OnLine Journal of Biological Sciences

Original Research Paper

Reduced Levels of Testosterone Induce LDL Oxidation and Atherosclerotic Lesions Involving Inflammatory Imbalance and Reduced Macrophage Apoptosis

[1]Placielle Fiorezi Filete, [1]Flávia de Souza Andrade Moraes, [1]Girlandia Alexandre Brasil, [1]Ewelyne Miranda de Lima, [1]Bianca Prandi Campagnaro, [1]Dominik Lenz, [1]Denise Coutinho Endringer, [2]Dulcineia Saes Parra Abdalla, [3]Nazaré Souza Bissoli, [1]Thiago de Melo Costa Pereira and [1]Tadeu Uggere de Andrade

[1]Pharmaceutical Sciences Graduate Program, University Vila Velha, Vila Velha, ES, Brazil
[2]School of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil
[3]Department of Physiological Sciences, Health Sciences Center, Federal University of Espirito Santo, Vitória, ES, Brazil

Article history
Received: 04-09-2019
Revised: 13-11-2019
Accepted: 05-12-2019

Corresponding Author:
Tadeu Uggere de Andrade
Pharmaceutical Sciences Graduate Program, University Vila Velha, Vila Velha, ES, Brazil
Tell: +552734212001
Fax: +552734212000
Email: tadeu.andrade@uvv.br

Abstract: Atherosclerosis is among the major cardiovascular diseases that cause death in the world. Men who are deficient in androgen hormones are more susceptible to this disease. The aim of this study was to evaluate the influence of reduced levels of testosterone on the lipid profile, pro and anti-inflammatory cytokines, apoptosis of peritoneal macrophages and the development of atherosclerosis in LDLr-/-mice, as well as on the levels of OxLDL in healthy male humans. Tissues and organs of the experimental animals were used to evaluate lipid deposition in aorta and apoptosis of peritoneal macrophages. In humans the levels of testosterone and OxLDL were analyzed in blood samples from healthy male subjects, who were divided into two groups: low level and high level of testosterone. Animals with decreased testosterone did not present changes in the lipid profile or the levels of IL-6 and TNF-α. However, a decrease in IL-10 and an increase in the IL-6/IL-10 and TNF-α/IL-10 ratios were observed. An increase in the aorta lipid deposition was observed and a decrease in macrophage apoptosis was also demonstrated. In humans, there was an increase of OxLDL in the group with lower levels of testosterone. The findings suggest that decreasing endogenous testosterone levels may induce the development of atherosclerosis, with participation of inflammatory response and macrophage apoptosis. This is reinforced by our human studies, where individuals with low testosterone levels had higher LDL oxidation, which may predict a high atherosclerotic risk for these men.

Keywords: Androgens, Atherosclerosis, Apoptosis, Oxidized LDL

Introduction

The leading causes of death in the world are related to cardiovascular diseases (Usman *et al.*, 2015). Some metabolic conditions may act as protectors and make some individuals less likely to develop these diseases. In this context, the protective effects of androgens on the cardiovascular system have been reported previously (Wu and von Eckardstein, 2003). A prospective study showed an increase in cardiovascular risk in men whose serum testosterone levels were decreased, which was independent of other risk factors (Kelly and Jones, 2013). Additionally, androgen deprivation therapy also increases the risk of cardiovascular disease (Rojas *et al.*, 2015). Androgen replacement in the population that is deficient in these hormones has been associated with a reduction in mortality (Frostegård *et al*., 1999).

Atherosclerosis is among the leading cardiovascular diseases in men with low testosterone levels (Araujo *et al*., 2011). Despite the higher incidence of cardiovascular disease in men compared to women, studies suggest that androgens, of which the major one is testosterone, protect

man against atherosclerosis (Wu and Von Eckardstein, 2003). Such protection is due to the fact that testosterone plays an important role in the regulation of carbohydrate, lipid and protein metabolism and positively affect the control of glucose, liver fat and cardiac biomarkers (Kirby *et al*., 2019; Ng Tang Fui *et al*., 2017; Rezanezhad *et al*., 2018), however, the mechanisms are not completely elucidated.

Atherosclerosis is an inflammatory and progressive disease characterized by endothelial dysfunction, vascular inflammation and accumulation of lipids in the intimal layer of medium- and larger-caliber arteries (Moore *et al*., 2013), where changes promote cardiac events, such as myocardial infarction and stroke, as a response to the rupture of atherogenic plaque (Usman *et al*., 2015).

Low density lipoprotein (LDL) is an acute phase mediator of atherosclerotic disease. At high levels, it is involved in the organization of the lipid nucleus and the polarization of circulating monocytes. In plaques, LDL is especially susceptible to oxidation, forming oxidized LDL (OxLDL) (Rojas *et al*., 2015). As a response to these molecules, T cells are activated and release adhesion molecules that provide recruitment of monocytes from the circulation to tissue, which is differentiated into macrophages (Frostegård *et al*., 1999; Ilhan and Kalkanli, 2015; Rojas *et al*., 2015). As a result, an increase in inflammation can be observed by the activation of T helper-1 (Th1) cells; the factors released by these cells contribute to the development of atherosclerosis (Frostegård *et al*., 1999).

Inflammatory mediators seem to initiate the phonotypical modulation of macrophages on the arterial wall during atherogenesis (Ley *et al*., 2011). Activated macrophages can increase plaque vulnerability, while an alternative phenotype can promote stability and regression of plaque for minimizing inflammation (Viola and Soehnlein, 2015). Cytokines are closely related to the progression of atherosclerotic lesions. According to Ait-Oufella *et al*. (2011) all cells involved with atherosclerosis are able to produce and respond to cytokines, which play a dual role in the disease, with pro- or anti-inflammatory responses.

Moore *et al*. (2013) reported that macrophages are important in inflammatory responses, which is attributed to their capacity to sustain pro-inflammatory factors and to their apoptosis, which contributes to a necrotic nucleus prone to rupture. Macrophage apoptosis has been identified as a key characteristic of atherosclerotic plaques in all phases of the lesion (Tabas, 2007). Macrophage apoptosis can be triggered by a combination of factors, according to the phase of the disease (Tabas, 2005) and this can affect plaque progression in different ways (Gautier *et al*., 2009). In early atherosclerotic lesions, macrophage apoptosis is associated with the reduction of plaque progression. However, in advanced atherosclerotic lesions, it promotes an increase in injury and an increased risk of plaque rupture (Lammers *et al*., 2011).

Among the in vivo experimental models that are used to study atherosclerosis, LDL receptor knockout (LDLr-/-) mice are widely used. This is due to the fact that these animals present metabolic changes and spontaneous lipid deposition in the aorta induced by aging or through interventions that characterize risk factors, such as the use of atherogenic diet (Hatch *et al*., 2012), castration (orchiectomy and ovariectomy) (de Oliveira *et al*., 2013; Hopmans *et al*., 2014) or treatment with supraphysiological doses of androgenic anabolic steroids (de Andrade *et al*., 2018). Moreover, the LDLr-/- are chosen for this study because mice present a lipoprotein profile similar to hyperlipidemic humans, since the cholesterol concentrates on the LDL fraction (Zadelaar *et al*., 2007).

Usually, many studies are performed using the induction of risk factors associated with the use of atherogenic diet (Hatch *et al*., 2012; Merat *et al*., 2000). However, atherogenic diets alone are responsible for metabolic changes that lead to the development of other pathogenic processes that may be confused with the object of study (Hopmans *et al*., 2014). Thus, the study of the development of atherosclerosis in experimental animals fed normal diet is justified, aiming to eliminate possible interferences that may be caused by the high fat diet (Hopmans *et al*., 2014).

Therefore, the aim of the present study was to evaluate the lipid profile, pro- and anti-inflammatory cytokines, apoptosis of peritoneal macrophages and the development of atherosclerosis in LDLr-/- and C57BL/6 mice subjected or not to orchiectomy. Additionally, we translationally evaluated the blood levels of testosterone and OxLDL in healthy, middle-aged men with different blood level patterns of testosterone.

Methods

Animals and Experimental Groups

Eight-week-old LDLr-/- and C57BL/6 Wild-Type (WT) mice were used, with a corporal mass between 20-30 g. The animals were separated into the following four groups (n = 8 each): WT and LDLr-/- (controls subjected to sham surgery); and WTOrx and LDLr-/-Orx (WT and LDLr-/-submitted to orchiectomy). The animals were obtained by the Research Laboratory of the Faculty of Pharmaceutical Sciences of USP/SP and were kept in a living room of the University Vila Velha (UVV). The animals were maintained in controlled conditions with a light-dark cycle of 12 h and a temperature of 22°C. The individual cages provided free access to water and food (Standard chow, Probiotério, Moinho Primor, S.A). All the experimental procedures were performed in accordance with the USA National Institutes of Health (Garber *et al*., 2010) and were approved by the Committee on Ethics in the Use of Animals of the University Vila Velha (CEUA-UVV, Protocol no. 191/2011).

Surgery Procedure

The animals subjected to orchiectomy were anesthetized with a mixture of ketamine and xylazine (11.5/1.15 mg/kg of body weight, intraperitoneal-i.p.) and after deep anesthesia, the scrotum was cleaned and a small incision was made. The tunic was opened and the testis, epididymis syrup, deferent duct and blood vessels were exteriorized. The blood vessels and deferent ducts were cauterized and the testis and epididymis were removed. The remaining tissue was returned to the scrotal sac and a suture was made. This procedure was repeated on the other testicle.

The local site of the incision was cleaned with iodopovidone and an injection of benzathine penicillin G and procaine penicillin G (60.000 U each, intramuscular) was applied in each mouse. After the surgical procedure, the animals were maintained in individual cages with free access to water and food until complete recovery (Zhang *et al.*, 1998).

Experimental Protocol

Four weeks after the surgical procedure, all the animals were anesthetized with a mixture of ketamine and xylazine (11.5/1.15 mg/kg of body weight, i.p.) and then euthanized. The blood was collected by a cardiac puncture to determine the serum levels of testosterone and cytokines as well as the lipid profile and the aorta was collected to quantify the lipid deposition. The other group of animals was separated to perform the isolation of the peritoneal cells to determine the level of macrophage apoptosis.

Biochemistry Analysis

The blood was collected in plastic dry tubes from the cardiac puncture. The plasma was separated by centrifugation (3500 rpm, 4°C, 15 min) and maintained at -20°C until used. The total cholesterol (TC) and HDL cholesterol (HDL-C) were determined using commercial kits (BIOCLIN, Minas Gerais, Brasil). In addition, the non-HDL cholesterol was determined by calculating the difference between the TC and the HDL-C (Daleprane *et al.*, 2012).

Quantification of Serum Testosterone

The determination of serum testosterone was made by ELISA using commercial kits in agreement with the manufacturing instructions (*In vitro* diagnostica, Minas Gerais, Brasil). The assay was made in duplicate and read at 450 nm using an ELISA Reader (Filter Max F5, Molecular Devices LLC, Austria).

Quantification of Pro- and Anti-Inflammatory Cytokines

Interleukin-6 (IL-6), Tumor Necrosis Factor alpha (TNF-α) and interleukin-10 (IL-10) were simultaneously quantified using the *Cytometric Bead Array Mouse* commercial kit (BD Biosciences, San Diego, CA, USA) in agreement with the description by Menezes *et al*. (2012). The analysis was made by flow cytometry using the animal's plasma. The ratio between pro-inflammatory (TNF-α and IL-6) and anti-inflammatory (IL-10) cytokines was obtained by division of the correspondent mean.

Determination of Apoptosis of Peritoneal Cells

Male LDLr-/- mice and wild-type C57BL/6 mice (WT) subjected to orchiectomy or sham surgery (sham) received an intraperitoneal injection with 1 mL of sterile thioglycolate (3% p/v. Sigma-Aldrich, St. Louis, MO, EUA) four days before their use. After, the animals were euthanized and the peritoneal cells were collected by washing the peritoneal cavity, using sterile cold PBS (Pedrosa *et al.*, 2010). To determine the apoptotic cells, anexin V (FITC) and Propidium Iodite (PI) were used as markers, which was followed by a flow cytometer analysis. Ten thousand events were registered from each sample and the macrophage populations were determined by morphological parameters, such as size and granularity. The procedure was performed in agreement with Tonini *et al*. (2013); briefly, 1×10^6 cells/mL were used in the assay and received 2 μL of anexin V and PI; after incubation, an analysis was performed using a flow cytometer (FACS Canto II; Becton Dickinson) at 585 nm and 530 nm. On the dot plot graph, the quadrants Q1 contains cells positive only to PI; Q2 contains necrotic or late apoptotic cells; Q3 contains live cells; and Q4 shows early apoptosis. Apoptosis was calculated as the sum of Q2 and Q4. For the positive control, H_2O_2 was used.

The Morphological Analysis of Lipid Deposition

For the analysis of lipid deposition, briefly, the aorta was completely dissected and maintained in a solution of PBS-formalin (10%; 15°C) for 24 h. After cleaning, a symmetric cut was performed and the aorta was fixed onto a surface of ethyl vinyl acetate with steel pins. After, the aorta was stained with Oil-Red-O (Sigma-Aldrich, St. Louis, MO, EUA) for *"en face"* analysis (Paigen *et al.*, 1987; Xu, 2006). The lipid deposition was evaluated by images obtained with a high-resolution camera (Canon T5 18-55; Canon, USA) and analyzed using the public software "ImageJ" (National Institute of Health, USA), by a researcher blind to experimental groups.

Human Testosterone and Oxidized LDL Analyses

To perform the analyses of testosterone and oxidized LDL on human serum, male healthy subjects were invited to participate. The research was performed following all the instructions of the Helsinki Declaration (WMA, 2008) and was approved by the Human Ethics Committee of University Vila Velha (#399751). All the participants provided informed consent and signed the consent form. The inclusion criteria were heathy men between ages 40-55 who had no apparent physical or physiological

problems. The exclusion criteria were previous use of alcohol, smoking or drug abuse, anabolic androgenic steroid use and other health problems. The patients' blood was collected and separated. The serum was maintained frozen (-80°C) until use. The serum testosterone and oxidized LDL were determined using ELISA commercial kits (Elabscience ® Houston, Texas, USA), following the manufacturer's instructions. The subjects were divided into two groups according to the level of testosterone as follows: Low level of testosterone (LT; below 350 ng/dL) and high level of testosterone (HT; above 350 ng/dL). This cut point of testosterone is in accordance with English *et al*. (2000), that showed men with arterial cardiovascular disease were under low total testosterone levels (around 12 nmol/L, which means 346 ng/dL). In both groups, the subjects were under the normal range of the testosterone levels, but with lower or higher levels.

Statistical Analyses

The results were expressed as the mean ± Standard Error of the Mean (S.E.M). The data were analyzed using a one-way Analysis of Variance (ANOVA) followed by the Tukey post hoc test, adjusted for multiple comparisons; significance was accepted when $p<0.05$. The statistical analysis was performed using Prism software (Prism6, GraphPad Software, Inc., San Diego, CA, USA), with the exception of the flow cytometry data, which was analyzed using DIVA software and FCS Express Flow Research Edition.

Results

Biochemical Analysis

Figure 1 shows the results of the cholesterol profile of the groups (TC, HDL-C and nHDL-C). The LDLr-/- group had an increase in TC compared to the others (WT and WTOrx) and the orchiectomy did not change the total cholesterol (Fig. 1, Panel A. WT = 114±11; WTOrx = 81.4±6.1; LDLr-/- = 174.4±14; LDLr-/-Orx=203.6±15 mg/dL) or the nHDL-cholesterol (Fig. 1, Panel B; WT= 86±13; WTOrx = 60±7; LDLr-/- = 148±13; LDLr-/-Orx = 185±22 mg/dL) between groups. The same observation was made for HDL cholesterol (Fig. 1, Panel C; WT = 28±3; WTOrx = 23±2; LDLr-/- = 26±1; LDLr-/-Orx = 21±8 mg/dL).

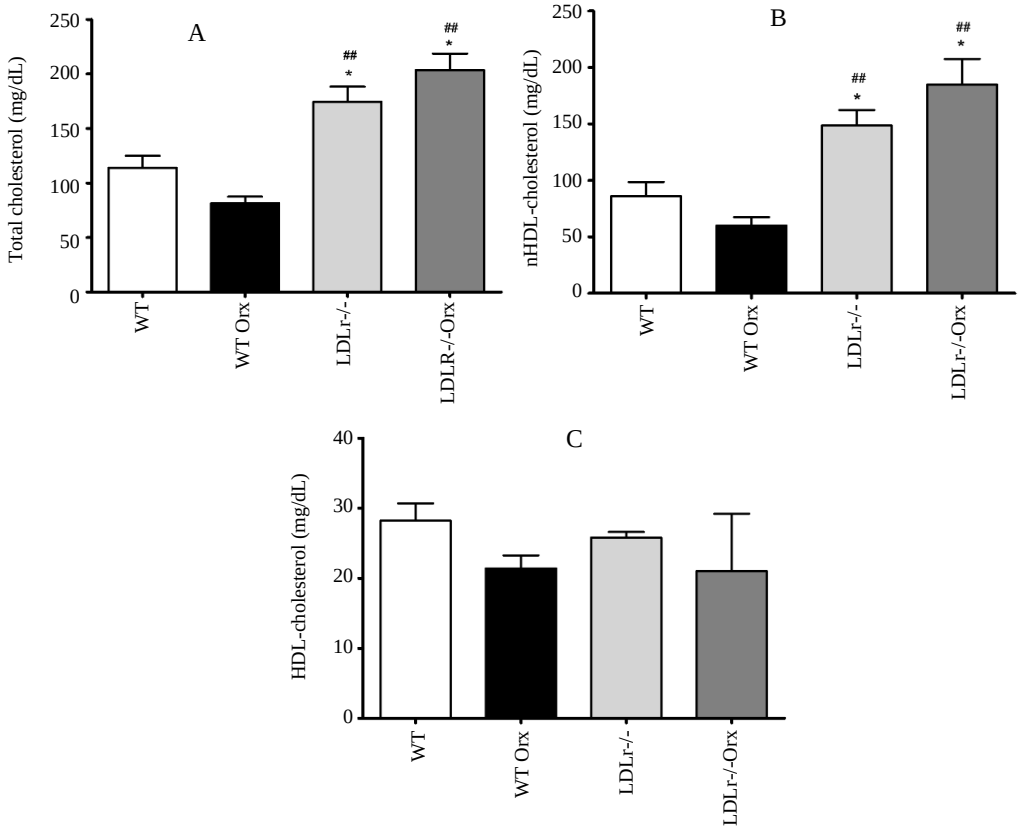

Fig. 1: Results from the biochemical analysis of the serum; an increase in both total cholesterol and n-HDL cholesterol in knockout mice; however, no changes are observed in HDL cholesterol. Panel A: Total cholesterol. Panel B: Values of non-HDL cholesterol. Panel C: Values of HDL cholesterol. Data are presented as the mean ± Standard Error of the Mean (S.E.M). $^{*}p<0.05$ compared to the WT group; $^{\#\#}p<0.01$ compared to the WTOrx group

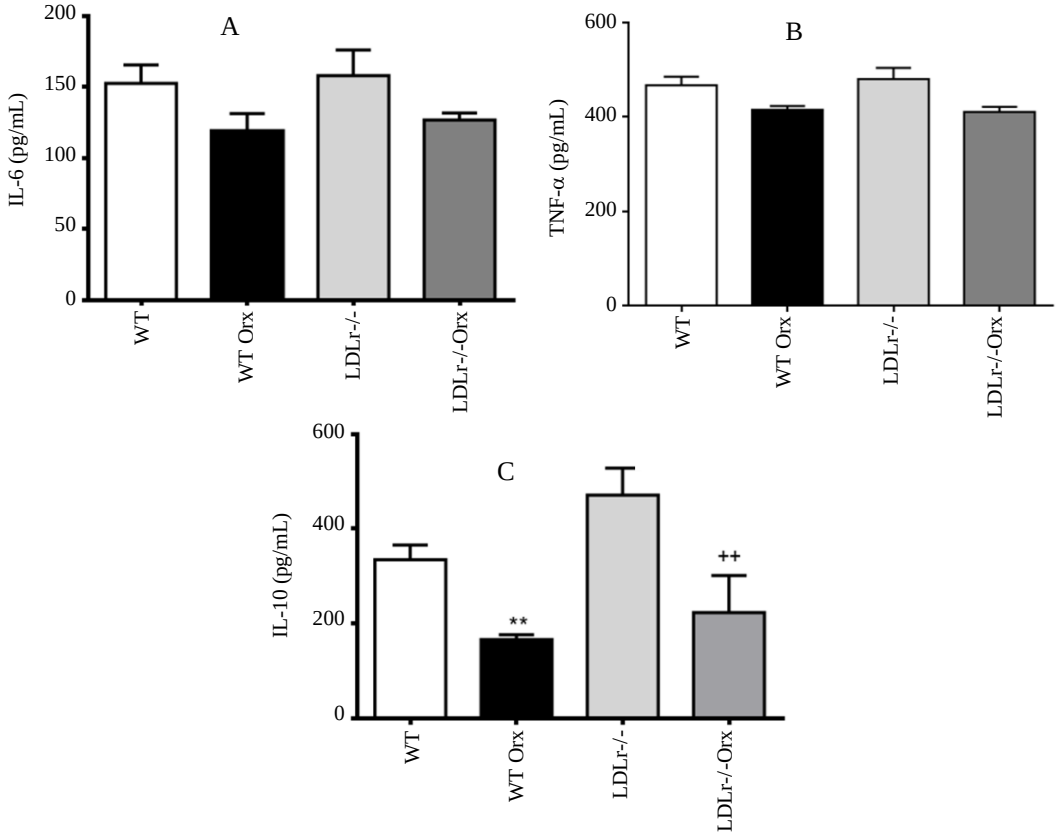

Fig. 2: Evaluation of the inflammatory profile of the animals. It is possible to observe that a decrease in testosterone promotes a change in the anti-inflammatory cytokine IL-10, while no changes in TNF-α and IL-6 are observed. Panel A: Values of IL-6 on the plasma. Panel B: Values of TNF-α on the plasma; Panel C: Values of the anti-inflammatory cytokine IL-10. Data are presented as the mean ± standard error of the mean (S.E.M). $^{**}p<0.01$ compared to the WT group; $^{++}p<0.01$ compared to the LDLr-/- group

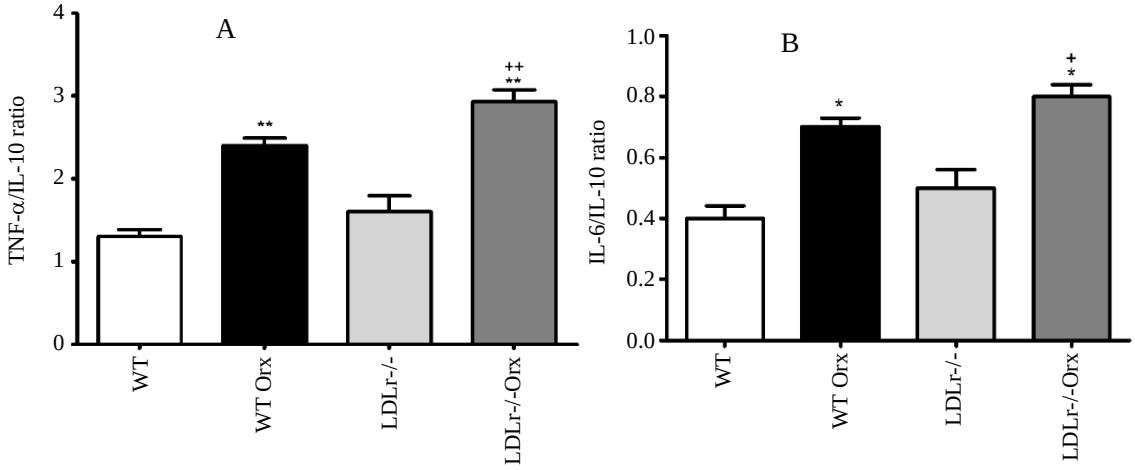

Fig. 3: Ratio of pro-inflammatory and anti-inflammatory cytokines on the serum of the animals. Despite no changes in the TNF-α and IL-6 amount, an imbalance of these cytokines is observed, demonstrating an increase in the inflammatory profile of the animals. Panel A: TNF-α/IL-10 ratio. Panel B: IL-6/IL-10 ratio. Data are presented as the mean ± standard error of the mean (S.E.M). $^{*}p<0.05$ compared to the WT group; $^{**}p<0.01$ compared to the WT group; $^{+}p<0.05$ compared to the LDLr-/- group; $^{++}p<0.01$ compared to the LDLr-/- group

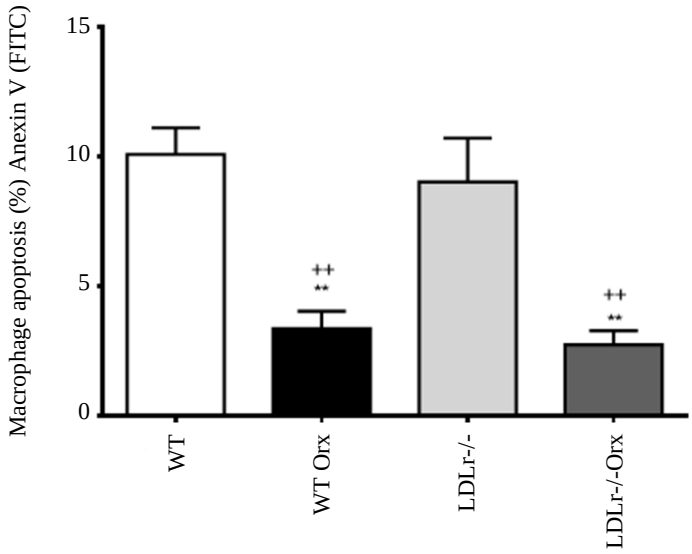

Fig. 4: Evaluation of macrophage apoptosis on peritoneal cells collected from the orchiectomized and sham groups. A decrease in apoptosis was observed in the orchiectomized groups. No difference was observed among the LDLr-/- and WT sham groups. Data are presented as the mean ± Standard Error of the Mean (S.E.M). $^{**}p<0.01$ compared to the WT group; $^{++}p<0.01$ compared to the LDLr-/- group

Amount of Animal Serum Testosterone

As expected, a decrease in serum testosterone was observed in all the orchiectomized animals (WT: 5.9±0.8; WTOrx: 0.35±0.05; LDLr-/-: 5.7±1.0; LDLr-/-Orx: 0.4±0.05 ng/mL), which confirms the surgical procedure.

Inflammatory Profile

The profiles of pro-inflammatory cytokines (IL-6 and IL-10) and anti-inflammatory cytokines (IL-10) are shown in Fig. 2. No differences were observed in IL-6 among the groups (Fig. 2. Panel A; WT = 152±13; WT Orx = 119±12; LDLr-/- = 158±18; LDLr-/- Orx = 127±5 pg/mL). The same observation was made for TNF-α (Fig. 2. Panel B; (WT= 466±18; WT Orx = 415±7; LDLr-/-= 480±23; LDLr-/- Orx= 411±10 pg/mL). However, IL-10 was decreased in orchiectomized animals compared to the control groups (Fig. 2, Panel C; WT = 387±26; WTOrx = 174±6; LDLr-/- = 433±56; LDLr-/- Orx = 145±7 pg/mL).

The ratios between TNF-α or IL-6 and IL-10 were observed. As can be seen in Fig. 3, Panel A, the ratio of TNF-α/IL-10 increased in both orchiectomized groups when compared to the controls (Fig. 3, Panel A; WT = 1.3±0.09; WTOrx = 2.4±0.09; LDLr-/- = 1.6±0.2; LDLr-/- Orx = 2.93±0.14 pg/mL). Additionally, the IL-6/IL-10 ratio increased in the same groups (Fig. 3, Panel B; WT= 0.45±0.04; WTOrx = 0.75±0.03; LDLr-/- = 0.43±0.06; LDLr-/- Orx = 0.75±0.04 pg/mL), indicating a pro-inflammatory status.

Macrophage Apoptosis

Orchiectomy of both wild-type and LDLr-/- animals indicated a reduction in macrophage apoptosis compared to the sham groups (WT 10.09±1.03% vs. WTOrx 3.38±0.68%; LDLr-/- 9.03±1.70%; LDLr-/- Orx 2.76±0.54%). However, no difference was observed between the WT and LDLr-/- groups (Fig. 4).

Aorta Lipid Deposition

As expected, the LDLr-/- animals showed an increase in lipid deposition when compared to the WT group. In addition, orchiectomy promoted an increase in lipid deposition in the orchiectomized groups when compared to the controls (Fig. 5; WT = 4.25±0.2; WTOrx = 8.4±0.4; LDLr-/- = 9.6±1.1; LDLr-/-Orx = 15.8±2%).

Human Testosterone and Oxidized Lipid Analyses

Forty-eight subjects signed the consent form and had their levels of testosterone and oxidized LDL measured. Eight subjects were excluded (one did not attend the day to collect blood sample; and 7 had declare previous health problem). The mean age was 47.5±0.7 years and the testosterone levels were considered normal in all the participants (361±23 ng/dL; reference values: 166 to 923 ng/dL). However, the testosterone levels between the two groups were different (LT: 232.7±12.16 (N = 23); HT: 398.1±16.41 ng/dL; (N = 17); P<0.05), which reflects the OxLDL levels, when two groups were compared (LT: 65.25±4.28; HT: 51.97±4.49 mU/L; p<0.01). The subjects from the group of low levels of testosterone showed higher levels of OxLDL compared with those from the HT group.

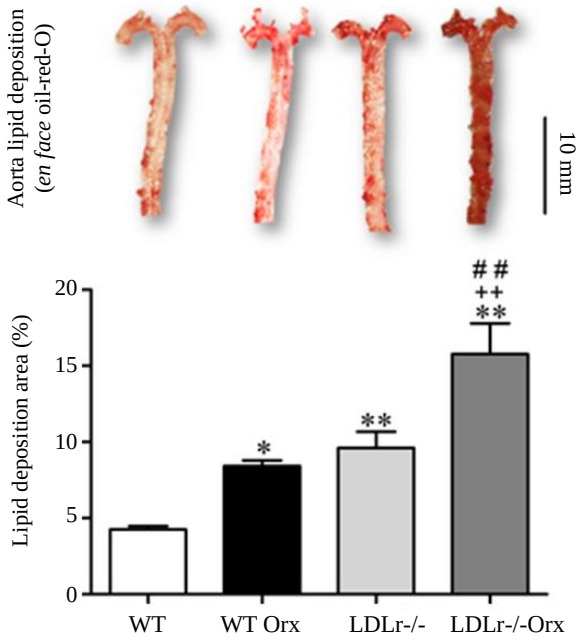

Fig. 5: Results of lipid deposition in the aorta of the animals. It is possible to observe an increase in lipid deposition after the removal of testosterone among the groups. Additionally, as expected in the LDLr-/- group, lipid deposition was greater than that of the WT group. On the upper panel are representative pictures of the aortas from each group. The graph presents the analyses of all the animals. Data are presented as the mean ± standard error of the mean (S.E.M). $^{*}p<0.05$ compared to the WT group; $^{**}p<0.01$ compared to the WT group; $^{\#\#}p<0.01$ compared to the WTOrx group; $^{++}p<0.01$ compared to the LDLr-/- group.

Discussion

The results of the present study demonstrated, for the first time, the influence of testosterone levels on atherosclerosis development in young male LDL receptor knockout mice that did not receive an atherogenic diet. Additionally, an imbalance was observed in pro- and anti-inflammatory cytokines, with a decrease in IL-10 levels and a decrease in apoptosis of macrophage cells. No change in the lipid profile was observed. Additionally, an increase in the oxidation of LDL was observed in human blood samples under low levels of testosterone.

The role of androgens on the pathogenesis of atherosclerosis has been the aim of many clinical (Khazai *et al*., 2016) and experimental investigations (Huang *et al*., 2014). However, the results are still controversial (Kapoor and Jones, 2008; von Dehn *et al*., 2001). Traish *et al*. (2009) reviewed the role of androgens in atherosclerosis and demonstrated that changes in the lipid profile promote oxidative stress and endothelial dysfunction, with increased release of pro-inflammatory factors, culminating in atherosclerosis development. Bourghardt *et al*. (2010) suggested that the effects on the lipid profile are not a prerequisite to the antiatherogenic effect of testosterone in LDLr^{-}-/- mice. Additionally, Nathan *et al*. (2001) used orchiectomized animals and observed no difference in the total cholesterol and triglyceride levels, but HDL levels were decreased. In that research (Nathan *et al.*, 2001), an occidental diet was used; thus, the decrease in HDL cholesterol could be attributed to the diet and not to testosterone levels.

However, our results demonstrated no changes in the lipid profiles. It seems reasonable to infer that the reduction in testosterone levels is more important than changes in the lipid profile to induce atherosclerotic lesions in orchiectomized animals. Our data corroborates the results found by Hatch *et al.* (2012), which demonstrated an increase in lipid deposition in LDLr-/- animals with a decrease in testosterone levels but under a lipid-rich diet.

The experimental model used in the present study indicates that levels of plasmatic cholesterol slightly increased when compared to wild-type animals and that there is a slowly developing atherosclerosis when using the chow diet (Merat *et al*., 2000), which mimics the development in humans (Emini Veseli *et al*., 2017). Therefore, the reduction in the male hormone, testosterone, should be as important as a lipid-rich diet for atherosclerosis development.

The inflammatory process associated with atherosclerosis has been studied for the last few years and has demonstrated importance in the physiopathology of the disease. Pro-inflammatory cytokines, such as IL-6 and

TNF-α, are produced by macrophages and are related to the transmigration of leukocytes (Ait-Oufella *et al*., 2011), which contribute to lesion progression. Additionally, IL-6 is considered to have a dual character (Ait-Oufella *et al*., 2011; Kleemann *et al.*, 2008) and has been involved in endothelial dysfunction, macrophage transition to foam cells and the acute response to inflammation (Keidar *et al*., 2001; Khan *et al*., 2015). In contrast, some cytokines are able to suppress inflammatory responses, such as IL-10, which is also secreted by macrophages and is considered atheroprotective (Kleemann *et al.*, 2008). Additionally, data in the literature has demonstrated that the balance between pro- and anti-inflammatory cytokines is crucial for determining the development of the lesion (Kleemann *et al.*, 2008).

Therefore, in the present study, we investigated the classical markers involved in atherosclerotic processes, such as IL-6, TNF-α and IL-10, which can be modulated by testosterone. Additionally, this was demonstrated by *in vitro* studies that showed the capacity of testosterone to suppress pro-inflammatory cytokines and stimulate anti-inflammatory cytokine production, especially IL-10 (Malkin *et al.*, 2004). Liva and Voskuhl (2001) observed that testosterone can act directly on CD4+ lymphocytes to promote an increase in the amount of IL-10 released and demonstrated that macrophages express the androgenic receptor, which does not exclude the direct action of the hormone in these cells. Moreover, the imbalance between IL-10 and TNF-α, with an increase in TNF-α, has been proposed as having an important role in the development of atherosclerotic lesions (Waehre *et al*., 2002).

Our study demonstrated no differences in pro-inflammatory cytokines; however, the decrease in testosterone produced a negative effect on IL-10 levels. Additionally, the TNF-α/IL-10 and IL-6/IL-10 ratios were increased in orchiectomized animals. Based on the inflammatory response observed, we investigated macrophage viability, since these cells are crucial for plaque development.

Macrophage apoptosis was evaluated by the isolation of peritoneal cells from mice. Zhang *et al*. (2008) demonstrated that among the peritoneal cells stimulated with thioglycolate, approximately 70% are macrophages. This technique is largely used in studies to evaluate molecular modulations involved with atherosclerosis lesion development (Hasegawa *et al.*, 2016; Liu *et al*., 2016). Our data demonstrated that animals with decreases in testosterone levels present a decrease in macrophage apoptosis simultaneously with an observed increase in lipid deposition. The development of plaques seems to have a paradoxical relationship with apoptosis that depends on the phase of the lesion (Lammers *et al*., 2011).

The initial phase of atherosclerosis seems to be closely linked to live macrophages, since many macrophages that are present at the site of the lesion are responsible for lipid and cell debris phagocytosis at the beginning of lesion development and macrophage apoptosis at this moment could have an anti-atherogenic effect (Ley *et al.*, 2009; Tabas, 2005). Gautier *et al*. (2009) demonstrated the importance of time on plaque development; according to their findings, a five-week lesion can be considered to be at an early stage and a 15-week lesion considered at an advanced stage. Therefore, we can infer that the lesion observed in the present study can be considered early, since it was evaluated in a period of four weeks without an atherogenic diet. Together, these results support the role of testosterone and macrophage activation in atherosclerosis development.

Additionally, Jin *et al*. (2006) verified the relationship between testosterone and apoptosis, demonstrating that testosterone is capable of promoting macrophage apoptosis in cells derived from bone marrow. Lammers *et al*. (2011) demonstrated an inverse relationship between atherosclerotic lesions and macrophage apoptosis in LDLr-/- mice. However, in both studies (Gautier *et al*., 2009), (*Lammers et al*., 2011) a western diet was used, which was enriched with fat and no hormonal evaluation was performed. Our results demonstrated for the first time the effect of testosterone on spontaneous plaque development in LDLr-/- mice and showed that in LDLr-/- mice, the decrease in the male hormone, testosterone, is just as important as diet in plaque development.

Atheroprotective effects of testosterone are attributed to many mechanisms and some authors suggest changes in the lipid profile (Malkin *et al*., 2004), inflammatory reduction (Li *et al*., 2008), the role of the aromatase enzyme (Nettleship *et al*., 2007) androgenic receptors and others. In a review, Vasconsuelo *et al*. (2011) showed that sexual hormones promote their action by apoptosis, which is an important cell event. Furthermore, they demonstrated the dual effect of testosterone, which can induce both apoptotic and anti-apoptotic effects that will depend on the cell type, hormone concentration and cellular environment.

Therefore, we can assume that the macrophages observed after induction with thioglycolate could reflect an inflammatory status of the orchiectomized animals, since the macrophages are probably maintaining the Th1 response, which contributes to plaque progression independently of lipid changes (Frostegård *et al*., 1999).

Together with inflammation, oxidation is also a mechanism reported in the development of atherosclerosis (Kattoor *et al*., 2017). As a translational study, we performed the evaluation of OxLDL in healthy middle-aged men under low and high levels of testosterone (both in the normal range of testosterone levels). It was possible to observe an increase in OxLDL when testosterone levels were decreased. The literature has demonstrated a high correlation of OxLDL levels

and atherosclerosis development (Gao and Liu, 2017; Linna *et al*., 2008). A negative correlation between testosterone levels and oxidized LDL, highlighting the atheroprotective effect of testosterone under physiological conditions, has been demonstrated (Barud *et al.*, 2002; Linna *et al*., 2008). Therefore, our study with human blood samples supports our findings with animals and macrophage cells, indicating that, under physiological conditions, testosterone can be a protective factor against plaque development involving antioxidant and anti-inflammatory mechanisms.

Conclusion

In summary, our results show that the reduction of endogenous testosterone levels can induce early atherosclerotic lesions in male LDLr-/- mice, which occurs with participation of inflammatory response and macrophage apoptosis, indicating that testosterone may play an atheroprotective role. This is reinforced by our studies in humans where individuals with low testosterone levels had higher LDL oxidation, which may predict a high atherosclerotic risk for these men. However, further studies are needed to further investigate these mechanisms.

Acknowledgment

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES; Finance Code 001), by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; grant number 482143/2012-6) and by the Fundação de Amparo a Pesquisa e Inovação do Espírito Santo (FAPES; grant number 0279/2016).

Author Contributions

Placielle Fiorezi Filete: Carried out the experimental; analysis and acquisition of data and analysis and interpretation of the data.

Flávia de Souza Andrade Moraes: Carried out the experimental analysis and drafted the manuscript.

Girlandia Alexandre Brasil: Supervised the care and treatment of the groups of animals and drafted the manuscript.

Ewelyne Miranda de Lima: Supervised the care and treatment of the groups of animals and drafted the manuscript.

Bianca Prandi Campagnaro: Coordinated the determination and analysis of apoptosis of peritoneal macrophages.

Dominik Lenz, Denise Coutinho Endringer and Nazaré Souza Bissoli: Participated in the design of the study and in the critical revision of the manuscript.

Dulcineia Saes Parra Abdalla: Study design and coordinated the oxidazided LDL techniques.

Thiago Melo Costa Pereira: Participated in the design of the study, coordinated the lipid deposition evaluation and has critically reviewed the manuscript.

Tadeu Uggere de Andrade: Study design and coordinator carried out the experimental analysis, drafted and critically reviewed the manuscript.

Conflict of interest

The authors disclose no conflict of interest.

References

Ait-Oufella, H., S. Taleb, Z. Mallat and A. Tedgui, 2011. Recent advances on the role of cytokines in atherosclerosis. Arteriosclerosis Thrombosis Vascular Biol., 31: 969-979. DOI: 10.1161/ATVBAHA.110.207415

Araujo, A.B., J.M. Dixon, E.A. Suarez, M.H. Murad and L.T. Guey *et al*., 2011. Clinical review: Endogenous testosterone and mortality in men: A systematic review and meta-analysis. J. Clin. Endocrinol. Metabolism, 96: 3007-3019. DOI: 10.1210/jc.2011-1137

Barud, W., R. Palusinski, J. Beltowski and G. Woójcicka, 2002. Inverse relationship between total testosterone and anti-oxidized low density lipoprotein antibody levels in ageing males. Atherosclerosis, 164: 283-288. DOI: 10.1016/S0021-9150(02)00069-2

Bourghardt, J., A.S.K. Wilhelmson, C. Alexanderson, K. De Gendt and G. Verhoeven *et al*., 2010. Androgen Receptor-Dependent and Independent Atheroprotection by Testosterone in Male Mice. Endocrinology, 151: 5428-37. DOI: 10.1210/en.2010-0663

Daleprane, J.B., V. da Silva Freitas, A. Pacheco, M. Rudnicki and L.A. Faine *et al*., 2012. Anti-atherogenic and anti-angiogenic activities of polyphenols from propolis. J. Nutritional Biochem., 23: 557-566. DOI: 10.1016/J.JNUTBIO.2011.02.012

de Andrade, T.U., S.C.G.C. Haguihara, R.M.P. Falsoni, C.L. da Silva and D.G. Dubois Filho *et al*., 2018. Stanozolol promotes lipid deposition in the aorta through an imbalance in inflammatory cytokines and oxidative status in LDLr knockout mice fed a normal diet. Basic Clin. Pharmacol. Toxicol. DOI: 10.1111/bcpt.13143

de Oliveira, F., L.B.M. Maifrino, G.P.P. de Jesus, J.G. Carvalho and C. Marchon *et al*., 2013. The role of cyclooxygenase-2 on endurance exercise training in female LDL-receptor knockout ovariectomized mice. Anais Da Academia Brasileira de Ciencias, 85: 1157-1164. DOI: 10.1590/S0001-37652013005000046

Emini Veseli, B., P. Perrotta, G.R.A. De Meyer, L. Roth and C. Van der Donckt *et al*., 2017. Animal models of atherosclerosis. Eur. J. Pharmacol., 816: 3-13. DOI: 10.1016/j.ejphar.2017.05.010

English, K.M., R.P. Steeds, T.H. Jones, M.J. Diver and K.S. Channer, 2000. Low-dose transdermal testosterone therapy improves angina threshold in men with chromic stable angina: A randomized, double-blind, placebo-controlled study. Circulation, 102: 1906-1911. DOI: 10.1161/01.CIR.102.16.1906

Frostegård, J., A.K. Ulfgren, P. Nyberg, U. Hedin and J. Swedenborg *et al*., 1999. Cytokine expression in advanced human atherosclerotic plaques: Dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. Atherosclerosis, 145: 33-43. DOI: 10.1016/S0021-9150(99)00011-8

Gao, S. and J. Liu, 2017. Association between circulating oxidized low-density lipoprotein and atherosclerotic cardiovascular disease. Chronic Diseases Translat. Med., 3: 89-94. DOI: 10.1016/j.cdtm.2017.02.008

Garber, J.C., R.W. Barbee, J.T. Bielitzki, L.A. Clayton and J.C. Donovan *et al*., 2010. National Institute of Health: Guide for the Care and use of Laboratory Animals. 8th Edn., The National Academies Press, Wasington D.C.

Gautier, E.L., T. Huby, J.L. Witztum, B. Ouzilleau and E.R. Miller *et al*., 2009. Macrophage apoptosis exerts divergent effects on atherogenesis as a function of lesion stage. Circulation, 119: 1795-1804. DOI: 10.1161/CIRCULATIONAHA.108.806158

Hasegawa, H., T. Watanabe, S. Kato, T. Toshima and M. Yokoyama *et al*., 2016. The role of macrophage transcription factor MafB in atherosclerotic plaque stability. Atherosclerosis, 250: 133-143. DOI: 10.1016/j.atherosclerosis.2016.05.021

Hatch, N.W., S.J. Srodulski, H.W. Chan, X. Zhang and L.R. Tannock *et al*., 2012. Endogenous androgen deficiency enhances diet-induced hypercholesterolemia and atherosclerosis in low-density lipoprotein receptor-deficient mice. Gender Med., 9: 319-328. DOI: 10.1016/j.genm.2012.08.003

Hopmans, S.N., W.C.M. Duivenvoorden, G.H. Werstuck, L. Klotz and J.H. Pinthus, 2014. GnRH antagonist associates with less adiposity and reduced characteristics of metabolic syndrome and atherosclerosis compared with orchiectomy and GnRH agonist in a preclinical mouse model contributed equally and share first authorship. Urologic Oncology: Seminars Original Invest., 32: 1126-1134. DOI: 10.1016/j.urolonc.2014.06.018

Huang, C.K., H. Pang, L. Wang, Y. Niu and J. Luo *et al*., 2014. New therapy via targeting androgen receptor in monocytes/macrophages to battle atherosclerosis. Hypertension, 63: 1345-1353. DOI: 10.1161/HYPERTENSIONAHA.113.02804

Ilhan, F. and S.T. Kalkanli, 2015. Atherosclerosis and the role of immune cells. World J. Clin. Cases, 3: 345-352. DOI: 10.12998/wjcc.v3.i4.345

Jin, L., X. Ai, L. Liu, Z. Wang and Y. Cheng *et al*., 2006. Testosterone induces apoptosis via Fas/FasL-dependent pathway in bone marrow-derived macrophages. Meth. Find. Exp. Anc Clin. Pharmacol.

Kapoor, D. and T.H. Jones, 2008. Androgen deficiency as a predictor of metabolic syndrome in aging men: An opportunity for intervention? Drugs. Drugs Ag.

Kattoor, A.J., N.V.K. Pothineni, D. Palagiri and J.L. Mehta, 2017. Oxidative stress in atherosclerosis. Curr. Atherosclerosis Rep., 19: 42-42. DOI: 10.1007/s11883-017-0678-6

Keidar, S., R. Heinrich, M. Kaplan, T. Hayek and M. Aviram, 2001. Angiotensin II administration to atherosclerotic mice increases macrophage uptake of oxidized ldl: A possible role for interleukin-6. Arteriosclerosis Thrombosis Vascular Biol., 21: 1464-1469. PMID: 11557673

Khan, R., V. Spagnoli, J.C. Tardif and P.L. L'Allier, 2015. Novel anti-inflammatory therapies for the treatment of atherosclerosis. Atherosclerosis, 240: 497-509. DOI: 10.1016/j.atherosclerosis.2015.04.783

Khazai, B., S.H. Golden, L.A. Colangelo, R. Swerdloff and C. Wang *et al*., 2016. Association of endogenous testosterone with subclinical atherosclerosis in men: The multi-ethnic study of atherosclerosis. Clin. Endocrinol., 84: 700-707. DOI: 10.1111/cen.12997

Kirby, M., G. Hackett and S. Ramachandran, 2019. Testosterone and the heart. Eur. Cardiol., 14: 103-110. DOI: 10.15420/ecr.2019.13.1

Kleemann, R., S. Zadelaar and T. Kooistra, 2008. Cytokines and atherosclerosis: A comprehensive review of studies in mice. Cardiovascular Res., 79: 360-376. DOI: 10.1093/cvr/cvn120

Lammers, B., P.G. Chandak, E. Aflaki, G.H.M. Van Puijvelde and B. Radovic *et al*., 2011. Macrophage adipose triglyceride lipase deficiency attenuates atherosclerotic lesion development in low-density lipoprotein receptor knockout mice. Arteriosclerosis Thrombosis Vascular Biol., 31: 67-73. DOI: 10.1161/ATVBAHA.110.215814

Ley, K., Y.I. Miller and C.C. Hedrick, 2009. Monocyte and macrophage dynamics during atherogenesis. Arteriosclerosis Thrombosis Vascular Biol., 42: 115-125. DOI: 10.1086/498510.Parasitic

Ley, K., Y.I. Miller and C.C. Hedrick, 2011. Monocyte and macrophage dynamics during atherogenesis. Arteriosclerosis Thrombosis Vascular Biol., 31: 1506-1516. DOI: 10.1161/ATVBAHA.110.221127

Li, S., X.Y. Li and Y. Li, 2008. Regulation of atherosclerotic plaque growth and stability by testosterone and its receptor via influence of inflammatory reaction. Vascular Pharmacol., 49: 14-18. DOI: 10.1016/j.vph.2008.03.004

Linna, M.S., M. Ahotupa, K. Irjala, P. Pöllänen and I. Huhtaniemi *et al*., 2008. Smoking and low serum testosterone associates with high concentration of oxidized LDL. Annals Med., 40: 634-640. DOI: 10.1080/07853890802161007

Liu, M., W. Zhang, X. Li, J. Han and Y. Chen *et al*., 2016. Impact of age and sex on the development of atherosclerosis and expression of the related genes in apoE deficient mice. Biochem. Biophys. Res. Commun., 469: 456-462. DOI: 10.1016/J.BBRC.2015.11.064

Liva, S.M. and R.R. Voskuhl, 2001. Testosterone acts directly on CD4+ T lymphocytes to increase IL-10 production. J. Immunol., 167: 2060-2067. DOI: 10.4049/jimmunol.167.4.2060

Kelly, M.D. and T.H. Jones, 2013. Testosterone: a vascular hormone in health and disease. J. Endocrinol., 217: R47-R71. DOI: 10.1530/JOE-12-0582

Malkin, C.J., P.J. Pugh, R.D. Jones, D. Kapoor and K.S. Channer *et al*., 2004. The effect of testosterone replacement on endogenous inflammatory cytokines and lipid profiles in hypogonadal men. J. Clin. Endocrinol. Metabolism, 89: 3313-3318. DOI: 10.1210/jc.2003-031069

Menezes, T.N., J.B.T. Carnielli, H.L. Gomes, F.E.L. Pereira and E.M. Lemos *et al*., 2012. Local inflammatory response induced by scorpionfish *Scorpaena plumieri* venom in mice. Toxicon, 60: 4-11. DOI: 10.1016/j.toxicon.2012.03.008

Merat, S., J. Fruebis, M. Sutphin, M. Silvestre and P.D. Reaven, 2000. Effect of aging on aortic expression of the vascular cell adhesion molecule-1 and atherosclerosis in murine models of atherosclerosis. J. Gerontol., 55: B85-94. PMID: 10737683

Moore, K.J., F.J. Sheedy and E.A. Fisher, 2013. Macrophages in atherosclerosis: A dynamic balance. Nature Rev. Immunol., 13: 709-721. DOI: 10.1038/nri3520

Nathan, L., W. Shi, H. Dinh, T.K. Mukherjee and X. Wang *et al*., 2001. Testosterone inhibits early atherogenesis by conversion to estradiol: Critical role of aromatase. Proc. Nat. Acad. Sci. USA, 98: 3589-3593. DOI: 10.1073/pnas.051003698

Nettleship, J.E., T.H. Jones, K.S. Channer and R.D. Jones, 2007. Physiological testosterone replacement therapy attenuates fatty streak formation and improves high-density lipoprotein cholesterol in the Tfm mouse. Circulation, 116: 2427-2434. DOI: 10.1161/CIRCULATIONAHA.107.708768

Ng Tang Fui, M., R. Hoermann, L.A. Prendergast, J.D. Zajac and M. Grossmann, 2017. Symptomatic response to testosterone treatment in dieting obese men with low testosterone levels in a randomized, placebo-controlled clinical trial. Int. J. Obesity, 41: 420-426. DOI: 10.1038/ijo.2016.242

Paigen, B., A. Morrow, P.A. Holmes, D. Mitchell and R.A. Williams, 1987. Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis, 68: 231-240. DOI: 10.1016/0021-9150(87)90202-4

Pedrosa, A.M.C., L.A. Faine, D.M. Grosso, B. de Las Heras and L. Boscá *et al*., 2010. Electronegative LDL induction of apoptosis in macrophages: Involvement of Nrf2. Biochimica et Biophysica Acta, 1801: 430-437. DOI: 10.1016/j.bbalip.2009.12.001

Rezanezhad, B., R. Borgquist, R. Willenheimer and S. Elzanaty, 2018. Association between serum levels of testosterone and biomarkers of subclinical atherosclerosis. Ag. Male, 21: 182-186. DOI: 10.1080/13685538.2017.1412422

Rojas, J., J. Salazar, M. Sofía Martínez, J. Palmar and J. Bautista *et al*., 2015. Macrophage heterogeneity and plasticity: Impact of macrophage biomarkers on atherosclerosis. Scientifica. DOI: 10.1155/2015/851252

Tabas, I., 2005. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis the importance of lesion stage and phagocytic efficiency. Arteriosclerosis Thrombosis Vascular Biol., 25: 2255-2264. DOI: 10.1161/01.ATV.0000184783.04864.9f

Tabas, I., 2007. Apoptosis and efferocytosis in mouse models of atherosclerosis. Curr. Drug Targets, 8: 1288-1296. DOI: 10.2174/138945007783220623

Tonini, C.L., B.P. Campagnaro, L.P.S. Louro, T.M.C. Pereira and E.C. Vasquez et al., 2013. Effects of aging and hypercholesterolemia on oxidative stress and DNA damage in bone marrow mononuclear cells in apolipoprotein e-deficient mice. Int. J. Mol. Sci., 14: 3325-3342. DOI: 10.3390/ijms14023325

Traish, A.M., R. Abdou and K.E. Kypreos, 2009. Androgen deficiency and atherosclerosis: The lipid link. Vascular Pharmacol., 51: 303-313. DOI: 10.1016/J.VPH.2009.09.003

Usman, A., D. Ribatti, U. Sadat and J.H. Gillard, 2015. From lipid retention to immune-mediate inflammation and associated angiogenesis in the pathogenesis of atherosclerosis. J. Atherosclerosis Thrombosis, 22: 739-749.

Vasconsuelo, A., L. Pronsato, A.C. Ronda, R. Boland and L. Milanesi, 2011. Role of 17b-estradiol and testosterone in apoptosis. Steroids, 76: 1223-1231. DOI: 10.1016/j.steroids.2011.08.001

Viola, J. and O. Soehnlein, 2015. Atherosclerosis - a matter of unresolved inflammation. Seminars Immunol., 27: 184-193. DOI: 10.1016/j.smim.2015.03.013

von Dehn, G., O. von Dehn, W. Völker, C. Langer and G.F. Weinbauer *et al*., 2001. Atherosclerosis in apolipoprotein e-deficient mice is decreased by the suppression of endogenous sex hormones. Hormone Metabolic Res., 33: 110-114. DOI: 10.1055/s-2001-12405

Waehre, T., B. Halvorsen, J.K. Damas, A. Yndestad and F. Brosstad *et al*., 2002. Inflammatory imbalance between IL-10 and TNFalpha in unstable angina potential plaque stabilizing effects of IL-10. Eur. J. Clin. Invest., 32: 803-810. DOI: 10.1046/j.1365-2362.2002.01069.x

WMA, 2008. WMA declaration of Helsinki - Ethical principles for medical research involving human subjects. World Medical Association General Assembly.

Wu, F.C.W. and A. Von Eckardstein, 2003. Androgens and coronary artery disease. Endocrine Rev., 24: 183-217. DOI: 10.1210/er.2001-0025

Xu, Q., 2006. A Handbook of Mouse Models of Cardiovascular Disease. 1 Edn., Wiley, ISBN-10: 0470016108, pp: 402.

Zadelaar, S., R. Kleemann, L. Verschuren, J. De Vries-Van Der Weij and J. Van Der Hoorn *et al*., 2007. Mouse models for atherosclerosis and pharmaceutical modifiers. Arteriosclerosis Thrombosis Vascular Biol., 27: 1706-1721. DOI: 10.1161/ATVBAHA.107.142570

Zhang, J., T.D. Pugh, B. Stebler, W.B. Ershler and E.T. Keller, 1998. Orchiectomy increases bone marrow interleukin-6 levels in mice. Calcified Tissue Int., 62: 219-226. PMID: 9501955

Zhang, X., R. Goncalves and D.M. Mosser, 2008. The isolation and characterization of murine macrophages. Curr. Protoc. Immunol. DOI: 10.1002/0471142735.im1401s83