Full Length Research Paper

Susceptibility to cardiac ischemia/reperfusion injury modulated by an estrogen derivative

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Several studies indicate that some steroid derivatives have activity at cardiovascular level; nevertheless, there is scarce information about the effects of the estradiol derivatives on cardiac injury ischemia/reperfusion (I/R). Therefore, in this study, an estradiol derivative was synthetized with the objective of evaluating its activity on I/R in an ischemia-reperfusion model. In addition, molecular mechanism involved in the activity of effect induced by estradiol derivative on perfusion pressure and coronary resistance was evaluated using the Langendorff technique by measuring left ventricular pressure in absence or presence of the following compounds; tamoxifen, yohimbine, ICI 118,551 and L-NAME. The results showed that estradiol derivative reduced infarct size compared with control. In addition, another results showed that the estradiol derivative significantly decrease the perfusion pressure and coronary resistance in isolated heart. Additionally, another data indicate that estradiol derivative low left ventricular pressure in a dose-dependent manner (1×10^{-9} to 1×10^{-4} mmol); however, this phenomenon was significantly inhibited by tamoxifen at a dose of 1×10^{-6} mmol and L-NAME (1×10^{-6} mmol). In conclusion, these data suggest that cardioprotective effect of estradiol derivative is through the interaction with estrogen receptor and activation of nitric oxide synthase. This phenomenon results in decrease of myocardial necrosis after ischemia and reperfusion.

Key words: Estradiol derivative, ischemia/reperfusion, tamoxifen, L-NAME.

INTRODUCTION

Data clinical and epidemiological indicate that myocardial infarction is a main cause of death worldwide (Yusuf et al., 2005; Thygesen et al., 2007). Myocardial infarction can be induced by prolonged ischemia which consequently brings in cell viability, and ultimately cardiac function. In addition, acute myocardial infarction can produce alterations in the topography of both the infarcted and non-infarcted regions of the ventricle (Pfeffer 1995). There are some reports which indicate the most effective method of limiting necrosis is restoration of blood flow; however, the effects of reperfusion itself may also be associated with tissue injury (Kloner et al., 1989). In addition, some studies suggest that there are differences between both men and women in the myocardial response to ischemia/reperfusion injury (Wang et al., 2005). These differences may be attributable to the effects of the sex hormones such as androgens and estrogens (Miejing et al., 2005). With respect to estrogens, there are studies which show that 17β-estradiol, but not 17α-estradiol, reduces miocardial necrosis in rabbits after ischemia and reperfusion (Hale et al., 1996). Other studies suggest that 17β-Estradiol prevents dysfunction of canine coronary endothelium and myocardium and reperfusion arrhythmias after brief ischemia/reperfusion (Kim et al., 1996). In addition, other studies showed that administration of 17β-estradiol reduces infarct size by altered expression of canine

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myocardial connexin43 protein (Tsung-Ming et al., 2004). Additionally, other reports indicate that effect of 17β-estradiol have a cardioprotective effect after myocardial ischemia and reperfusion by activation of the mitochondrial and sarcolemma ATP-sensitive K^+ channels (Chang-Her et al., 2002); this effect was independent of estrogen receptor. Nevertheless, some data indicate that estrogen receptor plays a role in the protective effects of estrogen following global, warm ischemia/reperfusion of the isolated mouse heart (Wang et al., 2008). Here, it is important to mention that some studies indicate that interaction of 17β-estradiol with receptor estrogen depend on the functional groups involved in its chemical structure (Anstead et al., 1997). Therefore, differences in the chemical structure of estradiol may be in part responsible of activity of 17β-estradiol on myocardial infarction/reperfusion effect. To test this information, the present study was designed to investigate the effects induced by an estradiol derivative in a myocardial infarction/reperfusion model. In addition, to evaluate the molecular mechanism involved in the activity of the estradiol derivative on left ventricular pressure which were used as pharmacological tools for blocking various biological systems; tamoxifen [estrogen receptor antagonist] (Shiau et al., 1998), yohimbine [α_2 adrenoreceptor antagonist] (Bruck et al., 2001), ICI 118,551 [selective β_2 receptor blocker] (Bilski et al., 1983), methoctramine [antagonist of the M2 receptor] (Watson et al., 1992), L-NAME [a selective inhibitor of nitric oxide synthase] (Moore et al., 1993).

MATERIALS AND METHODS

General methods

Estradiol-ethylenediamine (1) was prepared according to a previously reported method by Figueroa-Valverde et al. (2011). The other compounds evaluated in this study were purchased from Sigma-Aldrich Co., Ltd. The melting points for the different compounds were determined on an Electrothermal (900 model). Infrared spectra (IR) were recorded using KBr pellets on a Perkin Elmer Lambda 40 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Varian VXR-300/5 FT NMR spectrometer at 300 and 75.4 MHz in CDCl$_3$ using TMS as internal standard. EIMS spectra were obtained with a Finnigan Trace GCPolaris Q spectrometer. Elementary analysis data were acquired from a Perkin Elmer Ser. II CHNS/0 2400 elemental analyzer.

Succinic acid mono-{4-[(2-amino-ethylamino)-methyl]-17-hydroxy-13-methyl-7, 8, 9, 11, 12, 13,14 , 15, 16, 17-decahydro-6-H-cyclopenta[a]phenan- thren-3-yl }ester (3)

A solution of estradiol-ethylenediamine (100 mg, 0.29 mmol), succinic acid (70 mg, 0.59 mmol) and 1,3-dicyclohexylcarbodiimide (120 mg, 0.58 mmol) in 10 ml of methanol was stirred for 72 h to room temperature. The reaction mixture was evaporated to a smaller volume. After the mixture was diluted with water and extracted with chloroform. The organic phase was evaporated to dryness under reduced pressure; the residue was purified by crystallization from methanol : water (3:1).

Biological evaluation

All experimental procedures and protocols used in this investigation were reviewed and approved by the animal care and use committee of Universidad Autonoma de Campeche (UAC) and were in accordance with the guide for the care and use of Laboratory Animals (Bayne, 1996). Female rats (Wistar; weighing 200 to 250 g) were obtained from UAC.

Reagents

All drugs were dissolved in methanol and different dilutions were obtained using Krebs-Henseleit solution (≤ 0.01%, v/v).

Biological evaluation

First stage

Briefly, the female rats (200 to 250 g) were anesthetized by injecting them with pentobarbital at a dose rate of 50 mg/kg body weight. Then the chest was opened, and a loose ligature passed through the ascending aorta. The heart was then rapidly removed and immersed in ice cold physiologic saline solution. The heart was trimmed of non-cardiac tissue and retrograde perfused via a non-circulating perfusion system at a constant flow rate. It is important to mention that perfusion medium was the Krebs-Henseleit solution (pH 7.4, 37°C) composed of (mmol); 117.8 NaCl; 6 KCl; 1.75 CaCl$_2$; 1.2 NaH$_2$PO$_4$; 1.2 MgSO$_4$; 24.2 NaHCO$_3$; 5 glucose and 5 sodium pyruvate. The solution was actively bubbled with a mixture of O$_2$/CO$_2$ (95:5). The coronary flow was adjusted with a variable speed peristaltic pump. An initial perfusion rate of 15 ml/min for 5 min was followed by a 15 min equilibration period at a perfusion rate of 10 ml/min. All experimental measurements were done after this equilibration period.

Ischemia/reperfusion model

After of 15-min equilibration time, the hearts were subjected to ischemia for 30 min by turning off the perfusion system. After of this period, the system was restarted and the hearts were reperfused for 30 min with Krebs-Henseleit solution. It is important to mention that hearts were randomly divided into 2 major treatment groups:

Group I: Hearts were subjected to ischemia/reperfusion but received vehicle only (Krebs-Henseleit solution).
Group II: Hearts were subjected to ischemia/reperfusion and treated with estradiol derivative (1 × 10^{-9} mmol) before ischemia period (for 10 min) and during the entire period of reperfusion.

It is important to mention that at the end of each experiment, the perfusion pump was stopped, and 0.5 ml of fluorescein solution (0.10%) was injected slowly through a side arm port connected to the aortic cannula. The dye was passed through the heart for 10 min to ensure its uniform tissue distribution.

   The presence of fluorescein was used to demarcate the tissue that was not subjected to regional ischemia, as opposed to the risk region. The heart was removed from the perfusion apparatus and cut into two transverse sections at right angles to the vertical axis. The right ventricle, apex, and atrial tissue were discarded. The areas of the normal left ventricle nonrisk region, area at risk, and infarct region were determined using the technique reported by Boot et al., (2005). Total area at risk is expressed as the
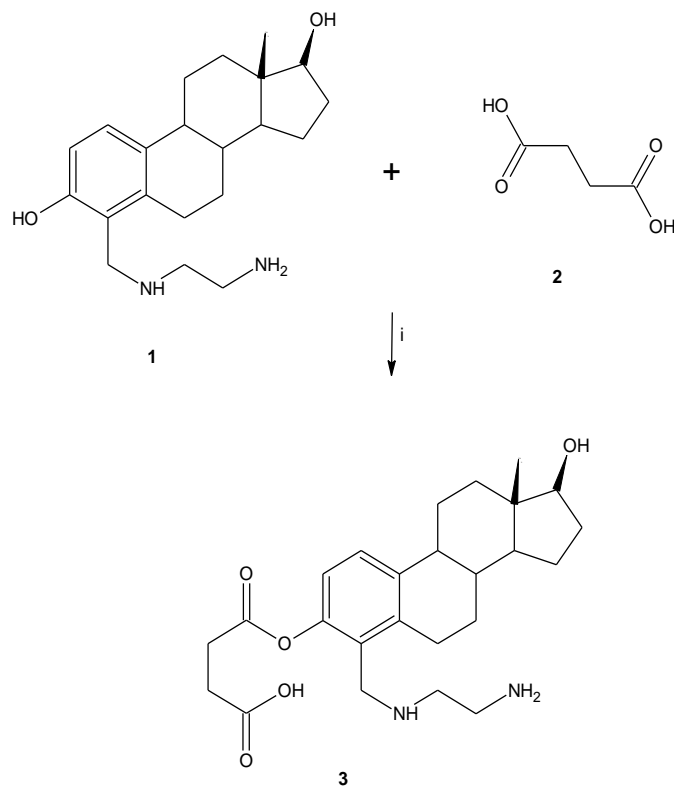
Figure 1. Synthesfis of succinic acid mono-{4-[(2-amino-ethylamino)-methyl]-17-hydroxy-13-methyl-7, 8, 9, 11, 12, 13, 14, 15, 16, 17-decahydro-6-H-cyclopenta[a]phenan-thren-3-yl}ester (3). Reaction of estradiol-ethylenediamine (1) with succinic acid (2) using 1,3-dicyclohexylcarbodiimide as catalyst (*i*).

percentage of the left ventricle.

Second stage

Perfusion pressure: Evaluation of measurements of perfusion pressure changes induced by drugs administration in this study were assessed using a pressure transducer connected to the chamber where the hearts were mounted and the results entered into a computerized data capture system (Biopac).

Inotropic activity: Contractile function was assessed by measuring left ventricular developed pressure (LVdP), using a saline-filled latex balloon (0.01 mm, diameter) inserted into the left ventricle via the left atrium. It is important to mention that latex balloon was bound to cannula which was linked to pressure transducer that was connected with the MP100 data acquisition system.

Effect induced by the estradiol derivative on perfusion pressure: Changes in perfusion pressure as a consequence of increases in time (3 to 18 min) in absence (control) or presence of the estradiol derivative at a concentration of 1×10^{-9} mmol were determined. The effects were obtained in isolated hearts perfused at a constant-flow rate of 10 ml/min.

Evaluation of effects exerted by the estradiol derivative on coronary resistance: The coronary resistance in absence (control) or presence of the estradiol derivative at a concentration of 1×10^{-9} mmol was evaluated. The effects were obtained in isolated hearts

perfused at a constant flow rate of 10 ml/min. Since a constant flow was used, changes in coronary pressure reflect the changes in coronary resistance.

Third stage

Effect exerted by the estradiol derivative on left ventricular pressurein the presence of α_2 adrenergic blocker: The boluses (50 μl) of the estradiol derivative (1×10^{-9} to 10^{-4} mmol) were administered and the corresponding effect on the left ventricular pressure was evaluated. It is important to mention that the bolus injection administered was done in the point of cannulation. The dose-response curve (control) was repeated in the presence of yohimbine at a concentration of 1×10^{-6} mmol (duration of preincubation with yohimbine was by a 10 min equilibration period).

Effects induced by the estradiol derivative on left ventricular pressurein the presence of β_2 adrenergic blocker: The boluses (50 μl) of estradiol derivative (1×10^{-9} to 1×10^{-4} mmol) were administered and the corresponding effect on the left ventricular pressure was evaluated. The dose-response curve (control) was repeated in the presence of ICI 118,551 at concentration of 1×10^{-6} mmol (duration of preincubation with ICI 118,551 was by a 10 min equilibration period).

Effects of estradiol derivative on left ventricular pressure in the presence of M_2 muscarinic blocker: The boluses (50 μl) of estradiol derivative (1×10^{-9} to 1×10^{-4} mmol) were administered and the corresponding effect on the left ventricular pressure was evaluated. The dose-response curve (control) was repeated in the presence of methoctramine at a concentration of 1×10^{-6} mmol (duration of preincubation with methoctramine was by a 10 min equilibration period).

Effects induced by the estradiol derivative on left ventricular pressure through estrogen receptors: Intracoronary boluses (50 μl) of the estradiol derivative (1×10^{-9} to 1×10^{-4} mmol) were administered and the corresponding effect on the left ventricular pressure was determined. The dose-response curve (control) was repeated in the presence of tamoxifen at a concentration of 1×10^{-6} mmol (duration of preincubation with tamoxifen was by a 10 min equilibration period).

Effects of estradiol derivative on left ventricular pressurein the presence of L-NAME: The boluses (50 μl) of estradiol derivative (1×10^{-9} to 1×10^{-4} mmol) were administered and the corresponding effect on the left ventricular pressure was evaluated. The dose-response curve (control) was repeated in the presence of L-NAME at concentration of 1×10^{-6} mmol (duration of preincubation with L-NAME wasby a 10 min equilibration period).

Statistical analysis

The obtained values are expressed as average ± SE, using each heart as its own control. The data obtained were put under ananalysis of variance (ANOVA) using the Bonferroni correction factor (Hocht et al., 1999). The differences were considered significant when *p* was equal or smaller than 0.05.

RESULTS

Chemical synthesis

The yielding of estradiol derivative (Figure 1) was 40%

0.89 (3H, s, 18 CH$_3$),
0.97-2.54 (m, 15H),
2.61 (t, 2H, J = 6.0 Hz, f-CH$_2$,),
2.65 (t, 2H, J = 5.8 Hz),
2.79 (t, 2H, J = 5.8 Hz),
2.84 (2H, t, J = 6.0 Hz),
3.67 (m, 1H),
3.82 (s, 2H),
4.58 (broad, 5H),
6.72 (1H, d, J = 8.55 Hz),
7.08 (1H, d, J = 8.55 Hz) ppm.

Figure 2. ^{1}H NMR (300 MHz, CDCl$_3$) data for the estradiol derivative.

11.3 (**C**-18), 23.57 (**C**-9), 26.23 (**C**-5), 27.57 (**C**-11), 27.70 (**C**-10), 29.22 (**C**-5), 29.70 (**C**-28), 30.83 (**C**-8), 37.41

(**C**-6), 39.03 (**C**-3), 41.57 (**C**-23), 43.60 (**C**-1), 44.42 (**C**-4), 44.95 (**C**-20), 50.50 (**C**-2), 53.33 (**C**-22), 81.41 (**C**-7),

118.06 (**C**-15), 129.70 (**C**-16), 135.52 (**C**-17), 137.61 (**C**-13), 138.45 (**C**-12), 144.84 (**C**-14), 171.83 (**C**- 26),

174.19 (**C**-30) ppm.

Figure 3. ^{13}C NMR (75.4 MHz, CDCl$_3$) data for the estradiol derivative.

with melting point of 174 to 176°C. In addition, the spectroscopic analyses show signals for IR (V_{max}, cm^{-1}) at 3380, 3330 and 1624. In addition, the chemical shifts of the spectroscopic analyses of ^{1}H NMR (Figure 2) and ^{13}C NMR for the estradiol-derivative is showed in Figure 3. Finally, the results of mass spectroscopy (MS) (70 eV) are shown: m/z 376.30 [M$^+$, 11] 273.30, 191.20 and 137.10. Additionally, the elementary analysis data for the estradiol derivative (C$_{25}$H$_{36}$N$_2$O$_5$) were calculated (C, 67.54; H, 8.16; N, 6.30; 0, 17.99) and found (C, 67.52; H, 8.15; N, 6.28).

Biological activity

First stage

Effect of estradiol derivative on ischemia/reperfusion: The results showed that estradiol derivative reduced infarct size expressed as a percentage of the area at risk compared with vehicle-treated hearts (Figures 4 and 5).

Second stage

In this study, the activity induced by the estradiol

derivative on perfusion pressure and coronary resistance in the isolated rats heart were evaluated. The results obtained from changes in perfusion pressure as a consequence of increases in the time (3 to 15 min) in absence (control) or in presence of estradiol derivative (Figure 6), showed that estradiol derivative (1 × 10^{-9} mmol) significantly decrease the perfusion pressure (p = 0.005) in comparison with the control conditions (1 × 10^{-9} mmol).

Additionally, another result showed that coronary resistance, calculated as the ratio of perfusion pressure at coronary flow assayed (10 ml/min) was low in the presence of estradiol derivative than in control conditions (p = 0.005) at a concentration of 1 × 10^{-9} mmol (Figure 7).

Third stage

Figure 8 shows that estradiol derivative decrease the left ventricular pressure in a dose dependent manner (1 × 10^{-9} to 1 ×10^{-4} mmol), and this effect was not inhibited in presence of yohimbine or ICI 118,551 (Figure 9) drugs at a concentration of 1 × 10^{-6} mmol. In addition, another results indicate that effect induced by the estradiol derivative on left ventricular pressure (Figure 10) in presence of methoctramine at a concentration of 1 × 10^{-6} mmol was not blocked.
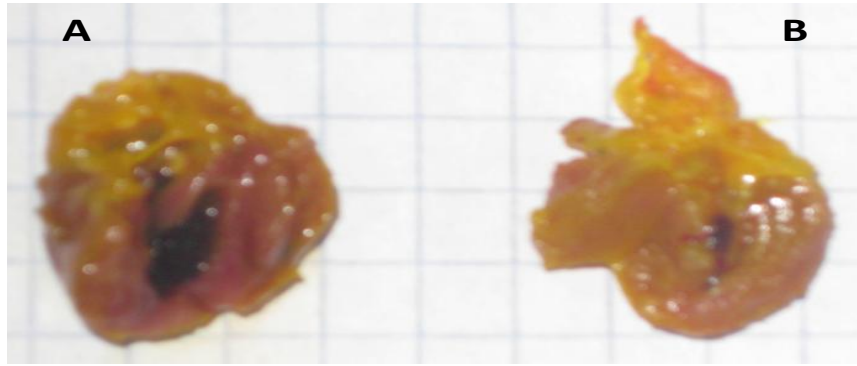
Figure 4. Comparison of cardioprotective effect of estradiol derivative (B) with control (A) on the functional recovery of rat hearts subjected to ischemia and reperfusion.
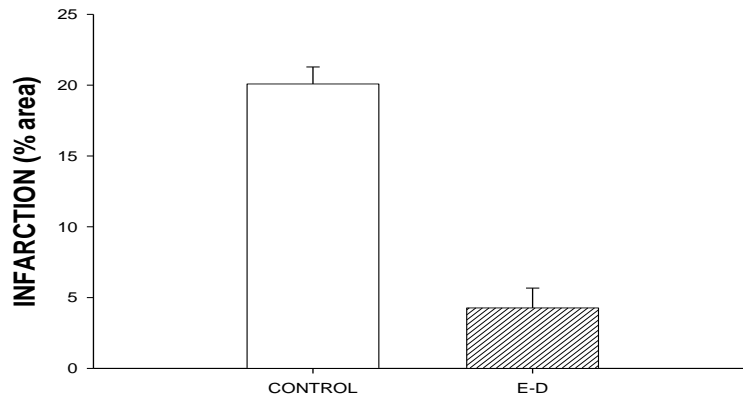

Figure 5. Effect exerted by estradiol (E-D) on cardiac ischemia/reperfusion with control. The results showed that estradiol derivative significantly reduced infarct size ($p = 0.005$) expressed as a percentage of the area at risk compared with vehicle-treated hearts. Each bar represents the mean ± S.E. of 8 experiments.
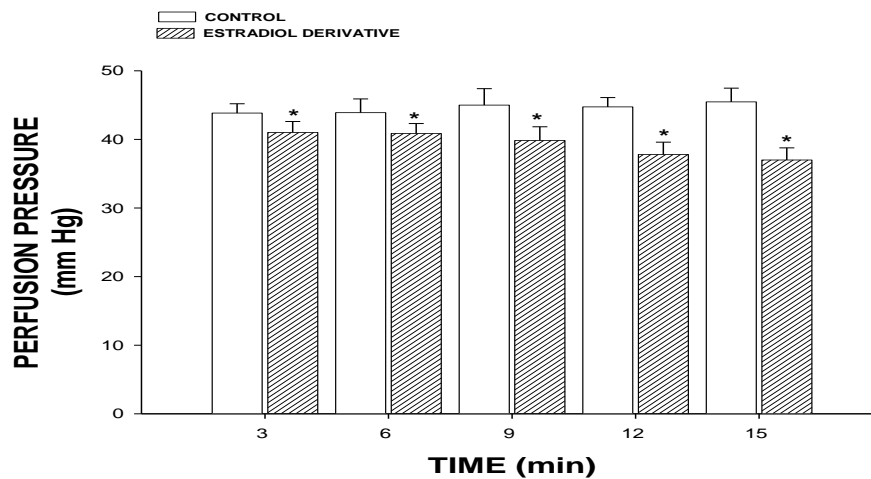

Figure 6. Effect induced by estradiol derivative on perfusion pressure. The results showed that estradiol-derivative significantly decreased the perfusion pressure ($p = 0.005$) through of time (3 to 18 min) in comparison with the control conditions. The effect is expressed as the area under the curve, and each bar represents the mean ± S.E. of 8 experiments.
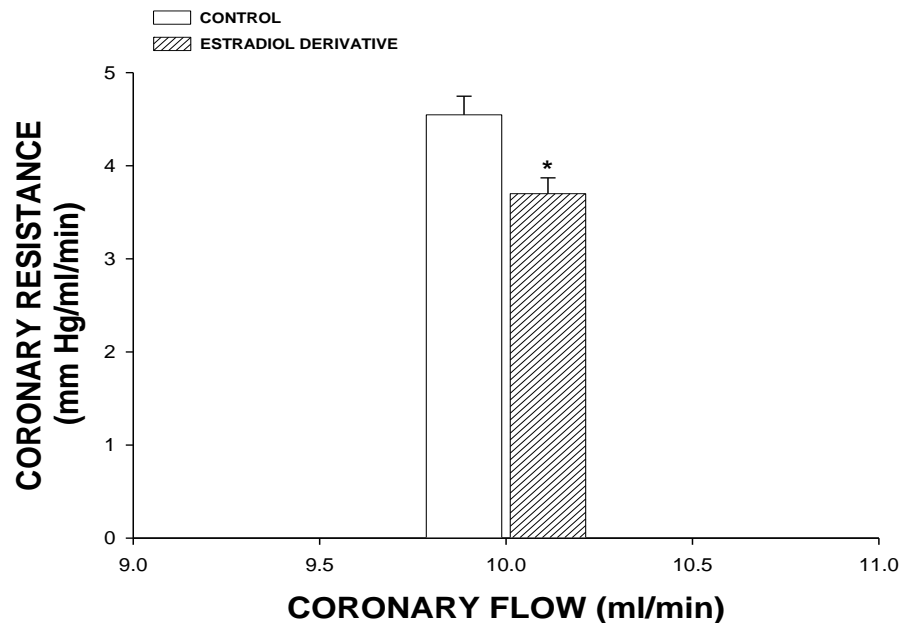
Figure 7. Activity induced by progesterone and estradiol derivative on coronary resistance. The results showed that coronary resistance was lower in presence of estradiol derivative ($p = 0.006$) in comparison with the control conditions. The effect it is expressed as the area under the curve, and each bar represents the mean ± S.E. of 8 experiments.
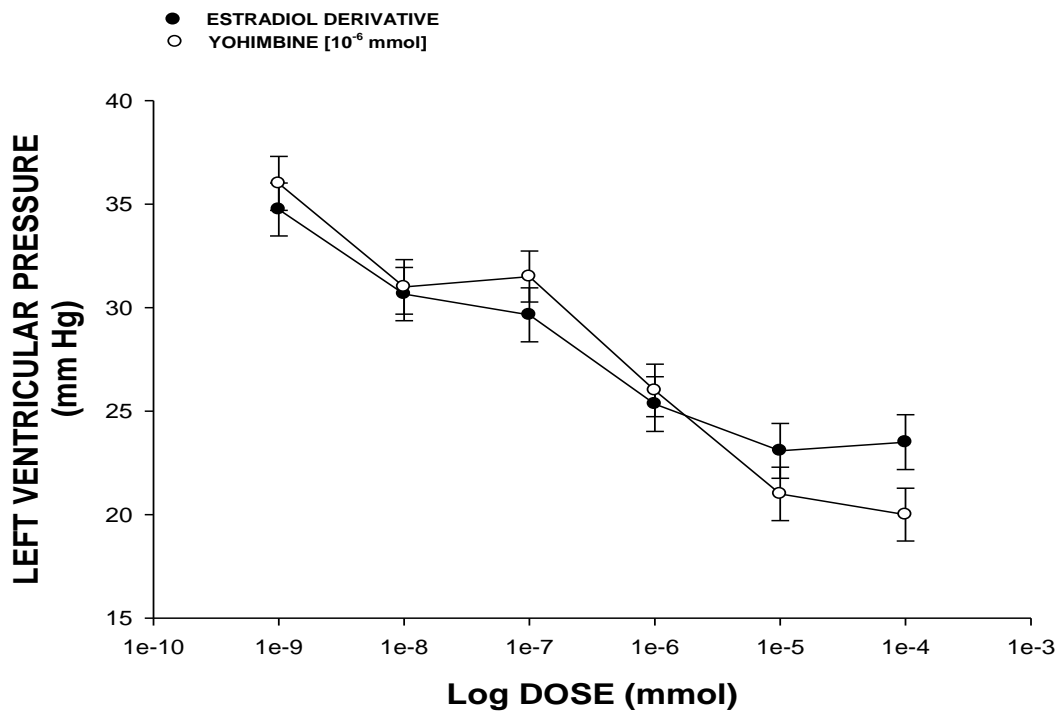

Figure 8. Effect exerted by estradiol derivative on left ventricular pressure through of α_2-adrenergic receptor. Estradiol derivative (1×10^{-9} to 10^{-4} mmol) was administered (intracoronary boluses, 50 µl) and the corresponding effect on the left ventricular pressure was evaluated in absence and presence of yohimbine (1×10^{-6} mmol). The results showed that activity induced by estradiol on perfusion pressure was not inhibited in presence of yohimbine. The effect it is expressed as the area under the curve, and each bar represents the mean ± S.E. of 8 experiments.
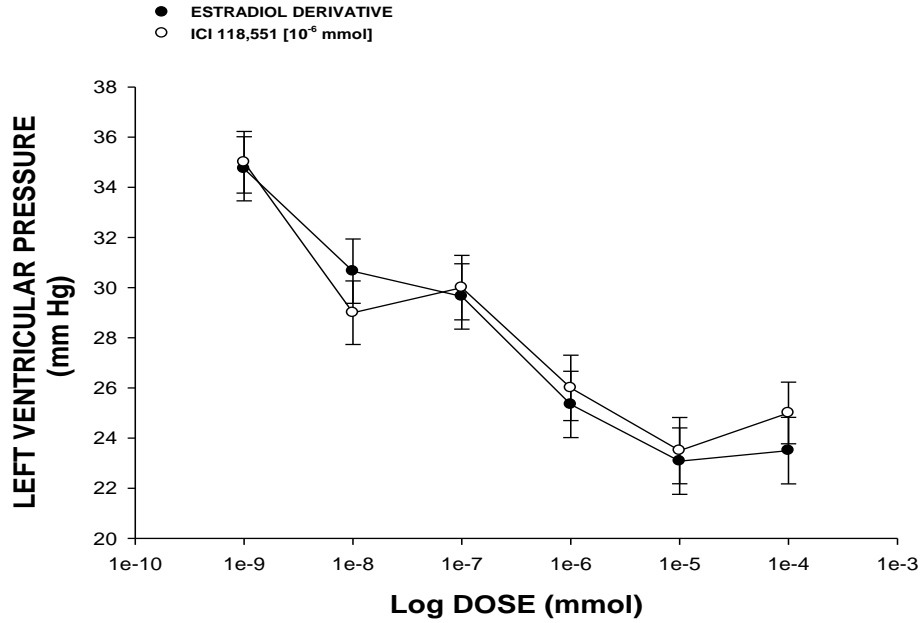
● ESTRADIOL DERIVATIVE
○ ICI 118,551 [10^{-6} mmol]

Figure 9. Activity induced by estradiol derivative on left ventricular pressure through of β_2-adrenergic receptor. Intracoronary boluses (50 µl) of estradiol-derivative (1×10^{-9} to 1×10^{-4} mmol) were administered and the corresponding effect on the left ventricular pressure was evaluated in absence and presence of ICI 118,551 (1×10^{-6} mmol). The results showed that activity induced by estradiol-derivative on left ventricular pressure was not inhibited in presence of ICI 118,551. The effects it is expressed as the area under the curve, and each bar represents the mean ± S.E. of 8 experiments.
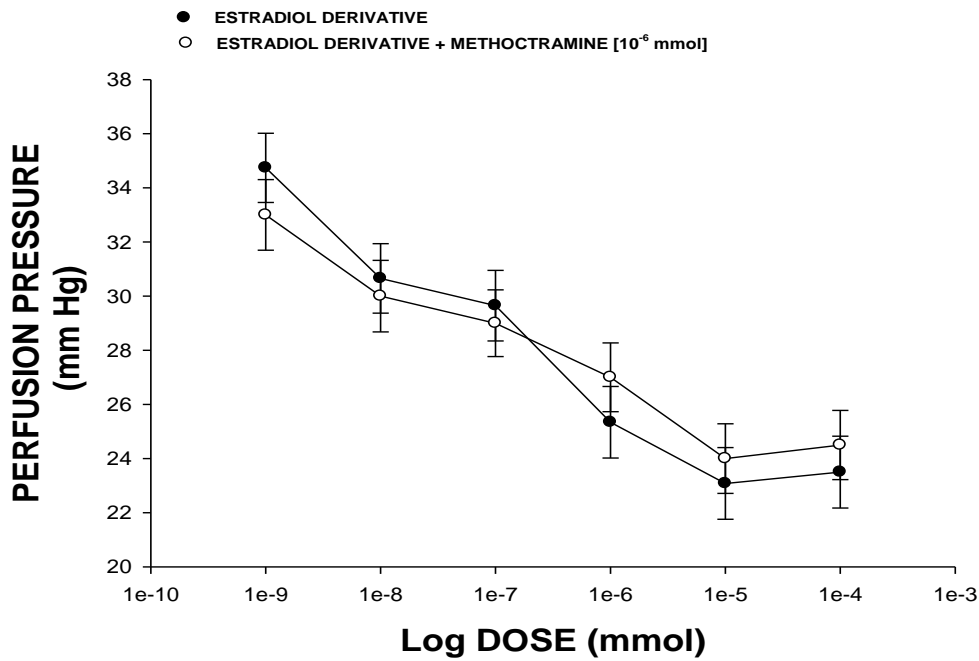
● ESTRADIOL DERIVATIVE
○ ESTRADIOL DERIVATIVE + METHOCTRAMINE [10^{-6} mmol]

Figure 10. Effects induced by estradiol derivative on left ventricular pressure through of muscarinic receptors. Intracoronary boluses (50 µl) of estradiol [1×10^{-9} to 1×10^{-4} mmol] were administered in absence and presence of methoctramine (1×10^{-6} mmol). The results showed that effect induced by estradiol derivative on left ventricular pressure in presence of methoctramine was not inhibited. The effect it is expressed as the area under the curve, and each bar represents the mean ± SE of 8 experiments.
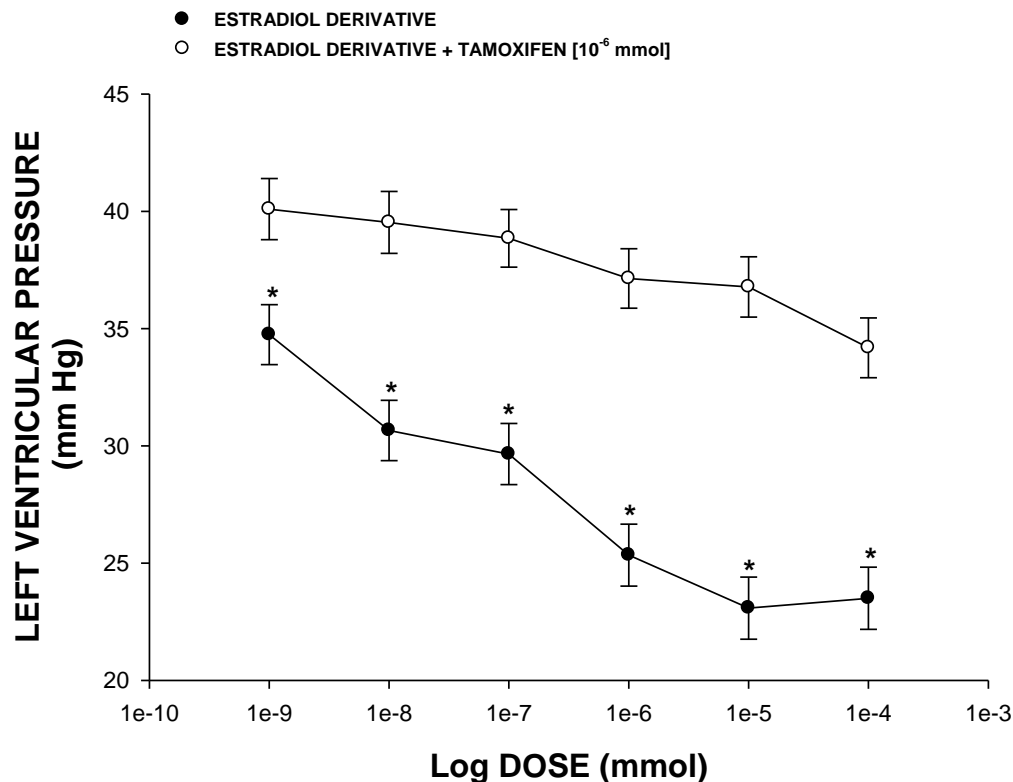
● **ESTRADIOL DERIVATIVE**
○ **ESTRADIOL DERIVATIVE + TAMOXIFEN [10^{-6} mmol]**

Figure 11. Effects induced by estradiol derivative on left ventricular pressure through estrogen-receptor. Intracoronary boluses (50 µl) of estradiol derivative (1×10^{-9} to 1×10^{-4} mmol) were administered and the corresponding effect on the perfusion pressure was determined. The results showed that estradiol derivative decreased the perfusion pressure in a dependent dose manner and this effect was inhibited in presence of tamoxifen (1×10^{-6} mmol). The effect it is expressed as the area under the curve, and each bar represents the mean ± S.E. of 8 experiments.

The results of alternative experiments (Figure 11) indicate that estradiol derivative induces a decrease in left ventricular pressure in a dose dependent manner (1×10^{-9} to 1×10^{-4} mmol) and that this effect was inhibited by tamoxifen (10^{-6} mmol). Finally, another results (Figure 12) showed that effect induced by the estradiol derivative (1×10^{-9} mmol) on perfusion pressure in presence of L-NAME (1×10^{-6} mmol) was inhibited.

DISCUSSION

It is important to mention that there are studies which indicate that ischemia/reperfusion (I/R) injury occurs when a tissue is temporarily deprived of blood supply and the return of the blood supply triggers an intense inflammatory response (Arumugam et al., 2004). Analyzing these data in this study, in the first stage, the effect of estradiol derivative was evaluated in anischemia-reperfusion model. The results showed that estradiol derivative reduced infarct size expressed as a percentage of the area at risk compared with vehicle-treated hearts. This phenomenon can be conditioned by

generation of some substances involved in endothelium of coronary artery (Bouis et al., 2001) or by the influence exerted by estradiol derivative on blood pressure which consequently bring reduce infarct size, and decrease the miocardial injury after ischemia-reperfusion such as happened with 17β-estradiol (Beer et al., 2002). To evaluate these hypotheses, the effect of estradiol derivative on blood vessel capacity and coronary resistance translated as changes in perfusion pressure in isolated rat heart was evaluated (Langendorff model). The results show that estradiol derivative significantly decreased the perfusion pressure over time (3 to 18 min) compared to the control conditions. These data suggest that estradiol derivative exerts effects on perfusion pressure which could subsequently modify vascular tone and coronary resistance. In this sense, the effects of estradiol derivative on coronary resistance were evaluated. We found that coronary resistance was decreased by the estradiol derivative. These data suggest that estradiol derivative exerts effect on vascular tone.

On the other hand, to characterize the molecular mechanism of this phenomenon, we analyzed the reports
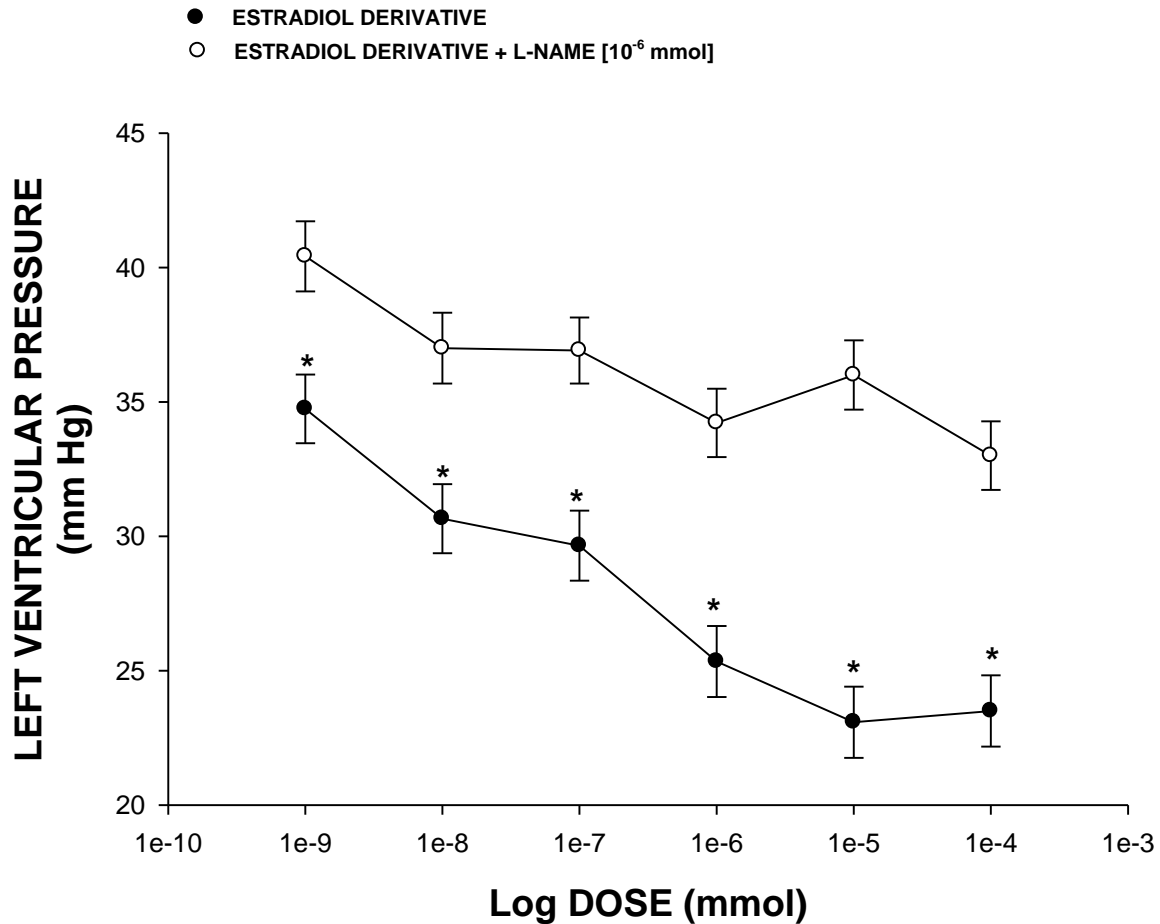
Figure 12. Effects exerted by estradiol derivative on left ventricular pressure through of synthesis of nitric oxide. Estradiol-derivative (1×10^{-9} to 1×10^{-4} mmol) was administered (intracoronary boluses, 50 µl) and the corresponding effect on the perfusion pressure was evaluated in absence and presence and L-NAME (1×10^{-6} mmol). The results showed that activity induced by estradiol derivative on perfusion pressure in presence of L-NAME was inhibited significantly ($p = 0.006$). The effect it is expressed as the area under the curve, and each bar represents the mean ± S.E. of 8 experiments.

of some investigations which indicate that estradiol can regulate adrenal catecholamine synthesis (Lilley et al., 1976), which has an important role in the development or maintenance of elevated blood pressure. In this study the biological activity of estradiol derivative on left ventricular pressure in the absence or presence of yohimbine or ICI 118,551 was evaluated. The results showed that the effect of estradiol derivative was not inhibited by yohimbine or ICI 118,551, indicating that the molecular mechanism involved is not through adrenergic activity.

Analyzing these results and other reports which indicate that some steroid derivatives induces activation of M_2 muscarinic (Figueroa-Valverde et al., 2012) that induces consequently a negative inotropic response and low blood pressure by activation of nitric oxide synthase (NOS) and increased production of cyclic GMP. In this study, the activity of estradiol derivative on left ventricular pressure was evaluated in the absence or presence of atropine. The results showed that the effect of estradiol derivative on left ventricular pressure was not inhibited in the presence of atropine; these data indicate that effect of estradiol derivative was not via muscarinic receptor.

On the other hand, in the search of the molecular mechanism involved in the effect of estradiol derivative, we also analyzed the reports of some investigations which indicate that estradiol induces its effect on blood pressure via activation of the estradiol receptor (Booth et al., 2005). For this reason, we used tamoxifen, an estradiol receptor blocker (Shiau et al., 1998), to determine if the effects of estradiol derivative on left ventricular pressure were via the estrogen receptor. It is important to mention that interaction of the estradiol derivative with the estrogen receptor may be a key requirement for the protective effect of this steroid on ischemic injury such as happening with activity exerted by 17β-estradiol (Gabel et al., 2005).

Our results showed that the effects of estradiol derivative were inhibited by tamoxifen, suggesting that the molecular mechanism is through estrogen receptor. Furthermore, to assess whether this phenomenon also involved the generation of nitric oxide, the compound L-NAME was used as pharmacological tool. The results indicate that effect of the estradiol derivate on left ventricular pressure was blocked with L-NAME, this data indicates that a part of interaction of estrogen receptor can induce activation of nitric oxide synthase and increase production of nitric oxide and consequently bring decrease of ischemia-reperfusion injury such as happening with other type of steroids (Gabel et al., 2005).

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