Tibolone Reverses NAFLD in Ovariectomised Rats by Reducing Adiposity and Insulin Resistance

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Abstract: This study aimed to investigate the effects of tibolone, a synthetic steroid, on several metabolic dysfunctions induced by oestrogen deficiency, in rats. Ovariectomised (OVX) rats were used as animal model of postmenopausal metabolic syndrome. The OVX rats were treated with daily doses of tibolone (0.16 mg/kg) and the results were compared with control (sham-operated) and OVX untreated rats. Tibolone reduced the adiposity and the visceral adipocyte size in OVX rats. The insulin sensitivity was also improved, and a decrease in the activity of the adipose tissue hormone-sensitive lipase enzyme was recorded. The lower lipolysis by visceral adipocytes, associated with the recovery of peroxisomal β-oxidation by tibolone may have contributed to the reversion of NAFLD in treated OVX rats. The reduction of liver lipid contents resulted in a general improvement in the liver redox state. In addition, tibolone reduced the mitochondrial ROS generation and restored the activity of glucose-6-phosphate dehydrogenase. Tibolone also exerted antioxidant effects on inguinal adipose tissue. Tibolone exerted several beneficial effects on cellular and metabolic dysfunctions induced by ovariectomy in rats. One important mode of action of tibolone was the reduction of the visceral adipocyte size, corroborating the relationship between this one and the development and progression of several comorbidities associated with metabolic syndrome.

Key words: Tibolone, ovariectomy, hepatic steatosis, obesity, oxidative stress.

1. Introduction

The postmenopausal period is commonly associated with a higher incidence of metabolic syndrome (MS), a group of metabolic and cardiovascular disturbances including visceral adiposity, insulin resistance (IR), dyslipidaemia, cardiovascular diseases and non-alcoholic fatty liver disease (NAFLD) [1]. The frequent association of these co-morbidities suggests that one or a reduced number of factors may underlie the appearance and complications of all these disorders [2].

In this context, visceral adiposity has been considered as an independent, determinant factor in the development of most complications associated with obesity, such as IR and NAFLD [2]. The hypertrophy of adipocytes induces important cellular dysfunctions, including over-secretion of tumour necrosis factor-alpha (TNF-α), leptin and other pro-inflammatory adipokines and diminished secretion of adiponectin [3–7]. At the cellular level, TNF-α-dependent activation of stress-related kinases inhibits insulin signal transduction, causing IR in both adipocytes and myocytes [8]. Insulin resistance, in turn, represents the “first-level” event, responsible for the development of several complications in obesity or “IR syndromes”, such as NAFLD [9, 10].

In addition, adipocytes secrete C-reactive protein and other cytokines, such as leptin and interleukin-6, which similarly to TNF-α, are pro-inflammatory [3, 4,
Thus, the development of a chronic low-grade inflammatory state represents the “second-level” contributing event for the pathogenesis of the metabolic and cardiovascular complications in obesity [2, 7].

Hormone replacement therapy (HRT) may improve many factors associated with MS, including fat mass and distribution, dyslipidaemia, IR and NAFLD [1, 11]. However, the time of exposure and initiation of postmenopausal oestrogen therapy may be an important factor in the development of cardiovascular diseases [12].

Tibolone is a synthetic steroid used as an alternative form of HRT and, similarly to conventional steroids, exerts positive effects on several abnormalities of the MS, including the prevention of weight gain both in postmenopausal women and in animal models of oestrogen deficiency [13, 14].

Rodent ovariectomy (OVX) is an approach used to model human menopause and to study the metabolic consequences of loss of ovarian function. Studies have demonstrated that, 13 weeks after surgical ovary removal, OVX rats develop obesity, IR and NAFLD [15]. Therefore, the purpose of this work was to evaluate the effects of tibolone on adiposity, plasma lipid profile, liver lipid metabolism and redox state in OVX rats. This study could also aid to clarify the relationship among adiposity, IR and NAFLD.

2. Materials and Methods

2.1 Materials

Substrates and reagents were purchased from Sigma Chemical Co. (Missouri, USA). Crystalline tibolone was obtained from New Cham S.P.A. (Italy). Kits from Gold Analisa® (Belo Horizonte, Brazil) were used for plasma biochemical analysis and to evaluate liver lipids levels. Insulin levels were measured using a Rat/Mouse Insulin ELISA Kit from Millipore (Missouri, USA). The levels of non-esterified free fatty acids (NEFA) in the serum were determined using a NEFA-HR (2) kit from Wako Chemical® (Osaka, Japan). The lipase activity was evaluated using a Lipase Activity Colorimetric Assay Kit II from BioVision (California, USA). All reagent-grade chemicals were obtained at the highest available grade.

2.2 Animals

Female Wistar rats (60 days old) weighing 160 to 180 g were obtained from the Central Bioterium of the University of Maringá. The animals were housed in polypropylene cages (maximum of four animals per cage) and had free access to standard rodent diet (Nuvilab®, São Paulo, Brazil) and tap water. Food consumption and animal weights were recorded throughout the entire experimental period (16 weeks). Animals were housed under controlled light (12 h light:12 h dark cycle starting at 06:00 h), humidity and room temperature (20-23 °C). All experiments were conducted in strict adherence to the guidelines of the Ethics Committee for Animal Experimentation of the University of Maringá (certified 149/2014).

2.3 Surgical Procedures and Animal Treatment

Two days after their arrival at the laboratory, batches of female Wistar rats were randomly designated for surgical procedures of sham-operation (control) or ovariectomy (OVX). For surgery, OVX rats were anesthetized with an i.p. injection of 10 mg xylazine + ketamine 50 mg/kg and had their ovaries removed by bilateral ovariectomy. Sham-operated (control) rats underwent the same surgical procedure but removal of the ovaries was omitted.

Thirteen weeks after ovary removal, the OVX rats were subdivided into two groups: untreated ovariectomised rats (OVX) and OVX rats treated with daily doses of tibolone (0.16 mg/kg per day: OVX + T). The dosage of tibolone was chosen based on preliminary dose-response studies of the beneficial effects of tibolone on the most important hepatic parameters according to the aim of this work (data not shown). The results revealed that from 0.16 mg/kg tibolone was effective in reducing the liver fat
accumulation and improving the general oxidative state of the liver.

Tibolone was administered as a suspension in an aqueous solution of 1.0% gum arabic, by oral gavage over a period of 21 days. The OVX and sham-operated groups (control) received daily similar volumes of Arabic gum (vehicle). After this period (16 weeks after the surgical procedure), the animals were anesthetized with pentobarbital sodium (50 mg/kg i.p.) for blood collection and removal of liver, adipose tissue and uterus.

2.4 Sample Collection Process

Blood was collected from fasted rats by cardiac puncture to obtain serum and plasma. The uterus was collected and weighed, and the results were expressed in g per 100 g of BW. Uterine atrophy was used as a marker for a well-established condition of oestrogen deficiency.

The liver samples were removed and, depending on the protocol, were used freshly or frozen in liquid nitrogen and stored at -80 °C. The retroperitoneal, uterine, mesenteric and inguinal fat deposits were weighed and expressed in g per 100 g of body weight (BW). The ratio between the sums of the weights of these tissues per 100 g of BW was defined as the adiposity index. The retroperitoneal and inguinal fat deposits were also frozen in liquid nitrogen for measurements of oxidative stress and hormone-sensitive lipase (HSL) activity.

2.5 Blood Biochemical Dosages

Total cholesterol, high-density lipoprotein (HDL cholesterol), triacylglycerols (TAG) and non-esterified free fatty acids (NEFA) were analysed in the serum, and glucose was analysed in the plasma by standard methods using assay kits. The levels of very-low-density lipoprotein (VLDL cholesterol) were calculated by Friedewald’s equation, and low-density lipoprotein (LDL cholesterol) levels were determined by subtracting HDL and VLDL cholesterol from total cholesterol. The fasting plasma insulin dosages were assessed by an ELISA assay. Insulin resistance was measured in terms of the homeostasis model assessment (HOMA) index [16] using the following formula:

\[
\text{Homa index} = \frac{\text{fasting plasm insulin (mU} / \text{L)}}{[\text{fasting plasm glucose (mmol} / \text{L)} / 22.5]} 
\]

2.6 Adipocyte Isolation and Adipocyte Cell Size

Adipocytes were isolated from retroperitoneal fat deposits according to the method described by Rodbell [17]. The isolated adipocytes were fixed in 4% formaldehyde in phosphate buffer saline, and images were captured with a high resolution digital camera (Pro-Series Media Cibertecnics®) coupled to an optical microscope Olympus BX40®. Image-Pro-Plus 4.1® was used to measure the area of 200 adipocytes. The images were used to calculate the frequency of distribution of adipocyte sizes and their average sizes.

2.7 Hormone-Sensitive Lipase Activity in Adipose Tissue

Hormone-sensitive lipase (HSL) activity was evaluated in retroperitoneal and inguinal fat deposits using a Lipase Activity Colorimetric Assay Kit II from BioVision (California, USA) according to the manufacturer’s instructions.

2.8 Liver Lipid Contents and Histochemical Analysis

Total lipid, TAG and total cholesterol liver contents were quantified after extraction of total lipids by homogenization of liver fragments in a chloroform-methanol mixture (2:1) following the method described by Folch [18]. After drying, the organic phase was weighed for determination of the total lipid contents, and afterwards, the pellet was resuspended in 200 μL of 2% Triton for quantifying the content of TAG and total cholesterol, using enzymatic assay kits.

Histochemical analyses were conducted by sampling in each batch of animals to prove the existence of
steatosis. These fragments were processed in semi-serial histological sections (10 µm thick) using a cryostat (Leica® CM1850) and stained with Sudan III, which specifically detects lipids.

2.9 Isolation of Liver Compartments

Liver mitochondria were isolated according to the method described by Bracht et al. [19]. The suspensions of intact mitochondria were used for ROS generation, mitochondrial β-oxidation, carbonylated protein content and nicotinamide nucleotide transhydrogenase (NNT) activity measurements. The supernatant was reserved to obtain the cytosolic fraction, as described below.

Freeze-thaw disrupted mitochondria were used for assays of reduced glutathione (GSH) levels. Mitochondria disrupted by sonication were used to measure the activities of the mitochondrial antioxidant enzymes, glutathione peroxidase 1 (GPx1), glutathione reductase (mGR) and manganese superoxide dismutase (MnSOD).

The post-mitochondrial supernatant was centrifuged at 15,000 × g for 10 min to eliminate contaminations by peroxisomes and plasma membrane vesicles. Finally, the supernatant was centrifuged at 105,000 × g for 60 min to precipitate microsomes. The final supernatant was the cytosolic fraction and was used to evaluate the following antioxidant enzyme activities: glutathione peroxidase (GPx3), glutathione reductase (GR) and Cu,ZnSOD.

Peroxisomes were isolated according to the method described by Natarajan et al. [20] to measure catalase (CAT) and peroxisomal fatty acyl-CoA oxidase activities (peroxisomal β-oxidation).

Total liver homogenates obtained from freeze-clamped liver of overnight fasted rats were used for GSH content and TBARS (thiobarbituric acid-reactive substances) measurements.

2.10 Protein Determination

Protein concentrations were determined according to the method of Lowry et al. [21] using bovine serum albumin as a standard.

2.11 Mitochondrial and Peroxisomal β-Oxidation

Mitochondrial fatty acid oxidation was measured polarographically at 37 °C, using a Teflon-shielded platinum electrode [22]. Medium-chain (octanoate) and long-chain (palmitate) fatty acids were used in these assays as acyl-CoA derivatives in the presence of L-carnitine, and the rate of oxygen consumption was expressed as nmol/min × mg of mitochondrial protein. The peroxisomal fatty acyl-CoA oxidase activity was measured fluorometrically [23] by the oxidation of DCFH-DA by H₂O₂ generated during the oxidation of palmitoyl-CoA in a highly fluorescent compound (DCF). The increase in fluorescence (excitation, 503 nm; emission, 529 nm) was recorded over a period of 10 min, and the activity of fatty acid acyl-CoA oxidase was expressed as pmol DCF produced/min×mg of peroxisomal protein.

2.12 Mitochondrial Reactive Oxygen Species (ROS) Generation

The mitochondrial ROS generation was monitored by the oxidation of DCFH-DA by reactive oxygen species and was expressed as pmol DCF produced/min×mg of mitochondrial protein [24].

2.13 Determination of GSH, TBARS and Carbonylated Protein Contents in the Liver

The GSH levels in liver homogenates and freeze-thaw disrupted mitochondria were measured fluorometrically according to the method described by Hissin & Hilf [25] and expressed as µg GSH/mg protein.

The carbonylated protein contents were determined in liver homogenates and freshly mitochondria as described by Hermoso et al. [26]. The carbonyl group levels were calculated using the molar absorption coefficient for aliphatic hydrazones at 370 nm of 22,000 M⁻¹ cm⁻¹ and expressed as nmol carbonyl/mg protein.
proteins.

Lipid peroxidation levels were measured spectrophotometrically by the TBARS method [27] in liver homogenates, and the results were expressed as nmol of malondialdehyde (MDA)/mg protein using a molar extinction coefficient for MDA of 1.56 × 10^5 M^{-1} cm^{-1}.

2.14 Hepatic Antioxidant Enzyme Activities

CAT activity was measured in isolated peroxisomes according to the method described by Aebi [28]. Glucose-6 phosphate dehydrogenase (G6PD) activity was measured in the 30,000 × g supernatants obtained from homogenates of freeze clamped livers in 0.1 M Tris/HCl buffer [29].

The activities of other antioxidant enzymes were measured in the cytosolic and mitochondrial fractions. Glutathione peroxidase (GPx1 and GPx3) activity was measured by the rate of NADPH oxidation in the presence of H_2O_2 [30]. Glutathione reductase (GR) activity was determined spectrophotometrically by decreases in NADPH concentrations in the presence of oxidized glutathione (GSSG) [31]. The activities of both isoforms of SOD, mitochondrial (MnSOD) and cytosolic (Cu,ZnSOD), were estimated spectrophotometrically (420 nm) by the capacity to inhibit pyrogallol autoxidation and were expressed as U of superoxide dismutase/mg protein [32].

The activity of the NNT enzyme was measured in isolated liver mitochondria by direct spectrometry using a combination of previously described methods [33]. The slopes of absorbance over time were converted to mmol APAD produced/min×mg protein using the molar extinction coefficient of 5.1 M^{-1} cm^{-1} for reduced APAD.

2.15 Oxidative Stress in Adipose Tissue

The retroperitoneal and inguinal fat deposits were clamped in liquid nitrogen and used for the evaluation of oxidative state, following the same protocols used for the livers. The following parameters were evaluated:

GSH content, lipid peroxidation levels and the activities of antioxidant enzymes, namely, GPx1, GR, Cu,ZnSOD and CAT.

2.16 Data Analysis

The data in the figures and tables are presented as mean ± standard error (SE) and were subjected to analysis of normality using the Shapiro-Wilk test. The experimental groups were compared using analysis of variance (ANOVA), followed by Newman-Keuls post hoc tests. The compared values were provided in the text as probability values (p), and the minimum criterion of significance was p ≤ 0.05. Statistical analyses were performed with GraphPad Prism 5.0 software (GraphPad Software Inc.).

3. Results

3.1 General Features, Adiposity and Adipocyte Cell Size

Sixteen weeks after the ovariectomy surgery, the OVX group presented a pronounced reduction in uterine wet weight (g/100 g BW) by approximately 73% compared to control rats (0.068 ± 0.012, n = 7, and 0.260 ± 0.024, n = 8, respectively), indicating oestrogen deficiency. Treatment of the OVX rats with tibolone did not affect uterine atrophy (0.089 ± 0.016, n = 6).

Fig. 1 shows the weight gain of the three animal groups studied throughout the experimental period of 16 weeks. The OVX rats exhibited higher body weight gain than control animals by approximately 31%. In contrast, in the OVX rats treated with tibolone (OVX + T), the weight gain was similar to that found in control rats. These alterations could not be attributed to differences in food ingestion (data not shown).

Fig. 2 presented the weight of four fat deposits of these animals: uterine (panel A), inguinal (panel B), retroperitoneal (panel C) and mesenteric (panel D). Although a clear increasing trend could be observed in these fat deposits, in OVX rats, only in inguinal and
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Fig. 1  Body weight gain. The body weights of sham-operated (control), bilateral ovariectomised (OVX), and OVX treated with tibolone (OVX + T) rats, measured during 16 weeks from the surgical procedures until the end of the treatment are shown. The results represent the means of the body weight gain (g) of 10 animals. Vertical bars represent the standard errors. The asterisks indicate significant differences between the values as revealed by ANOVA (*p < 0.05).

Fig. 2  Fat deposits weights and adiposity index. The weights of uterine (panel A), inguinal (panel B), retroperitoneal (panel C) and mesenteric (panel D) fat deposits were expressed in terms of weight per 100 g body weight. The adiposity index was defined as the ratio between the sums of the weights of these tissues per 100 g body weight (panel E). The values were expressed as the means and the vertical bars represent standard errors. The asterisks indicate significant differences between the values as revealed by ANOVA (*p < 0.05; **p < 0.01; ***p < 0.001).

Retroperitoneal fat deposits these increases were significant (+24 and +36%, respectively). Tibolone administration suppressed all the changes induced by ovariectomy, and significant reductions in all fat deposit weights, including in uterine and mesenteric fats, could be detected in the OVX + T group. These increases in adipose tissue weights resulted in higher adiposity indexes of the OVX rats compared to the control group (+27%), and treatment with tibolone reduced these indexes to values significantly lower than those of the control rats (Fig. 2E).

Adipocytes isolated from retroperitoneal fat deposits were used for adipocyte size analyses. Fig. 3 shows representative photomicrographs of adipocytes from control (panel A), OVX (panel B) and OVX + T (panel C) rats. The frequency distributions of adipocyte sizes of these animals are presented in panels D to F. As shown, 80% of the adipocyte areas of control rats were between 2,000 to 3,000 μm² (panel D). Compared to the control group, the OVX group (panel E) exhibited a higher incidence of hypertrophic cells, presenting more than 50% of cells with an area over 3,000 μm². Treatment with tibolone reduced the adipocyte sizes of the OVX rats such that the curve of the frequency distribution shifted toward the distribution of the control rats (panel F). These results were corroborated by the calculation of the average cell adipocyte sizes, as presented in panel G.
Fig. 3  The areas of retroperitoneal adipocytes. Images of adipocytes of control, OVX and OVX + T animals were captured at 40× original magnification with a high resolution digital camera (Pro-Media Series of Cibertecnics®) coupled to an optical microscope Olympus BX40®, with scale bar of 50 μm. Representative images of control, OVX and OVX + T rats are presented in panel A to C, respectively. Adipocyte area was measured in 200 cells per rat using Image-Pro Plus 4.1® program. These measurements were used to calculate the frequency of the distribution of adipocyte sizes of control (panel D), OVX (panel E) and OVX + T (panel F) rats. In panel G, the average adipocyte sizes of these animals are presented. The values are expressed as the means and the vertical bars represent standard errors. The asterisks indicate significant differences between the values as revealed by ANOVA (***p < 0.001).
3.2 Blood Biochemical Dosages

The blood biochemical analyses are presented in Table 1. The glycaemia and insulinemia of the OVX group differed significantly from those of the control rats. The peripheral insulin sensitivity was also altered in these animals, as demonstrated by the HOMA-IR index. All these undesirable alterations were completely reversed by treatment with tibolone.

The lipid profile was also altered in the OVX rats: these animals showed a significant increase in TAG, LDL cholesterol and VLDL cholesterol levels compared to control rats (increases of 38%, 35% and 36%, respectively). However, these results were not accompanied by changes in HDL cholesterol and total cholesterol levels. Treatment of OVX rats with tibolone did not alter TAG levels compared to untreated OVX rats, which remained significantly increased by approximately 40% compared to control rats. In relation to cholesterol fractions, the OVX + T rats presented significant reductions of total, HDL cholesterol and LDL cholesterol levels compared to the OVX group. The treatment with tibolone did not change the values of VLDL cholesterol, which was significantly increased by approximately 45% compared to control rats. NEFA was evaluated in serum but did not differ among these animal groups.

3.4 Hormone-Sensitive Lipase (HSL) Activity of Adipose Tissue

Fig. 4 shows HSL activity measured in the inguinal and retroperitoneal adipose tissues. Although a slight increase in HSL activity was observed in the OVX rats, only in the inguinal adipose tissue did these values reach statistical significance compared to control rats (approximately +8%). Treatment of these animals with tibolone significantly reduced HSL activity in both adipose tissues evaluated in this work.

3.5Liver Lipid Content Measurements, Histochemical Analysis and Fatty Acid Oxidation

The results of the analyses of liver lipid contents are presented in Fig. 5. In panels A to C are presented the representative photomicrographs of the livers from control, OVX and OVX + T rats, respectively. The histochemical analyses (Sudan III) show large amounts of lipid inclusions (orange) in the OVX rats, comparatively to the control and OVX + T rats. In panel D are presented the results of the liver total lipid content measurements, which were increased in OVX rats, in approximately 42%, comparatively to control and OVX + T rats, indicating extensive steatosis. The measurements of liver TAG (panel E) and cholesterol contents (panel F) revealed that among them, only TAG

Table 1  Blood biochemical analysis.

|                  | Control       | OVX           | OVX + T        |
|------------------|---------------|---------------|----------------|
| Glycaemia (mg/dL; n = 6–8) | 101.80 ± 2.42 | 131.5 ± 3.48*** | 105.5 ± 3.83   |
| Insulinemia (ng/dL; n = 6–8) | 0.35 ± 0.09  | 1.75 ± 0.21*** | 0.46 ± 0.06    |
| HOMA-IR index     | 2.17 ± 0.54   | 14.44 ± 2.28*** | 3.03 ± 0.42    |
| Triacylglycerols  | 23.59 ± 2.04  | 32.44 ± 2.29*  | 32.99 ± 2.26   |
| Total cholesterol | 63.32 ± 3.24  | 70.40 ± 1.84   | 27.47 ± 2.10†  |
| HDL-cholesterol   | 33.73 ± 2.46  | 30.81 ± 0.89   | 12.88 ± 1.39‡  |
| LDL-cholesterol   | 24.08 ± 1.81  | 32.56 ± 1.45*** | 9.32 ± 1.26†  |
| VLDL-cholesterol  | 4.77 ± 0.41   | 6.49 ± 0.46‡   | 6.92 ± 0.66*   |
| NEFA (mmol/L; n = 7–8) | 0.68 ± 0.05  | 0.70 ± 0.05    | 0.64 ± 0.03    |

Glycaemia (mg/dL; n = 6–8), insulinemia (ng/dL; n = 6–8), HOMA-IR index (n = 6–8), triacylglycerols (mg/dL; n = 8–9), total cholesterol (mg/dL; n = 8–9), high-density lipoprotein (HDL; mg/dL; n = 8–11), low-density lipoprotein (LDL; mg/dL; n = 8–11), very low density lipoprotein (VLDL; mg/dL; n = 10–11), non-esterified fatty acids (NEFA; mmol/L; n = 7–8) are expressed as the mean ± SEM. The symbol in the same line shows the statistical significance of the values calculated by ANOVA. ***p < 0.001 vs. Control and OVX + T; *p < 0.05 vs. Control; †p < 0.001 vs. Control and OVX.
Fig. 4  The adipose tissue HSL activity. The HSL activity was evaluated in retroperitoneal and inguinal fat deposits using a lipase colorimetric assay kit. Values are expressed as the means of individual experiments with different inguinal ($n = 7-8$) or retroperitoneal ($n = 7-8$) preparations. The vertical bars represent the standard errors. The asterisks indicate significant differences between the values as revealed by ANOVA ($^* p < 0.05; ^{**} p < 0.01$).
is increased in the livers of OVX rats, in approximately 30%, comparatively to control and OVX + T groups.

To investigate whether the fatty liver accumulation was the result of impaired β-oxidation, the fatty acid oxidation capacity of isolated mitochondria and peroxisomes were measured and the results are presented in Fig. 6. No difference was observed between the groups regarding mitochondrial β-oxidation of both octanoate and palmitate (Fig. 6A). However, peroxisomal β-oxidation of palmitate (Fig. 6B), which was decreased by approximately 25% in the OVX group compared to control rats, was completely restored by treatment with tibolone, and in the OVX + T rats, the β-oxidation capacities reached values close to those of the control group.

3.6 Liver Redox State Parameters

Biomarkers of oxidative stress were measured in liver homogenates and in cytosolic or mitochondrial fractions (Fig. 7, panels A to D). The mitochondrial ROS generation (Fig. 7A) was approximately 79% higher in the OVX rats than in control rats. Treatment with tibolone significantly reduced mitochondrial ROS generation by 25%, although this value remained higher than those of control rats.

The lipid peroxidation levels of the liver homogenate (Fig. 7B) and the carbonylated protein contents measured in the cytosol and in isolated mitochondria, (Fig. 7C) were all increased in the OVX rats compared to controls, but they were restored to values similar to those found in the control rats with tibolone treatment (OVX + T). The GSH contents were also evaluated in the total liver homogenates and in isolated mitochondria, and they were reduced in the OVX group in both (-26% and 16%, respectively).

Similarly, treatment with tibolone restored the GSH levels in both compartments (Fig. 7D).

In another series of experiments, the activities of antioxidant enzymes were evaluated in different liver compartments, and the results are presented in Table 2. MnSOD and GPx1 did not differ among these animals. However, the activity of mGR and NNT were significantly higher (both in approximately 33%) in OVX + T rats than in the control and OVX untreated groups. Among the cytosolic enzymes evaluated, GPx3 and G6PD activities were reduced in the OVX rats compared to control rats by approximately 20% and 62%, respectively. Treatment with tibolone restored the G6PD activity to levels similar to those found in control rats, although it did not affect GPx3 activity. Moreover, tibolone reduced Cu,ZnSOD activity by approximately 12% compared to the control and OVX groups. The cytosolic GR activity was affected neither by ovariectomy nor by tibolone treatment. Finally, CAT activity was significantly reduced to similar extents in the OVX and OVX + T rats by approximately 33% compared to the control group.

3.7 Oxidative Stress in the Adipose Tissue

Oxidative stress parameters were measured in the inguinal and retroperitoneal fat deposits, and the results are presented in Table 3. Contrary to the result of the redox state of the liver, no differences were observed in GSH content between the three groups of animals in either fat deposit. Lipid peroxidation levels (TBARS) were increased only in inguinal fat of OVX rats (+23%). Therefore, with respect to the biomarkers of oxidative stress evaluated in this work, no significant change was observed in the retroperitoneal fat.
Liver fatty acid β-oxidation. Liver mitochondrial fatty acid β-oxidation (Panel A) was measured by polarography in the presence of 100 μM DNP. Reactions were initiated by the addition of the following: 20 mM octanoyl-CoA + 2.0 mM L-carnitine (Oct-CoA) or 20 mM palmitoyl-CoA + 2.0 mM L-carnitine (Pal-CoA). The peroxisomal fatty acyl-CoA oxidase activity (Panel B) was measured by fluorimetry. The reactions were initiated by the addition of 30 μM palmitoyl-CoA (Pal-CoA). Values are expressed as the means of individual experiments with different mitochondrial (n = 9–13) or peroxisomal (n = 9–14) preparations. The vertical bars represent the standard errors. The asterisks indicate significant differences between the values as revealed by ANOVA (*p < 0.05; **p < 0.01).
Fig. 7  Liver redox state. The mitochondrial ROS generation was evaluated by fluorimetry (Panel A; n = 5–7), lipid peroxidation was measured by TBARS method (Panel B; n = 6–8), the levels of carbonylated proteins were measured in the cytosol and mitochondria using by DNPH method (Panel C, n = 6–8), the reduced glutathione contents were evaluated in the liver homogenate and in isolated mitochondria using OPT as fluorescence probe (Panel D, n = 6–7). Values are expressed as the means and vertical bars represent the standard errors. The asterisks indicate significant differences between the values as revealed by ANOVA (*p < 0.05; **p < 0.01; ***p < 0.001).

Table 2  Effects of tibolone on liver antioxidant enzymes.

| Enzyme activity (per mg protein) | Control | OVX | OVX + T |
|---------------------------------|---------|-----|---------|
| Mitochondria                    |         |     |         |
| MnSOD (U)                       | 1.06 ± 0.07 | 1.06 ± 0.04 | 1.04 ± 0.05 |
| mGR (nmol/min)                  | 11.56 ± 0.61 | 11.64 ± 1.02 | 15.51 ± 1.22 |
| GPx1 (nmol/min)                 | 222.70 ± 18.10 | 198.40 ± 12.41 | 193.20 ± 15.41 |
| NNT (mmol/min)                  | 57.17 ± 3.91 | 58.04 ± 3.05 | 76.59 ± 5.14 |
| Cytosol                         |         |     |         |
| Cu, ZnSOD (U)                   | 1.91 ± 0.03 | 1.89 ± 0.08 | 1.66 ± 0.05 |
| GR (nmol/min)                   | 27.20 ± 1.48 | 24.09 ± 1.28 | 24.09 ± 1.16 |
| GPx3 (nmol/min)                 | 298.50 ± 18.89 | 238.50 ± 11.38 | 238.00 ± 9.75 |
| G6PD (nmol/min)                 | 26.03 ± 1.35 | 9.74 ± 1.14 | 28.10 ± 2.47 |
| Peroxisome                      |         |     |         |
| CAT (µmol/min)                  | 428.80 ± 25.24 | 284.80 ± 15.51 | 286.70 ± 8.81 |

Manganese superoxide dismutase (MnSOD; n = 7–10), mitochondrial glutathione reductase (mGR; n = 7), glutathione peroxidase-1 (GPx1; n = 6–8), nicotinamide nucleotide transhydrogenase (NNT; n = 7), copper zinc superoxide dismutase (Cu, ZnSOD; n = 10–11), glutathione reductase (GR; n = 9–11), glutathione peroxidase-3 (GPx3; n = 9–10), glucose 6-phosphate dehydrogenase (G6PD; n = 6), catalase (CAT; n = 6) are expressed as the mean ± SEM. The symbol in the same line shows the statistical significance of the values calculated by ANOVA. *p < 0.05 vs. Control and OVX; **p < 0.01 vs. Control and OVX; ***p < 0.001 vs. Control and OVX + T; †p < 0.05 vs. Control; ††p < 0.001 vs. Control.

Table 3  Evaluation of oxidative stress parameters in adipose tissue.

| Parameter (per mg protein) | Control | OVX | OVX + T |
|---------------------------|---------|-----|---------|
| Inguinal fat               |         |     |         |
| GSH levels (µg)            | 1.92 ± 0.16 | 2.05 ± 2.05 | 2.04 ± 0.22 |
| TBARS (nmol)               | 18.32 ± 1.34 | 22.50 ± 0.92 | 17.11 ± 1.62 |
| Cu,ZnSOD (U)               | 1.55 ± 0.04 | 2.70 ± 0.22 | 2.95 ± 0.25 |
| GR (nmol/min)              | 8.17 ± 0.82 | 10.93 ± 1.09 | 12.33 ± 1.03 |
| GPx3 (nmol/min)            | 49.54 ± 3.63 | 61.58 ± 3.98 | 80.03 ± 4.49 |
| CAT (µmol/min)             | 104.70 ± 7.03 | 111.50 ± 7.92 | 123.40 ± 10.06 |
| Retropertoneal fat         |         |     |         |
| GSH levels (µg)            | 2.24 ± 0.16 | 2.25 ± 0.22 | 2.45 ± 0.11 |
| TBARS (nmol)               | 15.34 ± 0.24 | 14.27 ± 0.49 | 14.39 ± 0.43 |
| Cu,ZnSOD (U)               | 1.88 ± 0.13 | 1.74 ± 0.09 | 1.50 ± 0.13 |
| GR (nmol/min)              | 16.82 ± 2.10 | 13.94 ± 0.93 | 17.04 ± 1.13 |
| GPx3 (nmol/min)            | 87.07 ± 12.80 | 73.93 ± 7.69 | 72.86 ± 7.13 |
| CAT (µmol/min)             | 149.60 ± 5.20 | 171.70 ± 6.34 | 167.20 ± 10.94 |

Copper zinc superoxide dismutase (Cu, ZnSOD; n = 6–9), glutathione reductase (GR; n = 7–9), glutathione peroxidase-3 (GPx3; n = 9–11), catalase (CAT; n = 7–9) are expressed as the mean ± SEM. The symbol in the same line shows the statistical significance of the values calculated by ANOVA. †p < 0.05 vs. Control; ††p < 0.001 vs. Control; **p < 0.01 vs. Control and OVX; §p < 0.05 vs. Control and OVX + T.
Among the antioxidant enzymes evaluated in the inguinal fat deposit, Cu,ZnSOD was significantly increased by approximately 74% in the OVX rats compared to control rats. However, the OVX + T group did not differ from untreated OVX rats, which remained increased compared to control rats. Progressive and significant increases in GPx3 activity were observed between control rats and both OVX and OVX + T rats. In addition, CAT activity in the inguinal adipose tissue did not differ among these three animal groups, but GR activity was significantly increased in the OVX + T group compared to the control and OVX groups.

4. Discussion

The results of the present study revealed that, 16 weeks after ovariectomy, rats presented uterine atrophy and developed several characteristics of MS, including increased adiposity associated with adipocyte hypertrophy, peripheral IR, dyslipidaemia and NAFLD (Fig. 8). Liver fat accumulation was followed by a worsening of the redox state, as could be demonstrated by the increases in ROS generation, lipid peroxidation and carbonyl protein contents. However, the increase in the fat deposits did not reflect impacts on redox state, again demonstrating that ectopic fat accumulation exerts more damaging cellular effects [34].

OVX rats exhibited increases in fat deposits and hypertrophy of adipocytes. Tibolone reversed all these conditions, reducing the fat deposits and adipocyte sizes. Our results are in accordance with a previous study in which it was demonstrated that daily administration of tibolone (2 mg/kg) to OVX-aromatase knockout (ArKO) mice resulted in an obvious decrease in the adipose phenotype, reducing adipocyte size [35].

It has long been known that the hypertrophy of adipocytes leads to important cellular dysfunctions, including overproduction of TNF-α [5, 7], and higher expression of TNF-α [36, 37], which induces IR in both adipocytes and myocytes [8]. In this way, one important mode of action of tibolone was probably the reduction in the visceral adipocyte size, as demonstrated here and previously described for postmenopausal women [38].

The OVX rats also presented extensive NAFLD, which has been considered as an IR-syndrome. In insulin resistant adipocytes, the inhibition of HSL enzyme is suppressed and the lipolysis increases [39]. The increased release of free fatty acids directly into the portal vein overloads the liver, inducing NAFLD [40]. In the present work, an increase in the HSL enzyme activity was, in fact, observed in the OVX rats, a condition that was reversed by the treatment with tibolone.

Besides, the increased delivery of fatty acids to hepatocytes is sufficient to induce hepatic IR [41] and the inhibition of gluconeogenesis by insulin is impaired thus resulting in hyperglycemia, at the same time that put more substrate available to hepatic FA synthesis.

![Fig. 8 Effects of tibolone treatment in ovariectomised rats.](image-url)
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[42, 43]. This was another finding in the current work: the OVX rats were hyperglycaemic, a condition that was also reversed by tibolone.

However, IR could trigger NAFLD by other mechanisms, beyond the increased lipolysis. In the liver, insulin robustly promotes liver FA synthesis and decreases FA oxidation. In fact, the peroxisomal β-oxidation was impaired in OVX rats. Therefore, the liver fat accumulation could be attributed, at least in part, to a reduction in this pathway. Similarly, the increase in peroxisomal β-oxidation induced by treatment with tibolone could be another contributing factor to the improvement of steatosis in these animals. The oestrogen deficiency could also be involved in the reduction in the peroxisomal β-oxidation [11, 44] and, in this sense, tibolone could be exerting oestrogenic actions.

Recent studies performed in our laboratory revealed for the first time that tibolone was also effective in reversing NAFLD in hypertensive, OVX rats [45], by reducing the blood arterial pressure. So, although NAFLD has been considered, for a long time, as an IR-syndrome, recent studies have noted the importance of hypertension as a triggering factor of NAFLD [46, 47]. In this regard, it should be noted that in those animals, NAFLD arose very early after the ovariectomy surgery (3 weeks), and independent on the IR, which was absent [15, 45, 48]. Thus, tibolone has been proven to be effective in reversing steatosis associated with IR, as evidenced in this work, as well as to be associated with hypertension, as previously demonstrated by us [45].

With respect to the lipid profile, tibolone exerted the undesirable effect of lowering HDL cholesterol levels, as described before [14, 49]. The mechanisms by which tibolone exerts this effect are uncertain, but they could involve androgenic actions of the tibolone metabolites or a more rapid HDL clearance [50, 51]. Furthermore, similar to oestrogen [52], the oral administration of tibolone leads to increases in the bile concentrations of cholesterol and trihydroxy acids [53], and this could also contribute to the observed reductions in HDL levels.

However, tibolone was also effective in reducing LDL levels and, in doing this, restored the HDL/LDL ratio. This index of cardiovascular risk, which was significantly reduced in the OVX rats (0.996 ± 0.061), returned to values close to those found in control rats (1.409 ± 0.147) in the OVX + T rats (1.370 ± 0.048), which implies in a cardiovascular protective action. These alterations in the lipid profile were also accompanied by reductions in lower total cholesterol levels without interference in the plasmatic TAG levels, and the results agree with those described by Henriques et al. [14].

The impacts of oestrogen deficiency on the oxidative state of the liver and adipose tissues (retroperitoneal and inguinal) were also investigated. Oestrogen is a powerful antioxidant [54], and thus, the oestrogen deficiency has been associated with disturbances in redox state in OVX rats [48, 55] and postmenopausal women [56].

Among the effects of tibolone in the liver redox state, the increases in GSH levels stand out, which were reduced in both the liver homogenate and isolated mitochondria of the OVX rats and were restored by treatment with tibolone. These results agree with previous studies performed by our team [45, 48, 55] and could be largely attributed to increases in mitochondrial ROS generation in the OVX rats [45, 48, 57]. Moreover, oxidative damage in the liver was also evidenced in the OVX rats by the higher levels of lipid peroxidation and carbonylated protein contents. Similar to oestrogen, tibolone improved the redox state of the liver by reducing ROS generation, as well as, the oxidative damage associated, as demonstrated by reduction of the lipid peroxidation and carbonylated protein levels.

Alterations in the activities of antioxidant enzymes, especially those involved in the GSH cycle, could also contribute to oxidative damage. In this respect, among the observed alterations, a factor could contribute to the
worsening of the liver redox state of OVX rats and its improvement by treatment with tibolone, namely, the reduction of G6PD and GR activities in OVX rats and its restoration upon treatment with tibolone. These results are expected, as it has long been known that G6PD is activated by oestrogens [58], and its reduced activity in OVX rats was already described by us [45, 48, 55]. In addition, in mitochondria, the activities of NNT were also increased by tibolone. The NNT enzyme has also been associated with IR [59], and the importance of the increase in its activity by tibolone should not be minimized. Therefore, the overall effects of treatment with tibolone in the redox state of the liver were beneficial. Even a reduction in the activity of Cu,ZnSOD was expected in the liver of treated rats, as the expression of this enzyme is controlled by the presence of ROS [60].

As evidenced in this work, the redox state of the retroperitoneal adipose tissue was not altered by oestrogen deficiency or by tibolone treatment, whereas the inguinal adipose tissue showed higher lipid peroxidation levels in the OVX rats. In agreement with this, differences have been described between visceral and subcutaneous fat deposits with respect to their antioxidant state and responses [34]. Treatment with tibolone also showed beneficial effects in the redox state of the inguinal adipose tissue, as could be detected by the reduction in TBARS levels. The alterations observed in the activities of Cu,ZnSOD and GPx3 of inguinal adipose tissue were likely adaptive.

In summary, the metabolic alterations induced by ovariectomy resulted in the development of obesity, visceral adipocyte hypertrophy, IR and NAFLD, which was associated with a decline in the liver redox state but not in the adipose tissue. On the other hand, tibolone reduced the adiposity and adipocyte size, which was accompanied by improvements in several metabolic disorders, including IR and NAFLD. One important mode of action of tibolone was to reduce the visceral adipocyte size and these results reinforce the relationship between visceral adipocyte size, IR and NAFLD. Some actions of tibolone, however, could involve the binding to oestrogen receptors, in especial those antioxidant effects.

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**Declarations of Interest**

None.

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