Contribution of Testosterone in Attenuating Atherosclerotic Events and Adipose Tissue Inflammation in Male Rats

MAGDA M. EL-HAMZAWY, M.D.; HEBA M. SHAWKY, M.D.; HANY EL-SEBAIE, M.D.; HEBA S. SHOUKRY, M.Sc.; LAILA A. RASHED, M.D.; MAHA B. ZICKRI M.D. and HALA GABR, M.D.

The Departments of Physiology, Medical Biochemistry, Histology and Clinical Pathology, Faculty of Medicine, Cairo University

Abstract

The existence of a possible role of androgens has long been recognized in cardiovascular disorders in particular atherosclerosis. The aim of this study was to assess this role after castration and in response to testosterone replacement therapy (TRT) in an experimental rat model of atherosclerosis and to examine the impact of mechanisms involved. After 8 weeks of a hyperlipidemic regimen, the castrated rats showed a highly significant elevation in the studied serum lipid profiles (total cholesterol (TC) and triglycerides (TG) and the inflammatory markers [high sensitivity C-reactive protein (CRP) and interleukin-6 (IL-6)] compared to sham-operated hyperlipidemic animals (p<0.01), whereas serum adiponectin was markedly reduced. At tissue level, the vascular and adipose tissue displayed a significant increase in IL-6 gene expressed levels and in the inflammatory cell infiltration (macrophages and foam cells) in contrast to sham operated rats. Also marked deterioration was depicted in the gene expressed levels of estrogen receptor alpha (ER-α) and adiponectin receptor (ADEPR1) rather than ADEPR2 (p<0.01). Supplying the castrated hyperlipidemic rats with TRT with or without aromatase inhibitor (Letrazole) resulted in a significant attenuation of the pathogenic events, which was reflected in the significant reduction of studied serum lipid profile and inflammatory markers and the marked increase in the adiponectin levels. Histological and biochemical analysis also revealed marked decrease of IL-6 gene expressed levels with significant decreased infiltration of inflammatory cells in adipose and vascular tissue (p<0.05). On the other hand, the ER-α and adiponectin receptor 1 showed upregulation in their gene expressed levels (p<0.01) although these results were observed to be less significant after the aromatase inhibition with letrozole. In conclusion the present study confirms a potential role of androgen in atheroprotection via an androgen receptor mediated action as well as through mechanisms initiated by its aromatization to estrogen.

Key Words: Atherosclerosis – Testosterone – Aortic tissues – Adipose tissues – Adiponectin.

Introduction

RECENT work on cholesterol-fed animals exposed to social isolation or chronic stressful conditions, lack the ability to clear cholesterol from their circulation. This subsequently results in elevated levels of lipid profiles in their plasma and development of extensive atherosclerosis throughout the arterial wall [1].

The over-recruitment and activation of leucocytes which surround a lipid core which characteristic early atherosclerosis is considered the driving force behind atheroma development and is regulated by the concerted activities of several cytokines (e.g. TNF-α, interleukins (IL1-β, IL-6), chemokines and adhesion molecules expressed by endothelial cells [2]. Nevertheless, the release of these harmful cytokines, on the other hand is associated with the release of anti-inflammatory cytokines as IL-4 & IL-10 [3], in addition to increased adiponectin synthesis from adipose tissue [4].

Adiponectin through binding with its receptors (1&2) has attracted much attention because of its multiple actions especially as an anti-inflammatory [4] and anti-atherosclerotic agent [5]. In-vitro studies reported that adiponectin inhibits monocyte attraction to the vascular bed by attenuating TNF-alpha-induced expression of adhesion molecules. Consequently, the balance of the stimulatory and the inhibitory cytokines is crucial to the stability of the atheroma plaque and eventually to the slowing of the pathogenic events [6].

Although sex hormones have been proposed to play a dynamic role in the pathological events of several cardiovascular diseases including atherosclerosis, yet, the impact of androgens in males in...
Contribution of Testosterone in Attenuating Atherosclerotic Events

Relation to cardiovascular disease has still been neglected. Low serum testosterone has been found in previous reports to associate increased incidence of aortic atheroma in men [7]. Nevertheless, it is still not yet established the exact modifying role of androgens through the events of the disease in particular that mediated after aromatase inhibition. It is proposed however that testosterone may have an impact in limiting the vascular inflammation and cytokine activity underpinning the pathophysiology of atherosclerosis since macrophages, lymphocytes and vascular smooth muscle cells were all demonstrated to possess androgen receptors [8].

Thus, the present study aimed to elucidate the possible role of testosterone involved in these events through an in-vivo animal model of experimental stress atherosclerosis applied in castrated rats. To achieve this, the levels of the serum lipid profiles, high sensitive CRP, IL-6 and adiponectin were assessed. Since inflammation of adipose tissue is a common observation in the events of atherosclerosis thus, the pathogenesis of the disease and the effect of hormonal treatment in combating its events were further explored through evaluation of the gene expressed levels of IL-6, ER-alpha and adiponectin receptors 1&2 (ADIPR1, ADIPR2) in the adipose as well as vascular tissue, in addition to histopathological and morphometric evaluation of associated signs of inflammatory cell infiltration in these tissues.

Material and Methods

Experimental animals:

This study was performed using 44 adult male albino rats aging 12-14 weeks and of approximate body weights ranging from 180-200 grams. The animals were purchased from the Animal House of Faculty of Medicine, Cairo University during 2010. Experimental procedures and follow-up management of the animals were accomplished in the laboratories of the Physiology, Biochemistry, Clinical Pathology and Histology Departments, Faculty of Medicine, Cairo University. All procedures were carried out in compliance with the guide for care and use of laboratory animals published by the US National Institutes of Health (NIH publication 85-23 revised 1985) and in compliance with the Local Animal Ethics Committee of Kasr Al Aini, Faculty of Medicine, Cairo University.

The rats were placed under ordinary living conditions for acclimatization (i.e. room temperature, humidity and dark/light cycle) for 7 days before initiation of the experimental procedures. During this period of adaptation and the following 8 weeks of the study duration, the animals had free access to food and water.

After one week of purchase, the animals were randomized into:

Group I (n=10): Control group of non-atherosclerotic uncastrated rats which received ordinary diet of animal chow throughout the experimental period of the study.

In the remaining rats, atherosclerosis was induced experimentally using a high fat diet regimen for 8 weeks. To further participate the pathogenic events, groups of 5 rats each, were housed under stressful social conditions in confined cages [9].

The rats were then divided into:

Group II (n=10): Atherosclerotic non-castrated group in which the animals were subjected to sham operation.

Group III (n=24): Represented atherosclerotic castrated rats in which castration was achieved by bilateral orchiectomy performed 5 days before starting the atherosclerotic regimen [10]. These animals were then included in the following subgroups:

Group III A (n=7): Atherosclerotic castrated group, treated with maize oil as a vehicle supplied in an intramuscular dose of 0.5ml, twice per week.

Group III B (n=10): Atherosclerotic castrated rats testosterone-treated. According to Paget table the rats were supplied with a single intramuscular injection of testosterone oenanthate (Cidotestone; Cid drug Company, Egypt) in a dose of 6.25mg/Kg given in maize oil vehicle twice weekly [11,12].

Atherosclerotic castrated rats were treated with testosterone co supplemented with the nonsteroidal aromatase inhibitor letrozole (Femara; Novartis drug Company, Egypt), in a dose of 2.5mg/Kg body weight via subcutaneous injection [13]. Each tablet of the supplemented letrozole (2.5mg) was grinded into powder, weighed, dissolved in a few drops of alcohol, then diluted with 3.5ml of distilled water. The rats were then injected subcutaneously, from this mixture in a dose of 0.5ml each twice per week.

Experimental induction of atherosclerosis:

The rats for a duration of 8-9 weeks were kept on a feeding regimen of high fat diet documented and applied in previous animal studies. The diet comprised an animal chow containing atherogenic high-fat content in the form of 60% fat in each 100 gram of animal chow [9,14].
**Surgical procedure of bilateral orchiectomy:**

The rats were anaesthetized with 40mg/Kg body weight of sodium phenobarbital intraperitoneally. Surgical castration was performed via a midline scrotal incision allowing bilateral access to the hemiscrotal contents. The spermatic cord was ligated then the testicle was removed. The skin was closed with silk sutures, and a local antibiotic skin ointment (terramycin) was then applied twice daily for the next 5 days [10].

**Sham operation:**

The 2nd group of atherosclerotic rats was subjected to a sham operation in which a scrotal incision was made and then was closed with silk sutures.

**Mortality rate:**

Throughout the period of the study, 5 rats which were afterwards replaced died either from a wrong surgical procedure (3 rats) or from failure to adapt to the high-fat regimen and drug supplementation (2 rats).

**Body weight:**

The animals were weighed initially before starting the experimental procedures (week 0), then body weights were recorded weekly through the study duration. The weight was used as a marker of general health and follow-up status, in addition to its indication of the animal response to the high-fat diet regimen and drug treatment.

**Blood sampling:**

Rats in all experimental groups were allowed to fast for 7-8 hours before blood sampling using heparinized capillary tubes. Samples were withdrawn retroorbitally in 10ml eppendorf tubes. At the endpoint (week 8), blood withdrawal was achieved directly from the thoracic aorta immediately after sacrifice of the animals for assessment of serum lipids [i.e total cholesterol (TC) and triglycerides (TG)], serum levels of testosterone, circulating inflammatory markers [high sensitive C-reactive protein (CRP) and IL-6] and serum adiponectin levels.

**Tissue collection:**

On the day of sacrifice, after cervical dislocation of the animals and collection of blood samples, a median incision was performed. Visceral fat and aortic strips were extracted for histopathological, morphometric and biochemical studies for assessment of the signs of inflammation which included the rate of macrophage infiltration and foam cells formation, and estimation of the gene expressed levels of IL-6, estrogen receptor alpha (ER-α) and adiponectin receptors 1&2.

The visceral fat was separated, dissected and was left to dry on filter paper. It was weighed, then preserved in foil paper. After removing the heart and thoracic aorta, the latter was dissected free. It was then fixed to a piece of paper on a cork board and the surface area was determined. The aortic tissue was stripped of adventitia and was cut longitudinally for enface preparation. Tissues were stored in 10% buffered formalin for later staining and quantification of atherosclerotic disease [15].

The prostate was dissected from the surrounding connective tissue, the bladder (caudally) and from the seminal vesicles (cranially). The prostatic weight was then used as a marker of the gland response to the present androgenic effect [16].

**Histopathological of atherosclerosis and visceral fat inflammation:**

After dissection and separation of the aortic and adipose tissues, specimens were fixed in 10% formal saline for 24 hours. Paraffin blocks were prepared and 5 micrometer thick sections were subjected to histopathological study after haematoxylin and eosin-staining [17]. The sections were then photographed using Olympus Microscope computer-assisted digital camera.

The histopathological study included assessment of diameter of adipocytes, thickness of the media, count of apoptotic nuclei and the area of foam cells were estimated in aortic and adipose tissues. The measurements were done in 7 low power fields (LPF) using interactive measurements menu. A reference aorta template was created from the average size and shape of all the aortas and was laid onto each aortic image. Present lesion areas were then calculated.

**Biochemical study:**

Plasma total cholesterol and triglycerides were measured by quantitative - Enzymatic - Colorimetric determination by commercial available kits [18]. High sensitive C-Reactive Protein was estimated by immunoenzymometric assay (Immuno-Biological Laboratories, Inc. Minneapolis, USA) [15]. Measurement of Adiponectin and IL-6 in the blood was achieved using ELISA kit (B-Bridge International, Inc. USA) [19,20]. Gene expression levels of IL-6, ER-α and ADEPR1 and ADEPR2 were detected in adipose and aortic tissues using Real Time-Polymerase Chain reaction (RT-PCR).
Total RNA was extracted from aorta and adipose tissue homogenate using RNeasy purification reagent (Qiagen, Valencia, CA). The cDNA was generated from 5 μg of total RNA extracted with 1 μl (20pmol) antisense primer and 0.8 μl superscript AMV reverse transcriptase for 60min at 37°C. The relative abundance of mRNA species was assessed using the SYBR® Green method on an ABI prism 7500 sequence detector system (Applied Biosystems, Foster City, CA). PCR primers were designed with Gene Runner Software (Hasting Software, Inc., Hasting, NY) from RNA sequences from GenBank. All primer sets had a calculated annealing temperature of 60°. Quantitative RT-PCR was performed in duplicate in a 25-μl reaction volume consisting of 2X SYBR Green PCR Master Mix (Applied Biosystems), 900 nM of each primer and 2-3 μl of cDNA. Amplification conditions were 2min at 50°, 10min at 95° and 40 cycles of denaturation for 15s and annealing/extension at 60° for 10min. Data from real-time assays were calculated using the v1.7 Sequence Detection Software from PE Biosystems (Foster City, CA). All values were normalized to the beta actin genes.

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<th>Primer sequence</th>
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<th>Reverse primer</th>
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<td>3’-AGGCTCAAGAAGGTTGCA-5’</td>
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<tr>
<td>Adiponectin receptor 2</td>
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<td>3’-GTAGCAGATCGGAGGGACT-5’</td>
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<td>Beta actin</td>
<td>5’-TGTGCTCCGAGTCCCT-3’</td>
<td>3’-TAAATGCACGCAGATTCC-5’</td>
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Statistical analysis:

The results were analysed using SPSS computer software package version 10.0 (Chicago, IL, USA). Data were presented as mean±SD. Comparisons evaluated by one-way ANOVA followed by post hoc, Kruskal-Wallis and Mann-Whitney tests. Differences of p<0.05 were considered significant [22].

Results:

A- Effects of High-Fat Diet regimen on the experimental study parameters in control group (Group I) and sham-operated group (Group II):

- Serum level of testosterone, lipid profile (TC, TG), CRP, adiponectin and IL-6 level:

The high-fat diet for 8 weeks resulted in significant decline in the levels of serum testosterone of experimental sham-operated compared to normal rats, (2.5±0.55ng/ml versus 3.66±0.55ng/ml, p<0.05).

As regards the impact of 8 weeks duration of eating rich fat content, Table (1) revealed that, the rats showed signs of hyperlipidemia in the form of highly significant elevation in the mean values of TC and TG when compared to normal animals (61.4±5.1mg/dl and 150.67±10.2mg/dl respectively in group II versus 37.62±5.67mg/dl and 63.92±15.62mg/dl in group I, p<0.01).

Serum levels of adiponectin and high sensitive CRP in Fig. (1a) displayed excessive deterioration in serum levels of adiponectin reflected in the markedly significant decline in its mean value compared to control group I (6.15±1.12ng/ml versus 12.71±1.19 ng/ml respectively, p<0.01) with highly significant increase in the mean values of serum high sensitive CRP (1.72±0.30 µg/dl versus 0.09±0.02 µg/dl). As shown in Fig. (1b), highly significant increase in the mean values of serum IL-6 recorded in these animals compared to rats on ordinary chow (548.9±53.75pg/ml versus 118.13±3.52 pg/ml respectively, p<0.01).

- Gene expression levels of the IL-6, ER-α, ADIPR1 and ADIPR2 in adipose and aortic tissue:

In Fig. (2a,b), gene expression levels of the IL-6 showed significant increase with a mean value of 2.18±0.30 A.U in adipose tissue. In addition, a similar elevation in the expressed levels of IL-6 in aortic tissue was observed in the mean value which reached 2.17±0.52 A.U.

Gene expression levels of estrogen receptor alpha in Fig. (2a,b) showed a significant reduction in the levels of ER-α expressed in aortic and
adipose tissue in comparison to group I, thus reaching in adipose tissue a mean value of 0.72±0.135 A.U versus 1.98±0.43 A.U and in aorta a mean value of 0.62±0.16 A.U versus 1.88±0.11 A.U (p<0.05).

As depicted in Fig. (2a), a highly significant decrease in the gene levels expressed of both adiponectin receptors (ADIPR1 and ADIR2) was recorded with ADIPR1 reaching in sham-operated atherosclerotic rats a mean value of 0.84±0.132 A.U compared to normal rats (1.66±0.293 A.U, p<0.01), ADIPR2 showed a mean value of 0.38±0.218 A.U compared to group I levels (0.54±0.116 A.U, p<0.01). In addition, ADIPR1 and ADIPR2 gene expression levels in aortic tissue as shown in Fig. (2b) were down regulated in comparison to group I thus ADIPR1 reached a mean value of 0.43±0.12 versus 0.94±0.1 A.U respectively and the mean value of ADIPR2 was 0.56±0.12 versus 0.68±0.2 A.U respectively (p>0.05).

- **Body weight, visceral fat weight and prostatic weight:**

  In response to this regimen, a significant increase in the mean values of the body and visceral fat weights were recorded in the sham-operated (group II) compared to normal rats (260±12gm and 7.86±1.1gm versus 210±13gm, 3.79±1.1gm respectively, p<0.01), whereas the prostatic weight did not show any significant variation between the two groups (36±7mg in group II versus 40±5gm in group I, p>0.05).

B- **Effect of Castration, Testosterone replacement therapy (TRT) and TRT in combination with aromatase blocker in castrated atherosclerotic rats (Group IIIA, IIIB & IIIC):**

- **Serum level of testosterone, lipid profile (TC, TG), CRP, adiponectin and IL-6 level:**

  As a result of bilateral orchiectomy, the serum levels of testosterone decreased to reach a minimum mean value of 0.3±0.01ng/ml in castrated vehicle-treated rats in group III A. On the other hand, in response to TRT, the castrated atherosclerotic rats of group III B showed a highly significant rise in the mean testosterone value thus reaching 10.8±1.3ng/ml. Our findings depicted a highly significant elevation in the levels of serum testosterone in group IIIIC (TRT+letrazole) with a mean value of 17±1.3ng/ml compared to group III B (p<0.01).

  Our findings showed a highly significant elevation in the mean values of TC and TG as seen in Table (2) after castration in oil-treated rats, in which mean values reached 96.06±5.69mg/dl and 200.30±7.47mg/dl respectively. However, upon TRT, the present results recorded a highly significant reduction in TC & TG in the castrated testosterone-treated rats compared to castrated vehicle-treated in which mean values reached 51.57±10.2mg/dl and 121.93±9.7mg/dl respectively (p<0.01). In Table (2), our results showed also a highly significant reduction in the levels of TC & TG in testosterone+letrazole-treated (67.53±10.31mg/dl and 156.80±5.45mg/dl respectively) compared to oil-treated (p<0.01).

  As shown in Fig. (3a,b), castration resulted in a significant elevation in serum levels of CRP in which mean values reached 2.28±0.50 µg/ml. In response to TRT, our results depicted highly significant reduction in the levels of serum CRP compared to oil-treated and reaching mean value 1.13±0.42 µg/ml a highly significant reduction in the levels of CRP in TRT+letrazole-treated group with mean values of 1.49±0.26 compared to oil-treated, p<0.01.

  Castration resulted in a significant reduction in the serum levels of adiponectin and reaching mean value 5.01±0.523pg/ml. The effect of testosterone administration on the serum levels of adiponectin in our experiments showed a significant elevation in the mean value of testosterone-treated group 8.60±0.85pg/ml compared to oil-treated, (p<0.01), our results recorded a highly significant reduction in the levels of CRP in TRT+letrazole-treated group with mean values of 1.49±0.26pg/ml compared to oil-treated, p<0.01. As shown in Fig. 3b, castration resulted in a significant elevation in serum levels of IL-6 in which mean values reached 870.55±107.68pg/ml. In response to TRT, our results depicted highly significant reduction in the levels of IL-6 compared to oil-treated (p<0.01). IL-6 in TRT+letrazole-treated group with mean value of 348.54±86pg/ml in comparison to oil-treated (p<0.01) but a significant elevation was observed when compared to testosterone-treated (p<0.05).

- **Gene expression levels of IL-6, ER- α and ADEPR1 and ADEPR2 levels in adipose and aortic tissue:**

  As shown in Fig. (4a,b), gene expression levels of the IL-6 in castrated vehicle-treated group showed significant increase with a mean value of 2.88±0.42 A.U in adipose tissue. In addition, a similar elevation in the expressed levels of IL-6 in aortic tissue was observed and the mean value reached 2.92±0.58 A.U. Our results depicted a significant reduction in the levels of IL-6 gene expression in adipose and aortic tissues in TRT+letrazole-treated rats with mean values of 1.68±0.75
and 1.13±0.3 A.U compared to oil-treated (p<0.05). On the other hand, there was no significant elevation when compared to testosterone-treated group (p>0.05).

Our findings depicted a significant decline in ER-α gene expression in adipose and aortic tissue in castrated vehicle-treated group 0.51±0.13 A.U and 0.34±0.05 A.U respectively. With TRT, a significant elevation in the expressed levels of ER-α in adipose and aortic tissues was recorded with mean values of 0.72±0.135 A.U and 1.12±0.34 A.U respectively compared to castrated-oil treated (p<0.01). Our findings showed a significant elevation in the gene expressed levels of ER-α in testosterone+letrazole-treated in adipose and aortic tissues (1.39±0.52 A.U and 1.53±0.32 A.U) compared to oil-treated (p<0.05). Similarly, these mean values displayed a significant elevation when compared to testosterone-treated (p<0.05).

Castration was revealed also to have an inhibitory impact on ADIPR1 rather than on ADIPR2 gene expression levels in adipose and aortic tissues since mean values of ADIPR1 reached in castrated vehicle-treated group 0.46±0.621 A.U and 0.24±0.17 A.U respectively. As regards ADIPR2, mean values reaching 0.35±0.143 A.U and 0.54±0.12 A.U respectively in adipose and aortic tissues. Upon TRT, results showed a significant elevation in the levels of ADIPR1 in adipose and aortic tissue with mean values of 0.67±0.182 A.U and 0.56±0.15 A.U respectively compared to oil-treated (p<0.05), whereas, ADIPR2 after TRT in adipose and aortic tissues, recorded insignificant difference when comparing the testosterone treated 0.41±0.173 A.U and 0.58±0.14 A.U with the oil-treated (p>0.05).

There was a significant increase in the levels of ADIPR1 in TRT+letrazole-treated in adipose and aortic tissues (0.63±0.32 A.U and 0.48±0.15 A.U respectively) compared to oil-treated (p<0.05). Nevertheless, these values did not display any significant difference compared to testosterone-treated (p>0.05). As regards ADIPR2 there was no significant difference between testosterone + letrazole (0.43±0.59 A.U and 0.60±0.11 A.U respectively) compared to oil and testosterone treated groups (p>0.05).

- Body weight, visceral fat weight and prostatic weight:

The castrated oil-treated animals displayed significant increase in the body weight and visceral fat weight depicted in the recorded mean values 350±12.1gm and 17.4±3.2gm. The prostatic weight in response to castration was significantly declined in its mean value which is 21±3mg.

In response to TRT, throughout experimental period (i.e 8 weeks) the animals showed a significant decrease in the mean values of body and visceral fat weights (220±9gm and 5.6±1.3gm respectively) when compared to castrated-vehicle-treated group (p<0.05). The prostatic weight in response TRT, on the other hand was recorded to show a highly significant elevation in its mean value (60±23mg) compared to groups of oil (p<0.05), there was a significant reduction in the levels of body and visceral fat weights in testosterone+letrazole-treated (238±3gm and 5.8±3.1gm respectively) compared to oil-treated rats of III A (p<0.05). However, there was no significant variation in body weights and visceral fat in testosterone+letrazole-treated group compared to testosterone-treated (p>0.05). As regards the prostatic weight, there was a significant elevation in testosterone+letrazole treated (72±21mg) compared to oil-treated and to testosterone-treated (p<0.01).

C- Histopathological analysis:
Changes in adipose tissues:

In normal group I, adipocytes showed normal structure and of average size adipocytes with normal vascularity and connective tissue. In group II atherosclerotic tissues revealed that some adipocytes were distended with, mild vascular were congestion and minimum infiltrating cells (Fig. 5a). As a result of castration inflammation was clearly apparent since the adipose tissue showed multiple distended adipocytes, obviously congested vessels and dense cellular infiltration (Fig. 5b). In group III B in response to TRT a high improvement was recorded since adipocytes were observed to acquire the average size with diminished congestion of blood vessels and inflammatory infiltration average sized adipocytes and minimal infiltration (Fig. 5c). Similarly in group III C after use of aromatase inhibitor with TRT only, some adipocytes were distended and minimal infiltrate was evident (Fig. 5d).

- Changes in the aorta:

In the control group (group I), the normal structure of vascular intima was demonstrated. In sham-operated group (group II) some foam cells and few dark nuclei of smooth muscle cells (SMCs) were observed indicating progress of the disease in the media (Fig. 6a). In response to castration (group III A) the media revealed more prominent picture since multiple foam cells and multiple dark nuclei of SMCs with apparent increased thickness were revealed (Fig. 6b).
When the atherosclerotic castrated group was treated with testosterone (IIIB) a great attenuation in the infiltrating inflammation cells was observed since a few foam cells and few dark nuclei of SMCs were detected (Fig. 6c). Inhibition of aromatization was induced in atherosclerotic castrated group treated with testosterone and letrozole (group III C) histopathological analysis showed multiple foam cells, some dark nuclei of SMCs and some thickened parts of media (Fig. 6d).

Table (1): Comparison between mean values ± SD of serum total cholesterol and triglycerides in groups normal control (group I) and sham-operated (group II).

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<th>Parameters</th>
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<td>Group I</td>
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<td>37.62±5.67</td>
<td>63.92±15.62</td>
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<tr>
<td>Group II</td>
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<td>61.4±5.1*</td>
<td>150.67±10.2*</td>
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* Significant (p<0.05) in comparison to group I.

Table (2): Comparison between mean values ± SD of lipid profile (total cholesterol and triglycerides) in groups II, IIIA (saline-treated) and IIIB (OT-treated).

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<td>Group III A</td>
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# Significant (p<0.05) in comparison to group III A.
@ Significant (p<0.05) in comparison to group III B.

Fig. (1 A): Comparison between the mean values ± SD of serum levels of CRP and adiponectin in groups I (control) and II (sham-operated). Values are expressed as µg/ml for CRP and ng/ml for adiponectin.

Fig. (1B): Comparison between the mean values ± SD of serum levels of IL-6 in groups I (control) and II (sham-operated). Values are expressed as pg/ml.

Fig. (2A): Comparison between the mean values ± SD of the gene expression levels of IL-6, ER-α, ADIPR1 and ADIPR2 in adipose tissue in groups I (control) and II (sham-operated). Values are expressed as arbitrary unit (A.U).

Fig. (2B): Comparison between the mean values ± SD of the gene expression levels of IL-6, ER-α, ADEPR1 and ADEPR2 in the aorta in groups I (control) and II (sham-operated). Values are expressed as arbitrary unit (A.U).
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II (Oil-treated) IIIA (Testosterone-treated) IIIB (Testosterone + Letrazole-treated)

CRP (µg/ml) Adiponectin (ng/ml)

@: Significant difference ($p<0.05$) in comparison to group III A.
#: Significant difference ($p<0.05$) in comparison to group III A and IIIB.

Fig. (3A): Comparison between the mean values ± SD of serum levels of CRP and adiponectin in groups II (sham operated), IIIA (oil treated), IIIB (testosterone treated) and IIIC (testosterone + letrozole treated). Values are expressed as µg/ml for CRP and ng/ml for adiponectin.

II (Sham) IIIA (Oil-treated) IIIB (Testosterone-treated) IIIC (Testosterone+ Letrazole-treated)

IL-6 (pg/ml)

@: Significant difference ($p<0.05$) in comparison to group III A.
#: Significant difference ($p<0.05$) in comparison to groups III A and IIIB.

Fig. (3B): Comparison between the mean value ± SD of IL-6 in groups II (sham-operated), IIIA (oil-treated), IIIB (testosterone-treated) and IIIC (testosterone + letrozole-treated). Values are expressed in pg/ml.

II (Sham-operated) IIIA (Oil-treated) IIIB (Testosterone+ Letrazole-treated) IIIC (Testosterone+ Letrazole-treated)

IL-6, ER-α, Adiponectin 1 receptor, Adiponectin 2 receptor

@: Significant difference ($p<0.05$) in comparison to group III A.
#: Significant difference ($p<0.05$) in comparison to groups III A and IIIB.

Fig. (4A): Comparison between the mean values ± SD of the gene expression levels of IL-6, ER-α, ADEPR1 and ADEPR2 I in the adipose in groups II (sham-operated), IIIA (oil-treated), IIIB (testosterone-treated) and IIIC (testosterone + letrozole treated). Values are expressed as arbitrary unit (A.U).

II (Sham-operated) IIIA (Oil-treated) IIIB (Testosterone-treated) IIIC (Testosterone+ Letrazole-treated)

IL-6, ER-α, Adiponectin 1 receptor, Adiponectin 2 receptor

@: Significant difference ($p<0.05$) in comparison to group III A.
#: Significant difference ($p<0.05$) in comparison to groups III A and IIIB.

Fig. (4B): Comparison between the mean values ± SD of the gene expression levels of IL-6, ER-α, ADEPR1 and ADEPR2 I in the aorta in groups II (sham operated), IIIA (oil treated), IIIB (testosterone treated) and IIIC (testosterone + letrozole treated). Values are expressed as arbitrary unit (A.U).
Discussion

Since the dietary high-fat content is one of the most participating factors of experimental atherosclerosis [23], thus we applied a high fat-diet regimen for a duration of 8 weeks. This regimen promoted a subsequent state of hyperlipidemia which were depicted in the estimated highly significant lipid profiles (Total Cholesterol and Triglycerides), also a significant progressive elevation in the mean body weight of the high-fat fed rats was demonstrated. This emphasizes the current concept in
which the rich cholesterol content in the diet may be a critical contributer to whole-body obesity and metabolic syndrome [24].

To further participate the atherosclerotic events, the present study carried out the procedure of housing the experimental animals in groups of five confined in narrow cages. This procedure has established that the effects of stressful chronic social conditions, even if mild, can attribute with the whole-body obesity to an increased incidence of atherosclerosis and other cardiovascular diseases [25].

The present assessment of the marked elevation in the serum levels of IL-6 as well as its gene expression, supports previous data that concluded that about one third of the circulatory IL-6 at high levels arises from adipocytes and associates adipose tissue inflammation [26].

The present work revealing significantly augmented levels of the high sensitive CRP in the hyperlipidemic animals, focus the attention that biomarker is a strong independent predictor of the subsequent events in cardiovascular patients and even mortality. In addition, since CRP is a marker of cytokines, its significant increase suggests a direct potential proinflammatory role on the endothelial cells and the mediation of LDL uptake by macrophages [27].

In response to high-fat diet regimen, the present study recorded significantly reduced levels of serum adiponectin as well as the gene expression levels of its receptors 1&2. Thus, in view of recent reports we tend to believe that associating this diet regimen, the drastic state of hypoadiponectinemia which developed, may have predisposed or even participated in the initiation and acceleration of the atherosclerotic progress [28].

Several explanations have been proposed to explain the atheroprotective properties related to adiponectin. The latter reduces lipid accumulation in macrophages, and inhibits their proliferation thus preventing their transformation into foam cells. Furthermore, it promotes endothelial differentiation, controlling vascular healing and stimulating angiogenesis [29,30].

It was earlier proposed by interesting studies, that estrogen through its binding with ER- a , can act directly through its genomic action to abolish the atherosclerotic effects [31], and indirectly by activating other sex steroid hormone receptor expression [32]. This incited us to assess ER-alpha gene expressed levels not only the existence of these receptors in adipose and vascular tissues was identified but also, the estimated levels displayed significant deterioration in the sham-operated rats remove in response to hyperlipidemia.

Our findings conveyed early infiltration of increased numbers of macrophages in the visceral tissue. These invading macrophages are suggested to be initiated by the increase in the adipocytic size. Apparently, the latter helps to evaluate the extent of the adipocyte inflammation that joins atherogenesis [33]. Once inside the tissues, the differentiated macrophages significantly secrete large quantities of proinflammatory cytokines mainly TNF-α, IL-6 and IL-1 [34].

To further confirm the initiation and progress of these pathogenic events, significant numbers of foam cells originating from scavenging macrophages, the development of these lipid-laden macrophages is a hallmark of both early and late stages of atherosclerosis [35].

Hyperlipidemia reflected in the current work led to a state of lipid deposition in the vascular wall in the form of cholesterol-ester-enriched fatty streaks rather than the mature plaque observed in the human atheroma.

The present findings are confirming to the fact that castration per se results in deterioration and early significant elevation in all the parameters related to initiation and progression of atherosclerotic pathogenesis. These findings emphasizes the concept that testosterone is a potent regulator of lipolysis that influences catecholamine signal transduction in fat cells, by increasing β3-adrenergic receptor-mediated signals to lipolysis at multiple steps in the lipolytic cascade [36].

In this study, supra-physiological doses of testosterone were chosen as a replacement therapy it seems to be the most appropriate in having a significant impact on the serum lipids, lipoproteins and apolipoproteins [37].

The significant reduction in the serum lipid profile, CRP and IL-6 levels in letrozole-treated, although did not yield the same significance as that of testosterone-treated rats, yet it still signifies these testosterone-mediated CV benefits, and ultimately confirms the undeniable significant impact of estrogen via aromatization in these pathological events. Thus, the impaired estrogen production after castration can partially explain the high serum lipid profile.
Reduced estrogen production results in lowering hepatic triglyceride lipase which degrades LDL, stimulates the synthesis of HDL and apolipoprotein A and also improves the reverse cholesterol transport [38].

The present study revealed TRT with or without letrozole resulted in a significant increase in the circulating adiponectin and stimulated the expressed levels of adiponectin receptor 1. However, opposing data hypothesized that adiponectin expression and secretion are unaffected by sex steroids [39], they were suggesting the existence of a serum factor that is differently regulated by sex steroids. We may explain however, this discrepancy by the fact that the hyperlipidemic state and the inflammatory markers participated in this state of hypoadiponectinemia or may even have initiated it.

Whereas, the impact of TRT on adiponectin secretion and its receptor 1 gene expression may not be direct, but rather through its inhibitory role on the inflammatory markers and through its reduction of the mean body weight and visceral fat mass which seemed to improve the adipocytic differentiation.

In our study ER-α gene expression levels have shown a significant increase compared to the oil-treated, and testosterone-treated which may be explained these findings by an upregulation of these expressed receptors by the decreased estrogen levels.

The histological findings also confirmed in the letrozole-treated rats the marked improvement which was reflected in the reduced signs of inflammation (macrophages and foam cells formation) infiltrating the vascular and adipose tissues. Nevertheless, these results did not reach the same significant values compared to the atherosclerotic rats treated with testosterone only, which confirm the significance of aromatization in these events.

In conclusion, the high fat diet and social stress play a vital role in the development of atherosclerosis. Moreover, androgen deprivation resulted in deterioration of the condition. The results also emphasize the suggestion that testosterone replacement therapy could be a potential prophylactic and even a therapeutic agent in controlling the disease, further studies are still needed to help guide the evolution of treatment recommendations. In particular, these studies may contribute to setting research priorities for clinical, translational and basic scientists to further explore the area of testosterone therapy in cardiovascular diseases, and especially in the events of atherosclerosis.

References


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