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Nandrolone decanoate is able to modulate proliferation and adhesion of myoblasts

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Introduction. The search for a more efficient repair process of muscle injuries has become evident in clinical practice. The aim of the present study was to evaluate the effect of nandrolone decanoate (ND) on the proliferation, adhesion, and expression of myogenic regulatory factors (MRFs) in C2C12 cells.

Methods. Cell proliferation and adhesion were assessed using an MTT assay. The expression of MRFs was assessed by real-time PCR.

Results. ND applied at 10 or 25 μM concentration induced after 60 min an increase in adhesion, at 5 μM concentration induced after 5 days an increase in cell proliferation, and ND at 50 μM concentration led after 5 days to a decrease in cell proliferation in comparison with other groups. The steroid did not alter the expression of MRFs.

Conclusions. The positive effects of ND regarding the proliferation and adhesion of C2C12 cells suggest that this steroid may have positive effects following a muscle injury.

Key words: nandrolone decanoate, satellite cells, myoblasts, proliferation, differentiation, adhesion

Skeletal muscle has a characteristic plasticity, i.e. the ability to adapt to different conditions, including the postnatal growth, variations in overload, hormonal changes, muscle injuries (Charge and Rudnicki 2004; Marqueti et al. 2006; Piovesan et al. 2013), and in others allows a proper performance to the requested functional demand.

Muscle injuries are common among athletes and therefore the search for a faster and more efficient repair process has become increasingly evident in the clinical practice. As the muscle repair process is slow, depending on the severity of the injury, often achieves only a partial recovery. Uncovering of the best treatment for all stages of muscle repair is a principal challenge in the rehabilitation procedure (Piedade et al. 2008; Piovesan et al. 2013).

Following a muscle injury, satellite cells are activated, proliferate, fuse and form myotubes, and differentiate either to fuse with the pre-existing fibers or form new muscle fibers to replace the injured tissue (Dogra et al. 2007; Zammit 2008). This process is known as myogenesis, which is essential in the repairing of the injured muscle fibers (Parker et al. 2003). During this process, satellite cells express myogenic regulatory factors (MRFs), including MyoD, which mark the activation of satellite cells (proliferation), and myogenin, which indicate the onset of the differentiation (Muller 1999).

The adhesion processes between the satellite cells and between these cells and extracellular matrix components during myogenesis are essential for modulating the differentiation process, thereby allowing the fusion

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of myoblasts with primary myotubes and following the fusion with the primary myotubes to form myofibers (Hirsch et al. 1998; Taulet et al. 2009; Hsiao and Chen 2010; Papini et al. 2012).

Studies have found that the nandrolone decanoate (ND), an anabolic androgenic steroid (AAS), affects both the proliferation and differentiation (Liu et al. 2012) of C2C12 myogenic cells. In a previous *in vivo* study conducted by our group, ND has been found to induce a significant increase in MyoD mRNA after seven days and myogenin mRNA after 21 days during skeletal muscle repair process following a cryoinjury. Furthermore, the morphological analysis revealed no edema or myonecrosis after seven days and no edema or inflammatory infiltrate after 14 days in the cryoinjured ND group (Piovesan et al. 2013). Thus, ND seems to have beneficial effects on the resolution of the inflammatory process and reparation of muscle tissue.

Nandrolone decanoate is a derivative of testosterone with anabolic properties that modulates the cell cycle. The AAS is one of the most widely used in the world and compared to testosterone has higher anabolic and lower androgenic activity (Wilson 1988). Studies have shown that ND is related to changes in the size and muscle mass (Johansen et al. 2006; Wu et al. 2012) and the process of repair after injury (White et al. 2009; Piovesan et al. 2013), but the mechanisms involved still need to be clarified regarding the effects of this steroid on the muscle satellite cells.

The aim of present study was to contribute to the understanding of the effects of different concentrations of ND on muscle cells by analyzing the proliferation, adhesion, and expression of MRFs in different incubation periods.

Materials and Methods

Cell culture. C2C12 cells, a cell line derived from satellite cells of adult mice, were grown in Dulbecco's modified Eagle's medium (DMEM, Cultilab, Campinas, SP, Brazil), supplemented with 10% fetal bovine serum (FBS, Cultilab) and 1% antibiotic-antimycotic solution (Cultilab) at 37°C in a humidified atmosphere of 5% CO_2. Cell viability was determined with Trypan blue dye (0.4%). Cells with greater than 95% viability were used in the experiments.

Cell proliferation, viability, and adhesion. Cell proliferation was evaluated 1, 3, and 5 days after incubation with ND (Deca-durabolin®, Organon, Brazil) at final concentrations of 5, 10, 25, and 50 μM or vehicle drug (1.5:1 - benzyl alcohol/peanut oil) (Marqueti et al. 2006; D´Ascenzo et al. 2007) at concentrations of 5, 10, 25 and 50 μM.

Cell viability and proliferation was assessed using the MTT (3 - 4,5-Dimethylthiazol-2yl -2.5 - diphenyltetrazolium bromide; Thiazolyl blue) assay (Mossmam 1983). A total of $1x10^3$ cells/well were added to flat bottom sterile 96-well culture plates (TPP, Brazil) and incubated at 37°C in a 5% CO_2 atmosphere for the different incubation periods studied (1, 3, and 5 days). The cells were maintained in DMEM supplemented with 10% FBS.

At the end of each incubation period, the wells were washed with PBS (140 mM NaCl, 2.5 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO, pH 7.4) to remove dead cells and MTT (0.5 g/ml) (Sigma-Aldrich, St. Louis, MO, USA) was added. Incubation was carried out for 4 hours. Isopropanol was added to solubilize the crystals formed and absorbance was read at 620 nm using a plate reader (Anthos 2020, Anthos Labtec Instruments, Wals, Austria) (Mosmann 1983).

For adhesion assays, cells were cultured using the same conditions as described for the proliferation assay and $0.5x10^5$ cells/well were incubated in sterile 96-well flat-bottom culture plates (TPP, Brazil) with ND at final concentrations of 5, 10, 25, and 50 μM or the vehicle for 20, 40, and 60 min.

The analysis was performed using the MTT assay. In each evaluation, period following the method described for the proliferation assay and absorbance was read at 620 nm using a plate reader (Anthos 2020, Anthos Labtec Instruments, Wals, Austria) (Mosmann 1983). Cells cultured in the absence of steroid or vehicle were used as controls.

RNA extraction and real-time qPCR. For total RNA isolation, cDNA synthesis and real-time polymerase chain reaction (qPCR) C2C12 cells were cultured in DMEM containing 10% FBS and $1.5x10^6$ were placed in sterile dishes (TPP, Switzerland) 4 cm in diameter at 37°C in a 5% CO_2 atmosphere to obtain total RNA using the TRIzol reagent (Invitrogen, Sao Paulo, Brazil), following the manufacturer's guidelines. Three different conditions were analyzed: (a) control myoblasts (untreated); (b) myoblasts treated with ND (5 μM); and (c) myoblasts treated with vehicle alone. The choice of this concentration was based on proliferation results. All groups were evaluated after 2, 4 and 8 h.

Total RNA was used for cDNA synthesis and the real-time qPCR analysis of gene expression. Contaminated DNA was removed using DNase I (Invitrogen, Brazil)

and the reverse transcription was carried out using Moloney murine leukemia virus-reverse transcriptase (Invitrogen, Brazil).

Real-time qPCR was performed using the SYBRGreen kit (Applied Biosystems, USA) in a 7000 Sequence Detection System (ABI Prism, Applied Biosystems, Foster City, CA). The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Experiments were performed in triplicate for each data point. Myogenin and MyoD mRNA abundance was quantified as a relative value compared with an internal reference (GAPDH). The primers used for real-time PCR were as follows: GAPDH (GenBankTM NM 017008) forward primer 5'- TGCACCACCAACTGCTTAGC -3' and reverse primer GCCCCACGGCCATCA -3'; MyoD sense 5' GGA GAC ATC CTC AAG CGA TGC and antisense ACC AGC TGG GGA TAA ATC TTG (product: 80 pb) (Durigan et al. 2008); myogenin ACTACCCACCGTCCATTCAC sense 5 `-3 'and antisense 5` 3'TCGGGGCACTCACTGTCTCT (product 233pb) (Caiozzo et al. 2004).

The relative expression level of the target gene was normalized based on GAPDH expression as the endogenous control. ΔCt values of samples were determined by subtracting the average Ct MyoD/myogenin mRNA Ct mean value of the GAPDH internal control. As ΔCt data are rarely used due to this characteristic logarithmic parameter, 2 ΔCt was used to express the relative expression data.

Statistical analysis. The Shapiro-Wilk test was used in statistical analysis to determine the distribution of the data. Data with parametric distribution were tested using one-way ANOVA, followed by Tukey's test for comparisons between groups. Confidence levels were adjusted to 95% ($p<0.05$). Data analysis was performed with the aid of the GraphPad Prism 4.0 statistical program (GraphPad Software, San Diego, CA, USA). All samples were analyzed in quadruplicate and 3 independent experiments performed. However, only a single representative experiment is shown in each figure.

Results

The results of the assays revealed that ND used at 5 μM concentration induced a significant increase in the cell proliferation after five days, whereas a decrease in cell growth was observed at 50 μM concentration (Fig. 1).

As expected, there was an increase in the number of cells with the increase in incubation period (i.e. between days 1 and 3 and between days 3 and 5) in both the control and treated cells (Fig. 1). Due to a lack of differences, vehicle data are not shown.

After 20 and 40 min of incubation, there were no differences in adhesion among the ND, control, and vehicle groups. An increase in adhesion was found after 60 min incubation in cells that received ND at 25 and 50 μM concentrations in comparison with the control and vehicle groups in the same evaluation period (Fig. 2).

The results of MyoD and myogenin mRNA revealed no changes between the groups, as illustrated in Fig. 3

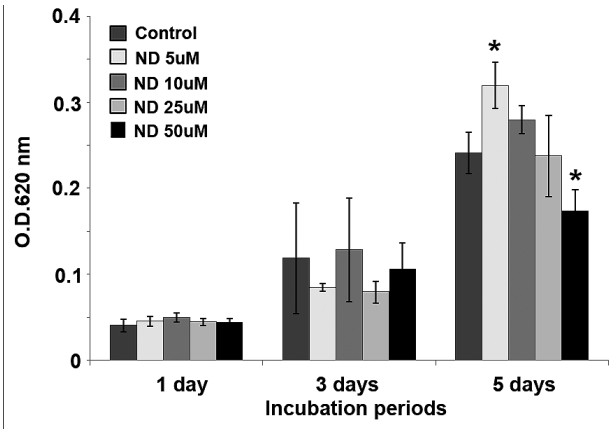

Fig. 1. Evaluation of cell proliferation measured as absorbance (O.D. 620 nm) by MTT of myoblasts cultured for 1, 3 and 5 days in absence (control) and presence of different concentrations of ND; * p≤0.05 denotes significant difference (ANOVA/Tukey) between control and ND groups in the same period.

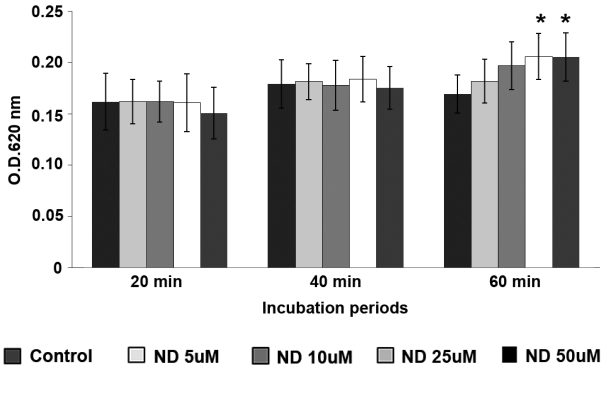

Fig. 2. Evaluation of cell adhesion measured as absorbance (O.D. 620 nm) by MTT of myoblasts cultured for 20, 40 and 60 min in absence (control) and presence of different concentrations of ND; *p≤0.05 denotes significant difference (ANOVA/Tukey) between control and ND groups in the same period.

and Fig. 4, respectively. MyoD mRNA expression was similar in all evaluation periods (Fig. 3). However, myogenin expression exhibited a significant increase at 8 h in comparison with the other periods, with no differences found between the control, ND, and vehicle groups (Fig. 4).

Discussion

Cell culture studies make possible to have a tight control over different variables in the experimental process and allows addressing questions more systematically. Therefore, *in vitro* studies evaluating the potential of ND are important to complement *in vivo* findings and offer further knowledge regarding the use of steroids in a more effective and safer manner.

A number of *in vitro* and *in vivo* studies have reported that androgen receptors are required to be present during the development and maintenance of skeletal muscle mass and muscle strength due to constant protein synthesis (Herbst and Bhasin 2004).

The present results demonstrate that ND increased the proliferation of muscle cells after 5 days at a concentration of 5 μM and a decrease in muscle cell proliferation was found only after 5 days at the highest ND concentration used. Adhesion was also modulated by ND, as the results revealed an increase in treated cells in comparison to the control and vehicle groups at 25 and 50 μM. Nogueira et al. (2012) have found that the adhesion of the osteoblasts was stimulated after 60 min following incubation with ND at 5, 10 and 25 μM combined with low-level laser irradiation. However, other periods and ND concentrations need to be studied to determine whether these results can be confirmed. The positive effects of ND regarding the proliferation and adhesion of C2C12 cells suggest that this steroid may have positive effects following a muscle injury.

In vivo studies have demonstrated that the effects of ND are dependent on the muscle evaluated, treatment time, concentration, and combination with exercise, all of which promote increased muscle mass and bone mineral density and the receiver androgen exerts a major influence on these effects (Lewis et al. 1999; Lee et al. 2002; Frisoli et al. 2005). The results of the present study are consistent with these findings, as cell proliferation and adhesion increased in the presence of ND and was affected by the different incubation periods and ND concentrations used.

Furthermore, our results showed that ND did not alter the expression of the regulatory myogenic factors studied. However, after 8 h, there was a significant increase in the expression of myogenin in all experimental groups, suggesting that after this period the cells were able to increase the expression of this marker, which is related to differentiation. It is difficult to compare these data with the literature data, as the majority of *in vitro* studies evaluate muscle cells during the differentiation process inducible with horse serum and the purpose of present study was to determine whether ND stimulus

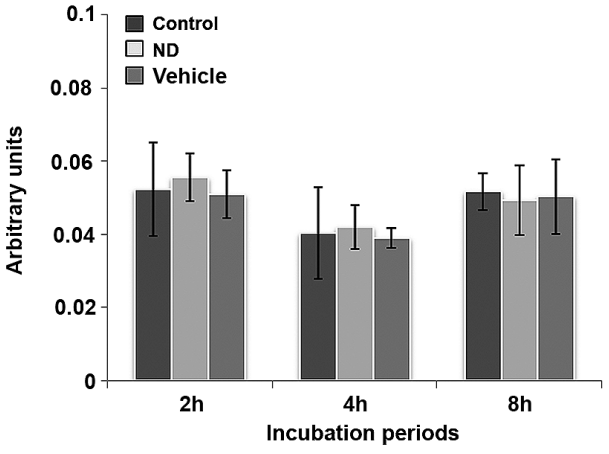

Fig. 3. MyoD mRNA analysis comparing control condition and myoblasts having received ND in different incubation periods; *p≤0.05 denotes significant difference (ANOVA/Tukey) between control and ND groups in the same period. Values are expressed as mean ± SD, corrected for GAPDH.

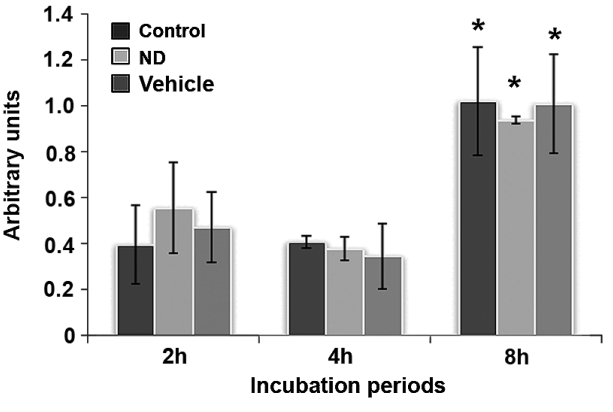

Fig. 4. Myogenin mRNA analysis comparing control condition and myoblasts having received ND in different incubation periods; # p≤0.05 denotes significant difference at 8 h in comparison to 2 and 4 h (ANOVA/Tukey). Values are expressed as mean ± SD, corrected for GAPDH.

alone would be sufficient to induce this complex process. Lee et al. (2002) have induced the differentiation of C2C12 cells with 3% horse serum and added ethanol or 10 μM testosterone to analyze the expression of the myogenic markers MyoD and myogenin after 12, 24, and 48 h and found that MyoD in C2C12 was not affected in the presence of 10 μM testosterone, but this concentration promoted an increase in myogenin expression in 24 h, indicating that androgens accelerate the differentiation of myoblasts. Thus, ND alone under the conditions tested was unable to alter the expression of MRFs and in this way was unable to induce a differentiation of myoblasts. The reduction in proliferation could be due a cytotoxic effect or a differentiation effect. Together with the proliferation findings, the results regarding the expression of MRFs suggest that the reduction in proliferation observed at the highest concentration of ND (50 μM) was possibly due to a cytotoxic effect. Nogueira et al. (2012) have correlated the reduction in proliferation observed at high ND concentrations (25 and 50 μM) in osteoblasts submitted to low-level laser irradiation to a differentiated effect, as the authors also found an increase in the alkaline phosphatase. On the other hand, Martins et al. (2010) have found chromosome damage and cytotoxicity in oral mucosa cells two months after ND administration.

In our previous *in vivo* study, we have found that ND at supraphysiological dose (5 mg/kg of the body mass) induced beneficial effects on the resolution of the inflammatory process, with a decrease in edema, myonecrosis, and inflammatory infiltrate during the muscle repair process and a significant increase in MyoD mRNA after 7 days as well as an increase in myogenin mRNA after 21 days (Piovesan et al. 2013). In another *in vivo* study has been found that the administration of ND to rats with injuries caused by a myotoxin (bupivacaine) induced an increase in muscle MyoD and cyclin D1 mRNA expression at 14 days and after 42 days of recovery. ND increased the incidence of large-diameter myofibers in comparison with injury alone, suggesting that this anabolic steroid affects the proliferation of satellite cells and consequently the muscle regeneration process (White et al. 2009).

According to Diel et al. (2008), the differentiation of C2C12 cells is modulated by androgens. Measuring CK activity and analyzing proliferation after 1, 3, and 6 days, the authors found that androgens stimulated the proliferation of C2C12 cells, accelerated the differentiation into myotubes, and stimulated myostatin expression in differentiated and undifferentiated cells in the presence of the steroid dihydrotestosterone (DHT).

Wannenes et al. (2008) have found that the expression of androgen receptors in the culture during the proliferation of C2C12 myoblasts was achieved by using plasma levels of testosterone in 0.1, 1, and 10 μM concentrations, suggesting that in this phase the skeletal muscle cells may be able to increase their responsiveness to androgen even at the presence of low concentrations of hormones. The results of the present study are in agreement with this finding, as there was increased proliferation of muscle precursor cells at lower concentrations of ND, while a significant reduction in the proliferation of these cells was found at the highest concentration (50 μM) of ND used.

Conclusion. ND induced an increase in cell proliferation and adhesion but did not alter the expression of MRFs.

Acknowledgments

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