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Effect of Supra-Physiological Doses of Anabolic-Androgenic Steroids on the Neuronal Density of the Central and Basolateral Amygdala in Mice

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Abstract: Anabolic-androgenic steroids (AAS), despite being controlled by government agencies, are illegally marketed and heavily consumed among youngsters that want a lean, muscular appearance and improved physical performance. Behavioral changes mediated by the amygdala, such as depression, aggressiveness and anxiety, are reported among users. The main scope of this work is to quantify the number of neuron cells on the nuclei of the central (CA) and basolateral (BLA) mice's amygdala submitted to a chronic supra-physiological doses of Durateston® (a combination of testosterone propionate, testosterone isocaproate and testosterone decanoate) and Deca Durabolin® (nandrolone decanoate), administered isolated and combined. For this purpose, 40 Swiss male mice were taken and divided into 4 (four) groups (n = 10): a control group (C), in which mice received saline solution; a Dura group, in which mice received Durateston®; a Deca group, in which mice received Deca Durabolin®; and a Dura-Deca group, in which mice received both drugs. Mice were treated with AAS for a period of 60 days and underwent exercises (swimming). After this time, mice were euthanized and had their brains removed. The material thus obtained was processed in a conventional histological routine. For the neuron cells quantitative analyses, the simple random sampling count method was used. The results showed that there was a significant decrease in the number of neuron cells in all AAS treated animals comparing to the control group; concerning the amygdala basolateral nucleus, the decrease ranged from 23% to 36%, according to the AAS selected group; regarding the amygdala central nucleus, the decrease ranged from 20% to 29%, according to the AAS selected group. One can conclude that the administration of supra-physiological doses of these AAS causes decrease in the number of neuron cells on the central and basolateral nuclei of Swiss male mice's amygdala.

Keywords: Anabolic-Androgenic Steroids, Central Amygdala, Basolateral Amygdala, Neuron Density

1. Introduction

Also called Anabolic Steroids Androgens or Steroids Anabolic Androgens [1] the AAS refer to steroid hormones belonging to the male sex hormone class [2]. Responsible for androgenic (masculinizing) effects, they act both on promotion and maintenance of male sexual characteristics. Among the androgenic hormones, the testosterone, produced in the testicles by the Leydig cells and in less quantity in the ovaries and suprarenal gland, stands out [3]. In addition to acting on reproduction and libido, testosterone exerts somatic changes in several structures, being well known its action upon muscular development, bone growth and decreasing of body fat, as well as in the renal, hematopoietic and nervous tissues [3, 4].

Testosterone's therapeutic potential has been widely explored in medicine since its synthesis from cholesterol in 1935 [5]. In this context, its synthetic derivatives, so-called AAS, began to be

developed with molecular alterations to reduce the androgenic effects and increase the anabolic ones [3]. These synthetic derivatives are used in the control of hormonal deficiencies and as adjuvants in the treatment of debilitating chronic diseases, in some cases of AIDS (acquired immunodeficiency syndrome), certain anemias, osteoporosis and cancer [6].

In recent years, some countries have adopted strict rules concerning distribution and commercialization of these substances; however, AAS are illegally marketed and their consumption among non-athletes began to intensify [7, 8].

The abusive and illicit use of AAS occurs among academy goers that aim muscular mass increase. Under these conditions, AAS are generally administered for periods (cycles) that last on average 8 to 16 weeks, separated by a rest period, in an attempt of reducing the adverse effects of these drugs [9]. In general, the drug is self-administered by two methods: the first is called "stacking", in which there is a simultaneous consumption of two or more drugs during the cycle; in the second one, called "pyramiding", the drug is taken initially in a low dosage and this is increased until reaching levels that can exceed 10 to 100 times the recommended therapeutic doses, with a gradual return to the initial dose [9, 10].

In a hurry to obtain the "perfect body", the deleterious effects of such AAS are neglected by the people that make use of them. Many consumers present themselves with acne, testicular atrophy and gynecomastia. In addition, clitoris hypertrophy, arterial hypertension and left ventricular hypertrophy may occur, as well as damage to the liver tissue, hematological and neurological disorders [10]. Among the effects influencing the central nervous system, functional and behavioral changes are highlighted, especially irritability, aggression, anxiety, depression and cognitive symptoms such as forgetfulness, distractions and confusion [8, 10].

The regulation of emotion and behavior related to disorders such as anxiety, depression and aggression depend on neuronal circuits involving the amygdala [11, 12]. The amygdala (=almond) comprises a bilateral complex of nuclei located in the anteromedial region of the temporal lobe that can be organized into three regions: Basolateral Amygdala, Central Amygdala, and a sparse cell group called "intercalated cell masses" [12, 13]. In animals, its location is extremely similar to that described and has a similar organization in all mammals [13].

The aim of this study was to quantify the number of neuron cells on nuclei of basolateral (BLA) and central (CA) amygdala in mice submitted to chronic use of supra-physiological doses of Deca Durabolin® and Durateston®, administered isolated and combined, compared to the control group.

2. Material and Methods

2.1. Animals and Treatment

The study is in accordance with the ethical principles, having been appraised and approved by the Committee of Ethics on Animal Experimentation (CEUA/Unifal-MG) under registry nº 479/2012.

A total of 40 Swiss male mice with 90 days of age (young adults) and weighing from 40 to 50 grams were used. They were housed in individual boxes, fed with commercial ration, water ad libitum (at will) and kept in a 12-hour light-dark cycle. The pharmacological treatment consisted of the isolated and combined intraperitoneal application of two AAS: Deca Durabolin® and Durateston®, both from *Landerlan Laboratory*. The doses are described in Table 1 [14, 15]. AAS were given at the same time, once a week, for 60 days. In the control group, saline solution was administered in the same volume as the isolated drug applications [16]. During AAS treatment, mice underwent swimming for 5 minutes three times a week in a plastic container measuring 43 x 34 x 26 cm, containing water at a temperature of 24-26ºC and at a height sufficient that the animals could not get out of the container nor to rely on its bottom [17]. After 60 days, mice were euthanized by inhalation of isoflurane for subsequent craniotomy.

Table 1. *Animal groups according to injected substance and its dosage.*

Groups	Injected substance	Dosage	Exercise
Control	Saline solution 0.9%	0.02ml	Swimming
Dura	Durateston®	83.3mg/Kg	Swimming
Deca	Deca Durabolin®	16.7mg/kg	Swimming
Dura-Deca	Dura® + Deca®	83.3mg/Kg+16.7mg/kg	Swimming

2.2. Histology and Cell Count

After 60 days, mice were euthanized by inhalation of isoflurane for subsequent craniotomy. The brains were removed entirely in a block, washed in saline solution and fixed in a 4% paraformaldehyde solution in phosphate buffer for 24 (twenty-four) hours. After this period, the standardized sequence for the conventional histological process was followed: alcohol dehydration, diaphanization in xylol and inclusion in paraffin. From each brain, serial and homotypic samples were taken in frontal sections [18] with a thickness of 7μm in a Yidi YD-315 microtome. To evaluate the areas established [20], the material was stained with cresyl violet [18, 20]. For the quantitative analysis of neuron cells, the methodology of simple random sampling count method was used [21-24]. All analyzes were performed on a computer by an image analyzer system, the Axiovision4 Module Interactive Measurement, coupled to an AxioScope A1 microscope by Carl Zeiss®. The study was conducted in a completely randomized design. The statistical analysis was performed by means of Analysis of Variance (One-Way ANOVA), followed by the Tukey average comparison test. GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA) was used and the significance level of 5% or less was adopted for all analyzes.

3. Results

3.1. Basolateral Amygdala (BLA)

Animals treated with both the AAS, isolated or combined, presented a significant reduction on the mean number of neuron cells (Figure 1). It is possible to observe in the Table 2 the absolute mean values and the standard deviation. The combination of both AAS led to the greatest loss of neuron, of 36.35%.

Table 2. *Mean and standard deviation (SD) of Basolateral Amygdala (BLA) neuron cells quantification.*

Groups	BLA neuron cell quantification	Percentage of reduction
Control	10.95 ± 2.62^{a}	-
Dura	8.38 ± 1.88^{b}	23.47%
Deca	8.20 ± 1.91^{b}	25.11%
Dura-Deca	6.97 ± 1.85^{b}	36.35%

Basolateral Amygdala (BLA) neuron cells quantification exhibited as mean value ± standard deviation. Different letters in superscript refer to statistical difference using Tukey test at the significance level of 5%.

3.2. Central Amygdala (CA)

There was a significant reduction on the mean number of neuron cells in the groups that received AAS compared to the control group (Figure 1). It is possible to observe the absolute values of the mean of quantification with the standard deviation, Tukey test's results and the percentage of reduction on the number of neuron cells for each AAS group (Table 3).

Table 3. *Mean and standard deviation (SD) of Central Amygdala (CA) neuron cells quantification.*

Groups	CA neuron cell quantification	Percentage of reduction
Control	24.78 ± 2.36^{a}	-
Dura	19.73 ± 3.02^{b}	20.38%
Deca	18.92 ± 1.77^{b}	23.65%
Dura-Deca	17.61 ± 2.18^{b}	28.93%

Central Amygdala (CA) neuron cells quantification exhibited as mean value ± standard deviation. Different letters in superscript refer to statistical difference using Tukey test at the significance level of 5%.

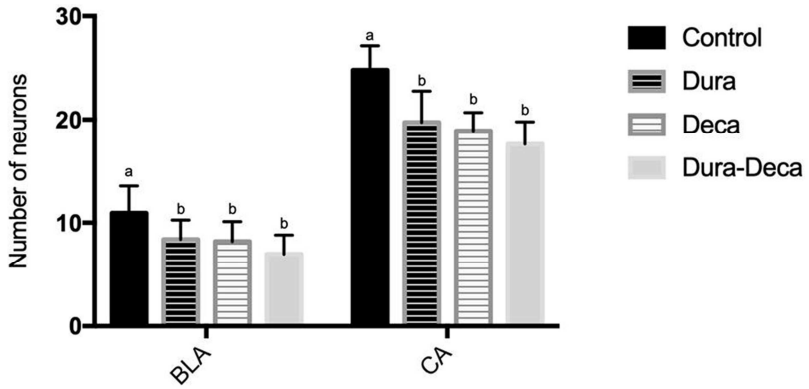

Figure 1. *Male mice Basolateral and Central Amygdala neuron cells quantifycation after treatment with Durateston® ($p<0.05$), Deca Durabolin® ($p<0.01$) and Durateston®+Deca Durabolin® ($p<0.001$). Equal letters refer to data without significant difference, while different letters refer to data with significant difference.*

4. Discussion

The reduction on neuron cells density with the use of supra-physiological doses of AAS in Basolateral (BLA) and Central Amygdala (CA) suggests a neurotoxic effect of these substances [8] and are indicative of neuronal death [25]. Another paper that studies the potentially neurodegenerative effects of AAS demonstrates that high concentrations of its metabolites and of testosterone's can alter neurons and cause biochemical, functional and morphological changes, culminating in cell death [26], while another one reports that high doses can lead to an increase in neuronal apoptotic stimuli susceptibility [27]. Such studies corroborate with the findings obtained in the present study.

Brain areas fundamentally involved in emotional responses and aggressive behavior present dopamine and serotonin [28, 29] among their neurotransmitters, and the presence of both are confirmed in the amygdala [30, 31]. These monoamines are affected in animal experiments using AAS in which it can be found reduced levels of dopamine and serotonin in the forebrain of rats under chronic abusive use of stanozolol [7]. Ricci et al. [32] worked with doses of three AAS in hamsters and observed decreased levels of serotonin in the amygdala of the animals. These studies are probably related to the findings in the present work.

The abusive use of AAS reduces the levels of messenger RNA of serotonin receptors in the prefrontal cortex, hippocampus, hypothalamus, and amygdala. The lower uptake of this substance provides clues about the behavioral changes (anxiety, aggressiveness, and competitive behavior) presented by the users of AAS in supra-physiological doses [8, 10, 33].

Although subcortical, BLA neurons closely resemble those of the cerebral cortex, with two outstanding subpopulations: a major group of projection neurons, which uses glutamate as an excitatory neurotransmitter, and a minor group consisting of sparse interneurons that use gamma-aminobutyric acid (GABA) as an inhibitory neurotransmitter [3]. The CA neurons have multiple dendrites which branch out profusely, most of which are projection neurons, although there is a cell group related to internal circuits of the amygdala. Although mainly GABAergic, glutamatergic neurotransmission is also reported in the nuclei of central amygdala, with an increase in response to anxiety and acute stressors [35, 36].

The amygdala excitability is mediated primarily by glutamatergic signaling that can be increased by different conditions [37-39]. The decrease in amygdala serotonin concentration caused by the use of AAS [7, 32] may induce changes such as increased expression of GluR1 (glutamate receptor) which favors glutamatergic neurotransmission and generates neuronal hyperexcitability [39]. There are papers suggesting that consumption of supra-physiological doses of AAS increases glutamatergic transmission and also the likely binding with its agonist receptor, N-Methyl-D-Aspartate (NMDA) [40-42]. Such studies may justify the cell death evidenced in this experiment.

Orlando et al. [49] described the effect of testosterone as well as three of its derivatives (nandrolone, stanozolol and gestrinone) on NMDA-induced neuronal death in primary cultures of rat cortex cells, suggesting that high doses of testosterone increase the neuronal vulnerability, occurring greater cytotoxicity and facilitating cell death. In another study the authors observed similar results of AAS neurotoxicity in cultures of cortical neurons [26]. Excessive release of glutamate and over activation of NMDA receptors that occurs under certain conditions cause an increase in intracellular Ca^{2+}. If not prevented, the ions concentration may reach a point of no return in cell death triggering 43-45]. The results obtained by these studies corroborate with the results which show how much AAS supra-physiological doses can interfere in the neuronal mechanism.

Many youngsters and adults make irregular use of AAS when searching for physical performance improvement and a muscular appearance in an immediate way without worrying about the negative effects that these drugs can entail on health [8]. Mental states of anxiety, aggressiveness and depression depend on circuits mediated by the amygdala [12] that undergo changes arising from lesions in their nuclei. These brain lesions caused by the abuse of AAS most often associated with behavioral disorders such as anxiety, aggressiveness and depression, so the results of neural loss presented here may be directly related because the neuronal loss can directly influence the neural circuit.

Molecular brain mechanisms are related to the behavioral effects of high doses of AAS in rodents [46]. Rodents that received AAS injections at supra-physiological doses [47] showed increased social aggression [48], with effects often dependent on the animal's species and lineage, as well as the type of AAS administered [47]. High concentrations of testosterone and its AAS' metabolites can lead to functional, morphological and biochemical changes on cortical neuron cells, culminating in cell death [49]. These studies corroborate the results found in this recent research.

According to Ribeiro et al. [16], the use of testosterone cypionate and stanozolol in a supra-physiological dosage promotes the reduction of neuron cells in the basolateral amygdala and posterior ventral nucleus of the medial amygdala in mice. In another study, the treatment of mice with AAS led to a significant decrease in the number of neuron cells in the cerebral cortex, hippocampal areas and basal nuclei when compared to the animals in the control group, which were treated with saline solution [50-52]. However, Silva et al. [17] reported that there was no decrease in the density of neuron cells in the cerebellar cortex in mice submitted to AAS, while Ribeiro et al. [16] using the same analysis technique by Silva et al. [17] reported reduction of Purkinje cells in male mice treated with Deposteron® (testosterone cypionate).

It could be expected from the results of individually administered AAS, that the group treated with both substances in combination would lead to a greater reduction of neuron cells; however, there was no significant difference which can be explained due to a possible saturation of the receptors. The increase in the number of receptors did not occur in the same proportion as the ligand concentration (AAS) which may explain the non-significant result for this evaluation [47, 48].

5. Conclusion

The results of neuronal quantification showed a drastic decrease in the number of neuron cells in mice's Basolateral and Central Amygdala when these animals were treated with supra-physiological doses of two AAS. This allows us to suggest that the use of supra-physiological doses of these drugs can lead to toxicity and consequent neuronal death. Based on this, the authors agree that the concomitant use of these anabolic steroids, often used by athletes and non-athletes, can cause damages even more severe than the individual use of the substances.

Conflict of Interest

The authors declare that they have no conflict of interest.

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