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Anxiogenic effects of chronic exposure to nandrolone decanoate (ND) at supraphysiological dose in rats: a brief report

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Abstract

OBJECTIVE: Nandrolone decanoate (ND) is frequently used anabolic androgenic steroid (AAS) among the athletes. Despite the health risks, there is significant increase in prevalence of AAS abuse.

DESIGN: The aim of this study was to investigate the effects of chronic exposure to ND at supraphysiological dose (to mimic the doses for human AAS abusers) on anxiety levels in adult rats.

SETTINGS: We performed several behavioral tests (open field test, elevated plus maze test, beam-walking test, evoked beam-walking test and tail suspension test) for estimation of anxiety in rats. Adult rats received 20 mg/kg intraperitoneal injection of ND weekly for four weeks. Behavioral test were performed on the seventh day after the last dose of ND.

RESULTS: Anxiogenic-like pattern of behavior was clearly observed in several behavioral tests, such as open field test (decrease of total distance moved and cumulative duration of moving, decrease of an average velocity of the animals, decrease of frequency and total time in centre zone); elevated plus maze (decreased total time spent in open arms and the number of entries in open arms of the elevated plus maze); evoked beam-walking test (decreased time to cross the beam) and tail suspension test (increased latency to first immobility and decreased total duration of immobility).

MAIN FINDINGS: Results of this study show that four-week treatment with the supraphysiological dose of ND produced anxiogenic effects in sedentary male rats.

CONCLUSION: Our results show that rats after chronic treatment with a supra-physiological dose of ND exhibited anxiety-like behavior.

Abbreviations:

ND	- nandrolone decanoate	EPM	- elevated plus maze
AAS	- anabolic androgenic steroid	BW	- beam-walking
OF	- open field	EBW	- evoked beam-walking
TDM	- total distance moved	TST	- tail suspension test

ORIGINAL ARTICLE

INTRODUCTION

Anabolic androgenic steroids (AAS) are the group of synthetic derivatives of endogenous steroid testosterone. Because of both anabolic and androgenic effects on humans, AAS were primarily developed for treatment of hypogonadal dysfunction and delayed puberty, as well as for growth promotion (Basaria *et al.* 2001). However, AAS are not used only for medical purposes. Instead, due to their anabolic effects (increase in muscle mass, strength and endurance; fasten recovery from injuries – Lukas 1993; Sjöqvist *et al.* 2008), AAS have been widely abused among the athletes. This phenomenon has been increased over the last decades (Evans 2004). AAS abuse has not necessarily been connected to sporting elites. Outside the sport, 4–6% of high school males admitted to the use of AAS at some time of their life (Bahrke & Yesalis 2004). Warnings about potential health risks that occur with AAS abuse are very old (Boje 1939), but it seems that they still do not have satisfying effects in the world of sport.

Nandrolone (19-nor-testosterone) and its esterified derivative nandrolone decanoate (ND) are among the most frequently used anabolic androgenic steroids among the athletes (Perry *et al.* 1990; Eklof *et al.* 2003) due to their relatively low androgenic effects (Eklof *et al.* 2003). The recommended therapeutic dose of ND for humans (Tamaki *et al.* 2003) is 0.4 mg/kg/day (i.m.), but the abusers of AAS usually receive supraphysiological doses that are up to 100 times the therapeutic dose. In this study, we applied dose of ND (20 mg/kg/week, i.p.) that is approximately 80 times greater than the therapeutic dose for humans. Such a high dose of ND was chosen to mimic administered doses for heavy abuse of AAS in humans (Kindlundh *et al.* 2004; Kurling *et al.* 2005).

There are numerous reports for the side effects of AAS abuse, such as liver and prostate tumors (Johnson 1985; Yesalis *et al.* 1993) and boldness accompanied with various dermatological problems (Scott 1992). Cardiovascular complications (including stroke, myocardial infarction, cardiac dysarythmia, cardiac hypertrophy and atherosclerotic cardiac disease with dislipidemia) have also been connected to AAS abuse (Akhter *et al.* 1994; Hartgens *et al.* 2004; Cheever & House 1992). Also, past AAS abusers have more psychiatric diagnoses than nonusers (Malone *et al.* 1985), such as manic-like states defined by irritability, aggressiveness, euphoria, grandiose beliefs, hyperactivity and reckless or dangerous behavior (Daly *et al.* 2003; Papazisis *et al.* 2007).

Studies performed on animal models confirm human studies for behavioral states related to depression and anxiety (Lumia & McGinnis 2010). However, there are a lot of contradictory results concerning overall effects of chronic nandrolone treatment. Anxiolytic-like effects (or no effects) of chronic nandrolone treatment were reported for adult male mice (Ambar & Chiavegatto 2009; Célérier *et al.* 2006) and rats (Kouvelas *et al.* 2008). On the other hand, anxiogenic effect of chronic exposure to nandrolone was observed in adult male rats (Minkin *et al.* 1993; Rocha *et al.* 2007) and adolescent Syrian hamster (Ricci *et al.* 2012). Possible explanations for contradictory results may be found in species differences and non specified age of the animals (Rainer *et al.* 2014), although different doses and protocols should be also taken into consideration concerning those discrepancies.

Several mechanisms have been postulated for explanation of neurobiochemical base of the behavioral effects of AAS in rats. Altered dopamine metabolism in specific brain regions (nucleus accumbens) was reported after subchronic nandrolone treatment in rats (Birgner *et al.* 2007). Dysfunction of serotonin neurotransmission was observed after exposure of younger animals to AAS, and was accompanied with disinhibitory behavior in rats (Kailanto *et al.* 2011) and mice (Chiavegatto *et al.* 2010). Also, noradrenergic system has been altered following acute injection of nandrolone in adult rats by means of increasing extracellular concentration of noradrenaline and its metabolites in the hypothalamus (Tamaki *et al.* 2003). Specific distribution of androgen and estrogen receptors in male rat brain, especially in dorsal raphe nucleus (Sheng *et al.* 2004) and locus coeruleus (Yamaguchi & Yuri 2012), seems to be involved in some behavioral changes, such as depression (Belmaker & Agam 2008). Addictive behavior after AAS abuse has been accompanied with alterations of brain opiod system (Nyberg & Hallberg 2012). Repeated injections of AAS (at doses that mimics human abuse of AAS) has been reported to induce, at the same time, behavior changes and changes in concentrations of neurotrophins, leading to the depressed state in rats (Matrisciano *et al.* 2010).

The aim of this study was to investigate the effects of chronic exposure to nandrolone decanoate at supraphysiological dose on anxiety levels in rats. For this purpose we performed several "standard" behavioral tests (open field test, elevated plus maze test) that allow obtaining the commonly used parameters for estimation of anxiety in rats. Also, we tried to introduce the possible application of modified beam-walking test (with additional anxiety-provoking pattern – evoked beam-walking test) as a tool for estimation of anxiety in animal behavioral models. Furthermore, we proposed that results obtained in the tail suspension test could be used as parameters not only for evaluation of depression, but also as potential indicators for estimation of anxiety.

MATERIAL AND METHODS

Animals and treatments

Male Wistar albino rats (3 months old, 350–400 g) were kept in permanent groups of four per (polycarbonate) cage. Animals were housed under controlled standard environmental conditions of temperature (23±1 °C)

and light (12/12h light/dark cycle). Rats had free access to food and water. Animals were divided in two groups (8 animals in each group). Test group (ND group) had received 20 mg/kg intraperitoneal injection of nandrolone decanoate weekly for four weeks (DEKA 300, SteroxLab, EU). Control group had received approximately the same amount of saline in the same manner as ND group received therapy. The supraphysiological dose of nandrolone was chosen on the base of literature data for heavy use of nandrolone (Long *et al.* 1996; Kurling *et al.* 2005) in order to mimic the doses for human AAS abusers.

Animals were trained (for the beam-walking test) six days after receiving the last dose of nandrolone decanoate, and then tested on the seventh day after the last dose. The rats were placed in the testing room for 1–2 h prior to the initiation of each training and/or testing session. All research procedures were carried out in accordance with European Directive for welfare of laboratory animals No. 86/609/EEC and principles of Good Laboratory Practice (GLP), approved by Ethical Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

Behavioral studies

Open field test

The open field (OF) test is one of the commonly used tests for the evaluation of general motor activity in animal models. The apparatus consisted of a square arena (60 × 60 × 30 cm) made of black wood. During a trial the experimenter was not present in the test room. At the beginning of test each rat was placed in the centre of arena. The moving pattern in arena shows information about anxious-like state. The time duration spent in the centre arena of the open field was determined as the major index for anxiety, and more ambulation towards the centre arena of the open field reflected less anxiety. The movements of the rats were recorded by a digital video camera (resolution of 30 samples per second) mounted centrally 150 cm above the open field. Activity of rats was recorded for a period of 5 minutes and then analyzed. The following parameters were scored: *total distance moved* (TDM, cm), *velocity* (cm/s), *cumulative duration of moving* (cm/s), *frequency in centre zone* and *cumulative duration in centre zone*. Frequency in centre zone and cumulative duration in centre zone are considered as indicators of anxiolytic-like effect (Heiderstadt *et al.* 2000; Prut & Belzung 2003). At the end of each session rats were removed from the open field and the experimental chamber was thoroughly cleaned with water and ethanol (70%) to remove possible interfering scents.

Elevated plus maze

Anxiety-like behavior was also evaluated in the elevated plus maze (EPM) test. Elevated plus maze for rats consisted of two open (50 × 20 cm) and two enclosed arms (50 × 20 × 30 cm) and an open roof with the entire maze elevated 100 cm from the floor. Each rat was placed in the centre of elevated plus maze with head facing toward the open arm, allowed 5 minutes for free exploration. This test allows determining emotional reactivity of animals by means of a conflict between secure parts of the maze (2 enclosed arms) and aversive parts of the maze (open arms). During the 5 minutes of test, the *number of entries (frequency) into the open* arms and the *total time spent in open arms (cumulative duration)* of the maze were recorded. Number of entries and cumulative duration in open arms are considered as indicators of anxiogenic effect (Pellow *et al.* 1985; Pellow & File 1986). The activity of the rats were recorded by a digital video camera (resolution of 30 samples per second) mounted centrally 250 cm above the elevated plus maze.

Beam-walking test

The beam-walking (BW) test is used to evaluate motor coordination, integration, balance performance and motor skills (Chen *et al.* 2001; Ohlsson & Johansson 1995). The beam-walking apparatus consisted of rectangular shaped base. In this test the ability of rats to pass through the beam to reach a goal box is evaluated. White wooden box (20 × 20 × 20 cm) with the black hole served as a nest for motivating the animal crossing from the beam. The stainless steel squared rubber topped beam (100 × 3 × 2 cm) was fixed between the base of the goal box (100 cm above the floor) and a vertical stainless steel pole (60 cm above the floor). The whole beam-walking apparatus was placed above the cushions which protected the fallen animals from injury. Rats were pre-trained to cross the beam six days after receiving the last dose of nandrolone decanoate. Four trials were performed each animal, and the interval between trials was 15 minutes. At the start of the test trial (seventh day days after receiving the last dose), the rat was placed at the end of the beam opposite to the goal box. The goal was to accustom the rats to the beam and to let them to know the existence of the goal box at the end of the beam. In this test *number of falls from the beam* and *time to cross the beam* were recorded. The beam-walking test was carried out under proper conditions of silence and illumination.

Evoked beam-walking test

Evoked beam-walking (EBW) test was performed in order to estimate emotional reactivity of animals by means of anxiety-provoking pattern effects on the performance in previously recorded beam-walking test. Thus, this test was performed at the same apparatus as beam-walking test. Rats were pre-trained to cross the beam with the same protocol as for beam-walking test. At the start of the trial, the rat was placed at the end of the beam opposite to the goal box, while the experimenter started tapping (every 3 seconds) with metal stick at the base of stainless steel pole while rat traverse

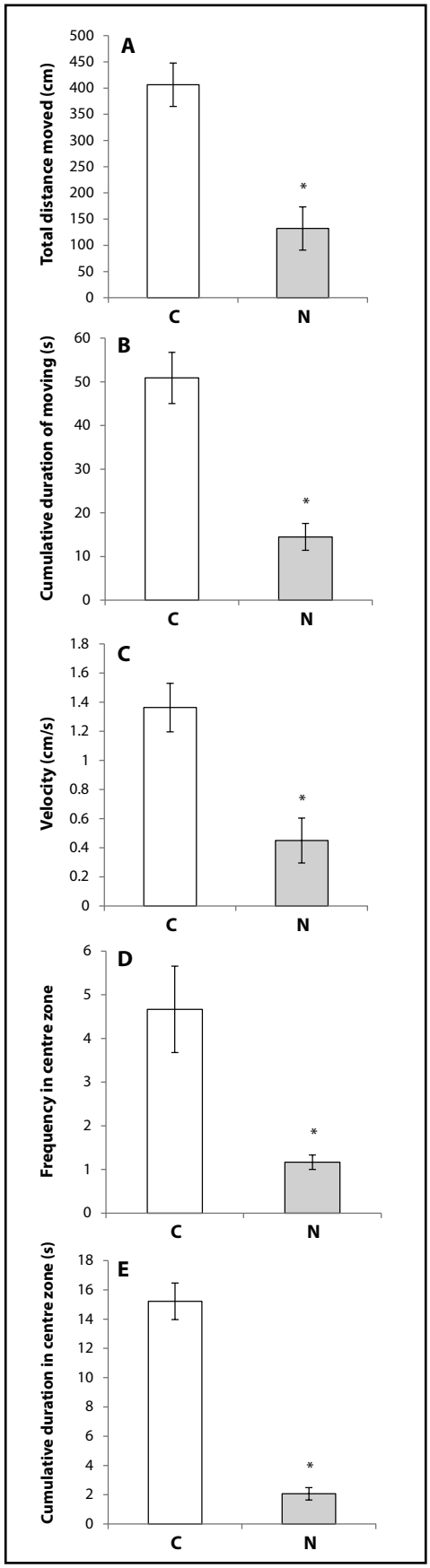

Fig. 1. Parameters calculated from the open field test (Mean ± SEM, * denotes a significant difference $p<0.05$). C – control group, N – nandrolone decanoate group.

the beam (anxiety-provoking pattern). Tapping was performed as long as the rat reached the goal box. Recorded parameters in this test were *number of falls from the beam* and *time to cross the beam*. The evoked beam-walking test was carried out under proper condition of illumination.

Tail suspension test

The tail suspension test (TST) is developed as a rodent screening test for mood disorders (Steru *et al.* 1985). In a TST rat is suspended by tail facing downward. The test is based on the fact that animals subjected to short term, inescapable stress of being suspended by their tail will develop immobile posture. Immobility was considered as a state of the animal with no visible voluntary movement (less than 1 cm) of head, body or limbs for at least 5 seconds. Involuntary swinging was considered as immobility. Cut off time in this trial was 6 minutes. During this period animal shows periods of agitation and immobility. In the present study apparatus consisted of metal frame (60 × 60 cm) with a hook in the centre. Behavior of the rats was recorded. The following parameters were scored: *latency to first immobility*, *number of episodes of immobility* and *total duration of immobility*.

Video recording system and analysis

All tests were recorded by digital video camera (Practika DVC 5.10 FHD). Video files (MPEG-2 format) were analyzed using Ethovision software [version XT 10 base], an integrating video tracking system for automatic recording of activity movement and interactions of animals [Noldus Information Technology, the Netherlands] (Noldus *et al.* 2001).

Statistical analysis

Results expressed as mean ± SE, and p-values <0.05 were considered significant in all tests. Analysis was performed with unpaired student t test between groups that follow normal distribution. Nonparametric Mann-Whitney U test were used for comparisons between groups that did not follow normal distribution. Variables were checked for normal distributions of the data with the Shapiro-Wilks test. A confidence level of 95% was accepted as significant. Analysis was performed with SPSS version 20.0 statistical package (IBM SPSS Statistics 20).

RESULTS

Open field test

The following parameters were obtained in the open field test: total distance moved, cumulative duration of moving, velocity, frequency in centre zone and cumulative duration in centre zone. Nandrolone decanoate (20 mg/kg/week for four weeks) significantly decreased total distance moved in the open field (Figure 1A) and cumulative duration of moving comparing to control group (Figure 1B). Also, nandrolone decanoate significantly decreased an average velocity of the animals in the open field comparing to control (Figure 1C). Frequency in centre zone (Figure 1D), as well as the total time in centre zone of the open field was significantly decreased comparing to control (Figure 1E).

Elevated plus maze

Cumulative duration in open arms and the number of entries in open arms were the parameters obtained in elevated plus maze test. Nandrolone decanoate significantly decreased total time spent in open

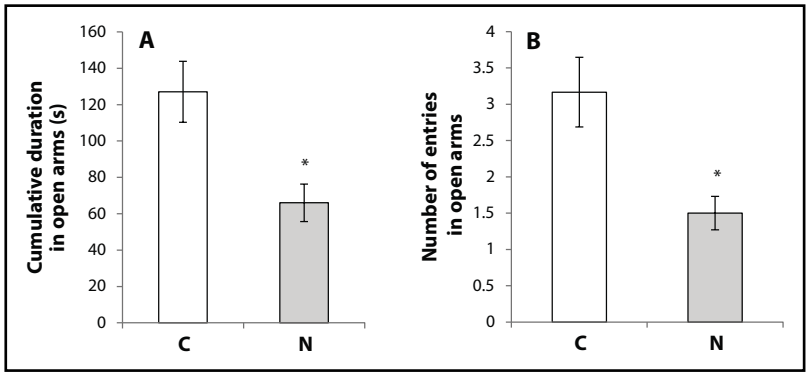

Fig. 2. Parameters calculated from the elevated plus maze test (Mean ± SEM, * denotes significant difference $p<0.05$). C – control group, N – nandrolone decanoate group.

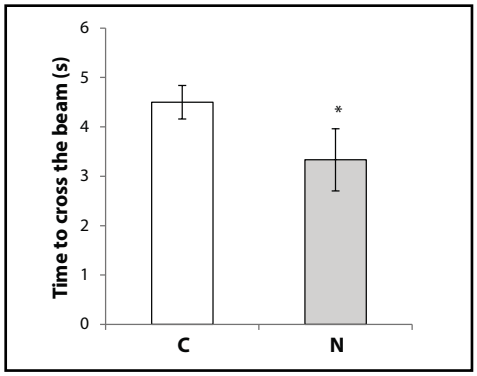

Fig. 3. Parameters calculated from the evoked beam-walking test (Mean ± SEM, * denotes a significant difference $p<0.05$). C – control group, N – nandrolone decanoate group

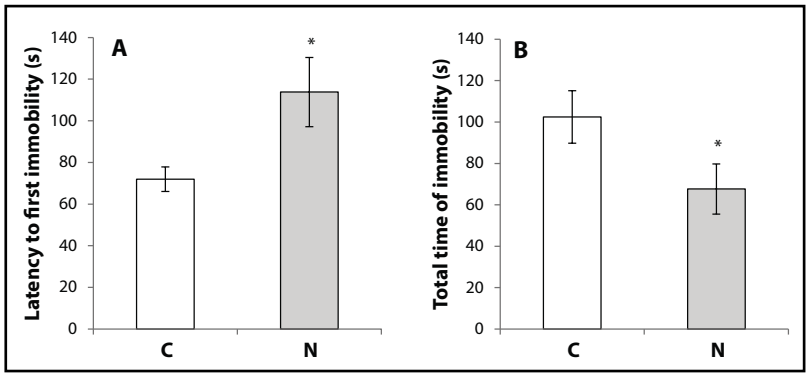

Fig. 4. Parameters calculated from the tail suspension test (Mean ± SEM, * denotes a significant difference $p<0.05$). C – control group, N – nandrolone decanoate group

arms of the elevated plus maze comparing to control group (Figure 2A). Also, the number of entries in open arms of the elevated plus maze was significantly lower in the nandrolone group when compared to control group (Figure 2B).

Beam-walking test and evoked beam-walking test

We followed two parameters in the beam-walking and in the evoked beam-walking test – time to cross the beam and number of falls from the beam. There was no difference between control and nandrolone group in time to cross the beam (14.17±1.96 s and 14.67±3.00 s, respectively) in the beam-walking test. Also, there was no falls from the beam in both groups during beam-walking test. Meanwhile, nandrolone decanoate significantly decreased time to cross the beam comparing to control group in evoked beam-walking test (Figure 3), with no falls from the beam in both groups during evoked beam-walking test.

Tail suspension test

The following parameters were obtained in the tail suspension test: latency to first immobility, number of episodes of immobility and total duration of immobility. Nandrolone decanoate significantly increased the latency to first immobility comparing to control group in tail suspension test (Figure 4A). There was no difference between control and nandrolone group in the number of episodes of immobility in tail suspension test (7.67±0.56 and 6.17±0.65, respectively). Total duration of immobility was significantly decreased in nandrolone group when compared to control group (Figure 4B).

DISCUSSION

Behavioral changes as consequences of AAS abuse in humans are still questionable due to lack of protocol standardization. It is very difficult to compare the results obtained from trials with different type of AAS, different dosage and/or frequency of AAS, and different duration of AAS abuse. Also, we are facing the data collected on persons with different fitness indexes. Furthermore, different types of tests used for evaluation of effects of AAS abuse on behavioral patterns in humans make the standardization of behavioral changes caused by AAS almost impossible. Animal experimental models for evaluation of behavioral effects of AAS offer more precise approach into this domain.

The main findings in this study are that rats after chronic treatment with a supraphysiological dose of nandrolone decanoate exhibited anxiety-like behavior changes. This pattern of behavior was clearly observed in several behavioral tests for evaluating anxiety, such as open field test, elevated plus maze, evoked beam-walking test and tail suspension test.

The results obtained in the open field test indicate that high dose of nandrolone decanoate produced anxiogenic effects in sedentary male rats. Rats receiving nandrolone decanoate in a

dose of 20 mg/kg/week, after four weeks of treatment, displayed the decrease in total distance moved, as well as in cumulative duration of moving in the open field. Those parameters are used as indicators of anxiety-related behavior (Prut *et al.* 2003; Kalueff & Tuohimaa 2004). Our findings support previous reports for AAS effects in the OF test (Minkin *et al.* 1993). Since the cumulative duration of moving in the OF was more markedly reduced than total distance moved, the velocity of moving in the OF was also decreased. The decrease in the velocity of moving in the OF, as a parameter of spontaneous locomotor activity in the OF that is affected by the level of anxiety, also indicate anxiety-like effect of ND. Furthermore, the decrease in both frequency in centre zone and cumulative duration in centre zone confirms higher level of anxiety in ND treated rats. Although, this is not in accordance with the results obtained with low doses of different AAS (Agren *et al.* 1999), our data are in line with recent results of Rainer (Rainer *et al.* 2014), who reported the same anxiogenic effects of chronic exposure of adolescence rats to ND in slightly lower doses and after a shorter trial.

The chronic exposure to a high dose of nandrolone decanoate induced anxiety-like consequences in ND treated rats during EPM test, as well. The cumulative duration in open arms, as well as the number of entries in open arms, showed decrease in treated animals. Both parameters are often used as indicators of behavior changes of anxiety origin, so that their decrease can be accepted as the evidence for anxiogenic-like effect of some drugs (Rocha *et al.* 2007). These findings do not correlate with Bitran (Bitran *et al.* 1993), who first reported anxiolytic-like effect of high dose AAS (testosterone propionate) after six days of treatment. This anxiolytic effect of AAS was attenuated after 14 days of treatment. They pointed bimodal effects of AAS that may lead to development of tolerance at $GABA_A$ receptors after their allosteric modulation induced by neurosteroid metabolites of AAS as a mechanism underlying this behavioral change. Still, it remains questionable whether this increased tolerance would lead into opposite (anxiogenic) effect of AAS if this protocol would last long enough (such as four weeks in our trial). Similar anxiolytic effects of nandrolone in the OF test were reported using different protocols (Kouvelas *et al.* 2008). On the other hand, results obtained from EPM test in this study are in accordance with numerous reports that confirm anxiogenic-like effects of nandrolone (Rainer *et al.* 2014) and other AAS (Olivares *et al.* 2014).

Results obtained in the beam-walking test suggest provide no evidence that four-week treatment with high dose of nandrolone decanoate affect motor coordination and balance performance. Since there is no data in the literature considering the influence of AAS on the results for the beam-walking test, we can only point out that chronic exposure to ND in our trial did not produce any change of both motor coordination and balance performance within four weeks. Nevertheless, even beam-walking test showed no effects of exposure to ND on motor coordination and balance performance, evoked (with anxiety-provoking stimuli) beam-walking test results clearly showed anxiogenic-like behavior of the rats after chronic exposure to ND. Shortening of the time to cross the beam in evoked beam-walking test may be, partly at least, the consequence of the persistence of anxiety-provoking stimuli (during beam-walking test) that is strong enough to trigger the fleeing reaction of the animals and to potentiate anxiety reaction (inappropriate) to the intensity of additional stimuli (Selakovic & Joksimovic 2014).

The tail suspension test is commonly used in behavioral studies for evaluation of depression and its treatment. Still, there are a few reports suggesting that this kind of test could be used in order to assess different mood disorders, such as anxiety (Mayorga & Lucki 2001; Kalueff & Tuohimaa 2004). Our basic idea was funded on the intention that parameters used for evaluation of antidepressant effects of drugs could be used, on the same ground, for testing the anxiety related patterns in behavior by means of quantification of the efforts that animals perform when facing both new and irritating situation (like tail suspension represents). Since antidepressant effects may be accompanied with anxiety-like behavior (Graeff *et al.* 1996; Cryan & Leonard 2000; Mombereau *et al.* 2004), we propose that increase in latency to first immobility (the time needed to give up in attempts to escape), decrease in number of episodes of immobility (the number of attempts to escape), and decrease in total duration of immobility (total time with no attempts to escape) could be considered as indicators of higher level of anxiety. As chronic treatment with high dose of nandrolone decanoate significantly increased the latency to first immobility and, also, significantly decreased the total duration of immobility when compared to control group comparing in tail suspension test (even with no significant change in number of episodes of immobility), we propose those results as a potential (indirect) evidence of anxiety-like behavior after chronic nandrolone treatment. The proposed conclusion is in accordance with the results of all previous tests in this trial.

In summary, results of this study show that four-week treatment with a high dose (20 mg/kg/week) of nandrolone decanoate produced anxiogenic effects in sedentary male rats. This effect was observed in all maze tests that were performed in this study. Consequently, this clearly demonstrated anxiogenic behavioral change after exposure to nandrolone that must be taken into consideration when even thinking about questionable beneficial AAS effects on physical performance.

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