Original Article

Effects exerted by an estrogen derivative against ischemia/reperfusion in an isolated heart model

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ABSTRACT

Several reports suggest that steroids can exert cardioprotective effects against ischemia/reperfusion injury; however, the molecular mechanism through which they exert such effects is not very clear. The aim of this study is to evaluate the biological activity of an estrogen derivative against ischemia/ reperfusion injury in an isolated heart model using noradrenaline, milrinone, dobutamine and levosimedan as controls. In addition, the effect exerted by the steroid derivative on left ventricular pressure was evaluated in the absence or presence of metoprolol, propanolol and nifedipine drugs. The results showed that 1) the steroid derivative significantly reduced ischemia-reperfusion injury, resulting in a reduction of the infarct area in a manner similar to the noradrenaline drug; 2) the steroid derivative increases the left ventricular pressure and this effect was inhibited in the presence of metoprolol and propanolol drugs. All these data indicate that the steroid derivative decreases the area of infarction and increases left ventricle pressure *via* β_1-adrenergic receptors; this phenomenon could constitute a new therapy for ischemia/reperfusion injury.

KEYWORDS: estrogen, ischemia, reperfusion, steroid, pressure.

1. INTRODUCTION

Several studies have shown that myocardial ischemia can be one of the main causes of death [1-3]. In order to reduce acute myocardial infarction, several procedures that promote the return of blood flow to the ischemic area of the myocardium can be performed [4]; however, these methods can induce reperfusion injury which can result in increased irreversible myocardial cell death. It is noteworthy that several pharmacological drugs such as amiloride (Na^+-H^+ exchange inhibitor) [5], sanglifehrin-A (inhibits opening of the mitochondrial permeability transition pore) [6], BW373U86 (δ-opioid agonist) [7], glibenclamide (ATP-regulated K^+ channels inhibitor) [8] etc. have been used to reduce reperfusion damage; however, some of these drugs can exert secondary effects such as hypoxic tolerance [9], intrahepatic cholestasis [10] and others. In the search of new drugs for the treatment of ischemia-reperfusion injury some

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steroids have been evaluated; for example, using an animal model a previous study showed that 17β-estradiol can decrease ischemia-reperfusion [11]; in addition, a report showed that 17β-estradiol can exert a cardioprotective effect against myocardial arrhythmias induced by ischemia/reperfusion injury [12]. Also, progesterone has been used in conjunction with estrogen in an ischemia/reperfusion model, resulting in significantly decreased myocardial injury [13]. Other data from studies using an isolated heart preparation suggest that testosterone may decrease some bioactive substances involved in the inflammation produced through ischemia/reperfusion injury [14].

On the other hand, to characterize the molecular mechanism involved in the biological activity exerted by the steroids against ischemia/reperfusion injury, several steroid derivatives have been used as pharmacological tools; for example, a study using an animal model showed that a progesterone derivative can exert cardioprotective effects against ischemia/reperfusion injury through calcium channel activation [15]. Additionally, one report has shown that treatment with medroxyprogesterone acetate can inhibit the biological activity of estradiol in ischemia/reperfusion injury, which can lead to changes in the concentration of neutrophils [16]. Also, some data suggest that an estrogen derivative may modulate the ischemia/reperfusion injury in an isolated heart model through calcium channel activation [17]. These studies suggest that steroids and their derivatives can exert effects against ischemia/reperfusion injury; however, the molecular mechanism involved

in its biological activity is not very clear; perhaps this phenomenon is due to the different research protocols used. To evaluate this hypothesis, in this study the main objective was to evaluate the biological activity of an estrogen derivative against ischemia/reperfusion injury in an isolated rat heart.

2. MATERIALS AND METHODS

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal care and use Committee of the Autonomous University of Campeche (No. PI-420/12) and were in accordance with the guidelines for the care and use of laboratory animals [18]. Male Wistar rats weighing 200-250 g were obtained from the Autonomous University of Campeche.

2.1. Reagents

The estrogen derivative (Figure 1) was prepared using a previously reported method [19]. In addition, all drugs used in this study were dissolved in methanol and different dilutions were obtained using Krebs-Henseleit solution ($\leq 0.01\%$, v/v).

2.2. Langendorff method

Briefly, the male rats (200-250 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 was passed through the ascending aorta. The heart was then rapidly removed and immersed in ice-cold physiologic saline solution. The heart was trimmed of noncardiac tissue and retrograde perfused *via* a non-circulating
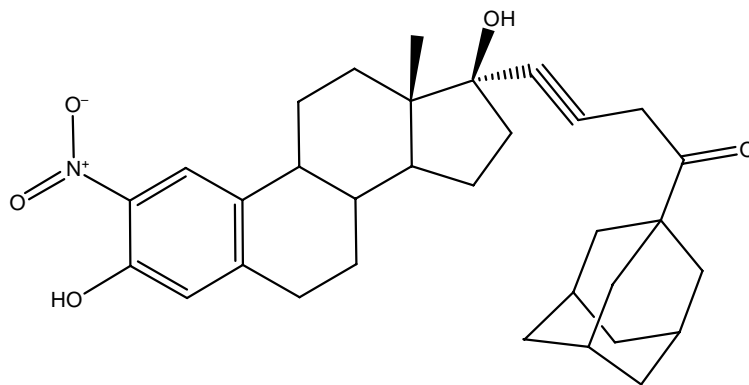

Figure 1. Chemical structure of the estrogen derivative.

perfusion system at a constant flow rate. It is important to mention that the perfusion medium was the Krebs-Henseleit solution (pH 7.4, 37 °C) composed of 117.8 mM NaCl, 6 mM KCl, 1.75 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 24.2 mM $NaHCO_3$, 5 mM glucose and 5 mM sodium pyruvate. The solution was actively bubbled with a mixture of O_2/CO_2 (95:5). The coronary flow was adjusted using a variable-speed peristaltic pump. An initial perfusion rate of 15 ml/min for 5 min was followed by a 25 min equilibration period at a perfusion rate of 10 ml/min. All experimental measurements were done after this equilibration period.

2.3. Evaluation of left ventricle pressure

To evaluate the biological activity of drugs involved in this study against left ventricle pressure, a latex balloon filled with saline solution (0.01 mm diameter) was inserted into the left ventricle through the left atrium. It is important to mention that the latex balloon was bound to a pressure transducer which was connected to a computerized data capture system (MP-100). Subsequently, the inotropic effect produced by compounds involved in this study was evaluated by determining left ventricular developed pressure (LV/dP) [20].

2.4. Experimental design

2.4.1. First stage

Effect exerted by an estrogen derivative against ischemia/reperfusion injury

After 15 minutes of equilibration time, the hearts were subjected to ischemia for 40 minutes by turning off the perfusion system [20]. Then, the system was restarted, and the hearts were re-perfused for 40 minutes with Krebs-Henseleit solution. The hearts were randomly divided into 7 major treatment groups that involved the control (without treatment), and the steroid derivative with n = 9 as follows:

Group I: Hearts that were subjected to ischemia/reperfusion but received vehicle only (Krebs-Henseleit solution).

Group II: Hearts that were subjected to ischemia/reperfusion and treated with the steroid derivative (0.001 nM).

Group III: Hearts that were subjected to ischemia/reperfusion and treated with the steroid derivative (0.01 nM).

Group IV: Hearts that were subjected to ischemia/reperfusion and treated with the steroid derivative (0.1 nM).

Group V: Hearts that were subjected to ischemia/reperfusion and treated with the steroid derivative (1 nM).

Group VI: Hearts that were subjected to ischemia/reperfusion and treated with the steroid derivative (10 nM).

Group VII: Hearts that were subjected to ischemia/reperfusion and treated with the steroid derivative (100 nM).

It is noteworthy 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 sidearm port connected to the aortic cannula. The dye was passed through the heart for 10 sec 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. Then, 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. It is important to mention that the non-infarcted areas and the infarcted regions were determined using a previously reported method [21].

2.4.2. Second stage

Effects induced by noradrenaline, milrinone, dobutamine, levosimendan and the estrogen derivative against the infarct area

The hearts were randomly divided into 6 major treatment groups with n = 9, as follows:

Group VII: Hearts that were subjected to ischemia/reperfusion but received vehicle only (Krebs-Henseleit solution).

Group VIII: Hearts that were subjected to ischemia/reperfusion and treated with noradrenaline (0.001 nM).

Group IX: Hearts that were subjected to ischemia/reperfusion and treated with milrinone (0.001 nM).

Group X: Hearts that were subjected to ischemia/reperfusion and treated with dobutamine (0.001 nM).

Group XI: Hearts that were subjected to ischemia/reperfusion and treated with levosimendan (0.001 nM).

Group XII: Hearts that were subjected to ischemia/reperfusion and treated with the estrogen derivative (0.001 nM).

Here, it is important to mention that the doses administered of the drugs noradrenaline, milrinone, dobutamine, and levosimedan were based on some previously reported methods [21]. Therefore, in this study the biological activity exerted by these drugs on ischemia/reperfusion injury was used as a control for comparison against the effect exerted by the steroid derivative against ischemia/reperfusion injury. Finally the non-infarcted areas and the infarcted regions were determined as mentioned above.

2.4.3. Third stage

Effects induced by the estrogen derivative on left ventricular pressure through β_1-adrenergic receptor activation

Intracoronary boluses (50 µL) of the estrogen derivative (0.001 to 100 nM) were administered in the absence or presence of metoprolol or propranolol at a concentration of 1 nM (duration of the preincubation with propranolol or metoprolol was 10 min). It is important to mention that dose of propranolol or metoprolol has been reported in a previous study [22].

Effect exerted by the estrogen derivative on the cAMP concentration

The hearts were perfused with the estrogen derivative (0.001 nM), isoproterenol (1 µM) and vehicle (control) for 2, 5, or 30 min. After the appropriate period of infusion, atrial tissue was removed, and the ventricles were immediately frozen using liquid nitrogen and stored at 270 °C until the assay. Tissue samples were homogenized with 6% trichloroacetic acid at 4 °C to give a 10% (w/v) homogenate, followed by centrifugation at 2000 rpm (Hettich Zentrifugen, EBA-270) for 15 min. Then supernatants were collected and washed with water:diethyl ether (5:1 v/v) five times. The extracts were lyophilized and processed

for the measurement of cAMP content by use of a standard ^{125}I radioimmunoassay kit supplied by Amersham International [23].

2.4.4. Statistical analysis

The obtained values are expressed as average ± SE, using each heart as its own control. The data obtained underwent an analysis of variance (ANOVA) using the Bonferroni correction factor [24]. The differences were considered significant when p was equal to 0.05.

3. RESULTS

3.1. Biological activity

In this study, the biological activity of the estrogen derivative against myocardial injury was evaluated using an ischemia/reperfusion model in rats. Hearts were subjected to ischemia/reperfusion and treated in the absence (received vehicle only; Krebs-Henseleit solution) or presence of the estrogen derivative (at a dose of 0.001 to 100 nM) before ischemia period (for 10 minutes) and during the entire period of reperfusion (30 minutes). The data shown in Figure 2 indicate that the estrogen derivative significantly reduced (p = 0.05) the infarct size (expressed as a percentage of the area at risk) compared to the control.

Other results showed that the estrogen derivative decreased (p = 0.05) the infarct area in a similar manner compared with both noradrenaline and dobutamine drugs (Figure 3). The effect of both noradrenaline and estrogen derivative on the left ventricular pressure was also evaluated. The results showed that both noradrenaline and the estrogen derivative increased (p = 0.05) the left ventricular pressure in a dose-dependent manner (Figure 4). However, the effect exerted by the estrogen derivative against left ventricular pressure was inhibited by metoprolol and propanolol. Finally, the results shown in Figure 5 indicate that the estrogen derivative did not induce changes in the cAMP concentration.

4. DISCUSSION

Some steroid derivatives have been developed against ischemia/reperfusion injury [11-15]; however, the molecular mechanism involved in their biological activity is not clear. These phenomena
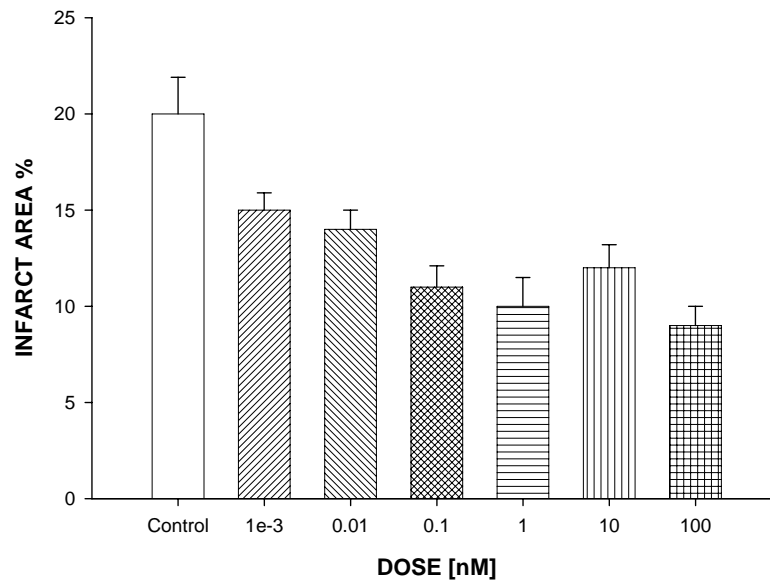
Figure 2. Biological activity induced by an estrogen derivative on infarct area. The results showed that the estrogen derivative significantly reduced ($p = 0.05$) the infarct size (size expressed as the percentage of the area at risk) in a dose-dependent manner (0.001-100 nM) compared with the control (group without treatment). Each bar represents the mean ± S.E. of 9 experiments.
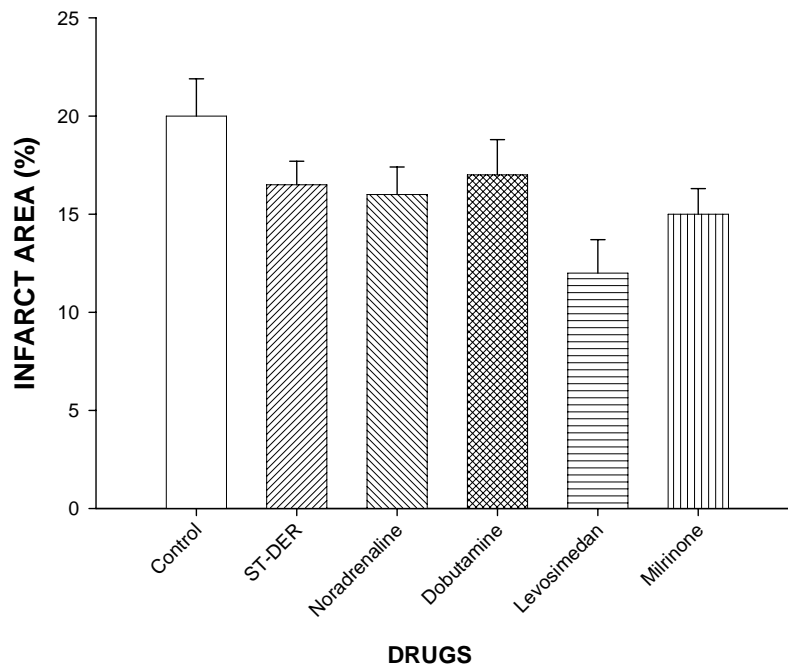

Figure 3. Effect exerted by noradrenaline, milrinone, dobutamine, levosimedan and the estrogen derivative (ST-DER) against the ischemia-reperfusion injury. The results showed that there are differences in the cardioprotective effects exerted by levosimedan and milrinone compared with the estrogen derivative; however, the biological activity ($p = 0.05$) exerted by the estrogen derivative was in a manner similar to that by noradrenaline and dobutamine. The infarct size is expressed as the percentage of the area at risk and each bar represents the mean ± S.E. of 9 experiments.

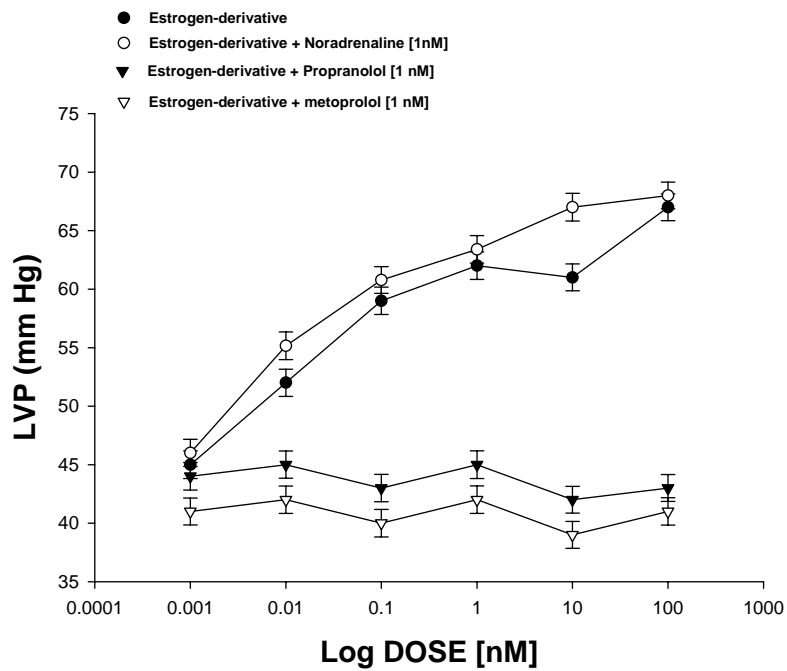**Figure 4.** Effects exerted by the estrogen derivative against left ventricular pressure (LVP) *via* β_1-adrenergic receptors. The scheme shows that both noradrenaline and the estrogen derivative increased LVP in a dose-dependent manner. Nevertheless, the biological activity of the steroid derivative was inhibited (p = 0.05) by propanolol and metoprolol. Each bar represents the mean ± SE of 9 experiments.
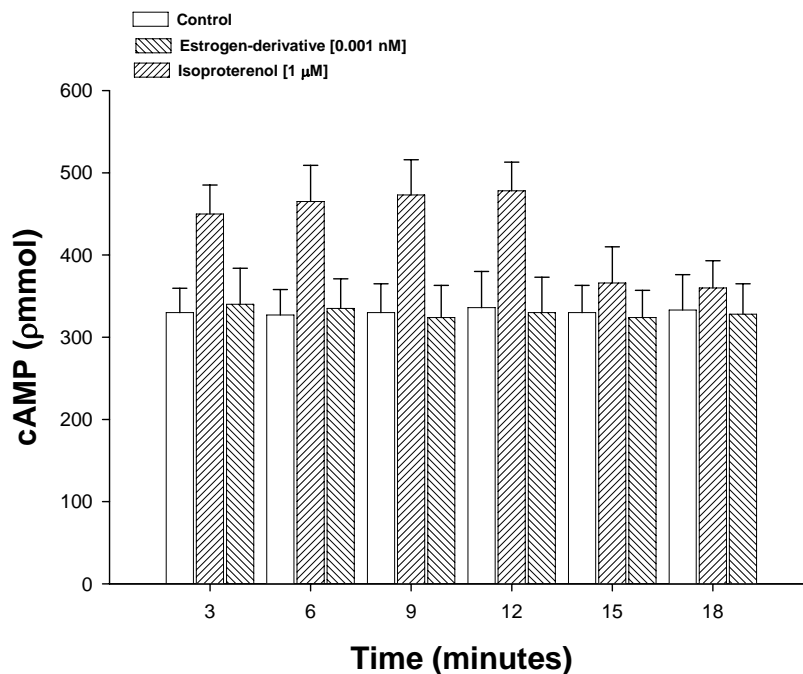

Figure 5. Effect exerted by the estrogen derivative and isoproterenol on cAMP levels over time. The results show that cAMP levels were higher (p = 0.05) in the presence of isoproterenol (3-12 min) in comparison with the estrogen derivative and the control (group without treatment). Each bar represents the mean ± S.E. of 6 experiments.

could be due to: i) the different protocols or biological models used and ii) the differences in the chemical structure of each drug. In this study, the biological activity of an estrogen derivative against ischemia/reperfusion injury was evaluated in an isolated rat heart. The results indicate that the estrogen derivative decreased the myocardial injury (translated as infarct area) in a dose-dependent manner compared with the control (without treatment). For characterizing the molecular mechanism involved in the biological activity exerted by the estrogen derivative on ischemia/reperfusion injury some drugs such as noradrenaline, digoxin, dobutamine, milrinone, and levosimedan were used as pharmacological tools. The results showed that both noradrenaline and dobutamine drugs significantly decrease the infarct area in a manner similar to the steroid derivative; however, this effect was different from the biological activity exerted by levosimedan, and milrinone against ischemia/reperfusion injury. These results suggest that the estrogen derivatives could exert cardioprotective effects through changes in left ventricular pressure, as occur with other types of drugs [19]. To evaluate this hypothesis, the biological activity of both noradrenaline and the estrogen derivative against left ventricular pressure was evaluated; the results showed that both noradrenaline and the estrogen derivative increased the left ventricular pressure in a dose-dependent manner. The data from the current study and other reports [25] which suggest that noradrenaline can exert its action *via* β_1-adrenergic receptor activation, opened new approaches to carry out alternative experiments to evaluate the biological activity exerted by the estrogen derivative on left ventricular pressure in the absence or presence of metoprolol (selective β_1-adrenergic receptor) [26] or propanolol (non-selective β_1-adrenergic receptor) [27]. The results showed that the effect exerted by the estrogen derivative against left ventricular pressure was inhibited by both propranolol and metoprolol drugs; these data suggest that the molecular mechanism involved in the biological activity of the estrogen derivative was *via* β_1-adrenergic receptor activation.

On the other hand, to evaluate the possibility that the estrogen derivative could exert changes in the levels of cAMP as do other drugs in some biological systems [23], in this study, alternative experiments were carried out using isoproterenol as a pharmacological tool. The results showed that isoproterenol increased cAMP levels (3-12 min) compared to the estrogen derivative and the control (without treatment) however, it is noteworthy that after 15-18 min the effect of isoproterenol decreased significantly ($p = 0.05$); this phenomenon is similar to that seen in other studies previously reported for isoproterenol [23]. All these results suggest that the biological activity exerted by the estrogen derivative is independent of cAMP levels.

5. CONCLUSIONS

The biological activity of the estrogen derivative is particularly of interest because of the cardioprotective effect it exerts against the ischemia/reperfusion injury. The compound decreases the area of infarction and increases the pressure of the left ventricle *via* β_1-adrenergic receptors. Therefore, this compound could constitute a new therapy for ischemia/reperfusion injury.

CONFLICT OF INTEREST STATEMENT

On behalf of all authors, the corresponding author states that there is no conflict of interest.

REFERENCES

1. Thygesen, D., Alpert, S. and White, D. 2007, J. Am. Coll. Cardiol., 50, 2173.
2. Lim, G. 2012, Nature Rev. Cardiol., 9, 186.
3. Pfeffer, M. 1995, Ann. Rev. Med., 46, 455.
4. Klone, R., Przyklener, K. and Whittaker, P. 1989, Circulation, 80, 1115.
5. Meng, H. and Pierce, G. 1990, Am J. Physiol., 258, 1615.

6. Halestrap, A., Clarke, S. and Javadov, S. 2004, Cardiovasc. Res., 61, 372.

7. McPherson, B. and Yao, Z. 2001, Anesthesiology, 94, 1082.

8. Pompermayer, K., Souza, D., Lara, G., Silveira, K., Cassali, G., Andrade, A. and Vieira, M. 2005, Kidney Int., 67, 1785.

9. Bofetiado, D., Mayfield, K. and D'Alecy, L. 1996, Anestes. Analg., 82, 1237.

10. Wongpaitoon, V., Mills, P., Russell, R. and Patrick, R. 1981, Postgrad. Med. J., 57, 244.

11. Hale, S., Birnbaum, Y. and Kloner, R. 1996, Am. Heart J., 132, 258.

12. Das, B. and Sarkar, C. 2006, J. Cardiovasc. Pharmacol., 47, 277.

13. Favre, J., Gao, J., Henry, J., Remy-Jouet, I., Fourquaux, I., Billon-Gales, A. and Richard, V. 2010, Arterioscler. Thromb. Vasc. Biol., 30, 2562.

14. Pongkan, W., Chattipakorn, S. and Chattipakorn, N. 2015, PLoS One, 10, e0122503.

15. Figueroa, L., Diaz, F., Rosas, M., Pool, E., Lopez, M., Sarabia, B. and Damian, I. 2014, Afr. J. Pharm. Pharmacol., 8, 157.

16. Jeanes, H., Wanikiat, P., Sharif, I. and Gray, G. 2006, Menopause, 13, 80.

17. Sarabia, B., Figueroa, L., Díaz, F., Hau, L., Rosas, M., García, E. and Zepeda, B. 2014, Pharmacol. Pharm., 5, 1130.

18. Bayne, K. 1996, Physiologist, 9, 208.

19. Figueroa, L., Diaz, F., Rosas, M., Mateu, V., Hernandez, P., Benitez, L., Pool, E., Lopez, M., Hau, L., López, T., Cauich, R. and Borges, Y. 2019, Biointerface Res. Appl. Chem., 9, 3860.

20. Figueroa, L., Diaz, F., Cervera, E., Gómez, P., Nexticapa, M. and Ramos, M. 2014, Acta Bioquímica Clínica Latinoamericana, 48, 203-211.

21. Figueroa, L., Díaz, F., López, M., García, E. and Quijano, K. 2011, Biomedica, 31, 232.

22. Figueroa, L., Diaz, F., Lopez, M., Garcia, E., Quijano, K. and Cordoba, J. 2011, Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech. Repub., 155, 27.

23. Figueroa, L., Díaz, F., García, E., Pool, E., Rosas, M., Hau, L. and Sarabia, B. 2015, Steroids, 95, 39.

24. Hocht, C., Opezzo, J., Gorzalczany, S., Bramuglia, G. and Tiara, C. 1999, Rev. Arg. Cardiol., 67, 769.

25. Silva, A. and Zanesco, A. 2010, J. Vasc. Br., 9, 47.

26. Johnson, J., Zineh, I., Puckett, B., McGorray, S., Yarandi, H. and Pauly, D. 2003, Clin. Pharmacol. Ther., 74, 44.

27. Atlas, D., Steer, M. and Levitzki, A. 1994, Proc. Natl. Acad. Sci., 7, 4246.