ACUTE EFFECTS OF NANDROLONE DECANOATE ON OXIDATIVE STRESS IN ISOLATED RAT HEART

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Abstract - Abuse of anabolic-androgenic steroids (AAS) produces side effects in different tissues, with oxidative stress linked to their pathophysiology, being involved in fibrosis, cellular proliferation, and tumorigenesis. The aim of this study was to examine the acute effects of nandrolone decanoate (ND) on oxidative stress in isolated rat heart. The hearts of male Wistar albino were excised and perfused according to the Langendorff technique at gradually increasing coronary perfusion pressures (40-120 cmH2O). The hearts were perfused with ND at doses of 1, 10 and 100 μM. Oxidative stress markers, including the index of lipid peroxidation (thiobarbituric acid reactive substances (TBARS)), nitric oxide (nitrites; NO2−), the superoxide anion radical (O2−) and hydrogen peroxide (H2O2) were measured in the coronary venous effluent. Our results showed that acute effects of ND do not promote the production of reactive oxygen species (ROS). Our finding pointed out that the highest concentration of ND may even possess some anti-oxidative potential, which should be examined further.

Key words: anabolic steroids; isolated rat heart; Langendorff technique; oxidative stress; nandrolone decanoate

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INTRODUCTION

Anabolic-androgenic steroids (AASs) are synthetic derivatives of testosterone and are important pharmacologically for their use in the treatment of various medical conditions, such as growth deficiency, certain blood disorders, osteoporosis, hypogonadal dysfunction and the commencement of delayed puberty in men and growth promotion. However, AASs have not always been used purely for medical purposes. Due to their anabolic effects, AASs became vastly popular among swimmers, weight lifters and both male and female professional athletes, recreational athletes, prepubescents and adolescents (Do Carmo et al., 2011; Tan et al., 1995). The use of AASs to enhance physical performance or appearance has greatly increased, and individuals usually administer doses that are 10 to 100 fold higher than the therapeutic dose. This abuse can cause many adverse effects (Yersalis et al., 1995). In the heart, AAS abuse
increases the risk of cardiovascular disease, possibly due to increased total cholesterol and low density protein levels, decreased high density lipoprotein levels, increased blood pressure, thrombosis, myocardial infarction and heart failure (Sullivan et al., 1998). On the other hand, little is known about influence of ND on different molecular interactions, such as the production of free radicals.

Developed by the laboratory Organon and introduced in the market in 1962, nandrolone decanoate (ND) or Deca-Durabolin™, whose active ingredient is nandrolone, is one of the most widely used AAS in the world. It is available commercially as an injectable anabolic preparation and its action lasts for up to three weeks, after intramuscular administration in humans (Tylicki et al., 2007). Compared to testosterone, ND shows more anabolic action and less androgenic activity (Piovesan et al., 2013; Phillis et al., 2007; Pozzi R et al., 2013).

Liver, heart and kidney pathophysiology are usually linked to oxidative stress, which is characterized by a disruption of redox signaling. Reactive oxygen species (ROS), such as the superoxide anion radical (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), can be formed by xanthine-oxidase, cytochrome P-450 or mitochondrial electron transport chain, as a by-product, or directly by the NADPH oxidase (NOX) family of enzymes (Aguirre et al., 2010). Intracellular ROS levels are maintained at adequate levels by antioxidant systems that react with these molecules producing less reactive compounds. Catalase (CAT) and glutathione peroxidase (GSHPx) are involved in H$_2$O$_2$ detoxification, producing H$_2$O directly or in a GSH-dependent reaction, while superoxide-dismutase (SOD) catalyzes the conversion of superoxide to H$_2$O$_2$ (Jones, 2008).

As the majority of side effects elicited by AAS have their etiology linked to oxidative stress, the aim of this study was to investigate the acute and direct effects of ND on oxidative stress in isolated heart of adult male rats.

MATERIALS AND METHODS

Isolated rat heart preparation

The experimental protocol was approved by the Faculty of Medical Sciences Ethics Committee for the welfare of experimental animals, University of Kragujevac. After a short ketamine/xylazine-induced narcosis, emergency thoracotomy was performed, and the hearts of male Wistar albino rats (n = 36; 12 in each experimental group; age 8 weeks; body mass 180–200 g) were attached to the Langendorff apparatus via an aortic cannula. The hearts were retrogradely perfused according to the Langendorff technique at gradually increasing perfusion pressure (40 cmH$_2$O–120 cmH$_2$O). The hearts were perfused with Krebs-Henseleit solution (118mM NaCl, 4.7 mM KCl, 2.5 mM CaCl$_2$, 1.7 mM MgSO$_4$, H$_2$O, 25 mM NaHCO$_3$, 1.2 mM KH$_2$PO$_4$, 5.5 mM glucose, equilibrated with 95% O$_2$/5% CO$_2$ and warmed to 37°C (pH 7.4).

Physiological assay and experimental protocol

After heart perfusion commenced, a 30 min period was allowed for stabilization of the heart. After an equilibration period (70 cmH$_2$O), CPP was lowered to 60 cmH$_2$O, and then gradually increased to 80 cmH$_2$O, 100 cmH$_2$O, and 120 cmH$_2$O, and finally lowered to 40 cmH$_2$O. After setting up the control experimental protocol (Krebs-Henseleit physiological solution [control group]), the hearts of the first group were perfused with 1 µM, the second group with 10 µM and the third group with 100 µM of ND. The flow was considered stable at each value of perfusion pressure, when three repeated values of CF were the same. In the collected samples of coronary venous effluent, following markers of oxidative stress were measured spectrophotometrically: (i) index of lipid peroxidation (measured as TBARS - thiobarbituric acid reactive substances); (ii) nitrites (NO$_2^-$); (iii) hydrogen peroxide (H$_2$O$_2$); (iv) superoxide anion radical (O$_2^-$).
Biochemical assays

TBARS determination (index of lipid peroxidation)

The degree of lipid peroxidation in the coronary venous effluent was estimated by measuring TBARS using 1% thiobarbituric acid in 0.05 NaOH, incubated with the coronary effluent at 100°C for 15 min and measured at 530 nm. Krebs-Henseleit solution served as a blank probe (Ohkawa et al., 1979).

Determination of nitrites (NO₂⁻)

Nitric oxide decomposes rapidly to form stable metabolite nitrite/nitrate products. The nitrite level (NO₂⁻) was measured and used as an index of nitric oxide (NO) production using Griess reagent. A total of 0.5 ml of perfusate was precipitated with 200 μl of 30 % sulphosalicylic acid, vortexed for 30 min, and centrifuged at 3 000 x g. Equal volumes of the supernatant and Griess reagent, containing 1% sulfanilamide in 5% phosphoric acid/0.1% naphthalene ethylenediamine dihydrochloride, was added and incubated for 10 min in the dark and measured at 543 nm. The nitrite levels were calculated using sodium nitrite as the standard (Green et al., 1982).

Determination of hydrogen peroxide (H₂O₂)

A measurement of hydrogen peroxide (H₂O₂) is based on the oxidation of phenol red by hydrogen peroxide, in a reaction catalyzed by horseradish peroxidase (HRPO). Two hundred μl of perfusate was precipitated with 800 ml of freshly prepared phenol red solution, followed by the addition of 10 μl of (1:20) HRPO (made ex tempore). For the blank, Krebs-Henseleit solution, instead of the coronary venous effluent, was used. The level of H₂O₂ was measured at 610 nm (Pick et al., 1980).

Determination of superoxide anion radical (O₂⁻)

The concentration of the superoxide anion radical (O₂⁻) was measured after the reaction of nitro blue tetrazolium in TRIS buffer with the coronary venous effluent, at 530 nm. Krebs-Henseleit solution served as a blank (Auclair et al., 1980).

Drugs

Nandrolone decanoate (DECA, 300mg /10ml) was purchased from Genox Laboratory, made in EU.

Statistical analysis

Statistical analysis of experimental data included the following basic descriptive statistics: the mean value (𝑥̅) standard deviation (SD) and standard error mean (SEM). For testing the normality of the distribution parameters, the Kolmogorov-Smirnov test was used. To test the statistical significance of the results and to confirm the hypothesis, the following statistical tests were used: Student's t-test (parametric test), for dependent and independent variables. A database analysis of the results was performed using software package SPSS 20 (SPSS Inc., Chicago, IL, USA). P < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The present study aimed to examine the effects of acute administration (1 µM, 10 µM, 100 µM) of nandrolone decanoate (Deca-Durabolin, DECA) on oxidative stress biomarkers in isolated rat hearts. ROS are normally produced by almost all cells of living organisms. ROS act in the redox-dependent regulation of different cellular functions, including response to stressors, angiogenesis, cell proliferation and other processes (Giorgio et al., 2007). There is insufficient data regarding ROS production in response to the application of steroid hormones, especially in heart muscle. ND is one of the most frequently used anabolic steroids, and it was used in our investigation. To maintain vitality of the heart, every group was perfused with one concentration of ND (1 µM, 10 µM, or 100 µM). As a result, the control values of all estimated parameters are different between groups (i.e. every group has its own control).

First, we investigated the influence of different concentrations of ND on lipid peroxidation by measuring the TBARS. There were no statistically significant changes in TBARS values during the application of 1 µM ND, 10 µM ND or 100 µM ND over the en-
None of the used concentrations induced significant changes in TBARS dynamics compared to their controls in the entire CPP range. This result may indicate that because of the short term application, ND did not have enough time to affect membranes and/or damage them. Literature data regarding the effects of anabolic steroids on cell membranes are scant. The prevailing opinion is that acute exposure to these agents does not provoke lipid peroxidation, and may even suppress it, for example after muscle denervation (Brazaluk et al., 1997). Otherwise, the newer studies suggest that chronic administration of ND is connected with increased lipid peroxidation in mice kidneys (Riezzo et al., 2014).

The second marker whose dynamics we investigated was NO. Administration of 1 µM ND induced a statistically significant increase in NO_2^- release at CPP = 60 cmH_2O, while administration of 100 µM ND induced a statistically significant decrease in NO_2^- release at CPP = 120 cm H_2O. Compared to the control conditions, the amount of NO_2^- released was not changed significantly during administration of 10 µM ND for any CPP value (Fig. 2A-C). Administration of the lowest concentration of ND (1 µM), was associated with higher values of NO_2^- than the control conditions at all pressures (except at CPP = 120 cmH_2O), with statistical significance at CPP = 60 cmH_2O (Fig. 2A); the highest concentration of this drug caused a significant decrease at the highest perfusion pressure (CPP = 120 cmH_2O) (Fig. 2C). A jump in NO release during application of the lowest dose may represent a beneficial effect in terms of increase of coronary perfusion mediated via NO. In that sense, the decrease in NO release after admin-
istration of the highest dose of ND, indicates that at this dose, anabolic steroids induce vasoconstriction and reduce heart muscle perfusion.

The dynamics of $\text{H}_2\text{O}_2$ was different compared to the above parameters. Administration of 10 µM ND induced a significant decrease in $\text{H}_2\text{O}_2$ release at CPP = 100 cmH$_2$O and CPP = 120 cm H$_2$O; administration of 100 µM ND induced a significant decrease in $\text{H}_2\text{O}_2$ release at CPP = 60 cm H$_2$O. There were no statistically significant changes in $\text{H}_2\text{O}_2$ release during the application of 1 µM ND over the entire CPP range, (Fig. 3A-C). Namely, administration of all concentrations of ND induced a decrease in $\text{H}_2\text{O}_2$ release (Fig. 3A-C). The reduction was most prominent (and significant) at CPP = 100 and 120 cmH$_2$O (Fig. 3B), and highest at CPP = 60 cmH$_2$O (Fig 3C). More recent studies (Frankenfeld et al., 2014) have shown that after 8 weeks of ND application (in similar range of values), the production of $\text{H}_2\text{O}_2$ in rat hearts was increased. The results of our and mentioned studies suggest that the oxidative potential of ND depends of the duration of exposure, i.e. acute application may even provide an anti-oxidant effect, whereas chronic application displayed a clear pro-oxidant potential.

Administration of 100 µM ND induced a statistically significant decrease in $\text{O}_2$ release at CPP = 120 cmH$_2$O. There were no statistically significant changes in $\text{O}_2$ release during the application of 1 µM ND and 10 µM ND over the entire CPP range, (Figs. 4A-C). The lowest dose of ND (1 µM) caused the greatest increase in $\text{O}_2$ release that was not statistically significant (Fig. 4A), while after administration
of 100 µM ND this marker decreased with statistical significance at CPP = 120 cmH 2O (Fig. 4C). Having in mind that the most pronounced effect on lipid membrane damage is caused by H₂O₂ and O₂⁻, the observed results regarding these two markers may helps us to explain the absence of membrane damage (no changes in TBARS values) after ND exposure. In summary, The acute application of ND affects the production of oxidative stress markers in a dose-dependent manner. The highest concentration of ND induced the most prominent changes in NO, O₂⁻ and H₂O₂ dynamics.

Martins et al. (2011) concluded that the therapeutic dose of ND was capable of neutralizing free radical production in blood serum. However, at present we cannot provide an unequivocal mechanism for the effects of ND. Our results are in agreement with Celec et al. (2003) who showed that ND was capable of partially protecting the liver and cerebellar tissue from ethanol-induced injury mediated by oxidative stress. On the other hand, many studies confirm the negative influence of ND on oxidative stress parameters (Sun et al., 2013; Chaves et al., 2013; Chaves et al., 2006; Liu et al., 2002; Frankenfeld et al., 2014). AASs increase anabolic effects and overcome catabolic pathways. Thus, it is possible that the net result of AAS administration is increased oxidative stress. Rat liver lysosomal and mitochondrial activities are modified by anabolic-androgenic steroids. It has been shown previously that prolonged administration of stanozolol (17-α-alkylated AAS) provoked dysfunction of mitochondrial respiratory chain complexes and mono-oxygenase systems. While it is possible that these alterations were accompanied by increased ROS generation, details of the mechanism through which intramuscular injection of ND is associated with free radical production remain to be identified (Molano et al., 1997; Saborido et al., 1993).

Finally, it is important to emphasize that the differences between the results of our study and other studies may be because of different experimental models and applied doses of ND. In that sense, we suggest that the chronic administration and higher exposure doses cause increased generation of ROS. Our study provides data regarding acute toxicity of ND, which may be of clinical interest when faced with overdose with this drug.

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Author’s contributions

Maja Jevđević performed the experiments on the Langendorff apparatus; Maja Jovanović performed the biochemical assays and statistical analysis; Nevena Jeremić performed the biochemical assays; Marija Canković performed the biochemical assays; Jovana Jeremić performed the biochemical assays; Vladimir Živković performed the experiments on Langendorff apparatus; Ivan Srejović performed the experiments on Langendorff apparatus; Dragan Đurić designed the study and prepared the manuscript; Vladimir Jakovljević designed the study and prepared the manuscript.

Conflict of interest disclosure

All authors of the present paper disclose no actual or potential conflicts of interest, including any financial, personal, or other relationships with people or organizations.

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