

# Effects of Nandrolone Stimulation on Testosterone Biosynthesis in Leydig Cells

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Anabolic androgenic steroids (AAS) are among the drugs most used by athletes for improving physical performance, as well as for aesthetic purposes. A number of papers have showed the side effects of AAS in different organs and tissues. For example, AAS are known to suppress gonadotropin-releasing hormone, luteinizing hormone, and follicle-stimulating hormone. This study investigates the effects of nandrolone on testosterone biosynthesis in Leydig cells using various methods, including mass spectrometry, western blotting, confocal microscopy and quantitative real-time PCR. The results obtained show that testosterone levels increase at a 3.9  $\mu\text{M}$  concentration of nandrolone and return to the basal level a 15.6  $\mu\text{M}$  dose of nandrolone. Nandrolone-induced testosterone increment was associated with upregulation of the steroidogenic acute regulatory protein (StAR) and downregulation of 17 $\alpha$ -hydroxylase/17, 20 lyase (CYP17A1). Instead, a 15.6  $\mu\text{M}$  dose of nandrolone induced a down-regulation of CYP17A1. Further in vivo studies based on these data are needed to better understand the relationship between disturbed testosterone homeostasis and reproductive system impairment in male subjects.

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Anabolic androgenic steroids (AAS) are probably the most commonly used drugs among athletes for improving physical performance (Gheshlaghi et al., 2015; Piacentino et al., 2015). The use of AAS was forbidden by the International Olympic Committee in 1976, and the World Anti-Doping Agency has included these compounds in the list of prohibited substances (Hartgens and Kuipers, 2004). Despite these bans, AAS consumption is constantly increasing particularly among the younger population at fitness centers (Hartgens and Kuipers, 2004), as well as among teenagers, also for aesthetic purposes (McPhail et al., 2014). Hence, the abuse of AAS is nowadays considered a major and widespread public health issue (Basaria, 2010).

Athletes usually take AAS in the form of intramuscular injections and it has been estimated that AAS abusers consume 600–5000 mg of AAS per week; this is 10–100 times more than the normal testosterone production level by testes (Fronczak et al., 2012). Such dosage may cause serious health consequences especially on male fertility, such as the suppression of the spermatogenesis, testicular atrophy, gynecomastia, and virilization (Basaria, 2010; Chimento et al., 2012; Fronczak et al., 2012). The use of synthetic testosterone and cognate substances leads to a reduction in natural testosterone production, mostly due to

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a disturbance of the feedback in the hypothalamic–pituitary–gonadal axis, with consequent decrease in its levels in circulation (Kostic et al., 2011; Rahnama et al., 2014). AAS disturb the endogenous production of gonadotrophins and androgens, which can in turn affect the ultrastructure of the testes (Naraghi et al., 2010; Rahnama et al., 2014). This effect may persist for a long time after drug withdrawal (Keeney et al., 1990). Moreover, there are clear evidence of other serious side effects of AAS administration, that include increased chance of heart attacks, mood changes, liver cancer, and premature arrest of bone growth in younger males (Bhasin et al., 2006; Basaria, 2010; Bhasin, 2010; Turillazzi et al., 2011; Pope et al., 2014).

Nandrolone is considered to be one of the most commonly abused AAS worldwide (Shahidi, 2001; Hartgens and Kuipers, 2004). It has a structure very similar to testosterone and it is included in the class II of AAS which includes 19-nor-testosterone-derivates. Its properties include boosting tissue-building, maintenance of strength and muscle mass, libido, and bone health. Endogenous nandrolone is produced as a byproduct during biochemical transformational reactions, and its main metabolite (norandrosterone) can be detected in human urine samples in a concentration range of 0.01–0.14 mg/l (Reznik et al., 2001; Bjelic et al., 2014). Despite the beneficial effects of nandrolone and its use in the treatment of many clinical conditions (Wood, 2004), nandrolone administration to male rats induced spermatogenic cell apoptosis (Shokri et al., 2010). Nevertheless, the mechanisms underlying nandrolone-induced testicular toxicity are not yet fully understood.

Testosterone, like other androgens, is produced within the testis by specialized steroidogenic cells known as Leydig cells (Kostic et al., 2011). The production of this hormone is regulated by a complex neuroendocrine mechanism which involves the pulsatile release of luteinizing hormone (LH) and subsequent cAMP activation of a steroidogenic cascade. Numerous steroidogenic stimuli, as well as intratesticular factors, play a role in the intricate regulatory network of testosterone (Wu et al., 2007; De Maddalena et al., 2012; Janjic et al., 2012). Cholesterol is the common substrate for all types of steroid hormone biosynthesis (including the testosterone one) which begins in the cytosol and is completed in the mitochondria. Steroidogenic acute regulatory protein (StAR) transfers cholesterol to the inner membrane of mitochondria and, through the mobilization and delivery from the outer to the inner mitochondrial membrane, cholesterol is converted to pregnenolone by the cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1) (Stocco et al., 2005). Pregnenolone is further metabolized to progesterone by mitochondrial or microsomal hydroxy-delta-5-steroid dehydrogenase, 3 beta-, and steroid delta-isomerase 1 (HSD3B1). In Leydig cells, maturation of progesterone to androstenedione is catalyzed by the 17 $\alpha$ -hydroxylase/17, 20 lyase (CYP17A1); further, the conversion of androstenedione to testosterone is dependent on the activity of 17 $\beta$ -hydroxysteroid dehydrogenase, a steroid dehydrogenase specific for androgen production (Kostic et al., 2011; Macaluso et al., 2013).

The aim of this study was to investigate the effects of nandrolone on testosterone production by Leydig cells, by evaluating levels, and expression of the main enzymes involved in testosterone biosynthesis.

## Materials and Methods

### Cell cultures

R2C cells (cat. No. 89031606, ECACC, Health Protection Agency Culture Collections, Salisbury, United Kingdom), a rat Leydig cell line used for testing the steroidogenic function, were cultured in M-199 medium (Invitrogen Corp., Carlsbad, CA) supplemented

with 15% horse serum (HS; Invitrogen Corp.), 2.5% fetal bovine serum (FBS; Invitrogen Corp.), and an antibiotic and antimycotic solution (Invitrogen Corp.) containing 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 0.25  $\mu$ g/ml amphotericin B. Cells were incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>, and maintained in culture as previously described (Macaluso et al., 2012; Barone et al., 2013). When cells reached approximately 80% confluence, they were treated for 48 h with different concentrations of nandrolone prepared by first dissolving Nandrolone Vetrinal analytical standard (Sigma–Aldrich, St. Louis, MO) in absolute ethanol to obtain a working solution, and then in the culture medium without horse serum to reach the desired concentrations (0–15.6  $\mu$ M).

### Cell viability test

3-[4,5-Dimethylthiazolyl]-2,5-diphenyltetrazolium bromide (MTT) assay was conducted to detect cell proliferation. A total of  $5 \times 10^3$  cells per well were seeded in 96-well plates, and after the incubation period of 24 and 48 h with serial dilutions of nandrolone working solution (0–500  $\mu$ M), fresh MTT solution (100  $\mu$ l), dissolved in PBS (0.5 mg/ml final concentration), was added to the cells. After 3 h of incubation at 37°C, the culture media were discarded and replaced with 100  $\mu$ l of Dimethyl Sulfoxide. Absorbance was measured at 570 nm in a spectrophotometer. The colorimetric assay was performed in triplicate. Absorbance of untreated cells (basal) was considered 100%.

### Liquid chromatography-mass spectrometry

Testosterone measurements were performed by “Locorotondo Labs S.r.l.,” Palermo, Italy. Testosterone in the cell culture medium was quantified using a validated method for serum/plasma analysis of testosterone by liquid chromatography-mass spectrometry (LC-MS/MS). The method, developed by Chromsystems<sup>®</sup>, was fully validated on an AB Sciex API 5500 QTrap<sup>™</sup> in terms of recovery (between 95% and 104%), linearity (0.01–6.00  $\mu$ g/L), CV percentage for intra-assay (2.6%), and CV percentage for inter-assay (5.2%) in serum. The apparatus employed consists of an HPLC Eksigent ultra LC 110-XL System, coupled with a AB Sciex API 5500 QTrap<sup>™</sup> mass spectrometer equipped with a TurbolonSpray<sup>®</sup> Ion Source, working in positive ion mode. The scan Mode used was Multiple Ion Monitoring (MRM) following the characteristic transition 289.1→96.9 (quantifier ion) and 289.1→109.0 (qualifier ion) for Testosterone and 292.2→96.9 (quantifier ion) and 292.2→109.0 (qualifier ion) for the Internal Standard (Testosterone d3). MS parameters, optimized in order to achieve an improved sensitivity, were the following: Curtain gas (CUR): 20 a.u.; Ion Spray Voltage (IS): 5500 V; Ion Source Gas 1 (GS1): 40 a.u.; Ion Source Gas 2 (GS2): 40 a.u.; Temperature (TEM): 650°C; Declustering Potential (DP): 90 for Testosterone and Testosterone d3; Entrance Potential (EP): 8 for Testosterone and 11 for Testosterone d9; Collision Energy (CE): 25 and 29 for the product ion 96.0 and 109.0 respectively, both for Testosterone and for Testosterone d3; Collision Exit Potential (CXP): 10 for Testosterone and Testosterone d3. Chromatographic elution was performed on a reversed phase column. Prior to be injected into the HPLC system, the samples were subjected to a complex process of extraction, purification, and concentration. This sample pre-treatment included the use of a reversed phase SPE plate (Solid Phase Extraction) that enabled an adequate degree of sensitivity even when analysing a complex matrix. Testosterone levels were also assayed in the culture medium alone as blank, and the value obtained was under the detection limit. Total testosterone analysis in cell cultures was performed in quadruplicate in four different independent experiments.

TABLE I. Forward and reverse primers used for qPCR

Primer	Forward	Reverse
GUSB	5'-ACCACCCCTACCACCTATATC-3'	5'-ATCCAGTAGTTCACCAGCCC-3'
HPRT1	5'-TGTCATGAAGGAGATGGGAG-3'	5'-ATCCAGCAGGTGAGCAAAG-3'
GAPDH	5'-GAAACCCATCACCATCTTCC-3'	5'-TCCACGACATACTCAGCAC-3'
CYP17A1	5'-TGATCCAAAAGTACCGCC-3'	5'-TCCACAGATTCTGTGCGCC-3'
STAR	5'-CCTTGGGCATACTCAACAAC-3'	5'-GCACCACCTTACTTAGCAC-3'

**Western blotting analyses**

Western blotting was performed as previously described (Barone et al., 2013). Equal amounts of proteins (40 µg) were separated on SDS-PAGE and transferred onto a nitrocellulose membrane (BioRad, Segrate, Italy). After blocking with 5% albumin bovine serum (Sigma-Aldrich), membranes were probed with primary antibodies [rabbit anti-CYP17A1 (M-80) clone polyclonal antibody, goat anti-HSD3B1 (P-18) clone polyclonal antibody, rabbit anti-StAR (FL-285) clone polyclonal antibody, rabbit anti-CYP11A1 (H-165) clone polyclonal antibody, and mouse anti-β Actin C-4 clone monoclonal antibody, Santa Cruz Biotechnology] diluted at 1:1000 overnight at 4°C. Protein bands were visualized using the enhanced chemiluminescence (ECL) detection system (GE Healthcare Life Sciences, Milan, Italy), the protein levels were normalized for β actin and the data were evaluated and quantified using ImageJ Free software (NIH, Bethesda, MD). Each experiment was performed in triplicate.

**Confocal analyses**

Immunofluorescence was performed as described previously (Gorska et al., 2013). Cells were fixed in ice-cold methanol, incubated with unmasking solution (10 mM trisodium citrate, 0.05% Tween-20) for 10 min, and treated with blocking solution (3% BSA in PBS) for 30 min at 24°C. Then, a primary antibody against CYP17A1 and StAR (Santa Cruz Biotechnology) was added and incubated in a humidified chamber overnight at 4°C, diluted 1:100. Cells were incubated for 1 h at RT with a conjugated secondary antibody (anti-rabbit IgG-FITC antibody produced in goat, Sigma-Aldrich, 1:200 dilution); followed by 15 min incubation with HOECHT33342 (Sigma-Aldrich) diluted 1:1000. Finally, slides were mounted with cover slips, and images were taken immediately with a Leica Confocal Microscope TCS SP8 (Leica

Microsystems, Heidelberg, Germany). The confocal microscopy analysis was carried out using the Leica application suite advanced fluorescences software. The staining intensity was measured as the mean pixel intensity (PI) normalized to the CSA (cross-sectional area) of all cells in five fields per slide. Each experiment was quadrupled.

**Quantitative real-time PCR (qRT-PCR)**

The qRT-PCR technique was performed as previously described (Barone et al., 2013; Di Felice et al., 2013). Total cellular RNA was isolated from cell cultures using TRIzol<sup>®</sup> REAGENT (Sigma-Aldrich), according to the manufacturer's instructions. RNA was retro-transcribed using the ImProm-II Reverse Transcriptase Kit (Promega Corporation, Madison, WI) to obtain cDNA, which was amplified using the StepOnePlus<sup>™</sup> Real-Time PCR System (Life Technologies, Monza, Italy). qRT-PCR analysis was performed using GoTaq qPCR Master Mix (A6001, Promega). The mRNA levels were normalized to the levels obtained for hypoxanthine phosphoribosyltransferase I (HPRT1), for beta-glucuronidase (GUSB) and for Glycereraldehyde 3-phosphate dehydrogenase (GADPH). Changes in the transcript level were calculated using the 2<sup>-ΔΔCT</sup> method (Livak and Schmittgen, 2001). The cDNA was amplified using the primers shown in Table I. cDNA was amplified using the Rotor-gene<sup>™</sup> 6000 Real-Time PCR Machine (Qiagen GmbH, Germany). Each experiment was performed in triplicate.

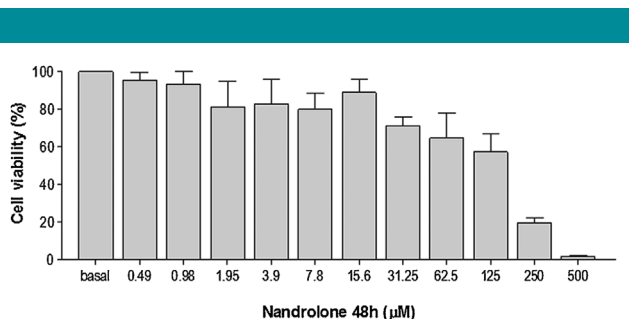


Fig. 1. Effects of nandrolone on R2C cell viability. Effects of treatment of R2C cells with different nandrolone doses (0–500 µM) for 48 h evaluated by MTT cell viability tests. Vertical axis: percentage of cell viability. Horizontal axis: nandrolone concentration, in µM. Basal: untreated cells. Data are presented as the mean ± SD of triplicate experiments.

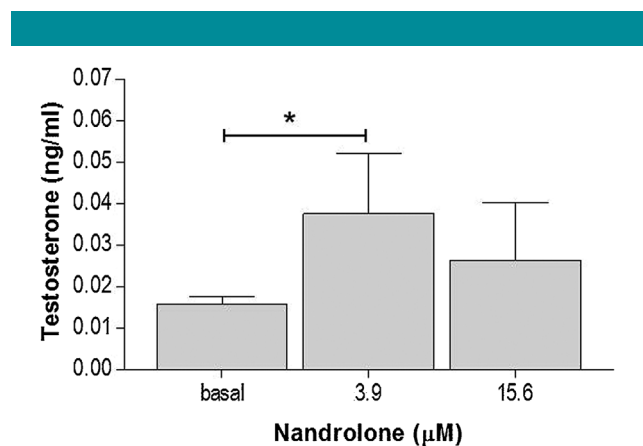
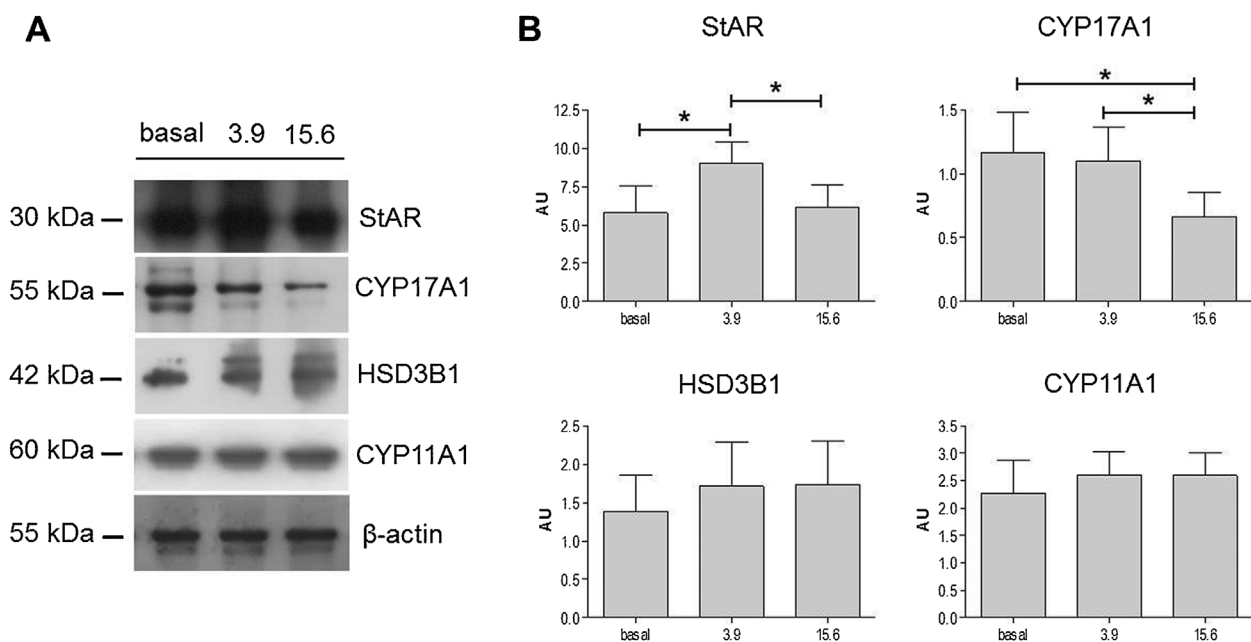


Fig. 2. Testosterone production. Testosterone production in cells treated with 3.9 and 15.6 µM nandrolone concentrations for 48 h. Vertical axis: testosterone levels (ng/ml). Horizontal axis: nandrolone concentration, in µM. Basal: untreated cells. Data are presented as the mean ± SD of quadruplicate experiments. \* = P < 0.001.



**Fig. 3.** Effect of nandrolone stimulation on steroidogenic proteins. **(A)** Representative cropped blots for StAR (30 kDa), CYP11A1 (60 kDa), HSD3B1 (42 kDa), and CYP17A1 (55 kDa). The gels were run under the same experimental conditions and  $\beta$ -actin was used as internal control. **(B)** Relative expression levels of StAR, CYP11A1, HSD3B1, and CYP17A1. Vertical axis: arbitrary units (AU). Horizontal axis: nandrolone concentration, in  $\mu$ M. Basal: untreated. Data are presented as the mean  $\pm$  SD of triplicate experiments. \* =  $P < 0.05$ .

### Statistical analyses

A one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons was used as an appropriate analysis for the data. All statistical analyses were performed using the GraphPad Prism<sup>TM</sup> 4.0 program (GraphPad Software Inc., San Diego, California). All data are presented as the mean  $\pm$  SD, and the level of statistical significance was set at  $P < 0.05$ .

## Results

### Cell viability

The inhibitory effect of nandrolone on R2C cell growth was evaluated using MTT cell viability assays. The cells were treated with serial dilutions of nandrolone within the range of 0–500  $\mu$ M, for 24 and 48 h. After 24 h of treatment, nandrolone did not induce significant cell death, while cell viability was affected by 48 h of nandrolone treatment at various concentrations. Figure 1 shows the mean value of cell viability after nandrolone treatment. To evaluate the effects of different nandrolone concentrations without significant cell death, 3.9 and 15.6  $\mu$ M of nandrolone were used for 48 h for further experiments.

### Testosterone production

To determine testosterone secretion, the medium was collected at the end of the incubation period and subsequently analyzed. The nandrolone working solution induced, after 48 h, an increase of the secreted testosterone in R2C cells treated with 3.9  $\mu$ M nandrolone, compared to the basal sample ( $P < 0.05$ ) (Fig. 2). Interestingly, a higher concentration (15.6  $\mu$ M) did not induce a significant testosterone increment compared to basal values.

### Levels of enzymes involved in testosterone synthesis

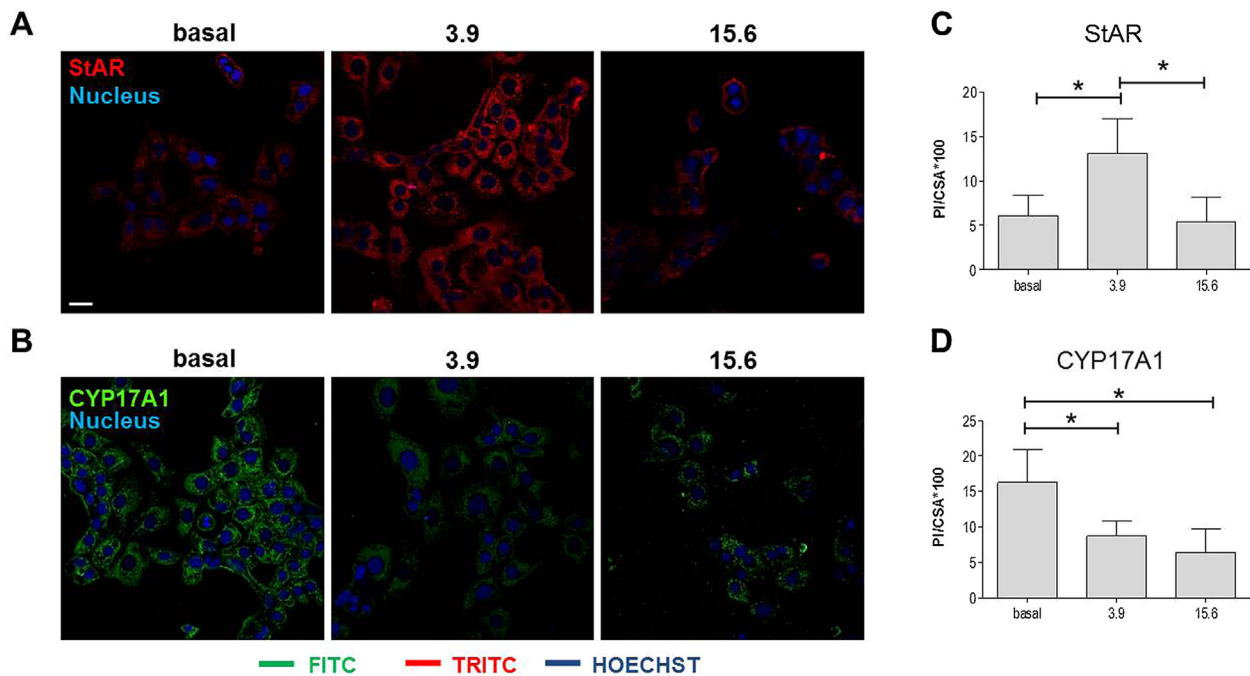
Lysates of R2C cells treated with different nandrolone concentrations were analyzed by western blotting analyses to verify the effects of nandrolone stimulation on the levels of proteins involved in testosterone synthesis. Our results showed that levels of StAR (Fig. 3) increased significantly in R2C cells treated with 3.9  $\mu$ M nandrolone compared to both basal and 15.6  $\mu$ M ( $P < 0.05$ ). These results were confirmed by confocal analyses (Fig. 4A and C). Interestingly, the levels of CYP17A1 decreased upon treatment with 15.6  $\mu$ M of nandrolone compared to both basal and 3.9  $\mu$ M ( $P < 0.05$ ) (Fig. 3). In addition, these results were in agreement with confocal analyses (Fig. 4B and D). Finally, western blotting analyses for CYP11A1 and HSD3B1 protein levels did not show any significant differences between untreated and treated cells (Fig. 3), and this datum was also in accordance with confocal analyses (not shown).

### Expression of StAR and CYP17A1 genes

In order to elucidate nandrolone-induced modification of the levels of StAR and CYP17A1, qRT-PCR analyses were performed for both molecules. The results showed that changes in both protein levels were related to variation in gene expression, as showed in Figure 5. In particular, expression of StAR mRNA increased significantly (Fig. 5A) while expression of CYP17A1 mRNA decreased significantly (Fig. 5B) in cells treated with both 3.9 and 15.6  $\mu$ M concentrations of nandrolone, compared to basal levels ( $P < 0.05$ ).

## Discussion

In many countries, the attention towards performance-enhancing drug use has focused on elite athletes and the illicit



**Fig. 4.** Effect of nandrolone supplementation on StAR and CYP17A1 levels: confocal analyses. (A and B) Representative microphotographs of immunofluorescence stain for StAR (A) and CYP17A1 (B) in cells treated with 3.9 and 15.6  $\mu\text{M}$  of nandrolone. Basal: untreated cells. Bar = 25  $\mu\text{m}$ . (C and D) Representative histograms of the immunofluorescence quantification of the staining intensity for StAR (C) and CYP17A1 (D). The stain intensity was expressed as the mean pixel intensity (PI) normalized to the cross-sectional area (CSA) using the Leica application suite advanced fluorescence software. Vertical axis:  $\text{PI/CSA} \times 100$ . Horizontal axis: nandrolone concentration, in  $\mu\text{M}$ . Data are presented as the mean  $\pm$  SD of quadruplicate experiments. \* =  $P < 0.001$ .

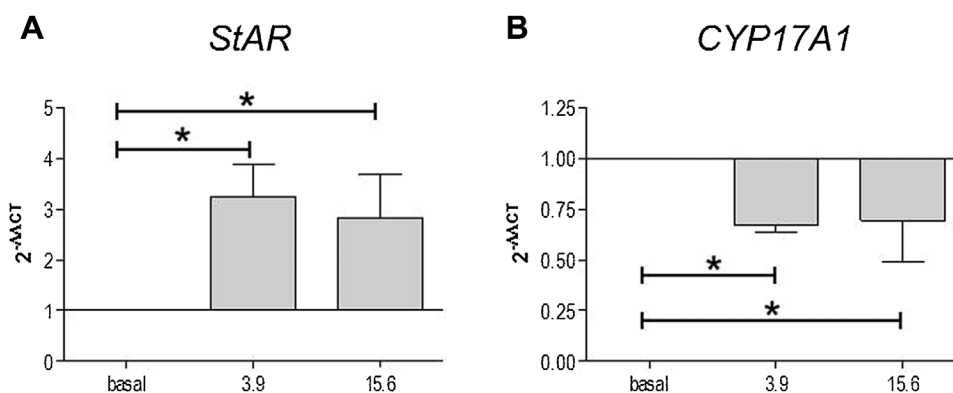
competitive advantage gained from these drugs (Pope et al., 2014). However, performance-enhancing drugs are also used by many non-athletes not only for enhancing performance, but also their personal appearance. Moreover, there is widespread misperception on the safety of performance-enhancing drug use or on the manageability of the adverse effects, when in fact these adverse effects still need to be better clarified (Pope et al., 2012). AAS is a category of performance-enhancing drugs widely used to enhance muscular mass, which are also used for the treatment of many clinical conditions (Pope et al., 2014).

In this study, we investigated the effects of nandrolone on the main enzymes involved in testosterone biosynthesis in Leydig cells. Testosterone is mainly produced by the Leydig cells of the testes in males and by the theca cells of the ovaries in females, although smaller amounts are synthesized by the adrenal gland in both sexes (Kostic et al., 2011). Testosterone plays a significant role in the growth and development of the male reproductive organs and it is generally accepted that AAS exert their effects via androgen receptor (Hyypää et al., 1997).

The present study demonstrates that nandrolone administration interferes with the biosynthesis of testosterone in a dose-dependent fashion. The results showed a significant increase of testosterone levels in the culture medium of R2C cells treated with 3.9  $\mu\text{M}$  of nandrolone, while the levels of this hormone did not change at higher doses (15.6  $\mu\text{M}$ ) of nandrolone, compared to basal condition. To the best of our knowledge, the present article is the first to describe the effects of nandrolone treatment on testosterone biosynthesis in Leydig cells, and our results reinforce the data available in the current literature on the deleterious effects of nandrolone on testis.

Recently, it was demonstrated that nandrolone decaonate, at the dose of 10 mg/kg/week for 8 weeks, induced a decrease of serum testosterone levels, a reduction of testicular weight, and an alteration of sperm characteristics in rats (Ahmed, 2015). Nandrolone administration in rats determines a number of morphological changes, such as reduction of the number and the size of Leydig cells, cytoplasmic vacuolization, and lipid droplet deposition. Moreover, from a biochemical point of view, it induces testicular damage by triggering oxidative stress, inflammatory cytokines, matrix metalloproteinases, cell-adhesion molecules, apoptotic markers, and DNA damage (Nagata et al., 1999; Noorafshan et al., 2005; Naraghi et al., 2010; Ahmed, 2015; Bjelic et al., 2015).

In our experimental model, the most important data obtained show that testosterone increases when Leydig cells are stimulated with a lower concentration of nandrolone (3.9  $\mu\text{M}$ ), but this increment disappears when the cells are treated with higher concentrations (15.6  $\mu\text{M}$ ). These changes are accompanied by modification at either protein or gene level of some of the main factors involved in testosterone production, such as StAR and CYP17A1. The latter data suggests that nandrolone is able to interfere with testosterone biosynthesis. Interestingly, we reported some discrepancies between protein and gene levels in certain conditions. These differences could be due to a number of causes (including miRNA regulations, post-translational modifications, protein degradation etc.) that are currently under investigation. However, the presence of these discrepancies could indicate that alternative (and probably unknown) routes for testosterone biosynthesis are stimulated by nandrolone, and should be further investigated in future studies.



**Fig. 5. Effect of nandrolone on StAR and CYP17A1 gene expression.** qRT-PCR evaluation of StAR and CYP17A1 gene expression after nandrolone stimulation at 3.9 and 15.6  $\mu\text{M}$ . The graphs show the normalization with the reference genes, according to the Livak Method ( $2^{-\Delta\Delta\text{CT}}$ ). Vertical axis:  $2^{-\Delta\Delta\text{CT}}$ . Horizontal axis: nandrolone concentration, in  $\mu\text{M}$ . Basal: untreated cells. Data are presented as the mean  $\pm$  SD of triplicate experiments. \* =  $P < 0.05$ .

In conclusion, our results support the hypothesis that nandrolone is able to modify testosterone production by interfering with StAR and CYP17A1 expression in Leydig cells. Further investigations are essential to better understand in vitro the molecular mechanisms responsible of these changes. Moreover, the data presented here also needs to be confirmed in in vivo models, in order to understand the relationship between AAS administration and testis impairment in human subjects.

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