic system. The drugs metoprolol [1 nM/min] or isoxsuprine [0.18 mg/min] or WB4-101[10 nM/min] were perfused in an ischemia-reperfusion injury model. In addition, he biological activity of diazoninol-steroid derivative was repeted in presence of butaxamine [50 pg/min]. The results showed that diazoninol-steroid derivative decreased the infarct area in a form similar to isoxsuprine and this effect was inhibited by butaxamide (p = 0.05). Each bar represents the mean ± S.E. of 9 experiments.

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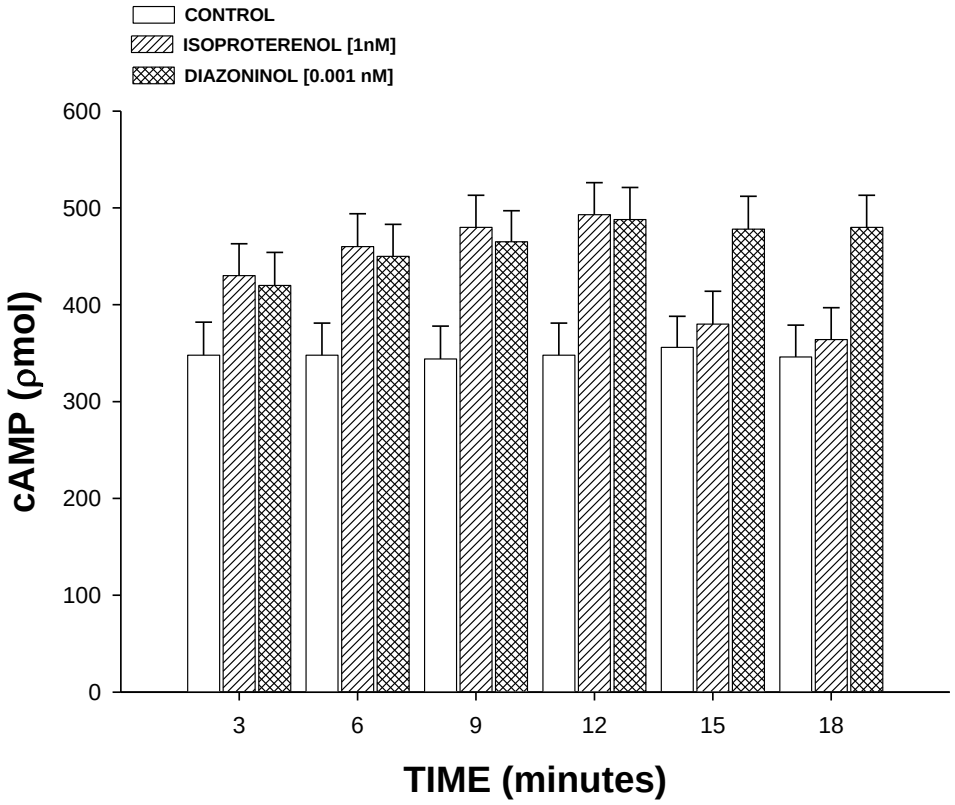

Figure 9: Biological activity induced by the diazoninol-steroid derivative and isoproterenol on cAMP levels through of time. The experimental data found shown that cAMP levels was lower (p = 0.05) in the presence of the diazoninol-steroid derivative compared with isoproterenol (3-18 min) and the control. Each bar represents the mean ± S.E. of 6 experiments.

Activity induced by the diazoninol-steroid derivative against ischemia-reperfusion injury via β_2-receptor activation.The experimental results (Figure 8) indicated that diazoninol-steroid derivative [0.001 nM/min] reduce the infarct area (**p** = 0.05) and this effect was inhibited by butaxamine (50 pg/min),

Biological activity exerted by the diazocinol-derivative and isoproterenol on the cAMP levels. The experimental data(Figure 9) shown that effect exerted by the diazocinol-derivative[0.001 nM]was lower (**p** = 0.05)compared with isoproterenol[100 nM]and conditions control.

DISCUSSION

Since several years ago, several drugs have been used for treatment of ischemia-reperfusion injury such as propofol [32], methylene blue [33],resveratrol [34], rapamycin[35],nitro-derivatives [36]and others. It is important to mention that these drugs exert their effect against ischemia-reperfusion injury throughof several molecular mechanisms; this phenomenon could be the result of its different chemical characteristics or to differences in the protocols used. Therefore, in this study the biological activity of a diazoninol-steroid derivativeagainst ischemia-reperfusion injury was evaluated and to characterize their molecular mechanism some pharmacological tools were used.

Biological activity

Effect induced by the diazoninol-steroid derivativeagainst ischemia injury.

Biological activities induced by the diazoninol-steroid derivativeat doses of 0.01 to 100 nMon infarct area were evaluated using an ischemia-reperfusion injury model.The experimental data found showed that the diazoninol-steroid derivativedecreased the infarct area in a dose-dependent manner compared with control

conditions(Figure 2 and 3). In the search of molecular mechanism involved in the biological activity of diazocinol-steroid derivative against ischemia-reperfusion injury translated as infarct area, several pharmacological drugs were used such as mifepristone [19, 20], flutamide [21], tamoxifen [22],levosimedan [23], nifedipine [24], digoxine [25], milrinone [26],metoprolol, WB-4101 [27], adenosine [28], isoxaprine [29], butaxamine [30] and ispoproterenol[18].

Characterization of molecular mechanism involved in the biological activityinduced by the diazoninol-steroid derivative against ischemia injury.

In the search of possible molecular mechanism involved in effect produced by the diazoninol-steroid derivativeon ischemia-reperfusion injury; some reports were analyzedwhich indicate that some drugs can exerttheir effects against ischemia-perfusion injury via activation of progesterone or estrogen or androgen receptors [19-22]. Analyzing these data, the effect exerted by diazoninol-steroid derivative in absence or presence of flutamide (androgen-receptor inhibitor) or tamoxifen (estrogen receptor antagonist) or mifepristone (progestin-receptor antagonist)against ischemia-reperfusion injury was determinate. The results shown that diazoninol-steroid derivative exert different biological activity was no inhibited by flutamide or tamoxifen or mifepristone (Figure 5); this resultssuggest that molecular mechanism involved in the effect induced by the diazocinol-steroid derivativeon ischemia-reperfusion injury was not via sex hormones activation.

In the search other possible mechanism molecular involved in the biological activity exerted by the diazocinol-steroid derivative against ischemia-reperfusion injury, also other studieswere analyzed, which indicate that some drugs can exert effectsonischemia-reperfusion injury through calcium channel activation [23, 24]. Therefore, in this experimental investigation, the biological activity exertedby nifedipine (calcium antagonist) or levosimedan (calcium sensitizer) against ischemia-reperfusion injury was evaluated and compared with the biological activity induced by the diazoninol-stroidderivative. The experimental data found shown that effect exerted by the diazoninol-steroid derivativewas lower in comparison with nifedipine and levosimedan; this data indicated that molecular mechanism involved in the biological activity exerted by diazoninol-steroid derivativewas not viacalcium channelsactivation.

On the other hand, was also validated the effect exerted by milrinone (phosphodiesterase inhibitor III)[26]on myocardial infarct size to compare withbiological activity exerted by the diazoninol-steroid derivative.The experimental data indicated that effect exerted by the diazoninol-steroid derivative on infarct area was lower compared with the effect produced by milrinone; these data indicated that molecular involved in the effect exerted by the diazoninol-steroid derivativewas not via phosphodiesterase III inhibition.

On the other hand, other reports were also analyzed; these reports indicate that ischemia-reperfusion in the subepicardial regions of ischemic tissue can be reduced in the presence of oubaine[25].Therefore, the biological activity of digoxine on ischemia-reperfusion injury was evaluated and compared with the effect exerted bythe diazoninol-steroid derivative. The experimental data found shown that digoxinedecreased the infarct area in comparison with control conditions; however, it is noteworthy that this effect was different compared with the diazoninol-steroid derivative; this results indicated that molecular mechanism was not via Na^+/K^+-ATPase inhibition.

All these results opened the way to evaluate a different mechanism; in this sense, there are some studies which suggest that adenosine can exert effect on ischemia-reperfusioninjury [28]; therefore, the biological activity induced by adenosine against ischemia-reperfusion injury was evaluated and compared with biological activity exerted by the diazoninol-steroid derivative. The experimental data found shown that adenosine decreased the infarct area in comparison with control conditions; however, this effect was different compared with the diazocinol-steroid derivative; this results indicated that molecular mechanism was not purinergic receptors activation.

Finally, analyzing other studies suggesting that ischemia-reperfusion injury [21] may involve the activation of the adrenergic system; in this experimental study, the pharmacological activity exerted by the diazocinol-steroid derivative against ischemia injury in absence or presence of metoprolol or WB-4101 or isoxsuprine was evaluated. The data showed that biological activity exerted by the diazocinol-steroid derivative was only inhibited by butaxamine; this data suggested that effect exerted by the diazocinol-steroid

derivative on ischemia-reperfusion injury could be through β_2 receptor activation. However, to check this hypothesis, in this study the biological activity of the diazocinol-steroid derivative against ischemia-reperfusion injury was evaluated in absence or presence of butaxamine (β_2-receptor antagonist) [27]; the results showed that effect produced by diazoninol-steroid derivative against ischemia-reperfusion injury was inhibited by butaxamine. Here, it is important to mention that some studies indicate that β_2-receptor activation could induce changes in cAMP levels [24]; therefore, also the effect exerted by the diazoninol-steroid derivative on cAMP concentration was evaluated in an ischemia-reperfusion injury model using isoproterenol as control. The experimental data showed that the diazocinol-derivativeincrease the cAMP levels (3-18 min) in comparison with isoproterenol and control conditions. All these results suggest that effect exerted by the diazocinol-steroid derivativeagainst ischemia-reperfusion injury translated as decreased of infarct area involves β_2-receptor activation and consequently brings changes of cAMP levels.

Conflict of interest: The authors declare that they have noconflict of interest with any institutions.

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