Attenuation of Oxidative Stress by Tamoxifen in the Heart of Ovarectomized Rats with Experimentally Induced Hyperthyroidism

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Abstract

Background: Postmenopausal estrogen deficiency increases the incidence of cardiovascular diseases. Although hormone replacement therapy can reduce cardiovascular risk, it is associated with an increased cancer risk. Selective estrogen receptor modulators (SERM), like tamoxifen and related compounds, have mixed estrogen agonistic/antagonistic effects. Tamoxifen may confer significant cardiovascular benefits without the estrogen-associated risks of endometrial and breast cancer. Hyperthyroidism has been recorded as subclinical conditions in postmenopausal women. Increased oxidative stress with high free radical generation has been described previously in animal models of hyperthyroidism. At the present time, the role of iNOS in hyperthyroidism is not clear, and the mechanisms responsible for the elevated NO activity of hyperthyroidism are not completely established.

Aim: The present study was designed to investigate the cardioprotective effects of tamoxifen on oxidative damage in heart of ovarectomized rats with experimentally induced hyperthyroidism.

Material and Methods: The study was conducted on 40 female albino rats. The experimental animals were divided into five groups, 8 rats each.

Group I: Control (sham operated).
Group II: Ovarectomized (OVX).
Group III: Ovarectomized and hyperthyroid (OVX-HT).
Group IV: Ovarectomized and tamoxifen treated (OVX-Tx).
Group V: Ovarectomized, hyperthyroid and tamoxifen treated (OVX-HT-Tx).

Hyperthyroidism was induced by intraperitoneal administration of L-thyroxine, 25 µg/100g BW/day for 4 weeks.

Tamoxifen was given SC. in a dose of 1mg /kg /day for 4 weeks. Morphometric parameters were evaluated at the end of the 4-week treatment period. The heart weight (mg) to body weight (g) ratio were calculated for evaluation of cardiac hypertrophy.

At the end of the experimental period, blood samples and heart of rats were taken for the determination of malondialdehyde (MDA), glutathione (GSH), and superoxide dismutase (SOD) levels and inducible nitric oxide synthetase (iNOS) gene expression in the heart tissue of studied animals. Thyroxin (T4) levels and serum cholesterol were determined using routine clinical-chemistry methods.

Results: The determinant ratio of cardiac hypertrophy the heart mg./body weight g. ratio significantly higher in OVX and OVX-HT rats compared to the control (p=0.02, p<0.01, respectively). This parameter showed insignificant difference between the control and tamoxifen treated groups.

Ovariectomy resulted in abnormal elevation of serum cholesterol and heart oxidative stress markers and changes in redox status of the heart tissue. Supplementation of tamoxifen partially alleviated these abnormalities and restored redox homeostasis of heart tissues after ovariectomy. Ovariectomy accompanied with hyperthyroidism also associated with significant increase in oxidative stress parameters. Among the studied oxidation parameters levels of the MDA in OVX rats and those OVX-HT were higher than those of the control groups (p<0.001). Tamoxifen therapy caused a decrease in MDA in the heart tissue of both OVX rats (p<0.001) and OVX rats with induced hyperthyroidism (p<0.001). In addition, tamoxifen therapy increased heart GSH (p<0.001) and SOD(p<0.001) in the previous groups. Indeed iNOS was significantly elevated in OVX rats and in OVX with hyperthyroidism, where tamoxifen significantly reduced it partially in both treated groups (IV and V).

Conclusion: The study showed the extent of changes in oxidative stress markers in this model of estrogen deficiency with induced hyperthyroidism. The protective effect of tamoxifen against oxidative damage in the heart suggests that tamoxifen as an estrogen modulator plays an important role within the antioxidant defense systems in the heart. The exact molecular mechanisms leading to these findings are not yet completely known. Meanwhile, tamoxifen as an estrogen modulator-therapy opening new avenues for pharmacological prevention of oxidative damage in the heart.


Introduction

ESTROGEN replacement therapy favorably influences several domains of vascular health, including
lipid metabolism, endothelial function and aspects of hemostasis [1]. However, estrogen replacement also carries an increased risk of endometrial [2] and possibly breast [3] carcinoma. Most women discontinue estrogen treatment because of its many side effects or the increased risk of breast and uterus cancer. This, in large part, has contributed to development of SERMs, such as tamoxifen and raloxifene, as alternative therapeutic agents.

A SERM, selective estrogen receptor modulators, is a molecule that binds with high affinity to estrogen receptors but has tissue-specific effects distinct from estrogen, acting as an estrogen agonist in some tissues and as an antagonist in others. Tamoxifen may confer significant cardiovascular benefits without the estrogen-associated risks of endometrial and breast cancer [4].

Despite long-standing use of tamoxifen, few studies have addressed its long-term impact on cardiovascular function.

There is considerable evidence that oxidative stress from superoxide and other reactive oxygen species (ROS) contributes to the development of cardiovascular diseases, diabetes and renal insufficiency [5].

There was relatively high prevalence of unrecognized hyperthyroidism in older adults, especially women, which necessitated routine screening of adults for thyroid disease by measurement of serum thyrotropin [6].

In women, the loss of estrogen may cause changes in redox homeostasis of proteins [7]. One or more reduced thiol (-SH) groups are essential for the function of many proteins. The thiol group on the side chain of the amino acid cysteine is particularly sensitive to redox reactions and is an established redox sensor. Proteins containing Cys thiol groups are particularly susceptible to oxidation by ROS [8]. Glutathione (GSH) can be reversibly bound to protein thiol groups by a mechanism called S-glutathionylation and leading to the formation of S-glutathionylated proteins [9]. Malondialdehyde (MDA) is a physiological ketoaldehyde produced by peroxidative decomposition of unsaturated lipids as a by-product of arachidonic acid metabolism. Excessive production of MDA, as a result of tissue injury, may combine with free amino groups of proteins. MDA reacts mainly with Lys residues by Michael type addition reaction and forms MDA-modified protein adducts [9].

Post-mitotic tissues of high-energy demand are at greater risk of damage by free radicals, consistent with the notion where signs of oxidative damage usually start to appear at these body sites. Due to the differing efficacy of redox homeostasis mechanisms, post-mitotic tissues are generally much more vulnerable to OPD than are mitotic cells [10].

Increased oxidative stress with high free radical generation has been described previously in animal models of hyperthyroidism [11].

At the present time, the role of iNOS in hyperthyroidism is not clear, and the mechanisms responsible for the elevated NO activity of hyperthyroidism are not completely established. Moreover, no data have been reported on the contribution of iNOS to the antihypertensive effect of NO in this endocrine disease. With this background, it was hypothesized that increased activity of iNOS, an isoform that plays an important role in renal function and BP regulation in various pathophysiological situations, might increase NO production and contribute to the homeostatic role of this factor in the hyperthyroid state [12].

In this study, we investigated:

1- The effects of chronic administration of tamoxifen on the redox status in heart tissues of Ovx rats. Thus, we examined whether tamoxifen therapy could reverse oxidation changes produced by ovariectomy.

2- The protective effects of tamoxifen on oxidative damage in rats with experimentally induced hyperthyroidism in ovarectomized rats.

3- The changes in iNOS gene expression in the ovarectomized rats and in ovarectomized rats with induced hyperthyroidism.

Material and Methods

Experimental animals:

40 female albino rats 6 months old with initial body weight range between 160-190g were used in this study. They were kept in the animal house of Kasr Al-Aini Faculty of Medicine, Cairo University. They were kept at 22 ± 1ºC temperature at 12h dark-light cycles.

Animal Groups:

They were randomly divided into five groups:

• Group I: Control, Sham operated (n=8).
• Group II: OVX: Ovarectomized rats (n=8).
• Group III: OVX-HT: Ovarectomized and hyperthyroid rats (n=8).
• Group IV: OVX-Tx: Ovarectomized and tamoxifen treated rats (n=8).
• Group V: OVX-HT-Tx: Ovarectomized, hyperthyroid and tamoxifen treated rats (n=8).

Animals were kept in conventional wire-mesh cages, four rats per cage, rats were given ad libitum access to conventional food and water from a drinking bottle throughout the course of the experiment.

Animals were anaesthetized by the administration of pentobarbital sodium (70mg/kg body wt ip).

The rats underwent either a bilateral ovariectomy via a dorsal incision or a sham surgical procedure and incisions were closed.

Hyperthyroidism was induced by intraperitoneal administration of L-thyroxine for 4 weeks [11].

Rats were treated for a total of 4 weeks. At the end of experimental period blood samples were drawn in the fasting state and processed within 1hr of collection, then the animals were sacrificed. After scarification, heart tissue of rats were taken for the determination of malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD) levels and gene expression of iNOS in the heart. The concentrations of thyroxine (T4) and total cholesterol level in serum were determined.

Induction of hyperthyroidism in group III and V was confirmed by measurement of thyroid hormone level in blood samples retroorbital veins at the end of the study by using serum T4 ELIZA kit according to the manufacturer's instructions (MONOBIND Co,USA). Rats with serum T4 ≥ 24 µg/dl were considered hyperthyroid one [11].

Two weeks after ovariectomy hormonal treatment of female rats was started with daily intraperitoneal administration L-thyroxine for 4 weeks either alone or combined with subcutaneous injection of tamoxifen. Ovarectomized control animals were injected with vehicle alone. Initial body weight and after the end of study, the body weight and heart weight were evaluated to determine cardiac hypertrophy.

Cardiac hypertrophy was evaluated by heart weight (mg) to body weight (g) ratio, according to Araujo et al. [13].

Drugs:
1-Thyroxine: L-thyroxin [Eltroxin; Sigma: St. Louis, MO] daily IP 25 µg/100g body weight [i.e. 40 µg/b.i.d in 0.5ml saline + NaOH solution] [11].

2- Tamoxifen: (Nolvadex [tamoxifen citrate-Trans isomer]. It was given SC. in a dose of 1mg/kg /day [i.e. 0.16mg each in 0.16ml saline + alcohol]. The doses of tamoxifen were chosen on the basis on previous work showing inhibition of mammary tumours in a rat model of breast cancer [14] and beneficial cardiovascular effects [15].

Preparation of tissue samples:

Segment of heart tissue from all the experimental groups were excised separately and rinsed in ice cold saline. A known weight of the tissue was homogenized in 200 µl buffer (0.05 M potassium phosphate and 0.1mM EDTA, pH 7.8) by a tissue homogenizer using a Teflon pestle at 4ºC at pH 7.4. The tissue homogenates obtained were centrifuged at 3,000-xg for 10min at 4ºC using Sorvall refrigerated centrifuge. The supernatant was stored in –20ºC for further studies.

Biochemical measurements:

Measurement of MDA:

The rate of lipid peroxidation was determined by the procedure of Beuge and Aust. 27 One of the major secondary products of lipid peroxidation is malondialdehyde (MDA). MDA was measured in tissue homogenate after precipitation of protein by addition of trichloroacetic acid (TCA)- then thiobarbituric acid (TBA) reacted with malondialdehyde (MDA) to form thiobarbituric acid reactive product, which was measured at 532nm according to Draper and Hadley [16].

Determination of reduced glutathione concentrations:

Reduced glutathione content was measured as described by method earlier [17]. Briefly, the reaction mixture containing 1.2ml EDTA (0.02M), 1ml distilled water, 250 µl Tris-chloroacetic acid and 50 µl Tris-buffer (0.4M, pH 8.9) was centrifuged at 300xg for 15min. The clear supernatant (500 µl) was mixed with 1ml of 0.4M Tris buffer (containing 0.02M EDTA, pH 8.9), 100 µl 0.01M DTNB [5,5'-dithio-bis-(2-nitrobenzoic acid)] and 100 µl enzyme source. The mixture was incubated at 37ºC for 25min. The yellow color developed was read at 412nm against a blank.

Determination of reduced glutathione concentrations:

Reduced glutathione content was measured as described by method earlier [17]. Briefly, the reaction mixture containing 1.2ml EDTA (0.02M), 1ml distilled water, 250 µl Tris-chloroacetic acid and 50 µl Tris-buffer (0.4M, pH 8.9) was centrifuged at 300xg for 15min. The clear supernatant (500 µl) was mixed with 1ml of 0.4M Tris buffer (containing 0.02M EDTA, pH 8.9), 100 µl 0.01M DTNB [5,5'-dithio-bis-(2-nitrobenzoic acid)] and 100 µl enzyme source. The mixture was incubated at 37ºC for 25min. The yellow color developed was read at 412nm against a blank.

Determination of SOD activity:

The SOD activity was measured according to the method described earlier [18]. Briefly, the reaction mixture (2.1ml) contained 24 µM sodium car-
bonate buffer (50mM), 30 µl Nitroblue tetrazolium (1.6mM), 6 µl Triton X-100 (10%) and 20 µl hydroxylamine-HCl (100mM). Subsequently, 100 µl of enzyme source (tissue homogenate) was added and absorbance (560nm) was read for 5min. against the blank (reaction mixture without enzyme source).

Detection of iNOS gene expression:

Heart tissue from studied groups, was homogenized in phosphate buffer saline, then centrifuged at 14000rpm for 10min. The supernatant of tissue homogenate was kept frozen at -80ºC till examined for gene expression of iNOS by RT-PCR.

Total RNA was extracted using total RNA isolation system (Promega, Madison, WI) according to manufacturer’s recommendations, the sample obtained was quantitated by absorbance at 260nm. cDNA was synthesized from 1 µg total RNA using 0.2 µl of moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI), 50pmol random hexamet, 1mM dNTPS, 2 µl RNase inhibitor (Promega) in total volume of 20 µl. The reaction was performed at 37ºC for 60min. PCR reaction was performed in a total volume of 50 µl in the presence of Tag DNA polymerase (Promega) 200 µmol/L d NTPs, 25pmol/L. 5' and 3' specific primer which have the following sequence: 5'-GAATCT AATTACAGTCATTAG-3' and 3' GATAGATC- TATAATGTTCATG-5'. Thermal cycling conditions were 35 cycles as follows, denaturation 1min at 94ºC, annealing for 1min at 64ºC and elongation at 72ºC for 1min and final extension for 7min at 72º.

Plasma cholesterol was detected by enzymatic colorimetric test [19].

Data analysis and statistics:

The data was coded and entered using the statistical package SPSS version 15. The data was summarized using descriptive statistics: mean ± standard deviation, minimal and maximum values for all variables. Statistical differences between groups were tested using ANOVA (analysis of variance) for quantitative normally distributed variables. When a significant F was obtained, multiple comparison post tests were used to determine which groups were significantly different. Correlations were done to test for linear relations between variables. p-values less than or equal to 0.05 were considered statistically significant.

Results

Animals’ weights, hearts' weights and heart/body weight ratio were summarized in Table (1).

Sham-operated and OVX animals gained weight (2.27% and 8.47% respectively) during the 4 week treatment period. But the OVX rats gained significantly more weight than the sham operated in the same period (p<0.01). On the other hand, OVX-HT rats lose weight (1.5%) when compared with the same group, and about (8%) when compared to the control rats after 4 weeks. While the weight of OVX-Tx rats didn't change over the same period, as there was insignificant change in the weight of this group over 4 weeks of treatment (p>0.05). but it appeared significantly low when compared to the control after 4 weeks (p<0.01). However, group (V) lose weight (p<0.01)compared to the same group and to the sham-operated animals after 4 weeks.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Group (I) (control)</th>
<th>Group (II) (OVX)</th>
<th>Group (III) (OVX-HT)</th>
<th>Group (IV) (OVX-Tx)</th>
<th>Group (V) (OVX-HT-Tx)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>176±14</td>
<td>177±15</td>
<td>177±16</td>
<td>178±16</td>
<td>176±14</td>
</tr>
<tr>
<td>After 4 weeks</td>
<td>180±16</td>
<td>192±18$^*$</td>
<td>170±15*#</td>
<td>177±17*#</td>
<td>168±15$^*$#</td>
</tr>
<tr>
<td>Heart weight</td>
<td>0.55±0.05</td>
<td>0.64±0.06$^*$</td>
<td>0.69±0.06*</td>
<td>0.55±0.04</td>
<td>0.53±0.06</td>
</tr>
<tr>
<td>Heart/body weight ratio</td>
<td>3.06±0.6</td>
<td>3.3±0.5$^*$</td>
<td>4.06±0.6*</td>
<td>3.11±0.5</td>
<td>3.15±0.5</td>
</tr>
</tbody>
</table>

$ = $Statistically significant as compared to same group.

$^* = $Statistically significant as compared to control [Group (I)].

$^# = $Statistically significant as compared to ovariectomized [Group (II)].

The heart weight of OVX rats and OVX-HT rats was significantly higher than that of the control (p<0.01). While the heart weight of other groups was insignificantly changed from that of the sham-operated animals. The determinant ratio of cardiac hypertrophy the heart mg. /body weight g. ratio
significantly higher in OVX and OVX-HT rats compared to the control ($p=0.02$, $p<0.01$, respectively). Heart/body weight ratio in the tamoxifen treated groups showed insignificant difference when compared to the sham-operated rats.

Serum total cholesterol level was higher in OVX rats and in OVX-HT rats than those of sham-operated control ($p<0.001$, $p=0.001$ respectively) (Table 2), Fig. (1). While it was significantly reduced in OVX-HT rats when compared to OVX rats ($p=0.001$). But there was insignificant difference neither in between group IV and control, nor in between group V and control as regard total serum cholesterol. The last two observations confirm the ability of tamoxifen to reduce serum cholesterol.

<table>
<thead>
<tr>
<th>Groups</th>
<th>CHOL</th>
<th>Range</th>
<th>Mean ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (I) (control)</td>
<td>119.2-132.4</td>
<td>124.2±4.67#</td>
<td></td>
</tr>
<tr>
<td>Group (II) (OVX)</td>
<td>159.5-209.1</td>
<td>181.7±14.99*</td>
<td></td>
</tr>
<tr>
<td>Group (III) (OVX-HT)</td>
<td>128.5-178.5</td>
<td>153 ±20.15*#</td>
<td></td>
</tr>
<tr>
<td>Group (IV) (OVX-Tx)</td>
<td>120.6-148.9</td>
<td>138.5±9.8#</td>
<td></td>
</tr>
<tr>
<td>Group (V) (OVX-HT-Tx)</td>
<td>126.3-126.3</td>
<td>138.2±10.95#</td>
<td></td>
</tr>
</tbody>
</table>

* = Statistically significant as compared to control [Group (I)].
# = Statistically significant as compared to ovarectomized [Group (II)].

The changes in the activities of anti-oxidant enzymes SOD, and GSH were shown in Table (3,4). Figs. (2,3). SOD, and GSH decreased significantly in OVX and OVX-HT rats compared to the control ($p<0.001$ in both groups). However, simultaneous administration of tamoxifen for 4 weeks to these animals reversed most of the aforementioned changes, as there was significant change between groups IV and II on one hand ($p<0.001$ for SOD and $p=0.008$ for GSH) and between group V and III on the other hand ($p<0.001$ for both antioxidants), indicating its potential to ameliorate oxidative stress in the heart induced by both ovarectomy alone or ovarectomy plus hyperthyroidism. The previous data are supported also by the presence of significantly higher MDA; which was used as a marker for lipid peroxidation, in groups II and III, ($p<0.001$ for both groups) as compared to control. These results were signifying oxidative stress as a result of either ovarectomy alone or when accompanied with hyperthyroidism, where hyperthyroidism was additionally increases MDA by about 18.87% in group III over group II Table (5).

iNOS is partly responsible for the increased NO production. Analysis of the results of iNOS were shown in Table (6) and Fig. (5). Table (6) showed significantly lower level in iNOS level in the control (sham-operated) as compared to groups II, III, IV & V ($p<0.001$). iNOS production was higher in group II (OVX rats) as compared to groups (IV & V), the p-values were <0.001. It also shows a high level of iNOS in OVX-HT rats (group III) which is statistically significant as compared to other groups, p-values were <0.001. These results indicate that the inducible isoform is activated in hyperthyroid animals. This suggests that thyroid hormone may be an important stimulus for inducible nitric oxide synthetase production.

Tamoxifen treated groups showed lower levels of iNOS than non treated groups. This indicated that, tamoxifen can reduce iNOS level partially but not to the control level as there was significant difference between them and the control.
### Attenuation of Oxidative Stress by Tamoxifen in the Heart of Ovarectomized Rats

**Table (3): Heart SOD (U/mg protein) in the studied groups (n=8 in each group).**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Range</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (I) (control)</td>
<td>0.58-0.87</td>
<td>0.77±0.1#§</td>
</tr>
<tr>
<td>Group (II) (OVX)</td>
<td>0.31-0.51</td>
<td>0.42±0.06*$§</td>
</tr>
<tr>
<td>Group (III) (OVX-HT)</td>
<td>0.21-0.42</td>
<td>0.3 ±0.09*$#</td>
</tr>
<tr>
<td>Group (IV) (OVX-Tx)</td>
<td>0.58-0.72</td>
<td>0.66±0.05 *§$</td>
</tr>
<tr>
<td>Group (V) (OVX-HT-Tx)</td>
<td>0.49-0.64</td>
<td>0.57±0.05*$# §</td>
</tr>
</tbody>
</table>

* = Statistically significant as compared to control [Group (I)].
§ = Statistically significant as compared to [Group (III)].
# = Statistically significant as compared to ovarectomized [Group (II)].

**Table (4): Heart GSH (mg/mg protein) in the studied groups (n=8 in each group).**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Range</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (I) (control)</td>
<td>12.3-18.7</td>
<td>14.8±2.12#</td>
</tr>
<tr>
<td>Group (II) (OVX)</td>
<td>5.7-9.04</td>
<td>7.3±1.19*</td>
</tr>
<tr>
<td>Group (III) (OVX-HT)</td>
<td>4.8-7.2</td>
<td>5.5 ±0.76*</td>
</tr>
<tr>
<td>Group (IV) (OVX-Tx)</td>
<td>8.6-10.7</td>
<td>9.6±0.67*§</td>
</tr>
<tr>
<td>Group (V) (OVX-HT-Tx)</td>
<td>7.7-10.02</td>
<td>8.6±0.76 *§</td>
</tr>
</tbody>
</table>

* = Statistically significant as compared to control [Group (I)].
§ = Statistically significant as compared to [Group (III)].
# = Statistically significant as compared to ovarectomized [Group (II)].

**Table (5): Heart MDA (nmol/mg protein) in the studied groups (n=8 in each group).**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Range</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (I) (control)</td>
<td>8.5-12.5</td>
<td>9.96±1.27#$§</td>
</tr>
<tr>
<td>Group (II) (OVX)</td>
<td>13.5-17.3</td>
<td>15.9±1.36*$§</td>
</tr>
<tr>
<td>Group (III) (OVX-HT)</td>
<td>16.8-20.7</td>
<td>18.9 ±1.47*$#</td>
</tr>
<tr>
<td>Group (IV) (OVX-Tx)</td>
<td>9.05-12.7</td>
<td>10.6±1.35#$§</td>
</tr>
<tr>
<td>Group (V) (OVX-HT-Tx)</td>
<td>10.4-14.6</td>
<td>12.3±1.53*$§</td>
</tr>
</tbody>
</table>

* = Statistically significant as compared to control [Group (I)].
§ = Statistically significant as compared to [Group (III)].
# = Statistically significant as compared to ovarectomized [Group (II)].

**Table (6): Heart iNOS (gene expression) in the studied groups (n=8 in each group).**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Range</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (I) (control)</td>
<td>0.01-0.03</td>
<td>0.02±0.01*$§</td>
</tr>
<tr>
<td>Group (II) (OVX)</td>
<td>0.43-0.68</td>
<td>0.54±0.08*$§</td>
</tr>
<tr>
<td>Group (III) (OVX-HT)</td>
<td>0.53-1.05</td>
<td>0.83±0.19*$#</td>
</tr>
<tr>
<td>Group (IV) (OVX-Tx)</td>
<td>0.18-0.34</td>
<td>0.25±0.06*$§</td>
</tr>
<tr>
<td>Group (V) (OVX-HT-Tx)</td>
<td>0.27-0.51</td>
<td>0.39±0.09*$§</td>
</tr>
</tbody>
</table>

* = Statistically significant as compared to control [Group (I)].
§ = Statistically significant as compared to [Group (III)].
# = Statistically significant as compared to ovarectomized [Group (II)].
Discussion

Selective estrogen receptor modulators, like tamoxifen and related compounds, have mixed estrogen agonistic/antagonistic effects. Tamoxifen may confer significant cardiovascular benefits without the estrogen-associated risks of endometrial and breast cancer [4].

In women, the loss of estrogen may cause changes in redox homeostasis of proteins [7]. There is considerable evidence that oxidative stress from superoxide and other reactive oxygen species (ROS) contributes to the development of cardiovascular diseases, diabetes, and renal insufficiency [5]. Reactive oxygen species–induced oxidation is considered to be a key factor in causing cardiac injury [5].

An efficient clinical laboratory diagnosis of altered redox status of the plasma and tissue proteins in menopausal stage is very important in
order to control the degenerative conditions associated with oxidative protein damage (OPD) [7]. On the other hand, an effective preventive and therapeutic strategy against oopherectomy-induced OPD has not been developed.

One of the interesting observations in this work reduced weight gain in tamoxifen treated rats. This finding was supported by many studies [20,21]. Although, the precise mechanisms that could explain the lower weight gain associated with tamoxifen or its metabolites are not well understood [20,21]. Numerous studies have documented the effects of ovariectomy on absolute body weight in female rats and interestingly, demonstrates in females that food consumption does not account for these differences in body weight [20].

Our study showed that cardiac hypertrophy was observed in OVX rats as well as in OVX and hyperthyroid rats at the end of treatment. The effect of cardiac hypertrophy induced by ovariectomy alone can be explained by the finding of Bottner et al. [22] who reported that ovariectomy increased pituitary TSH/β mRNA (thyroid stimulating hormone/β mRNA) levels and that this elevation was abolished by 17β-E2-3-benzoate treatment. Interestingly, the redundancy of DNA recognition and the common utilization of cofactors have been postulated to mediate crosstalk between the E-ER and T3-TR signaling pathways [23].

The effect of cardiac hypertrophy induced by hyperthyroidism was noticed also by Araujo et al. [24]. This suggests that cardiac hypertrophy could be contributed to the oxidative stress observed by the rise of oxidation product MDA and decline of antioxidants SOD and GSH in this model of hyperthyroidism associated with estrogen deficiency as well.

Recent data also suggest that low GSH concentration may activate JUN, which is a transcriptional complex important to gene expression induction in cardiac hypertrophy [25]. In addition, a decreased JUN expression prevents the development of cardiac hypertrophy through a ROS-dependent pathway [26]. The findings of Araujo et al. [5] corroborated these data, since a positive correlation was seen between FOS/JUN and cardiac hypertrophy.

Importantly, Kenessey and Ojamaa [27] demonstrated that thyroxine (T4) activates the AKT1 signaling pathway in myocardium and this process contributes to the cardiac hypertrophy associated with hyperthyroidism model. Recent evidence point to a non-transcriptional action of thyroid hormone through membrane-initiated processes or the cytosolic thyroid hormone receptor (TRα1) [27]. The author also mention the observation that there were conformational changes in membrane proteins due to cellular oxidative stress may activate or inhibit the AKT1 pathway, inducing phosphorylation and activation of endothelial nitric oxide synthase (NOS3).

It was proposed that thyroid hormone-induced physiologic cardiac growth is mediated by activating the PI3K (phosphatidylinositol 3-kinase)-Akt signaling pathway through cytosol-localized TRα1 [27].

This work detected a significant rise in serum total cholesterol level in OVX rats and in OVX-HT rats than those of sham-operated control. It is to be noted that the decline in cholesterol level observed in group III compared with group II occurred as result of excess thyroid hormone which was shown in previous studies to reduce plasma cholesterol [11]. On the other hand, tamoxifen was found to reduce serum cholesterol in the treated groups to the control level.

There is evidence suggesting that a novel mechanism is involved. Firstly, tamoxifen and raloxifene act as estrogen agonists in the liver, causing a decrease in total plasma cholesterol in rats and LDL in humans [28,29]. The study of Simoncini et al. and others [30-32] described non genomic agonistic effects of both tamoxifen and raloxifene via ER.

Early studies provided mechanistic clues as to how the estrogens mediate their effects on plasma lipids. It was shown that pharmacological doses of estrogens up-regulate LDL receptors in rat livers [33] and in human hepatoma cell lines [34]. Regulation of the LDL receptors has been shown to involve both transcriptional [14] and posttranscriptional [30] mechanisms. Lundeen et al. [35] showed that although the decrease in plasma cholesterol was significant, it was small compared to the decrease evoked by the estrogens examined under tamoxifen or raloxifene treatment.

The mechanisms to explain the cardioprotective effects of estrogen remain complex and elusive. Estrogen-induced changes in blood lipid profiles can only account for 30% of the decreased risk in premenopausal women [36].

Cardiovascular protective effects of tamoxifen could come partially from its lipid lowering effect, but this does not seem to explain all the potential cardiovascular benefit.
This work showed that the activities of antioxidant enzymes, SOD and GSH decreased significantly in OVX and OVX-HT rats compared to the control. The changes of antioxidant activity as a result of hyperthyroidism was supported by the work of Panda and Kar [37], who showed that SOD, GSH and CAT (catalase) were increased in hyperthyroid female rats in their study. The results of Araujo et al. [24] imply that hyperthyroidism generates myocardial dysfunction associated with oxidative stress inducing antioxidant enzyme activities and protein expression. This could explain the improved response with tamoxifen therapy in groups IV and V which showed insignificant difference in cardiac size compared to the control, as well as marked improvement in oxidative stress. In this view, we can suggest that tamoxifen has antioxidant capacity.

Our results also showed that iNOS gene expression of the heart was elevated in rats either ovariectomized alone or accompanied with hyperthyroid state. This proposition is supported by findings that hyperthyroidism causes an upregulation of NOS activity and NO production and that AG; an anti NOS, decreases plasma nitrite/nitrate in hyperthyroid rats [12].

In addition, Araujo et al. [13] were surprised on the finding that since even reducing oxidative stress in hyperthyroid animals, nitrite/nitrate levels persisted much higher than control; this indicates that other NO production pathways must be involved. In this regard, it has recently been demonstrated that thyroid hormones would activate the inducible NOS2 directly, enhancing additionally NO level [12].

Moreover, apoptosis has been postulated to be involved in the cardiac damage associated with dilated cardiomyopathy, diabetes and sepsis [38] which are all associated with an enhanced generation of ONOO− within the myocardium [39]. On the basis of these findings, Levrand et al. [39] propose that ONOO− may represent a major oxidant species involved in the process of cardiomyocyte apoptosis in these cardiac diseases.

As superoxide radicals may be enhanced in hyperthyroid rats, peroxyxinitrite (ONOO−) could also be produced, and may act to trigger the hypertrophic response [40].

These data imply that ONOO−-dependent oxidant stress is instrumental in activating proapoptotic signals (caspase-3 and PARP cleavage). The authors added that PARP cleavage as a consequence of ONOO− generation was secondary to the activation of caspase-3, but additional mechanisms may be implicated as well. Furthermore, Bojunga et al. [41] showed that antioxidative treatment was capable of reversing changes in NO-cGMP system. This can explain the decline of iNOS in tamoxifen treated groups, and again confirm its antioxidant capacity.

From other point of view, Rodríguez-Gómez et al. [12] hypothesized that nitric oxide generated by inducible nitric oxide synthase (iNOS) may contribute to the homeostatic role of this agent in hyperthyroidism and may, therefore, participate in long-term control of blood pressure (BP), as a consequence of the observation of marked increase in BP after inhibition of iNOS in hyperthyroid rats. The author also found that plasma nitrates (products of NO) were increased in the T4 group and reduced after inhibition of iNOS in T4-treated rats but not in normal rats. In conclusion, the results of the present paper indicate that iNOS activity may counterbalance the prohypertensive effects of T4.

Clarke et al. [42], showed that treatment with tamoxifen (40 mg/d) for 56 days caused an increase in endothelium-dependent flow-mediated dilatation (ED-FMD) of the brachial artery in men with angiographically proven coronary artery disease (CAD) and in men with angina-like symptoms but normal coronary arteries (as shown by angiography). In contrast, tamoxifen did not affect endothelium-independent FMD in the same subjects. The ED-FMD response to tamoxifen was unlikely to have been caused by the associated increase in plasma estradiol or a reduction in plasma cholesterol levels [42].

The authors suggested a possible mechanism for the above observed response with tamoxifen to the reduction in plasma total homocysteine (tHcy) levels. High tHcy levels are associated with increased risk for CAD and other atherothrombotic diseases [43]. The reduction of tHcy levels by tamoxifen (20 mg/d) was seen in women with moderate hyperhomocysteinemia and in women with normal tHcy levels [44]. On the basis of these considerations, it seems reasonable to hypothesize that the improvement of vascular endothelial function after treatment with tamoxifen, as described by Clarke et al. [42] can be attributed, at least in part, to the lowering effect of the drug on plasma tHcy levels.

However, Clarke et al. [42] did not take into account important safety issues regarding the long-term use of tamoxifen. Many studies performed in rats have shown an association between the admin-
istration of tamoxifen and the development of hepatocellular carcinoma (HCC) [45,46]. Moreover, several cases of liver cancer were recently reported after long-term treatment with tamoxifen [47,48]. It is of interest to note that the histological pattern of these lesions is similar to that seen in tamoxifen-induced HCC occurring in rat models.

It is to be noted that, no studies far have demonstrated an increased incidence of HCC in women treated with tamoxifen. Consequently, we think that although tamoxifen might have some beneficial effects on the coronary arteries, it may not be an appropriate treatment for men with CAD.

Several reports have described variable cardiovascular effects produced by tamoxifen [13,28]. The reasons for these conflicting results are poorly understood. However, no study has been conducted to examine the possibility that tamoxifen and its metabolites could produce different, contrasting effects on the cardiovascular system, although it is known that tamoxifen is metabolized to several metabolites some of which are more active (up to 100 times more potent) than tamoxifen itself [49].

In fact, the relationship between molecular events, such as control of redox regulation, and intracellular signaling pathways are still under investigation. Molecular mechanisms that control redox-regulation systems related to cellular proteins, and variation in the regulation of these controlling systems will contribute to the susceptibility of post-mitotic tissues to oxidative stress during aging and menopause [7]. As redox regulation mechanisms in aging and menopause become clearer, new therapeutic approaches and prospective solutions are coming into view. A major research and development effort is required to produce novel therapies related to redox regulation of proteins and make them available to elderly women. The findings of our study will, lead to new insight into the extent of oxidative protein damage in this model of estrogen deficiency. On the other hand inhibition of protein oxidation and lipid peroxidation by tamoxifen may be one of the anti-atherosclerotic effects of this drug. The underlying mechanisms need clarification. The molecular mechanisms are not clear and further study is required. Meanwhile, selective estrogen receptor modulators therapy to prevent oxidative stress damage in a tissue specific manner may be required.

References


