Role of Sex Hormones on Myocardial Ischemia Reperfusion Injury-Induced Calcium Overload: Possible Mechanisms

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Abstract

Background/Aim: The influence of sex on the response of the myocardium to ischemia-reperfusion (I/R) is under investigated. Sex hormones and their effects on intracellular calcium loading might be involved in gender difference. The aim of the present work is to study the effect of sex hormones on different mechanisms involved in calcium loading during myocardial I/R injury.

Methods: Rats were equally divided into male (gps 1–6) and female (gps 7–12) groups as follows: Sham groups (gps 1&7); gonadectomized groups (gps 2&8), gonadectomized with either testosterone (gp 3) or estrogen substitution (gp 9). Isolated Langendorff perfused hearts from all these groups were subjected to 45min. ischemia followed by 60min. reperfusion. To test the effect of sex hormones on I/R-induced calcium loading, hearts from gonadectomized and hormone substituted rats were perfused 15 minutes prior to ischemia with Verapamil (gps 4&10); the L-type calcium channel blocker, Amiloride; the Na+/H+ exchanger inhibitor (gps 5&11) and Thapsigargin; the sarcoplasmic reticulum calcium ATPase pump inhibitor (gps 6&12). At 5 minutes of reperfusion, creatine Kinase activity was assessed. Then, left ventricular developed pressure, dp/dt, Fas Ligand, cytosolic calcium and infarct size were measured at the end of reperfusion period.

Results: The left ventricular functional parameters were significantly higher in female sham-operated rats and female ovariectomized rats with estrogen substitution than their corresponding values in male gps. Such improvement was associated with less myocardial damage as indicated by less CK release, smaller infarcts, decreased Fas ligand and less cytosolic calcium. We also noticed that gonadectomy was detrimental to the female rats and beneficial to the male rats, supporting the important beneficial role for endogenous estrogen to resist I/R injury. The addition of either Amiloride or Thapsigargin to hearts of male gps 5&6 resulted in significantly greater left ventricular functional parameters and significantly lesser cytosolic calcium as compared to gp 3. Meanwhile, addition of Verapamil to the hearts of gp 4 caused insignificant change in any of the parameters measured. Concerning the female gender, Verapamil and Thapsigargin resulted in a more additional cardio-protective effects against I/R injury, while, Amiloride failed to cause any significant change as compared to gp 9.

Conclusions: Hearts of male rats have a higher vulnerability to I/R than hearts of female rats. The protective effect of estrogen is partly mediated via decreasing intracellular calcium accumulation through inhibition of Na+/H+ exchange mechanism. A supraphysiological dose of testosterone could afford a cardioprotective effect by inhibiting I/R-induced calcium load through L-type calcium channel blockade.

Key Words: Ischemia Reperfusion Injury – Sex difference – Calcium load – Na+/H+ exchanger – Sarcoplasmic reticulum calcium ATPase – L-type calcium channel.

Introduction

SEX hormones play an important role in ischemic heart disease [1]. Clinical findings indicate that females are protected from cardiovascular injury and that this protection is mediated via estrogen [2]. Estrogen replacement therapy has been reported to decrease the occurrence and mortality of myocardial infarction and increase survival in patients following coronary artery bypass surgery [3].

Anabolic androgenic steroids have been associated with myocardial infarction, ischemia, sudden cardiac death and hypertension in athletes [4]. In contrast, short term intracoronary administration of testosterone induces coronary artery dilatation and increases coronary blood flow in man with established coronary artery disease [5].

Few experimental studies have demonstrated male to female differences in the susceptibility to myocardial ischemia reperfusion (I/R) injury [6]. Irreversible damage inflicted on the heart by a prolonged ischemic period is effectuated by loss of cellular integrity [7], influx of calcium ions [8], oxygen-derived free radicals and their metabolites [9], loss of intracellular proteins and disruption of cell membranes & apoptosis [10]. This multifactor process, either elicited by ischemia alone or in combination with reperfusion, calcium overloading
Role of Sex Hormones on Myocardial Ischemia

plays a key role and therefore is an important target when measures have to be taken to decrease the vulnerability to I/R damage [8].

Intracellular calcium loading depends on both calcium influx from the extracellular space and calcium release from the sarcoplasmic reticulum stores. Altered Na+/Ca++ exchangers are also important factors because cytosolic calcium loading was temporarily associated with Na⁺ loading during early reperfusion [11].

High cytosolic calcium increased myocardial I/R injury in males more than females via a NO synthase-mediated mechanism [3]. In mice with cardiac overexpression of the Na⁺/Ca++ exchanger, myocardial I/R injury was exacerbated in males but not in females [12]. However, the effect of sex hormones on myocardial I/R injury and the corresponding intracellular calcium homeostasis is not yet understood.

The aim of the present study is to investigate the influence of sex hormones (Estrogen and Testosterone) on myocardial I/R injury and the associated intracellular calcium changes. The impact of sex hormones on different mechanisms involved in calcium loading during I/R injury was probed through investigating the effect of L-type calcium channels blocking, sarcoplasmic reticulum calcium ATPase pump inhibition and Na⁺/H⁺ exchange inhibition.

Material and Methods

Drugs and chemicals:
Estrogen, Testosterone, Verapamil hydrochloride, Amiloride hydrochloride hydrate and Thapsigargin were purchased from Sigma-Aldrich, Inc. St.Louis, USA. Creatine Kinase activity assay kit was supplied by Stanbio laboratory, Inc. (USA). Fas Ligand ELISA kit was supplied by Ray Biotech, Inc (USA). Cytosolic calcium was detected by using QuantiChromTM Calcium Assay Kit (DICA-01K). All other chemicals used in this study were of analytical purity and commercially available.

Animals:
One hundred and twenty adult albino rats (60 females and 60 males) weighing from 180-230gm were used in this study. Rats were housed in wire mesh cages at room temperature. All animals were kept under same environmental conditions and had free access to water and standard rodent chow. All experimental procedures were carried out in accordance with the guide for the care and use of laboratory animals published by the US National insti-
recovery period after gonadectomy; rats for estrogen substitution were treated with 17 beta-Estradiol (70 microg/kg/day) subcutaneous injection for 4 weeks [13]. This estradiol substitution in a physiological concentration for 4 weeks would be sufficiently long to ensure an influence of the hormone. Rats for testosterone substitution were treated with Dihydrotestosterone (DHT), a nonaromatizable androgen in a supraphysiological dose of 80 mg/kg/day. The hormone was given subcutaneously for 3 weeks [14].

Verapamil (1 µmol/Liter) [15], Amiloride (100 µmol/Liter) [16] and Thapsigargin (2.5x10^-8 M/Liter) [17] were added to the perfused hearts of different groups of rats according to the experimental protocol, 15 minutes prior to ischemia and at a flow rate of 7 ml/minute.

**Langendorff perfusion:**

At the end of each experimental protocol, rats of each group were anaesthetized with Ketamine hydrochloride 150 mg/kg and heparinized with 1000U/kg intraperitoneally, then, killed. Hearts were isolated, perfused with Krebs-Henseleit buffer solution and subjected to I/R protocol as follows:

A mid thoracotomy was performed to expose the hearts which were then immediately excised through a pericardial incision at their bases. Hearts were then immediately placed in sterile ice cold modified Krebs-Henseleit solution, transferred and attached to modified Langendorff apparatus by the aortic root through the perfusion cannula. The time between extraction of the hearts and their attachment to the Langendorff apparatus did not exceed one minute. Hearts were then perfused retrogradelly with non recirculating sterile modified oxygenated Krebs-Henseleit solution of the following concentrations (in mM): 118 NaCl, 25 NaHCO₃, 1.2 KH₂PO₄, 4.7 KCl, 1.2 MgSO₄, 2 CaCl₂ and 10 dextrose in 1000 ml distilled water. The perfusate was maintained at 38 °C and the hearts were also maintained at 38 °C using a water reservoir surrounding the hearts in which the open end was covered to maintain temperature and humidity.

**Recording of intraventricular pressures:**

To record intraventricular pressures, a saline filled latex balloon connected to a catheter was inserted into the left ventricle. The catheter was then connected to a pressure transducer (gold statum). Pressure changes were then analyzed and displayed on an electronic polygraph (NEC-Sanei instruments Ltd, 2238). The balloon was gradually inflated to attain end diastolic pressure (EDP) of (2-5 mmHg). Hearts were then left to stabilize for 30 minutes.

After stabilization, hearts were subjected to 45 minutes of no flow global ischemia by clamping the perfusion line. Then, the clamp was released and the hearts were reperfused for 60 minutes.

**Determination of left ventricular functional parameters:**

I- **Left ventricular developed pressure (LVDP):**

Left ventricular systolic pressure (LVSP) and end diastolic (LVEDP) pressures were measured at the end of reperfusion period. Left ventricular developed pressure (LVDP) was calculated as follows:

\[
LVDP = (LVSP - LVEDP) \quad [18].
\]

II- **Peak rate of maximum left ventricular pressure (dp/dt):**

The peak rate of maximum left ventricular pressure (dp/dt) which is considered as an index of contractility was measured at the end of reperfusion period.

**Following I/R protocol, these measures were taken:**

1- One ml of the coronary effluent was collected immediately at the beginning of reperfusion period (after 5 minutes) and frozen at −80°C for further analysis of Creatine kinase (CK) activity.

2- At the end of reperfusion, hearts were then immediately removed and frozen at 80°C for subsequent assessment of cytosolic calcium, Fas Ligand and area % of myocardial infarction in the cardiac tissue.

**Biochemical assays:**

I- **Determination of creatine kinase activity:**

Creatine kinase (CK) activity as an index of myocardial cell damage was determined in the coronary effluent using the assay kit.

II- **Detection of Cytosolic calcium level:**

Cytosolic calcium was assessed in the myocardial cytosolic fraction after being separated by the following procedure:

In the cold room, the heart was rinsed in PBS, and then rinsed in freshly prepared Homogenization buffer. The heart was weighed and the tissue was scissor minced and then transferred to a glass homogenizer. 1 volume of homogenization buffer containing 0.5 mM MgGTP was added. The homogenizer was immersed in slushy ice and homog-
Measurement of infarct size:

Parameters. In each chosen field the cardiac muscle was masked by a blue binary color to be measured. Using the measuring field menu, the area, area % and standard measuring frame of a standard analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. Controlled by Leica Qwin 500 software. The image analyzer computer system (England) and histopathological study:

Histopathological study:

Measurement of infarct size:

Hearts specimens of sham-operated and experimental groups were placed in 10% formol saline and allowed for paraffin block preparation. Serial sections were cut and stained with haematoxylin and eosin. Then, the area % of myocardial infarction was measured using Leica Qwin 500 image analyzer computer system (England) and controlled by Leica Qwin 500 software. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. Using the measuring field menu, the area, area % and standard measuring frame of a standard area equal to 118476.6 µm² were chosen from the parameters. In each chosen field the cardiac muscle tissue was enclosed inside the standard measuring frame & then the area of infarcted muscle fibers was masked by a blue binary color to be measured. These measurements were done using an objective lens of magnification 10, i.e. of total magnification 100. Ten readings were obtained in each specimen and their mean values were taken.

Results

1- Results of male groups at the end of reperfusion:

A- Left ventricular functional parameters:

1- Left ventricle dp/dt (Left ventricular contractility index):

As shown in Table (1), Fig. (1), the mean values of left ventricular dp/dt at the end of reperfusion were increased significantly from 41.7 ± 1.42 in male sham-operated rats (gp1) to reach 46.3 ± 2.79 and 50.9 ± 5.72 in male orchiectomized rats (gp2) and male orchiectomized rats with testosterone substitution (gp3), respectively (p < 0.05). Group 3 showed significantly increased left ventricular dp/dt as compared to group 2 (p < 0.05). The mean values of left ventricular dp/dt were 51.2 ± 4.51, 63.8 ± 2.57 and 54.9 ± 4.18 in male orchiectomized rats with testosterone substitution and addition of Verapamil (gp4), Amiloride (gp5) and Thapsigargin (gp6), respectively to the perfused hearts. Left ventricular dp/dt values in groups 5 and 6 were significantly higher than those in groups 1, 2 and 3 (p < 0.05), while dp/dt value in group 4 was significantly higher than those in groups 1, 2 (p < 0.05) and insignificant when compared to group 3 (p > 0.05).

2- LVDP = (LVSD-LVEDP) mmHg:

The mean values of LVDP, following reperfusion, were shown in Table (1) and Fig. (2). LVDP was increased significantly in male orchiectomized rats (gp2) and orchiectomized rats with supraphysiological testosterone substitution (gp3) as compared to male sham-operated rats (gp 1) (LVDP: 57.9 ± 3.03, 64.1 ± 3.11 versus 51.6 ± 2.59 mmHg respectively). The increase in LVDP in group 3 was significantly higher than its corresponding value in group 2. Verapamil, added to the perfused hearts of orchiectomized rats with testosterone substitution (gp 4), caused a rise in LVDP which was significantly higher than those in groups 1 and 2 but no significant change could be detected as compared to group 3. Indeed, addition of Amiloride (group 5) and Thapsigargin (group 6), to the perfused hearts of orchiectomized rats with testosterone substitution caused a significant rise in LVDP as compared to their values in group 1, 2 and 3.

B- Biochemical parameters:

1- Creatine kinase enzyme (CK) activity (u/ml) as index of myocardial ischemic injury:

To ascertain the effects of male sex hormone on the degree of I/R induced myocardial damage, we assessed the CK release in the coronary effluent of isolated hearts from orchiectomized rats (gp2)
and orchiectomized rats substituted with testosterone (gp3). Testosterone depletion in group 2 significantly attenuated I/R induced CK leakage as compared to sham-operated one (gp1) (p<0.05). Meanwhile, Testosterone substitution (gp3) resulted in more significant decrease in CK activity as compared to that in group 2 indicating that the severity of the ischemic insult was significantly reduced under the effect of high dose of Testosterone. When isolated hearts of orchiectomized rats with testosterone substitution were perfused with Verapamil (gp4), Amiloride (gp5) and Thapsigargin (gp6), the mean values of CK enzyme activity in group 5 and group 6 were significantly lower than those in groups 1, 2 and 3 (p<0.05), while group 4 receiving the L-type calcium channel blocker "Verapamil" showed significantly lower values than those of groups 1&2 (p<0.05) but did not show any significant change as compared to group 3 (p>0.05) (Table 1, Fig. 3).

2- Fas ligand (ng/mg tissue):

Table (1), Fig. (4), shows the mean values of Fas ligand- related apoptosis in the male studied groups. In male rat hearts, the Fas ligand level was significantly influenced by orchietomy (gp2) and orchietomy with testosterone substitution (gp3). Fas ligand level was decreased significantly in group 2 as compared to sham-operated rats (gp1) (p<0.05). Furthermore, its value in group 3 was significantly lower than that in groups 1 and 2 (p<0.05) suggesting that the supraphysiological dose of testosterone targets the death ligand, that initiate the extrinsic apoptotic pathway, as part of its potential cardioprotective effect. Perfusion of isolated hearts from orchiectomized rats with testosterone substitution with either Amiloride (group 5) or Thapsigargin (group 6) resulted in significantly lower Fas ligand value than that in groups 1, 2 and 3 (p<0.05), while perfusion with Verapamil in group 4 caused insignificant change as compared to group 3 (p>0.05) and significant decrease as compared to groups 1&2 (p<0.05).

3- Cytosolic calcium level (nMol):

The effect of Testosterone on cytosolic calcium level was shown in Table (1), Fig. (5). Following reperfusion, orchietomy of rats significantly attenuated the I/R-induced calcium overload from 59.6±2.50 in group1 to reach 52.4±2.63nMol in group 2, p<0.05. Further decrease in the cytosolic calcium level was observed in orchietomized rats substituted with testosterone (gp3) (p<0.05 Vs. gp2). The mechanism involved in the calcium-lowering effect induced by supraphysiological testosterone, was assessed by perfusing the hearts of orchietomized rats with testosterone substitution with the L-type calcium channel blocker; Verapamil (gp4), the Na-H exchange inhibitor; Amiloride (gp5) and the sarcoplasmic reticulum calcium ATPase pump inhibitor; Thapsigargin (gp6). The mean values of cytosolic calcium in groups 5 and 6 were significantly lower than the corresponding values in groups 1, 2 and 3 (p<0.05) suggesting that both Amiloride and Thapsigargin enhance the attenuating effect of testosterone on cytosolic calcium. While in group 4, a significant decrease in calcium level than that in groups 1 and 2 (p<0.05) has been demonstrated but no significant change could be detected as compared to group 3 (p>0.05). These results indicate that testosterone might inhibit the I/R-induced calcium overload via blocking the entry of extracellular calcium through L-type calcium channels.

C- Determination of infarct size:

The extent of myocardial necrosis was assessed by measuring the area % of infarction which decreased significantly in male orchiectomized rats (gp2) as compared to sham-operated one (gp1) (p<0.05). Hearts of orchiectomized rats substituted with testosterone developed smaller infarcts in group 3 as compared to groups 1 and 2 (p<0.05). Less infarcts were seen in hearts of groups 5&6 perfused with Amiloride and Thapsigargin respectively in comparison to groups 1, 2 & 3 (p<0.05). On the other hand, hearts of group 4 receiving Verapamil, showed a significant decrease in the area % of infarction versus those of groups 1&2, while no significant change in the area % of infarction compared to group 3 could be observed (Table 1, Fig. 6).

2- Results of female groups at the end of reperfusion:

B- Left ventricular functional parameters:

1- Left ventricle dp/dt (Left ventricular contractility index):

Following reperfusion, the postischemic contractile function detected by dp/dt was significantly deteriorated in the ovariecetomized females (gp8) as compared to sham-operated females (gp7). Substitution of the ovariecetomized rats with estrogen (gp9) significantly improve the contractile dysfunciton observed after ovariecetomy as indicated by significant increase in dp/dt in group 9 versus group 8 (p<0.05). Perfusion of isolated hearts of ovariecetomized and estrogen substituted female rats with either Verapamil (gp10) or Thapsigargin (gp12), augments the beneficial effect of estrogen substitution on cardiac contractility where dp/dt
values were significantly higher in these groups than those in groups 7, 8 & 9 (p<0.05). However, perfusion of hearts of group 11 with Amiloride did not evoke any significant change in dp/dt versus group 9 (p>0.05). All data obtained were shown in Table (2), Fig. (7).

2- LVDP = (LVSD-LVEDP) mmHg:

Table (2), Fig. (8) show that the mean values of LVDP were decreased significantly in female ovariectomized rats (gp8) as compared to female sham-operated rats (gp7). Estrogen substitution after ovariectomy (gp9) resulted in a significant increase in LVDP as compared to sham-operated and ovariectomized groups supporting the beneficial effect of estrogen on I/R induced cardiac dysfunction. The effect of addition of Verapamil (gp 10) and Thapsigargin (gp 12), to the perfused hearts of female ovariectomized rats with estrogen substitution caused a significant rise in LVDP as compared to the corresponding values in groups 7, 8 and 9 (p<0.05). Whereas, addition of Amiloride to the perfused hearts of group 11 caused significant increase in LVDP as compared to groups 7&8 but did not cause any significant change in LVDP versus that in group 9 (p>0.05).

II- Biochemical parameters:

1- Creatine kinase enzyme (CK) activity (u/ml):

After 5 minutes of reperfusion, CK leakage was significantly higher in ovariectomized rats versus sham-operated rats (p<0.05). Estrogen substitution in ovariectomized rats resulted in a significant decrease in CK enzyme level as compared to sham-operated (gp7) and ovariectomized rats (gp8) (p<0.05). Further significant decrease in CK release has been observed in the groups of isolated hearts perfused with either Verapamil or Thapsigargin (gps 10&12) when compared to group 9. Meanwhile, no significant change could be detected between group 11, which was perfused with Amiloride and group 9, the estrogen substituted ovariectomized group (P>0.05) (Table 2, Fig. 9).

2- Fas ligand (ng/mg tissue):

The changes in Fas Ligand level were shown in Table (2), Fig. (10). The mean values of Fas Ligand-related apoptosis increased significantly in hearts of ovariectomized rats (gp8), following reperfusion, as compared to the corresponding values in hearts of sham-operated rats (p<0.05). This increase was significantly attenuated when ovariectomized rats were substituted with estrogen in group 9 (p<0.05 Vs. gp8). Such decrease in Fas Ligand level was exacerbated in hearts of group 10 perfused with Verapamil & that in group 12 perfused with Thapsigargin. However, perfusion of hearts with Amiloride in group 11, did not affect significantly the Fas Ligand level versus that of group 9 (p>0.05) but it is still significantly lower than that in groups 7&8.

3- Cytosolic calcium level (nMol):

As shown in Table (2), Fig. (11), the I/R-induced calcium loading was significantly augmented in ovariectomized rats (gp8) as compared to sham-operated rats (gp7). The increase in cytosolic calcium was significantly decreased when ovariectomized rats received estrogen as substitution (p<0.05 Vs. gp8). In order to identify the mechanism by which estrogen inhibits the I/R-induced calcium loading, hearts of ovariectomized rats substituted with estrogen were perfused with Verapamil, Amiloride & Thapsigargin (gps 10,11,12). Our results with Verapamil, the L type calcium channel blocker and Thapsigargin, the sarcoplasmic reticulum calcium ATPase pump inhibitor, showed a further significant decrease in cytosolic calcium level than that in groups 7,8 &9 (p<0.05). While blockade of Na⁺/H⁺ exchanger by Amiloride in group 10 did not change significantly the cytosolic calcium level in comparison to group 9 indicating that inhibiting the Na⁺/H⁺ exchanger may be the mechanism of action by which estrogen decreases I/R-induced calcium loading.

III- Determination of infarct size:

The mean values of area % of myocardial infarction in the female studied groups were illustrated in Table (2), Fig. (12). There was significant increase in infarct size in ovariectomized rats (gp8) as compared to sham-operated rats (gp7) (p<0.05). Indeed, estrogen substitution after ovariectomy resulted in significant decrease in the area % of myocardial infarction in group 9 as compared to that in group 7 and group 8. Moreover, further reduction in the infarct size has been observed in groups 10 and 12 perfused with Verapamil and Thapsigargin respectively as compared to groups 7, 8 and 9. However, addition of Amiloride to perfused hearts of group 11 resulted in an insignificant decrease in the area % of myocardial infarction than that in group 9 (p>0.05).

Role of gender and sex hormones in I/R injury (Table 3, Figs. 13-18):

Following reperfusion, gender differences were observed in all the parameters studied. LVDP and left ventricular dp/dt values were significantly higher, while mean values of CK enzyme activity, Fas ligand, cytosolic calcium level and area % of
myocardial infarction were significantly lower in female sham-operated rats (gp7) than those in male sham-operated rats (gp1) \((p<0.05)\).  

However, the left ventricular functional parameters were significantly decreased in parallel with the significant increase in all myocardial injury parameters including: CK leakage, Fas ligand-related apoptosis, cytosolic calcium level and infarct size; in female ovariectomized rats (gp8) as compared to male orchiectomized rats (gp2) \((p<0.05)\).

Moreover, the results of the present study show that estrogen substitution to ovariectomized female rats (gp9) results in significantly higher left ventricular functional parameters and lower myocardial injury parameters, than those observed in male orchiectomized rats with supraphysiological testosterone substitution (gp3) \((p<0.05)\).

### Table (1): Comparison of all measured data at the end of reperfusion between the different studied male groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>LVdp/dt (mmHg)</th>
<th>LVDP (mmHg)</th>
<th>CK (u/ml)</th>
<th>Fas ligand (ng/mg tissue)</th>
<th>Cytosolic calcium (nmol/l)</th>
<th>Area % of infarction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sham-operated (group 1)</td>
<td>41.7 ± 1.42</td>
<td>51.6 ± 2.59</td>
<td>50.6 ± 1.84</td>
<td>0.29 ± 0.015</td>
<td>59.6 ± 2.50</td>
<td>22.65 ± 2.30</td>
</tr>
<tr>
<td>Male orchiectomy (group 2)</td>
<td>46.3# ± 2.79</td>
<td>57.9# ± 3.03</td>
<td>44.5# ± 1.63</td>
<td>0.25# ± 0.018</td>
<td>52.4# ± 2.63</td>
<td>19.47# ± 2.25</td>
</tr>
<tr>
<td>Male orchiectomy + Testosterone (group 3)</td>
<td>50.9#* ± 2.88</td>
<td>64.1#* ± 3.11</td>
<td>39.1#* ± 0.82</td>
<td>0.22#* ± 0.016</td>
<td>46.1#* ± 2.92</td>
<td>16.04#* ± 1.80</td>
</tr>
<tr>
<td>Male orchiectomy + Testosterone + Verapamil (group 4)</td>
<td>51.2#* ± 4.51</td>
<td>65.1#* ± 3.03</td>
<td>40.32#* ± 4.07</td>
<td>0.22#* ± 0.025</td>
<td>46.7#* ± 5.79</td>
<td>17.17#* ± 2.38</td>
</tr>
<tr>
<td>Male orchiectomy + Testosterone + Amiloride (group 5)</td>
<td>63.8#*#$ ± 2.57</td>
<td>79.9#*#$ ± 3.67</td>
<td>28.9#*#$ ± 2.48</td>
<td>0.16#*#$ ± 0.018</td>
<td>32.1#*#$ ± 2.13</td>
<td>11.18#*#$ ± 1.46</td>
</tr>
<tr>
<td>Male orchiectomy + Testosterone + Thapsigargin (group 6)</td>
<td>54.9#*#$ ± 4.18</td>
<td>68.1#*#$ ± 2.85</td>
<td>33.8#*#$ ± 3.36</td>
<td>0.19#*#$ ± 0.021</td>
<td>37.2#*#$ ± 2.48</td>
<td>14.06#*#$ ± 1.99</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD.
# Significant as compared to male sham-operated rats (group 1).
* Significant as compared to male orchiectomized rats (group 2).
$ Significant as compared to male orchiectomized rats with testosterone substitution (group 3).

Fig. (1): Mean values ± SD of LV dp/dt at the end of reperfusion for the different studied male groups.

Fig. (2): Mean values ± SD of LVDP mmHg at the end of reperfusion for the different studied male groups.
# Significant as compared to male sham-operated rats (group 1).
* Significant as compared to male orchiectomized rats (group 2).
$ Significant as compared to male orchiectomized rats with testosterone substitution (group 3).

Fig. (3): Mean values ± SD of Creatine kinase enzyme (u/ml) at 5min. of reperfusion for the different studied male groups.

# Significant as compared to male sham-operated rats (group 1).
* Significant as compared to male orchiectomized rats (group 2).
$ Significant as compared to male orchiectomized rats with testosterone substitution (group 3).

Fig. (4): Mean values ± SD of Cytosolic calcium level (nMol/L) at the end of reperfusion for the different studied male groups.

# Significant as compared to male sham-operated rats (group 1).
* Significant as compared to male orchiectomized rats (group 2).
$ Significant as compared to male orchiectomized rats with testosterone substitution (group 3).

Fig. (5): Mean values ± SD of Cardiac Fas Ligand (ng/mg tissue) at the end of reperfusion for the different studied male groups.

# Significant as compared to male sham-operated rats (group 1).
* Significant as compared to male orchiectomized rats (group 2).
$ Significant as compared to male orchiectomized rats with testosterone substitution (group 3).

Fig. (6): Mean values ± SD of Area % of myocardial infarction at the end of reperfusion for the different studied male groups.

Table (2): Comparison of all measured data at the end of reperfusion between the different studied female groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>LVdp/dt (mmHg)</th>
<th>LVDP (mmHg)</th>
<th>CK (u/ml)</th>
<th>Fas ligand (ng/mg tissue)</th>
<th>Cytosolic calcium (nmol/l)</th>
<th>Area % of infarction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female sham-operated (group 7)</td>
<td>47.1 ±3.18</td>
<td>58.9 ±3.21</td>
<td>41.3 ±1.76</td>
<td>0.24 ±0.017</td>
<td>50.3 ±2.75</td>
<td>16.81 ±2.53</td>
</tr>
<tr>
<td>Female gonadectomy (group 8)</td>
<td>40.2# ±3.46</td>
<td>50.2# ±2.53</td>
<td>48.1# ±2.61</td>
<td>0.28# ±0.019</td>
<td>60.1# ±2.60</td>
<td>23.84# ±2.94</td>
</tr>
<tr>
<td>Female gonadectomy + Estrogen (group 9)</td>
<td>56.8#* ±3.08</td>
<td>71.2#* ±2.12</td>
<td>35.3#* ±3.36</td>
<td>0.20#* ±0.022</td>
<td>43.3#* ±2.83</td>
<td>15.01#* ±1.61</td>
</tr>
<tr>
<td>Female gonadectomy + Estrogen + Verapamil (group 10)</td>
<td>64.1#*$ ±2.92</td>
<td>80.6#*$ ±2.63</td>
<td>30.2#*$ ±1.87</td>
<td>0.16#* $ ±0.016</td>
<td>37.8#*$ ±2.78</td>
<td>12.46#*$ ±1.09</td>
</tr>
<tr>
<td>Female gonadectomy + Estrogen + Amiloride (group 11)</td>
<td>58.2#* ±2.93</td>
<td>73.1#* ±2.60</td>
<td>33.5#* ±2.98</td>
<td>0.19#* ±0.021</td>
<td>41.1#* ±2.64</td>
<td>14.02#* ±1.85</td>
</tr>
<tr>
<td>Female gonadectomy + Estrogen + Thapsigargin (group 12)</td>
<td>60.9#*$ ±3.03</td>
<td>76.3#*$ ±2.54</td>
<td>32.6#*$ ±2.10</td>
<td>0.17#* $ ±0.029</td>
<td>41.1#*$ ±1.37</td>
<td>13.08#*$ ±1.54</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD.
$ Significant as compared to female ovariectomized rats with estrogen substitution (group 9).
# Significant as compared to female sham-operated rats (group 7).
* Significant as compared to female ovariectomized rats (group 8).
**Fig. (7):** Mean values ± SD of dp/dt at the end of reperfusion for the different studied female groups.

**Fig. (8):** Mean values ± SD of LVDP mmHg at the end of reperfusion for the different studied female groups.

**Fig. (9):** Mean values ± SD of Creatine kinase enzyme (u/ml) at 5 min. of reperfusion for the different studied female groups.

**Fig. (10):** Mean values ± SD of Cardiac Fas Ligand (ng/mg tissue) at the end of reperfusion for the different studied female groups.

**Fig. (11):** Mean values ± SD of Cytosolic calcium level (nMol/L) at the end of reperfusion for the different studied female groups.

**Fig. (12):** Mean values ± SD of Area % of myocardial infarction at the end of reperfusion for the different studied female groups.
Table (3): Comparison of all measured data at the end of reperfusion between the male studied groups (1, 2&3) and the female studied groups (7, 8&9).

<table>
<thead>
<tr>
<th>Groups</th>
<th>LVdp/dt (mmHg)</th>
<th>LVDP (mmHg)</th>
<th>CK (u/ml)</th>
<th>Fas ligand (ng/mg tissue)</th>
<th>Cytosolic calcium (nmol/l)</th>
<th>Area % of infarction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sham-operated (group 1)</td>
<td>41.7±1.42</td>
<td>51.6±2.59</td>
<td>50.6±1.84</td>
<td>0.29±0.015</td>
<td>59.6±2.50</td>
<td>22.65±2.3</td>
</tr>
<tr>
<td>Female sham-operated (group 7)</td>
<td>47.1±3.18</td>
<td>58.9±3.21</td>
<td>41.3±1.76</td>
<td>0.24±0.017</td>
<td>50.3±2.75</td>
<td>16.81±2.53</td>
</tr>
<tr>
<td>Male orchiectomy (group 2)</td>
<td>46.3±2.79</td>
<td>57.9±3.03</td>
<td>44.5±1.63</td>
<td>0.25±0.018</td>
<td>52.4±2.63</td>
<td>19.47±2.25</td>
</tr>
<tr>
<td>Female ovariectomy (group 8)</td>
<td>40.2±3.46</td>
<td>50.2±2.53</td>
<td>48.1±2.61</td>
<td>0.28±0.019</td>
<td>60.1±2.60</td>
<td>23.84±2.94</td>
</tr>
<tr>
<td>Male orchiectomy + Testosterone (group 3)</td>
<td>50.9±2.88</td>
<td>64.1±3.11</td>
<td>39.1±0.82</td>
<td>0.22±0.016</td>
<td>46.1±2.91</td>
<td>16.04±1.80</td>
</tr>
<tr>
<td>Female ovariectomy + Estrogen (group 9)</td>
<td>56.8±3.08</td>
<td>71.2±2.12</td>
<td>35.3±3.36</td>
<td>0.20±0.022</td>
<td>43.3±3.83</td>
<td>15.01±1.61</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD.
* Significant as compared to male orchiectomized rats with testosterone substitution (group 3).
# Significant as compared to male sham-operated rats (group 1).
@ Significant as compared to male orchiectomized rats (group 2).

Fig. (13): Mean values ± SD of LV dp/dt at the end of reperfusion in Male and Female groups.

Fig. (14): Mean values ± SD of LVDP at the end of reperfusion in Male and Female groups.

Fig. (15): Mean values ± SD of Creatine kinase (CK) (u/ml) at 5min. of reperfusion in Male and Female groups.

Fig. (16): Mean values ± SD of Fas Ligand (ng/mg tissue) at the end of reperfusion in Male and Female groups.
Discussion

Myocardial ischemia is the leading cause of death in both men and women. Although a great deal of data exists regarding the influence of sex on the development of coronary artery disease [19], very little information exists concerning the influence of sex on the response of the myocardium itself to an acute insult such as ischemia-reperfusion (I/R). Sex hormones and their effects on intracellular calcium loading may be involved in this gender difference.

First, concerning male sex tolerance to I/R injury, results of the present study demonstrated that left ventricular functional parameters including left ventricular dp/dt and LVDP values, following reperfusion, were significantly increased in male orchietomized rats (gp2) as compared to sham-operated rats (gp1). This has been associated with significant decrease in area % of myocardial infarction, Fas ligand level and the CK enzyme release. In analogy with findings in the present study, Remmers et al. [20] reported a protected cardiac performance in animals with testosterone depletion and suggested that endogenous testosterone may have a negative effect on the heart subjected to acute I/R. Similar improvement in ventricular performance in hearts of orchietomized male rats has been reported by Song et al. [13]. Furthermore, Wang et al. [21], found that post ischemic recovery of LVDP and dp/dt values were significantly higher in castrated males and males treated with testosterone receptor blockade than in normal rats. They also reported a decrease in TNF-alpha (TNFα), interleukin-1 beta (IL-1β) and IL-6 as well as a reduction in activated P38 MAPK, Caspases 1, 3, & 11 and increased Bcl-2 expression.

Of importance is our data indicating that, whereas depleting testosterone by orchietomy in group 2 improve postschismic contractile performance, the supraphysiological testosterone substitution in male orchietomized rats (gp3) significantly improved LVDP & left ventricular dp/dt and reduced the infarct size as end point to ischemic injury, when compared to group 2 and sham-operated rats (gp1). It also significantly inhibited the postschismic leakage of CK and the apoptosis-related Fas ligand. Callies et al. [14] and more recently Kuhar et al. [22] support our findings that testosterone in supraphysiological levels significantly decreased I/R injuries of isolated rat hearts. Other studies also indicate that testosterone administration is beneficial in alleviating myocardial ischemia in men with significant coronary artery disease (CAD), a condition which is associated with hypotestosteronemia, and that infusion of testosterone into coronary arteries at angiography results in rapid vasodilatation in patients with (CAD) [23]. Furthermore, men with CAD have lower levels of circulating testosterone compared with normal coronary arteries and it was demonstrated that one of four men of CAD are clinically hypogonadal [24].
Several mechanisms have been postulated to explain the cardio protective effect of high dose of testosterone: One postulated mechanism, suggested by Jones et al. [25] and by Li et al. [26], is based on its role in reducing serum levels of the pro-inflammatory cytokines TNF-α, IL-1β and increasing levels of the anti-inflammatory cytokine IL-10. In addition, supraphysiological testosterone has been reported to reduce vascular cell adhesion molecule (VCAM)-1 expression in aortic endothelial cells and promote vascular smooth muscle and endothelial cell proliferation. It also induces vasodilatation & improves vascular reactivity. It decreases serum levels of the pro-thrombotic factors plasminogen activator inhibitor & fibrinogen, reduces low density lipoprotein cholesterol, body mass index and visceral fat mass [27]. These actions of testosterone may confer cardiovascular benefit since testosterone therapy reduces atheroma formation in cholesterol-fed animal models and reduces myocardial ischemia in men with CHD [28].

As regards the female resistance to I/R injury, Zhai et al. [29], reported that ovariectomized rats had significantly lower coronary flow rate, left ventricular functional parameters, mitochondrial respiratory function and nitrite production as compared to sham-operated or estrogen treated ovariectomized rats. Additionally, they observed marked interstitial edema, contraction bands, fewer viable myocytes, more severely damaged mitochondria and ultrastructural damage to myocytes in hearts from the ovariectomized rats group but not in hearts from either of the other groups. Nikolic et al. [30], demonstrated that hearts from ovariectomized female mice had a significantly lower recovery of LVDP than the hearts from intact female mice and proved that treatment with estrogen receptor beta-selective agonist (2, 3-bis (4-hydroxyphenyl)-propionitrile, DPN) can provide cardioprotection in female mice lacking endogenous estrogen. In line with the findings of these authors, results of our study showed that left ventricular functional performance, at the end of reperfusion, were deteriorated as evidenced by significant decrease in left ventricular dp/dt and LVDP values in female ovariectomized rats (gp8) as compared to female sham-operated rats (gp7). A higher significant increase in CK leakage, Fas ligand and CK enzyme activity. Our results are in accordance to the findings of Patten et al. [31], who reported that hearts of estrogen treated rats are associated with less myocardial damage and cardiac dysfunction following I/R injury than hearts of ovariectomized rats. They stated that; 17 beta-estradiol reduces cardiomyocyte apoptosis and infarct size. Further studies by Anderson et al. [32] and Kuhar et al. [33] confirm the functional left ventricular recovery with decreased cardiac enzymes release after estrogen substitution. In cardiac myocytes, rapid actions of estrogen afford beneficial effects against heart failure and corresponding myocardial remodeling [33]. Several potential mechanisms have been reported; estrogen activates mitochondrial ATP sensitive potassium channels, decreases influx of calcium during ischemia and reduces the severity of contraction band necrosis. Additionally, estrogen increases the production of nitric oxide, reduces oxygen free radical formation and reduces leucocyte adhesion and migration in infarcted tissues through reducing expression of cytokines and leucocytes adhesion molecules [34,35,36]. It has to be noticed also that the improved ventricular performance associated with decreased parameters denoting myocardial cell injury observed in ovariectomized female rats with estrogen substitution could be explained on the basis of the beneficial cardioprotective effect of endogenous estrogen especially that the dose used in this study is in the upper physiological range as has been reported by Beer et al. [37].

In this study we tested the hypothesis that cardioprotection in females may be due to lesser susceptibility than males to the detrimental effects of calcium overload during I/R injury. To get some insight into its underlying mechanism, we use some inhibitors of sarcolemmal and sarcoplasmic reticulum membrane proteins which are involved in the regulation of intracellular calcium.

From the forementioned results in this study, we found that male orchietomy (gp 2) significantly decreased the cytosolic calcium as compared to sham group (gp1). When supraphysiological testosterone is administered to the male orchietomized rats (gp3), further depletion in cytosolic calcium has been observed as compared to group 2. This decrease in calcium load was associated with improved functional ventricular recovery. This led
Naga t Y. Mina, et al.

...to suggest that the higher male susceptibility to I/R injury can be partly explained by a higher tendency towards calcium overloading during reperfusion after global no flow ischemia. In deed, this effect is reduced by supraphysiological testosterone administration.

At the end of reperfusion, the present results demonstrate that addition of Amiloride (Na+/H+ exchange inhibitor) or Thapsigargin (sarcoplasmic reticulum calcium ATPase pump inhibitor) to the perfusated hearts of male orchiectomized rats with testosterone substitution (gps 5&6) resulted in a more additional cardio-protective effect as indicated by significantly higher ventricular performance and significantly lower cytosolic calcium, Fas ligand and CK leakage than that obtained by supraphysiological testosterone substitution alone in group 3.

In accordance with our results, several studies have provided a direct link between Na+/H+ exchange inhibition and improved postischemic contractile function. Avkiran et al. [38] reported that Na+/H+ exchange inhibition prevent excessive calcium accumulation within the myocardium and significantly attenuated the development of early apoptosis. Jian-Wen et al. [39], reported that reduction of intracellular calcium during reperfusion; reduces necrosis, diminishes ischemia and reperfusion-induced contracture and enhances/slow down systolic recovery after reperfusion by Na+/H+ exchange inhibition.

The effect of Thapsigargin addition (gp6) at the end of reperfusion is consistent with the study of Kumada et al. [40], that proved the beneficial effect of Thapsigargin-induced SR calcium diminution on the myocardial protection using the isolated working rat heart model under conditions of normothermic ischemia. Thapsigargin is a selective modulator of the SR Ca2+-ATPase. It interferes with the catalytic cycle of the pump by preventing Ca2+ and/or ATP binding [41]. These actions can be attributed to progressive depletion of the SR Ca2+ pool. In fact, during diastole, cytosolic Ca2+ is transported either into the SR or outside the sarclemma by the sarcosomal Ca2+-ATPase and Na+/Ca2+ exchanger. The extent of the SR Ca2+ pool depends on the ratio between the activity of the SR Ca2+-ATPase and the activity of the sarcosomal transport systems. Inhibition of the SR Ca2+-ATPase causes more Ca2+ to be extruded from the cell, leading to a reduced SR Ca2+ pool. As far as ischemia and post-ischemic recovery are concerned, less Ca2+ is available for release from the SR, which should be a beneficial effect [41].

Results of the present study demonstrate that addition of Verapamil (L-type calcium channels blocker) to the perfusated hearts of male orchiectomized rats with testosterone substitution (gp4) didn’t cause any significant change in cytosolic calcium, hemodynamic measures, CK and Fas ligand as compared to group 3.

Taken together, the findings of the present work strongly support that some of the beneficial cardiovascular effects of supraphysiological testosterone most likely arise from its ability to block L-type calcium channels and decrease calcium overload during I/R. This is based on the similar effects obtained in perfusated hearts of orchiectomized rats substituted with supraphysiological testosterone (gp3) and after addition of verapamil (gp4). Since addition of both Amiloride and Thapsigargin to perfusated hearts of groups 5&6 caused more beneficial effect on postischemic recovery than in group 3, we could exclude that the lowering effect of testosterone on calcium overload is through Na+/H+ exchange inhibition or sarcoplasmic reticulum calcium ATPase pump inhibition. These results are consistent with other studies in isolated vessel preparation that support the idea that testosterone acts as L-type Ca2+ channel antagonist in rat pulmonary and coronary artery [42]. Hall et al. [43] suggests selectivity of supraphysiological testosterone for L-type but not T-type calcium channel blockade to reduce Ca2+ influx into vascular smooth muscle and therefore promote vasodilatation. L-type calcium channels blockade by testosterone was rapid, making it unlikely to be mediated through a genomic effect. This nongenomic action of testosterone mediates its effects in a variety of cell types and play crucial pathophysiological roles in various tissues [43,44,45].

Signaling pathways for modulation of ion channels by hormones are still not well known. However, some authors proposed the signaling cascade for non genomic action is through the recruitment of various second messengers including PI3K-AKT and mitogen activated protein kinase (MAPK) signaling cascade [33]. In cardiac myocytes, testosterone via PI3K-Akt-signaling dependent activation of endothelial type nitric oxide synthase (eNOS) causes release of nitric oxide (NO). NO released by testosterone acts via a cGMP-dependent mechanism to antagonize PKA-dependent activation of the ICa,L channels [46,47,48].

Concerning the effect of estrogen on I/R-induced calcium loading, we observed that ovariectomy of female rats (gp2) resulted in significant increase in cytosolic calcium as compared to sham.
group. This has been associated with postischemic contractile dysfunction and increased myocardial injury. Substitution of ovariectomized rats with estrogen decreased significantly the cytosolic calcium, in parallel with enhanced postischemic recovery. These findings provide strong evidence that estrogen protects against I/R injury via decreasing intracellular calcium loading. However, the question is still raised about the possible underlying mechanism involved in this estrogen effect. In this study, we found that addition of Verapamil (group 10) and Thapsigargin (group 12), to the perfused hearts of female gonadectomised rats with estrogen substitution caused a significant rise in left ventricular functional parameters which was associated with a significant decrease in cytosolic calcium, area % of myocardial infarction, Fas ligand level and CK enzyme leakage as compared to their corresponding values in groups 7, 8 and 9. These results come in accordance with a study by Mandal et al. [49] that reported that verapamil significantly decreased I/R-induced cardiac dysfunction. In addition, Avellanal et al. [50] and Kumada et al. [40], reported that pretreatment with Thapsigargin (sarcoplasmic reticulum calcium ATPase pump inhibitor) improved recovery of LV functional parameters significantly as compared to non treated sham-operated rats. This improvement was correlated with lower cytosolic calcium and reduced Creatine kinase leakage in isolated hearts and in ventricular muscle preparations subjected to ischemia and reperfusion. Arrhythmias induced by ischemia or I/R were also decreased by Thapsigargin.

On the contrary, it has been observed that addition of Amiloride (Na+/H+ exchange inhibitor) to the perfused hearts of ovariectomized females with estrogen substitution (gp11) caused significant decrease in the cytosolic calcium that was associated with insignificant change in any other parameter measured as compared to group 9.

These findings indicate that the estrogen cardioprotective effect is mediated via lowering I/R-induced calcium overload. Such effect is mostly not through blocking L-type calcium channels or sarcoplasmic reticulum calcium ATPase pump inhibition as both resulted in a more additional cardio-protective effects against I/R injury. Meanwhile, we could suggest that estrogen effect is likely to be due to its ability to inhibit Na+/H+ exchanger as there were no further significant changes in both cytosolic calcium and all other parameters measured indicating contractile function and cell injury between groups 9&11. The inhibitory effect of estrogen on Na+/H+ exchanger is supported by Anderson et al. [32], who reported that estrogen stimulates the release of NO and that NO inhibits Na+/H+ exchanger in cardiac myocytes. So estrogen treatment of isolated rat hearts limits Na+ uptake during ischemia-reperfusion and thus limit Na+ dependent increases in intracellular calcium as well as associated I/R injury.

Opposite to our results, Zhai et al. [29] reported that estrogen transiently decrease the inward Ca2+ current, the intracellular free Ca2+ in ventricular myocytes and specifically inhibit L-type Ca2+ channel currents. In addition estrogen was shown, during I/R injury, to modify the function of a genetically overexpressed Na+/Ca2+ exchanger [33]. The discrepancies could be the result of different experimental protocol and/or different animal species.

Role of gender and sex hormones in ischemic reperfusion injury:

By comparing the results of male groups 1,2,3 with their corresponding in female groups 7, 8, 9 to illustrate the difference in tolerability of males and females to I/R injury, we detect that the left ventricular functional parameters were significantly higher in female sham-operated rats (gp7) and female ovariectomized rats with estrogen substitution (gp9) than their corresponding values in male gps 1 and 3. The improvement in the haemodynamic data in the female groups was associated with less myocardial damage as indicated by less CK release, decreased % area of myocardial infarction and less apoptosis with decreased Fas ligand. In addition, there is more significant decrease in cytosolic calcium level than that in males. On the contrary, female ovariectomy (gp8) resulted in a significant decrease in the left ventricular functional parameters compared to that in male orchietomized rats (gp2). This deterioration observed in gp8 was accompanied with more myocardial damage as indicated by significant increase in CK activity, area % of myocardial infarction as well as increased Fas ligand and cytosolic calcium level than that in gp2. As we see, gonadectomy was detrimental to the female rats and beneficial to the male rats, supporting the important beneficial role for endogenous estrogen to resist I/R injury. Furthermore, the cardioprotective effect of estrogen substitution in female ovariectomized rats is greater than that of supraphysiological testosterone substitution in male gonadectomized rats against I/R injury. Our results are supported by previous studies by Song et al. [13], Cavasin et al. [51] and Kuhar et al. [22], demonstrating such sex difference in the cardiac tolerance to ischemia and reperfusion injury which depends on the role of estrogens in cardioprotection.
Relative to the male gender, it has been suggested that females are protected in varying extents from cardiovascular injury under conditions of ischemia, hypoxia, oxidative stress, and senescence [52]. Males have a higher prevalence of cardiomyocyte death across species [53]. In hearts from human subjects without cardiovascular disease, the level of apoptotic myocyte death was 3-folds higher in men than women [54]. There has been solid evidence that estrogens have anti-apoptotic action [55]. In contrast, androgenic steroids have been shown to induce apoptosis in cultured cardiomyocytes in a dose-dependent manner [56]. Several factors have been found to be involved in estrogen-mediated cardiovascular protection, including Heat shock proteins (HSPs) [57], PI3K/Akt [58] and interaction of IGF-1 and estrogen which provides further protection to female gender against factors leading to senescence, such as oxidative stress [59].

Accumulating data show that during I/R injury, impaired myocardial function and calcium accumulation probably forms a vicious circle that leads to a progressive myocardial damage [22]. The accumulated Ca\textsuperscript{2+} may be deposited in myocardial mitochondria and leads to harmful effects on myocardial cells; It depletes ATP by activating Ca\textsuperscript{2+}-activated ATPases and inhibiting high-energy phosphate production in mitochondria [29], degrades cellular membrane systems by activating phospholipases and lipases [60] and accelerates oxygen free radical production via the endothelial xanthine oxidase system [22]. In our study, gender differences in cardioprotection could be explained by the ability of estrogen to lower calcium overload which is mostly due to inhibition of Na\textsuperscript{+}/H\textsuperscript{+} exchanger as previously discussed. Meanwhile, testosterone depletion in orchiectomized rats decrease calcium load and improves the cardiac tolerance to I/R injury, thereby, explaining the higher vulnerability of males to I/R injury than females. In addition, we proved that the supraphysiological testosterone replacement in male orchiectomized rats decrease calcium load mostly through blocking L-type calcium channels. However, this effect is less pronounced than the effect of estrogen on calcium load.

Summarizing, the data of the present work suggests that there is a sex difference in the cardiac tolerance to I/R injury due to the influence of sex hormones. Our data provide direct evidence that lowering calcium overload is crucial for postischemic myocardial recovery and salvage during I/R injury. Here, we demonstrated that the protective effect of estrogen is partly mediated via decreasing intracellular calcium accumulation through inhibition of Na\textsuperscript{+}/H\textsuperscript{+} exchange mechanism. Thus, short term estrogen therapy in females could be beneficial against myocardial ischemic injury in menopausal women. Furthermore, the acute beneficial effect of supraphysiological testosterone in cardioprotection appears to be due to a rapid non genomic mechanism through L-type calcium channel blockade. This cardioprotective effect appears to translate into a clinical benefit as supplementation of testosterone in hypogonadal male patients with coronary artery disease (CAD) to improve myocardial ischemia.

References


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