



Anabolic steroids and their effects of on neuronal density in cortical areas and hippocampus of mice

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Received: May 29, 2019 – Accepted: February 1, 2020 – Distributed: August 31, 2021
(With 6 figures)

Abstract

Anabolic substances have been increasingly used by bodybuilders and athletes with the goal of improving performance and aesthetics. However, this practice has caused some concern to physicians and researchers because of unknowledge of consequences that the indiscriminate and illicit use of these substances can cause. Thus, this study analyzed the effects of two commercially available anabolic steroids (AS), Winstrol Depot® (Stanozolol) and Deposteron® (Testosterone Cypionate), in the neuronal density of limbic, motor and sensory regions on the cerebral cortex and in CA1, CA2, CA3 regions of the hippocampus. A total of 60 Swiss mice were used (30 males and 30 females), separated into three groups: control and two experimental groups, which received the AAS. From each brain, homotypic and semi-serial samples were taken in frontal sections from areas established for the study. The results showed that females treated with testosterone cypionate presented a reduction in all regions tested and the ones treated with Stanozolol showed a decrease in some hippocampal areas. Regarding male animals, stanozolol led to a decrease in neuron number in one hippocampal region. These data allow us to conclude that supra-physiological doses of steroids used in this study, can cause considerable damage to nervous tissue with ultrastructural and consequently behavioral impairment. These changes could interfere with the loss of physical yield and performance of athletes and non-athletes and may cause irreparable damage to individuals making irresponsible use of anabolic steroids.

Keywords: anabolic steroids, neuronal density, cerebral cortex, hippocampus.

Esteroides anabolizantes e seus efeitos na densidade neuronal em áreas corticais e no hipocampo de camundongos

Resumo

As substâncias anabólicas tem sido cada vez mais utilizadas por fisiculturistas e atletas com o objetivo de melhorar o desempenho e a estética. No entanto, essa prática tem causado algumas preocupações aos médicos e pesquisadores, devido ao desconhecimento das consequências que o uso indiscriminado e ilícito dessas substâncias podem causar. Diante disso, este estudo analisou os efeitos de dois esteroides anabolizantes (EA) comercialmente disponíveis, Winstrol Depot® (Stanozolol) e Deposteron® (cipionato de testosterona), na densidade neuronal das regiões corticais límbica, motora e sensitiva bem como das áreas CA1, CA2, CA3 do hipocampo. Foram utilizados 60 camundongos Swiss (30 machos e 30 fêmeas), separados em três grupos: controle e dois grupos experimentais, que receberam o EA. De cada cérebro, foram coletadas amostras homotípicas e semi-seriadas em cortes frontais das áreas estabelecidas para o estudo. Os resultados mostraram que as fêmeas tratadas com cipionato de testosterona apresentaram uma redução em todas as regiões analisadas já as fêmeas tratadas com Stanozolol mostraram uma diminuição em algumas áreas do hipocampo. Em relação aos animais machos, o stanozolol levou a uma diminuição na densidade neuronal em uma região do hipocampo. Estes dados nos permitem concluir que doses supra fisiológicas de esteroides utilizadas neste estudo podem causar danos consideráveis ao tecido nervoso com comprometimento ultraestrutural e consequentemente comportamental. Essas alterações podem interferir na perda de rendimento físico e no desempenho de atletas e não atletas e podem causar danos irreparáveis a indivíduos que fazem uso irresponsável destes EA.

Palavras-chave: esteroides anabolizantes, densidade neuronal, cortex cerebral, hipocampo.

1. Introduction

Anabolic-androgenic steroids (AAS) represent a large group of synthetic testosterone derivatives, produced to maximize anabolic effects. These drugs can be administered orally, intramuscularly and transdermally (Fratl et al., 2015). They are promoters and maintainers of sexual characteristics associated with masculinity (including genital system organs, secondary sexual characteristics, and fertility) and anabolic status of somatic tissues (Evans, 2004; Tousson et al., 2018).

Approximately 60 types of AS are available in the market, varying in their chemical structures, implying different physiological and psychological effects (Basaria et al., 2001). All anabolic agents have an androgenic effect, and several of these compounds have receptors on brain tissue (Roselli, 1998).

The AAS have similar structures to endogenous testosterone (Clark and Henderson, 2003). The doses of AS used by bodybuilders and other athletes, professional or not, reach 10 to 100 times fold those indicated therapeutically (Van Breda et al., 2003; Fink et al., 2019). The illicit use of AS occurs by athletes aiming the increase in muscle mass, physical strength, and aggressiveness in competitions, and also decreasing recovery time between intense exercises (Alaranta et al., 2006). Anabolic steroids have also been abused by non-athletes for aesthetic purposes (Matsumoto, 1996).

Data from the Brazilian Center for Information on Psychotropic Drugs collected in the last decades show a frightening increase in the consumption of substances considered as “body image drugs” among gym users (Iriart and Andrade, 2002; Silva et al., 2007; Abrahim et al., 2014).

Studies reported that abusive use of AAS has adverse effects on mental health, ranging from morphological changes (Omar et al., 2017) and behavioral parameters, such as anxiety and aggressiveness (Martínez-Sanchis et al., 1998), up to deleterious effects on neuron number in mice brains (Damião et al., 2012; Freitas et al., 2017; Alves et al., 2018). The prevalence of side effects is directly related to the type of steroid, age, and gender of the user, and duration of administration (Parssinen et al. 2000; Sader et al., 2001; Perez-Laso et al. 2018).

2. Materials and Methods

2.1. Material and treatment

Sixty young-adults Swiss mice were used (30 males and 30 females, 90 days old) weighing between 40 and 50 grams. Animals were housed in plastic boxes containing 5 animals each, with commercial mice chaw and water ad libitum, and submitted to a 12-hour light-dark cycle.

The animals were divided into three groups: Control group, TC-group, and S-group. The treatments consisted in intraperitoneal (IP) application of physiological solution (Control group), Deposteron® (testosterone cypionate-0.8mg/kg) and Winstrol Depot® (stanozolol-1.8mg/kg) in the respective groups. The treatment lasted one month, with injections

performed twice a week, on Tuesdays and Thursdays. The amount of AS used was calculated from doses used by attendees of academies in Alfenas city (Brazil – Minas Gerais), using Allometric Extrapolation method (Mahmood, 2007).

In the days that interspersed injections (Mondays, Wednesdays, and Fridays), the animals were put to swimming for 15 minutes, inside a plastic container measuring 43x34x26cm, with enough water volume so that they could not get out of the container nor touch the bottom. The water temperature was kept at 24-26 °C.

The research is in accordance with the Ethics Committee in Animal Experimentation of Unifal-MG, under the protocol number 414/2012.

2.2. Sample collection and processing

The animals were euthanized using Halothane inhalation (Garcia et al. 2015) and the brains were removed and stored in glass containers with phosphate buffer 0.1M, pH 7 containing 4% formaldehyde 0.1% for 24 hours. From each brain, homotypic samples were taken from the middle region with frontal sections (Rabinowicz et al., 2002; Brown and Aggleton, 2001), aiming to evaluate the areas established in this study.

Samples were processed following the standardized sequence in conventional histological procedures: alcohol dehydration, xylol dyafanization, and paraffin inclusion. Each sample was cut in 7µm sections and mounted over glass slides using a Lupe microtome and stained with cresyl violet to facilitate the visualization of Nissl corpuscles in neurons bodies, thus enabling a strong and individual marking of each cell for further quantification.

2.3. Neuronal density estimation

The estimation of neuronal cell density was performed using the methodology known as simple random counting (Mandarim-de-Lacerda, 2003) (Figure 1). In this method, 2 random microscopic fields were acquired from 3 semi-serial sections of the areas, thus totaling six (6) fields analyzed for each chosen area. The regions chosen for the analysis were three cortical areas, limbic, motor and sensitive, as well as three hippocampal regions, CA1, CA2, and CA3 (Silva et al., 2018).

The analyses were performed using an Axiovision Rel. 4.8.2 and Axionvision 4 Module Interactive Measurement from Carl Zeiss, coupled to a Carl Zeiss AxioScope A1 microscope.

Statistical analyses were performed using ANOVA (One-Way ANOVA) followed by Tukey averages comparison test. P values <0.05 were considered significant.

3. Results

The results show a significant decrease in neuronal density, both in male and female groups treated with anabolic steroids.

There was a significant reduction in neuronal density in the limbic area in male mice, for both experimental groups (≈16%) and decreased numbers of neurons in CA1

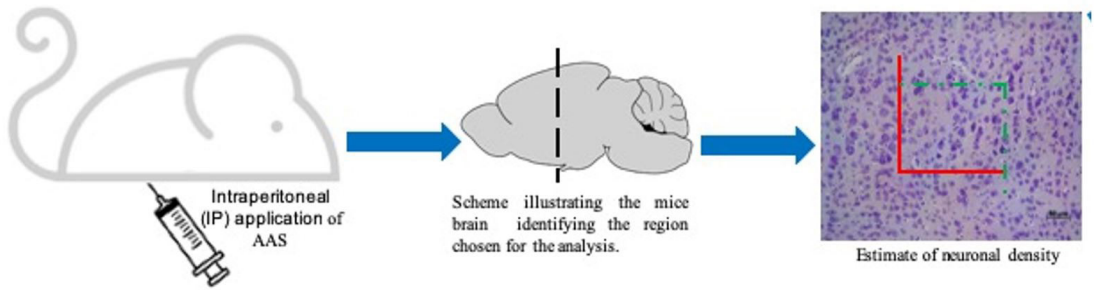


Figure 1. Summary of neuronal density estimation methodology. Being IP-Intraperitoneal application and AAS- Anabolic-androgenic steroids.

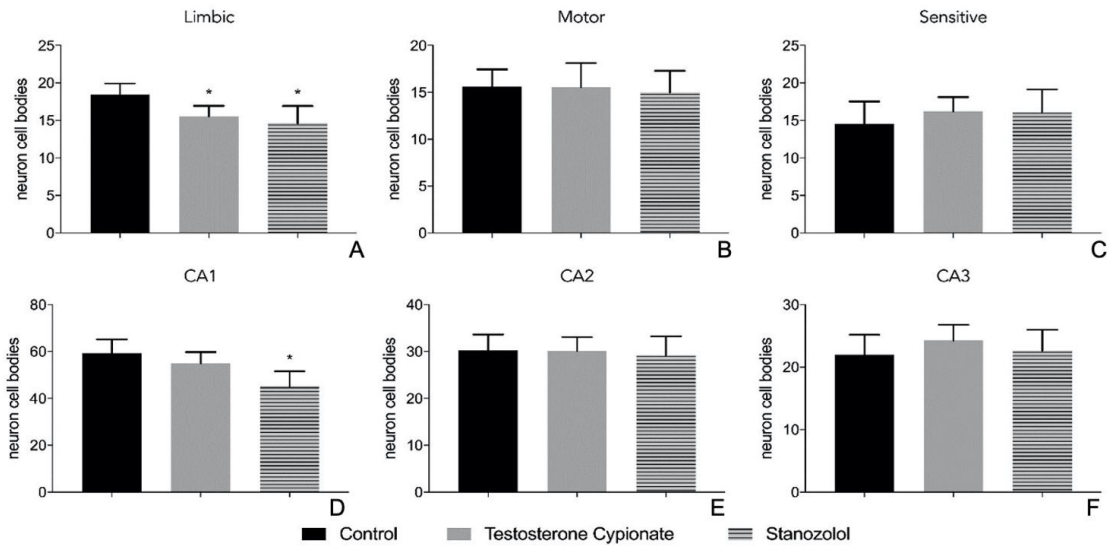


Figure 2. Estimation of neuronal density in cortical and hippocampal areas of male animals. Asterisk means the result is statistically different from Control ($p < 0.05$).

hippocampus area ($\approx 24\%$) in S-group. When comparing experimental groups, the treatment with stanozolol caused a greater reduction in neuronal density compared to testosterone cypionate, in hippocampus CA1 area (Figure 2D and 3).

For females, the results obtained show that testosterone cypionate caused an even more significant reduction in neurons number, with an approximate loss of 26% in the limbic area (Figure 4A), 30% in the motor area (Figure 4B) and 25% in the sensitive area (Figure 4C).

Regarding hippocampal regions, the neuronal loss was about 28%, 29%, and 18.5% in CA1, CA2, and CA3 areas respectively. In females, the testosterone cypionate induced a more expressive neuronal loss than the stanozolol considering the hippocampal areas CA2 ($\approx 13\%$), and CA3 ($\approx 34\%$) (Figure 4D, 4E, 4F, 5 and 6).

4. Discussion

Similar results were observed in some studies (Kochakian, 1993; Damião et al., 2012; Silva et al., 2018) revealing significant morphological changes in the cerebral

cortex of animals treated with supraphysiological doses of anabolic steroids. A study with cortical rats cell cultures (Orlando et al., 2007) examined the effect of testosterone and three of its synthetic derivatives (nandrolone, stanozolol, and gestrinone) on N-methyl-D-aspartate (NMDA)-induced excitotoxic neuronal death. The study led to the belief that testosterone, by itself, is toxic, but the action of aromatase converting it to 17β -estradiol could protect the neurons.

Unlike testosterone, its synthetic derivatives have increased NMDA toxicity at low concentrations and some are not substrate of aromatase. None of the anabolic agents showed to be toxic without the presence of NMDA.

People who abuse anabolic agents have micromolar concentrations of these substances in the brain (Yang et al., 2002), which makes the results presented in this research even more worrying, given the uncontrolled and excessive use of these drugs. Stanozolol is not a substrate for aromatase enzyme (Orlando et al., 2007), which may, even in need of further studies, help us to understand the effect of anabolic agent Winstrol Depot®, which showed a significant reduction of neuronal density.

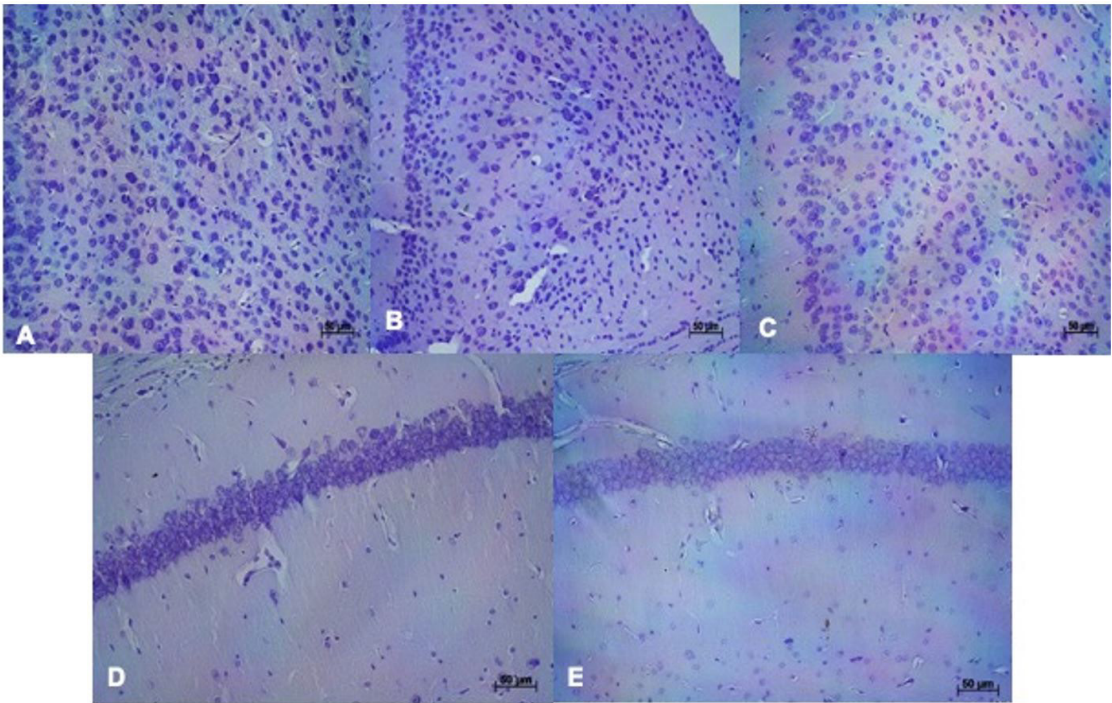


Figure 3. Histological sections of the limbic and hippocampal (CA1) areas of male animals. (A) limbic area of control group; (B) limbic area testosterone cypionate group and (C) limbic area stanozolol group; (D) CA1 control group and (E) CA1 stanozolol group. Violet cresil. Objective of 20x.

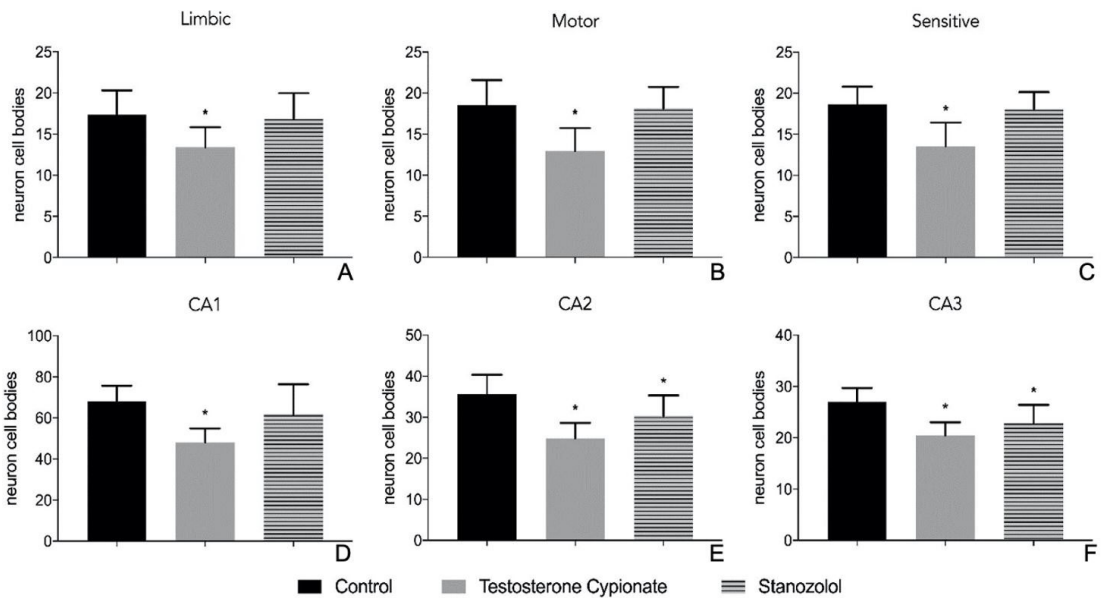


Figure 4. Estimation of neuronal density in cortical and hippocampal areas of female animals. Asterisk means the result is statistically different from Control ($p < 0.05$).

Testosterone cypionate (Deposteron®) has a high affinity for androgenic receptors (Kochakian, 1993), which facilitates its conversion in more mild products by aromatase. Even so, our results indicate that the doses tested exceeded the enzyme catalytic capacity since there

was a significant reduction in the number of neuronal cell bodies in cortical areas. A similar pattern could be observed in the hippocampal areas studied, where researchers have concluded that aromatase also protects neurons in such regions in cases associated with epilepsy

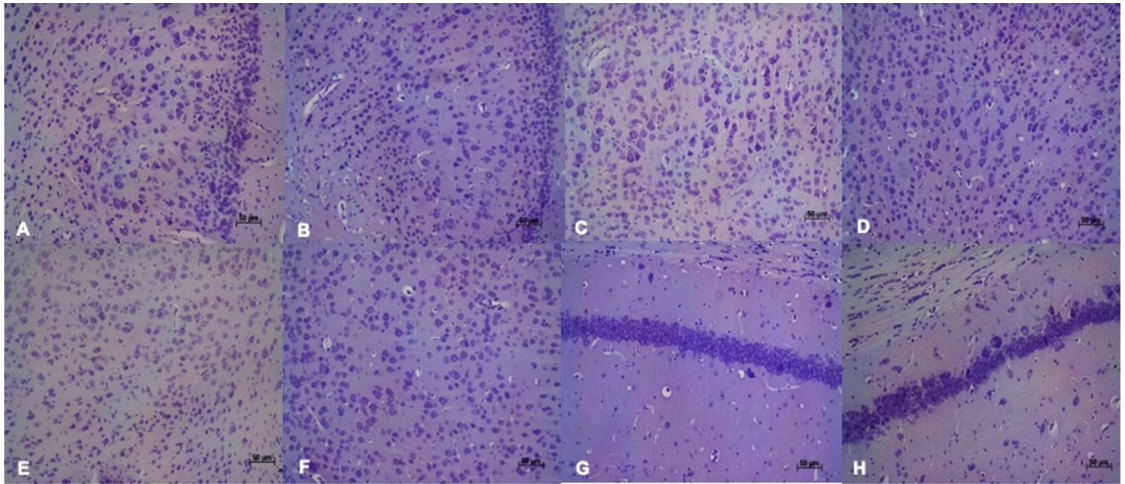


Figure 5. Histological sections of the cerebral cortex and hippocampal areas of female animals. (A) limbic area (LA) of control group; (B) LA testosterone cypionate group; (C) motor area (MA) control group; (D) MA testosterone cypionate group; (E) sensitive area (SA) control group; (F) SA testosterone cypionate group; (G) CA1 control group and (H) CA1 testosterone cypionate group. Violet cresil. Objective of 20x.

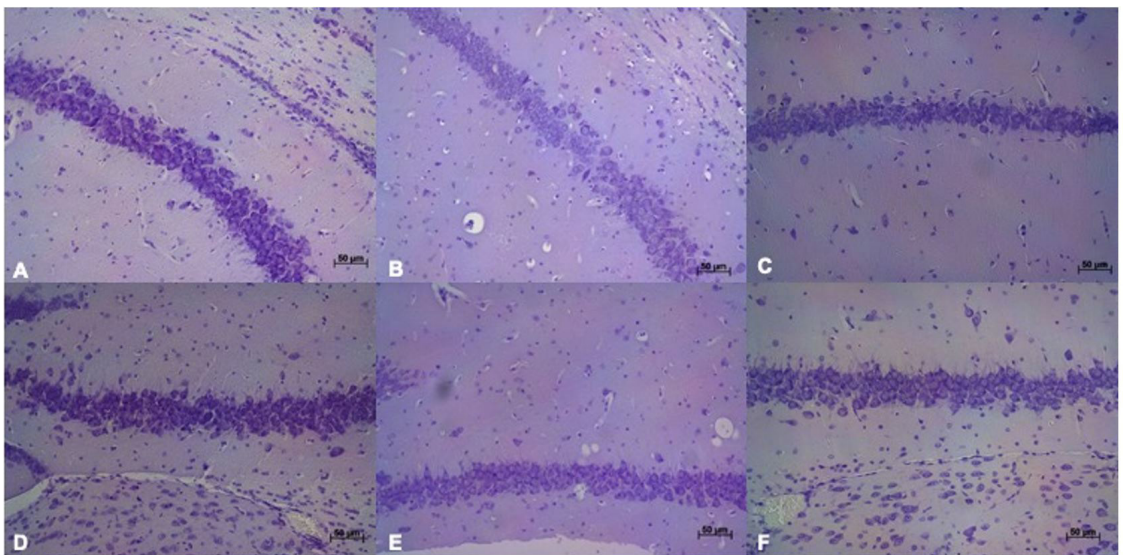


Figure 6. Histological sections of the hippocampal areas (CA2 and CA3) of female animals. (A) CA2 control group; (B) CA2 testosterone cypionate group; (C) CA2 stanozolol group; (D) CA3 control group; (E) CA3 testosterone cypionate group; (F) CA3 stanozolol group. Violet cresil. Objective of 20x.

(Zhou et al., 2007). Even with the protective effect of the enzyme, supraphysiological doses of Stanozolol led to a reduction of the number of neurons in CA1, CA2, and CA3.

There is evidence that the use of synthetic androgens reduce the threshold for emotional reactivity, motor response, alertness to sensory stimuli, and disrupt inhibitory control over some behaviors (Selakovic et al., 2017; Hildebrandt et al., 2018; Tobiansky et al., 2018). These alterations result from a change in basic neurocircuits that amplify limbic activation and reduce cortical control. Our findings seem to be in accordance with that affirmation, given the

neuronal damage and quantitative differences of nerve cells found exactly in hippocampal and cortical areas.

Another study reveals that use of androgenic anabolic steroids, at supra-physiological doses, even for a short period, caused severe disorder in the hypothalamic-pituitary-gonadal axis of a bodybuilder. Endogenous testosterone synthesis was severely compromised with a significant decline in serum luteinizing hormone levels (Vilar Neto et al., 2018). The decrease of this hormone may be one of the causes that leads to the reduction of neuronal density in the measured areas since a smaller amount of this hormone can promote

deficiency in neurons receptors and consequent alteration in neural connectivity. The limbic and sensitive areas participate effectively in the hypothalamic-pituitary-gonadal axis.

Studies show that anabolic steroids have detrimental and often irreversible effects on the brain and affect this organ primarily through two mechanisms: 1) Neural hyperstimulation by increasing the activation of specific neurotransmitters, such as norepinephrine, dopamine, and serotonin; 2) Significant reduction in neural connectivity of the central nervous system affecting several regions of brain, including the basal ganglia, hippocampus and cortical areas (Damião et al., 2012; Creagh et al., 2018; Silva et al., 2018; Freitas et al., 2017). The results here obtained can have serious implications in the second mechanism, since researchers observed a significant decrease in the neuronal population in some of the areas mentioned.

5. Conclusion

These data show that supra-physiological doses of stanozolol and testosterone cypionate can cause considerable damage to the nervous tissue, with structural and probable sequential behavioral impairment. Such damage leads to loss of efficiency and performance, as well as behavioral disorders, both in athletes and non-athletes, and can cause irreparable damage to the individuals who use it illegally, without a prescription and medical support.

Acknowledgements

This work was supported by the National Council of Scientific and Technological Development (CNPq) and Federal University of Alfenas (Unifal-MG).

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