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High-dose Nandrolone Decanoate induces oxidative stress and inflammation in retroperitoneal adipose tissue of male rats

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ABSTRACT

The non-therapeutic use of the androgenic anabolic steroid Nandrolone Decanoate is popular due to its effects on physical performance and body composition, especially for its lipolytic and anabolic effects associated. However, high doses of such drugs are often associated with a series of pathologies related to unbalanced redox homeostasis, which, in turn, can be linked to inflammation. The oxidative stress onset could deregulate the secretion of cytokines, evidencing a dysfunctional adipocyte. Thus, the aim of this study was to investigate the effect of supraphysiological doses of Nandrolone Decanoate on redox homeostasis of retroperitoneal fatpad of male rats and its relationship with cytokines-based inflammatory signaling. Hydrogen peroxide production was assessed in the retroperitoneal fat pad of adult male rats which received either 10 mg kg of Nandrolone Decanoate or only a vehicle. Also, catalase, superoxide dismutase and glutathione peroxidase activities were measured, together with total reduced thiols and protein carbonylation, as well as IL-1β, TNF-α, and IL-6 local levels. High doses of Nandrolone Decanoate caused an increase in the hydrogen peroxide production, together with lower activities of the antioxidant enzymes and lower levels of total reduced thiol. There were also higher protein carbonylation and greater levels of IL-1β, TNF-α, and IL-6 in the treated group compared to control group. Therefore, it was possible to verify that high doses of Nandrolone Decanoate cause oxidative stress and induce higher inflammatory signaling in retroperitoneal fat pad of male rats.

1. Introduction

Nandrolone Decanoate (ND) is a popular injectable drug that belongs to the so-called Androgenic Anabolic Steroids (AAS), which are synthetic molecules chemically similar to testosterone that were originally synthesized for treating hypogonadism. Intending to optimize anabolic over androgenic effects, the synthesis of ND is based on the substitution of a methyl group to the carbon atom at position 19 by a hydrogen atom in the testosterone molecule [1,2]. The use of ND for improving physical performance and body composition has increased in the past years, with doses reaching 100 times higher than the therapeutic one. Moreover, it is well established that AAS abuse can cause health risks, such as increased blood pressure, thrombosis, myocardial infarction, heart failure and central-nervous, hepatic and renal

Abbreviations: AAS, Androgenic Anabolic Steroids; CAT, catalase; DNPH, dinitrophenyl hydrazine; DTNB, 5,5-dithionitrobenzoic acid; DUOX, dual oxidase 1; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; EP, epididimal; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; IL-1β, interleukin 1 beta; IL-6, interleukin 6; MF, microsomal fraction; NADPH, nicotinamide adenine dinucleotide phosphate; ND, Nandrolone Decanoate; NOX, nicotinamide adenine dinucleotide phosphate oxidase; NTB-, 2-nitro-5-thiobenzoate; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; RP, retroperitoneal; SC, subcutaneus; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SOD, superoxide dismutase; TNF-α, tumor necrosis factor; WAT, white adipose tissue; XOD, xanthine oxidase

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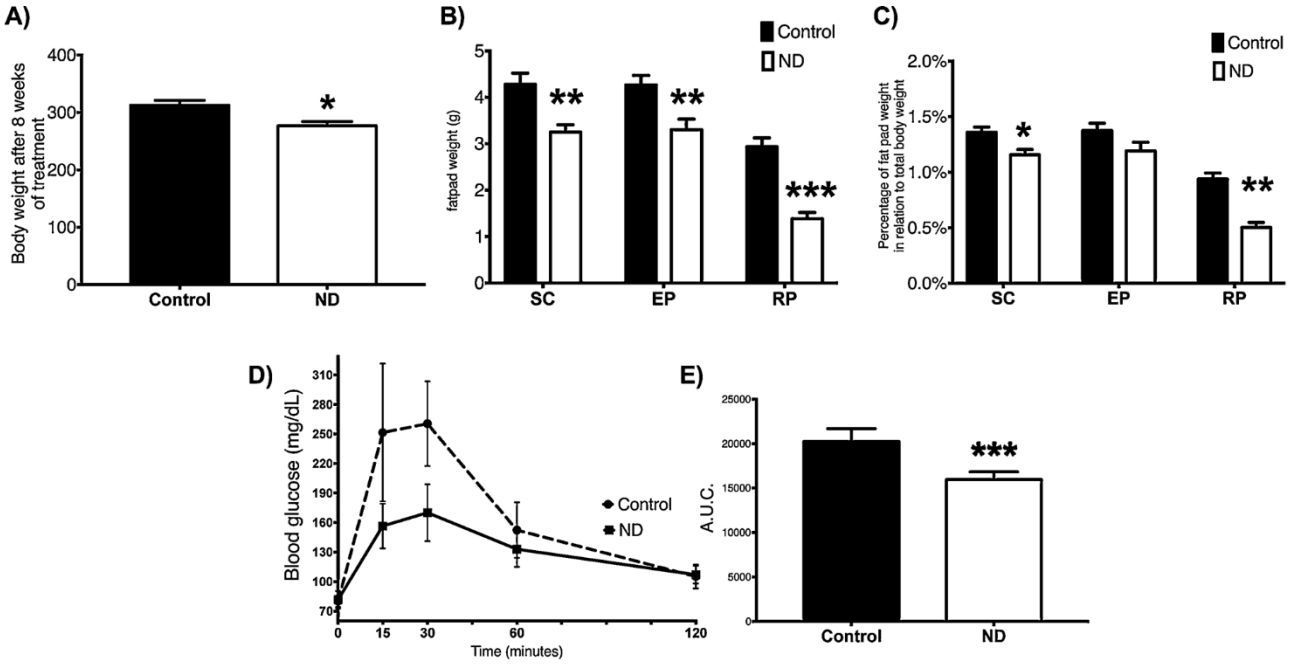

Fig. 1. Effect of nandrolone decanoate (ND) on body weight, adiposity and glucose tolerance of male rats. Final body weight (A); subcutaneous (SC), epididymal (EP) and retroperitoneal (RP) fat pads weight (B), and intraperitoneal glucose tolerance (D and E) of the animals after 8 weeks. Data are expressed by mean ± standard error of the mean. A.U.C. = area under the curve. * $p < 0.05$ ** $p < 0.001$ *** $p < 0.0001$ (unpaired Student *t*-test) ND group (n = 8) vs. Control group (n = 8).

dysfunction, which are generally related to a oxidative stress caused by these drugs [3].

Reactive oxygen species (ROS), produced by the activity of the mitochondria and of the NADPH oxidases (NOX), plus other sources, can cause oxidative stress when the antioxidant systems cannot proportionally neutralize these molecules, which may harmfully cause membrane lipid peroxidation, protein carbonylation and DNA damage [3,4]. Still, the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) have their activities crucially involved in those implications caused by oxidative damage, taking into account their capacity of detoxifying and/or decreasing the reactive potential of ROS, such as superoxide and hydrogen peroxide [3,4].

Although oxidative stress triggered by AAS in the adipose tissue was not yet characterized, it is known how a disrupted redox homeostasis is involved in the dysfunction of the white adipose tissue (WAT) and how it is deeply involved in inflammatory reactions [5–8]. Among the fat pads that constitute the WAT, the retroperitoneal (RP) fat pad is known for its role in metabolism regulation and its greater androgens sensitivity [9,10]. So, taking into account the importance of such tissue in regulating the systemic metabolism, and that a dysfunctional adipose tissue would be capable of triggering-off and/or dysregulating the cytokine secretion by adipocytes [11], our study aimed to investigate the effect of supraphysiological doses of ND on redox homeostasis and inflammatory cytokines levels of the WAT's RP fat pad.

2. Materials and methods

2.1. Animals and treatment

Sixteen male Wistar rats, four months-old, weighing 200–250 g, were maintained in an animal house with controlled lighting (12 h light-dark cycle) and temperature (23 − 24 °C). They were weighed weekly and divided into 2 groups: Control (n = 8), whose animals didn`t receive ND, but a vehicle (peanut oil) for 8 weeks, and ND (n = 8), whose animals received a weekly intramuscular high dose (10 mg kg) of ND for 8 weeks.

10 mg kg of ND (Deca Durabolin®, Organon, SP) was administered as a single weekly intramuscular injection on the left hind limb, always at 8:00am. The doses administered in the present study are consistent with previous reports of their efficacy, especially in increasing serum testosterone levels and reducing adipose tissue mass [3]. After 8 weeks of treatment, the rats were euthanized by anesthesia followed by decapitation. Retroperitoneal (RP), Subcutaneous (SC) and Epididymal (EP) fat pad were excised and weighed. The RP fat pad was frozen for further analysis.

The institutional committee for use of animals in research approved the study (#3211216/2018). All procedures were in compliance with the International Guiding Principles for Biomedical Research Involving Animals of the Council for International Organizations of Medical Sciences (Geneva, Switzerland) and the guiding principles for care and use of animals from the American Physiological Society.

2.2. Intraperitoneal glucose tolerance test (IPGTT)

Two days before the euthanasia, after a twelve-hour fast, the animals received a dose of glucose (75 mg/100 g) by intraperitoneal injection. Caudal capillary blood glucose was measured using a glucometer (One Touch Ultra, Johnson & Johnson's) at different times: 0 (fasting), 5, 15, 30, 60 and 120 min after glucose administration. From the values of the glycemic curves of each animal, the incremental area under the curve was calculated.

2.3. Antioxidant enzyme activities and total reduced thiol levels measurements

RP samples (400 mg) were homogenized in 5 mM Tris − HCl buffer (pH 7.4), containing 0.9 % NaCl (w/v) and 1 mM EDTA, followed by centrifugation at 750 × *g* for 10 min at 4 °C. The supernatant aliquots were stored at −80 °C. The same amount of tissue was used for each group. Protein concentration was determined by the Bradford assay [12] for normalization of the results.

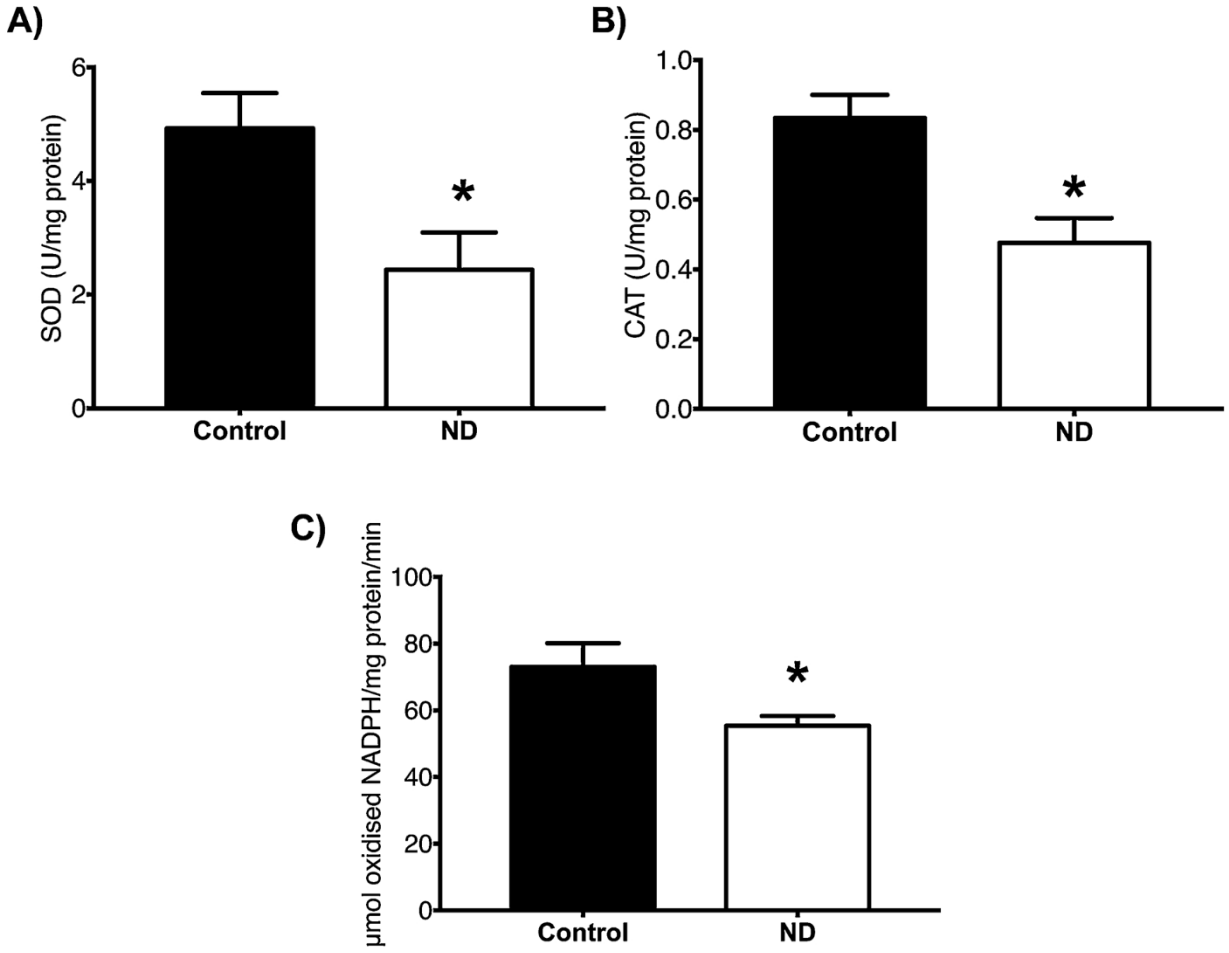

Fig. 2. Effect of nandrolone decanoate (ND) on antioxidant enzymes activities of retroperitoneal adipose tissue of male rats. Superoxide Dismutase (A), Catalase (B) and Glutathione Peroxidase (C) activities were measured by spectrophotometry. Data are expressed by mean and standard error of the mean. * $p < 0.05$ (unpaired Student *t*-test) ND group (n = 8) vs. Control group (n = 8).

2.3.1. Catalase activity

CAT activity was assayed following the method of Aebi [13]. Briefly, it measures CAT by the disappearance of hydrogen peroxide (H_2O_2), forming water and oxygen. The assay evaluates the decrease in light absorption by 240 nm for 1 min, with reading points at 0, 30 and 60′. CAT activity was obtained through the difference between the decrease in absorbance rate with and without sample, which allows to calculate the amount of H_2O_2 consumed per minute, using its molar extinction coefficient (43.6 M^{-1}). The enzyme activity was expressed in units per milligram of protein (U mg^{-1}), in which one unit represents 1 micromol of H_2O_2 consumed per minute.

2.3.2. Glutathione peroxidase

GPx activity was determined by the measurement of cytochrome C reduction at 550 nm [14]. Briefly, the method is based on hydroperoxide conversion to water by GPx, oxidizing reduced or free glutathione (GSH) to oxidized glutathione (GSSG) at the expense of NADPH oxidation to NADP. The decrease in light absorption by 340 nm promoted by NADPH oxidation was evaluated for 5 min, and NADPH oxidation rate obtained without the sample was subtracted from the one with the sample for GPx calculation, expressed in units micromol of oxidized NADPH per minute.

2.3.3. Superoxide dismutase activity

Total SOD activity was determined using the Superoxide Dismutase (Ransod) assay kit, according to the manufacturer's protocol (Randox Laboratories Ltd., UK), which employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazilium chloride to form a red formazan dye. SOD activity is measured by the degree of this reaction. SOD was expressed as units per milligram of protein (U.mg^{-1}).

2.3.4. Total reduced thiol groups

Total reduced thiol groups were determined in a spectrophotometer (Hitachi U-3300) using 5,5-dithionitrobenzoic acid (DTNB). Thiols react with DTNB, cleaving the disulfide bond to give 2-nitro-5-thiobenzoate (NTB^-), which ionizes to NTB^{2-} in water at neutral and alkaline pH. The NTB^{2-} was quantified in a spectrophotometer at 412 nm [15].

2.4. NADPH oxidase activity and H_2O_2 generation

NOX activity was quantified in particulate fractions of RP fat pad by the Amplex red®/horseradish peroxidase assay (Molecular Probes, Invitrogen) [16]. RP tissues were homogenized and then centrifuged at 720 × *g* for 15 min at 4°C. After that, the pellet was collected and resuspended. Then, the supernatant was centrifuged twice at 100,000 × *g* for 35 min at 4 °C for obtaining the microsomal fraction (MF) and resuspended in 50 mM sodium phosphate buffer, pH 7.2, containing 0.25 M sucrose, 2 mM $MgCl_2$, 5 mg/mL aprotinin and 34.8 mg/mL phenylmethylsulfonyl fluoride (PMSF), and stored at −80 °C. In order to evaluate NOX activity, MF was incubated with sodium phosphate buffer containing SOD (100 U/mL; Sigma, USA), horseradish peroxidase (0.5 U/mL, Roche, Indianapolis, IN), Amplex red (50 mM;

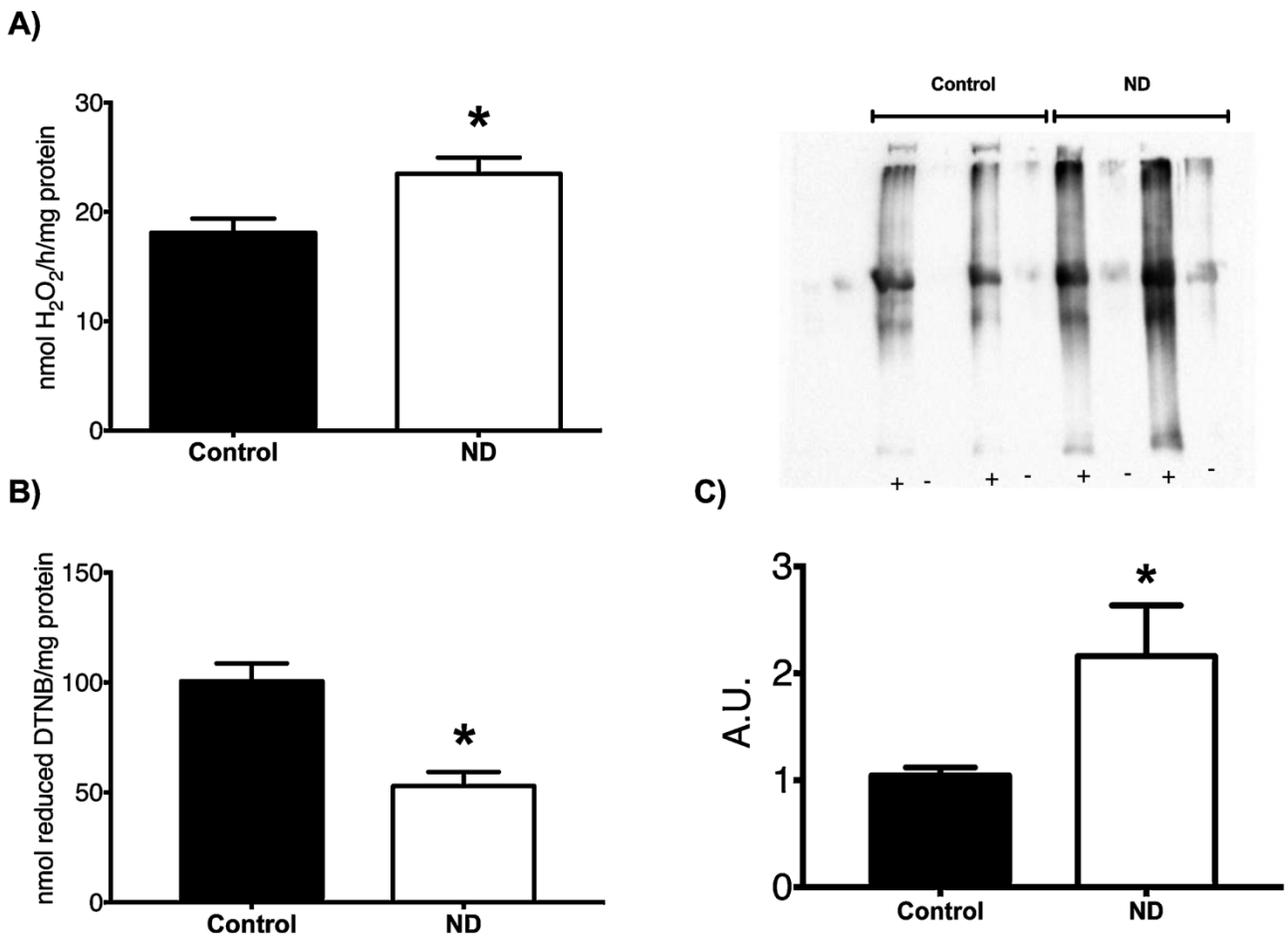

Fig. 3. Effect of nandrolone decanoate (ND) on reduced thiol, NOX activity and protein carbonylation. Reactive protein thiol levels (A) was assessed by measurement of total sulfhydryl groups by the reaction of thiols with DTNB, evaluated in a spectrophotometer at 412 nm. Protein concentration of the samples was fixed. H_2O_2 production (B) was determined in the microsomal enriched fraction by the Amplex red/horseradish peroxidase assay. Carbonylated protein and representative blot of carbonylated protein residues (C). Residues of carbonylated protein were evaluated based on the reaction with dinitrophenyl hydrazine (DNPH) and quantifies by Oxyblot assay (values were normalized by control mean of samples of each oxyblot gel). In the representative blot of the expression of carbonylated protein in control and ND groups (C), $^{+}$ indicates the representative lanes with DNTB-derivatization reaction, and – indicates the respective control lanes. * $p < 0.05$ (unpaired Student *t*-test) ND group (n = 8) vs. Control group (n = 8).

Molecular Probes, Eugene, OR), 1 mM EGTA, and 1 mM NADPH. The fluorescence was immediately measured in a microplate reader (Victor X4; PerkinElmer, Norwalk, CT) at 30 °C, using excitation at 530 nm and emission at 595 nm. The specific enzymatic activity was expressed as nanomoles of H_2O_2 per hour per milligram of protein (nmol h^{-1} mg^{-1}).

2.5. Protein carbonylation assay by Oxyblot

Protein concentration of the samples was fixed (10 μg per μL of sample) in order to ensure that there was no difference in the protein contents between control and ND group. Carbonylated protein residues were evaluated based on the reaction with dinitrophenyl hydrazine (DNPH), using the Oxyblot-kit (Chemicon®, Milipore), according to the manufacturer's protocol. SDS-PAGE was prepared according to a previously described protocol [17,18]. The proteins were transfed from SDS-PAGE to a nitrocellulose membrane (Amersham™ Protran®).

After that, nonspecific binding blockade was performed in a blocking solution for 1 h, then washed with PBS. Then, the membrane was incubated with the primary antibody (Rabbit Anti-DNP provided by the kit, 1:150) for 1 h, then washed again and incubated for a further 1 h with the secondary antibody (Goat Anti-Rabbit IgG [HRP-conjugate, provided by the kit], 1:300 dilution).

2.6. Cytokine dosage

TNF-α, IL-1β, and IL-6 levels were measured in the tissue's homogenate by ELISA kits (R&D Systems Inc., Minneapolis, MN, USA), following the manufacturer's instructions. Equal amounts of homogenized tissue were used in each experimental group (50 uL). 96-well plates were sensitized with anti-mouse monoclonal antibody and incubated overnight in a humid chamber at room temperature. Then the wells were washed 3 times and the plate was blocked to avoid nonspecific binding and incubated 1 h at room temperature, then washed again.

After blocking, the samples were added and placed back in a humid chamber and incubated overnight at room temperature. Then, detection antibody in dilution reagent were added to each well and, after that, the plate was then incubated at room temperature for 1 h. After washing the wells again, streptoavidin was added, then the plate was incubated in the dark at room temperature for 30 min. After that, and after a further wash of the wells, the substrate solution was added, followed by a 30-minute incubation at room temperature in the dark. The reaction was stopped with H_2SO_4. Absorbance was measured by spectrophotometry at a wavelength of 490 nm. The result was normalized by the amount of sample's protein.

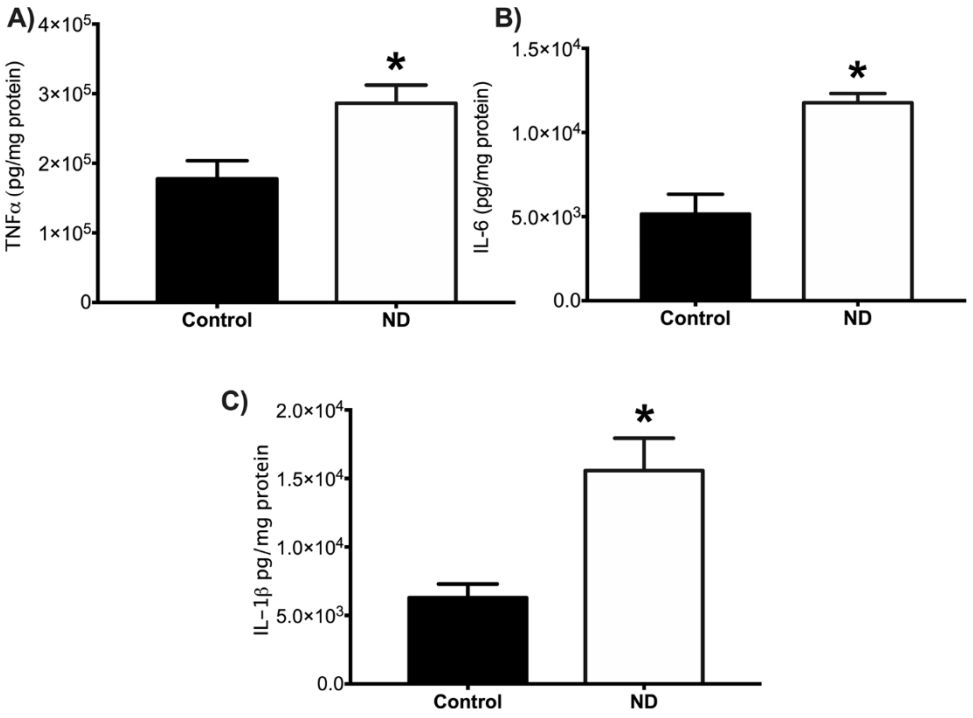

Fig. 4. Effect of nandrolone decanoate (ND) on the levels of inflammatory cytokines in the retroperitoneal adipose tissue. TNF-α (A), IL-6 (B), and IL-1β (C) were measured in retroperitoneal fat pad by ELISA. Data are expressed by mean and standard error of the mean. * $p < 0.05$ (unpaired Student *t*-test) ND group (n = 6) vs. Control group (n = 5).

2.7. Statistical analysis

All the results are expressed as mean ± standard error of the mean and were analyzed by unpaired Student's *t*-test. Statistical analyses were carried out using GraphPad Prism (version 7.01, GraphPad Software Inc., San Diego, USA) and the minimum level of significance was set at 5% ($p < 0.05$). Shapiro-Wilk normality test was used for normality assumption.

3. Results

3.1. Body weight, adiposity and glucose tolerance

The body weight after 8 weeks of treatment was lower in ND group when compared to control ($p = 0.0042$) (Fig. 1A). The weight of all three fat pads, RP, EP and SC, were decreased in DN compared to Control ($p < 0.0001$, $p < 0.001$ and $p < 0.001$, respectively). When relativized to body weight, the weights of RP and SC were decreased in ND group ($p < 0.001$ and $p = 0.0115$, respectively), while EP weigh were not different ($p = 0.088$) (Fig. 1C). As shown in Fig. 1D and E, ND group had higher glucose tolerance, based on the area under the IPGTT curve.

3.2. Redox homeostasis

For analyzing redox homeostasis, we assessed different redox parameters, such as NOX activity, antioxidant enzymes activities, residues of reduced thiol, and protein carbonylation.

SOD (Fig. 2A), CAT (Fig. 2B) and GPx (Fig. 2C) activities were lower in ND group compared to control ($p = 0.020$, 0.003 and 0.049, respectively).

ND had a greater H_2O_2 production by NOX activity ($p = 0.02$) in RP (Fig. 3A), together with lesser total reduced thiol residues ($p = 0.001$) (Fig. 3B) and a greater amount of carbonylated protein residues ($p = 0.02$) (Fig. 3C).

3.3. Inflammatory cytokines levels

To investigate the inflammatory profile in response to a chronic treatment with high dose of ND, we evaluated the levels of TNF-α (Fig. 4A), IL-6 (Fig. 4B) and IL-1β (Fig. 4C). All of the cytokines investigated were higher in ND group compared do control group ($p = 0.022$, 0.0028 and 0.013, respectively).

4. Discussion

Confirming their action in the organism, supraphysiological doses of ND reduced the animals' body weight, as well as SC, EP, and RP fat pads weight. Although reduction of the body weight is often related to a decreased food intake in response to ND administration [19,20], those results could be mainly related to the already well-characterized metabolic effects of AAS in WAT [21,22]. AAS are known for stimulating lipolysis by increasing beta-adrenergic receptors, adenylate cyclase and protein kinase A, and also inhibiting adipogenesis by decreasing lipid uptake, lipoprotein lipase activity, leptin production and differentiation of adipocyte precursors [9,23]. Moreover, proinflammatory cytokines can also be strong stimulators of lipolysis, which were higher in ND group. Besides the weight reduction in all of the tested fat pads, the greater response of RP can be related to the different density of androgen receptors among fat pads. Androgen sensitivity is more pronounced in deep fat depots in comparison to other adipose compartments, due to a higher expression of androgen receptors [9,10]. Those expected features are also the reason why RP was selected as the investigated tissue by our study, besides its importance as a visceral fat pad. The effects of high-doses ND on lowering glycemia were expected as well, possibly explained by mechanisms evolving higher serum insulin together with a less effective gluconeogenesis [24].

In order to determinate the effect of ND on WAT redox homeostasis, we investigated ROS production, reduced thiol levels, enzymatic antioxidant activity and oxidative damage to proteins. NOX activity was higher in WAT of ND treated rats. Once ND is an agonist of androgens receptor, NOX activity was likely increased by either genomic or non-genomic mechanisms. It has already been demonstrated that NOX mRNA levels and activity can be induced by AAS, such as ND, testosterone and its metabolites [24–26]. Androgens are also capable of up-regulating the expression of NOX subunits, and of increasing NOX activity through p47phox phosphorylation [25,27]. Such features could explain the higher NADPH oxidase-dependent H_2O_2 generation

observed in ND group in our study.

ROS availability in a given tissue depends on their production and detoxification rates. We observed that high-doses of ND lead to a decrease of three important antioxidant enzymes, SOD, CAT, and GPx. Decreased activity of the antioxidant enzymes has already been associated with administration of high doses of ND in various tissues [28–31]. A single supraphysiological dose of testosterone is already proven to decrease SOD, CAT, and GPx expression in some tissues, such as vascular and cardiac, besides increasing ROS production [32,33]. The explanation for the reduced expression of these antioxidant enzymes is not yet very clear. It could be due to the cytotoxic metabolites of ND and directly related to the condition of being under the effect of a larger amounts of ROS [29].

It is well known that exposure to high ROS levels can induce cytotoxic effects, apoptosis, cell death and necrosis in a variety of cell types [34–36]. In order to assay the redox-status and protein oxidative damage, we quantified total thiol levels and protein carbonyl content. ND group showed a lower total reduced thiol levels, together with higher levels of carbonyl groups in WAT. Lower thiol levels indicate a higher availability of ROS in RP [29], what could cause oxidative damage to the macromolecules of this tissue. Studies have linked protein carbonylation in adipose tissue to redox-based cell dysfunctions [37–40], although an association to AAS was yet to be demonstrated.

Since oxidative damage is related to cell injury, the adipocyte itself likely secretes proinflammatory cytokines, or even triggers signaling pathways for the production of proinflammatory cytokines. Once activated, many immune cells produce ROS, so inflammation itself would reinforce the oxidative stress, creating a vicious cycle. Inflammatory cytokines also play a role in the activation of NOX enzymes, what could also explain the higher NOX-dependent H_2O_2 production found in rats treated with ND [41,42]. The levels of proinflammatory cytokines are closely linked to adipose inflammation. Thus, we measured the levels of three pro-inflammatory cytokines: tumor necrosis factor-alpha (TNF-α) and interleukins 6 (IL-6) and 1-beta (IL-1β). We observed higher levels of all cytokines tested in WAT of ND group, confirming the expected molecular link between AAS-induced oxidative stress and inflammation in RP.

It is known that a disrupted redox homeostasis is involved in inflammatory reactions [5–8], and the mutual association among ND, oxidative stress and pro-inflammatory cytokines were already described in various tissues [43–46]. Lower thiol levels have often been related to cell death induced by TNF-α [47–49]. Importantly, TNF-α can be related to the increased uncoupling of oxygen consumption and to the interaction of electrons with oxygen, increasing the proportion of the generation of superoxide anions [50,51], while antioxidant treatment in the adipocytes attenuates the expression of TNF-α genes [52]. IL-6 expression and secretion seem to be stimulated by H_2O_2 [53,54], and it was also confirmed that, in adipocytes culture, the adding of glucose oxidase increases IL-6 expression [55]. In turn, IL-1β, mainly released by monocytes in response to tissue damage, infection or immunologic stress has been proven to cause additional production of pro-inflammatory cytokines [56]. Although IL-1β secretion seems to be redox-independent in some cases [57], studies have found its relationship with redox-related mechanisms [58,59].

Besides their association to oxidative stress, proinflammatory cytokines are also known for their role on lipolysis stimulation in adipocytes [51,60]. Furthermore, the expression of genes related to the regulation of lipolysis can be regulated by TNF-α in fat cells [60–62]. It is also known that IL-6 stimulates lipolysis and fat oxidation [63,64]. Thus, the lipolytic stimuli of ND found herein should be related to the higher levels of TNF-α and IL-6 in RP.

Overall, our present study pioneerly shows that supraphysiological doses of ND disrupts redox homeostasis and triggers an inflammatory response in retroperitoneal white adipose tissue. These physiological alterations in WAT could lead to a series of health complications, including insulin resistance and metabolic syndrome. The warning about risks of the non-professionally-oriented use of such a popular substance should be reinforced.

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CRediT authorship contribution statement

Saulo Chaves Magalhaes: Conceptualization, Methodology, Investigation, Writing - original draft, Project administration. **Keciany Alves de Oliveira:** Methodology, Investigation, Data curation. **Paula Alexandre Freiras:** Methodology, Investigation, Data curation. **Maria Diana Moreira Gomes:** Methodology, Investigation, Data curation. **Leonardo Matta Pereira:** Methodology, Investigation, Data curation. **Luiz Fonte Boa:** Methodology, Investigation, Data curation. **Denise Pires de Carvalho:** Resources, Supervision. **Rodrigo Soares Fortunato:** Resources, Supervision, Writing - review & editing. **Adriano Cesar Carneiro Loureiro:** Supervision, Validation. **Luciana Catunda Brito:** Supervision, Validation. **Ariclécio Cunha de Oliveira:** Conceptualization, Methodology, Investigation, Data curation, Project administration, Supervision, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2020.105728.

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