



Instituto de  
Biociências



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UNIVERSIDADE ESTADUAL PAULISTA  
"JÚLIO DE MESQUITA FILHO"

CLASTOGENICIDADE E/OU ANEUGENICIDADE DO HORMÔNIO  
ANDROGÊNICO NANDROLONA (DECA-DURABOLIN®) EM CAMUNDONGOS.

**CAROLINA ALMEIDA DO CARMO**

Dissertação apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Mestre no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração Biomoléculas – Estrutura e Função.

*Orientador: Edson Luis Maistro*

**BOTUCATU – SP**

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## Resumo

Os anabolizantes esteróides têm sido amplamente utilizados por profissionais e atletas de elite para melhorar sua aparência e habilidades atléticas. Além disso, eles apresentam um importante papel quimioterapêutico no tratamento de vários tipos de distúrbios metabólicos, homeostáticos e sexuais, em ambos os sexos. Tendo em vista que muitas drogas esteróides têm apresentado diferentes resultados considerando efeitos genotóxicos e mutagênicos, o objetivo desse trabalho foi avaliar o potencial genotóxico do hormônio nandrolona (deca-durabolin<sup>®</sup>) *in vivo* em células da medula óssea e do sangue periférico de camundongos, usando o teste do micronúcleo e o ensaio do cometa, respectivamente. Os animais receberam injeção intradérmica de 3 concentrações do hormônio esteróide (1.0, 2.5 e 5.0 mg/kg peso corporal). As células foram coletadas 24 h após o tratamento hormonal para o teste do micronúcleo (avaliação da clastogenicidade) e o teste do cometa (avaliação da genotoxicidade). O teste do micronúcleo evidenciou que as duas maiores doses testadas da nandrolona induziram aumentos estatisticamente significativos de células micronucleadas e o teste do cometa não evidenciou aumento significativo de danos no DNA nos linfócitos do sangue periférico. Sob estas condições experimentais, conclui-se que o hormônio esteróide nandrolona apresentou efeito clastogênico e/ou aneugênico e, por outro lado, não foram observados efeitos genotóxicos quando o mesmo foi administrado intradermicamente em camundongos.

## Abstract

Anabolic androgenic steroids have been widely used by professional and elite athletes to improve their appearance and athletic abilities. Besides, they have an important place in the chemotherapeutic treatment of various types of metabolic, homeostatic, and sexual disorders in both sexes. Since many steroidal drugs have been found to be different results considering genotoxic and mutagenic effects, the aim of this study was to evaluate the genotoxic potential of nandrolone (deca-durabolin®) *in vivo* in bone marrow and peripheral blood cells of mice, using micronucleus and comet assays, respectively. The animals received intradermal injection of the 3 concentrations of the steroid (1.0, 2.5 and 5.0 mg/kg body weight). The cells were collected 24 h after the hormone-treatment for the micronucleus (clastogenicity endpoint) and comet assays (genotoxicity endpoint). Micronucleus test showed that the two higher tested-doses of the nandrolone induced statistically significant increase of the micronucleated cells and comet assay no evidenced significant increase in the DNA damage of the lymphocytes from peripheral blood. Under our experimental conditions, the nandrolone steroid hormone showed clastogenic and/or aneugenic effects and, on the other hand, no genotoxic effects when administered intradermally to mice.

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## 1. INTRODUÇÃO

### 1.1. *Ensaio de Genotoxicidade e Mutagenicidade*

Mutagenicidade refere-se a alterações permanentes na estrutura do material genético de célula ou de um organismo, que podem ser transmitidas e resultar em mudanças hereditárias nos organismos. Essas mudanças podem envolver um único gene, um segmento de genes, um bloco de genes ou até mesmo todo o cromossomo. Efeitos no cromossomo podem ser estruturais ou numéricos (SCCNFP 2003).

Genotoxicidade é um termo amplo e refere-se ao efeito potencial de danos no material genético, que não está necessariamente associado com mutagenicidade. No entanto, testes de genotoxicidade incluem testes que dão uma indicação de danos induzidos no DNA (mas não uma evidência direta de mutação) (SCCNFP 2003).

Há dois tipos de testes de genotoxicidade/mutagenicidade, *in vitro* e *in vivo*, muitos deles estão descritos no guia da OECD. Os testes *in vitro* referem-se a experimentos em um ambiente controlado, fora do organismo vivo, como por exemplo, em um tubo de ensaio. Já os testes *in vivo* referem-se à experimentação feita em organismos vivos. O ensaio do cometa e do micronúcleo *in vivo* tem grande aceitação na comunidade científica, por essa razão serão utilizados no presente estudo.

### 1.2. *Considerações sobre o Ensaio Cometa*

Para avaliar os danos no DNA de células individuais causados pelas substâncias testadas foi utilizada a técnica do cometa. A metodologia do cometa foi desenvolvida por Östling e Johanson (1984) e é baseada na eletroforese de células em lise embebidas no gel de agarose, resultando em uma imagem ao microscópico que lembra um cometa com cabeça e cauda (KLAUDE *et al.*, 1995).

A técnica mede o grau de migração do DNA nuclear durante a eletroforese (GIOVANNELLI *et al.*, 2002). O método é quantificado através da fluorescência da cabeça e da cauda do cometa (KLAUDE *et al.*, 1995). A extensão da migração é proporcional ao número de quebras no DNA, e sua avaliação permite calcular o número de quebras no DNA em nível de células individuais (GIOVANNELLI *et al.*, 2002).

Essa técnica é versátil e sensível para medir quebras em cadeias simples e duplas de DNA (COLLINS *et al.*, 2008).

O mecanismo principal sugerido por Östling e Johanson, é que o DNA é organizado em grandes estruturas supercoloidais, que, quando separadas por quebras na dupla-fita de DNA, podem migrar para o anodo através da eletroforese (TICE *et al.*, 2000; KLAUDE *et al.*, 1995). Com altas doses de irradiação gama a cauda do cometa consiste em fragmentos de DNA que migram mais livremente no gel do que os DNA inteiros (ÖSTLING & JOHANSON 1987).

A técnica foi modificada por Singh *et al.* (1988) que usou eletroforese alcalina para analisar danos no DNA de tratamentos com raio-x ou H<sub>2</sub>O<sub>2</sub>. O número de publicações baseadas na técnica do cometa cresceu nos últimos anos, sendo a alcalina a técnica mais usada, com pequenas variações em alguns passos. Desde que a técnica se desenvolveu de forma empírica, houve a necessidade de padronizar e assegurar o entendimento dos mecanismos em que esta se baseia (KLAUDE *et al.*, 1995).

Comparada com outras técnicas a técnica do cometa apresenta algumas vantagens, dentre elas: (1) Apresenta sensibilidade em apontar baixo nível de danos no DNA; (2) Requerimento de baixo número de células por amostras; (3) Flexibilidade; (4) Baixo Custo; (5) Fácil aplicação; (6) Habilidade de conduzir estudos utilizando pequenas porções de substâncias; e (7) Tempo relativamente curto para a realização de experimentos (TICE *et al.*, 2000).

### ***1.3. Aspectos envolvendo o teste do micronúcleo***

Outro teste utilizado no presente estudo foi o teste do micronúcleo em medula óssea de camundongo *in vivo*. Esse teste é um dos sistemas de testes disponíveis para avaliar a existência de aberrações cromossômicas em mamíferos *in vivo* (WAHNSCHAFFE *et al.*, 2005).

O teste do micronúcleo *in vivo* em mamíferos é usado para detectar danos induzidos pela substância testada nos cromossomos ou no aparato mitótico de eritroblastos. Os possíveis danos são analisados através de amostras de eritrócitos em células da medula óssea e/ou em células do sangue periférico de animais, sendo os roedores os mais utilizados (GUIA OECD 474, 1997).

A documentação dos procedimentos e a avaliação dos resultados são dadas no guia OECD (Organization for Economic Cooperation and Development). Esse teste é rotineiramente utilizado e tem grande aceitação entre as indústrias e autoridades. Micronúcleos são corpos de cromatina presentes no citoplasma, originários de fragmentos de cromossomos acêntricos ou de cromossomos inteiros que não foram incorporados no núcleo de células filhas durante os últimos estágios da mitose (WAHNSCHAFFE *et al.*, 2005).

Fragmentos de cromossomos estão associados a atividades clastogênicas (quebra de cromossomos) da substância testada, enquanto a presença de um ou mais cromossomos inteiros seja um indicativo de um efeito adverso da substância testada nas fibras do fuso, durante a mitose (efeitos aneugênicos). A diferença no tamanho dos micronúcleos é, portanto, um indicador de clastogenicidade (pequenos micronúcleos) ou aneugenicidade (grandes micronúcleos). No entanto, o tamanho dos micronúcleos são medidas imprecisas. Micronúcleos podem ser distinguidos por vários critérios, como por exemplo, pela identificação da presença de cinetócoro ou DNA centromérico, indicando atividade aneugênica. No total, um aumento na quantidade de micronúcleos é uma medida indireta da indução estrutural ou numérica de aberrações cromossômicas (WAHNSCHAFFE *et al.*, 2005).

No teste de micronúcleo, de acordo com o guia da OECD 474 (1997), eritrócitos na medula óssea de camundongos são usados como células alvo. Quando um eritroblasto da medula óssea se desenvolve em um eritrócito policromático, o núcleo principal é excluído. Qualquer micronúcleo que foi formado pode permanecer no lado anucleado do citoplasma e pode ser facilmente detectado. O aumento na frequência de micronúcleos de eritrócitos policromáticos em animais tratados é indicativo da indução de danos cromossômicos (KRISHNA & HAYASHI 2000; WAHNSCHAFFE *et al.*, 2005).

O teste de micronúcleo *in vivo* é especialmente relevante para indicar perigo mutagênico e permite considerar fatores metabólicos, farmacológicos e o processo de reparo no DNA *in vivo* e também é útil para promover investigações sobre efeitos mutagênicos detectados em testes genotóxicos *in vitro* (KRISHNA & HAYASHI 2000).

Nas últimas três décadas, toxicologistas têm frequentemente usado o teste de micronúcleo em medula óssea de camundongos, porque 1) é parte da toxicologia regulatória no procedimento de admissão para químicos e drogas e 2) tem vantagens na rapidez, simplicidade e custo efetivo em comparação com outros sistemas *in vivo* para testar aberrações cromossômicas (WAHNSCHAFFE *et al.*, 2005).

#### **1.4. Os hormônios esteróides**

Os hormônios esteróides são produzidos pelo córtex da supra-renal e pelas gônadas (ovário e testículo) (SILVERTHORN, 2003). Os esteróides anabolizantes ou esteróides anabólico-androgênicos (EAA) referem-se aos hormônios esteróides da classe dos hormônios sexuais masculinos, promotores e mantenedores das características sexuais associadas à masculinidade (incluindo o trato genital, as características sexuais secundárias e a fertilidade) e do *status* anabólico dos tecidos somáticos. Os esteróides anabólicos incluem a testosterona e seus derivados. Entretanto, alguns autores referem os esteróides anabolizantes como os derivados sintéticos da testosterona que possuem atividade anabólica (promoção do crescimento) superior à atividade androgênica (masculinização) (SILVA *et al.*, 2002).

A testosterona foi pela primeira vez sintetizada, em 1935, e, desde então, os andrógenos, tornaram-se disponíveis para utilização com fins terapêuticos e experimentais (SAMUELS *et al.*, 1942; SIMONSON *et al.*, 1944 *apud* CUNHA *et al.*, 2004).

Em 1945, houve a popularidade no meio atlético através da publicação do escritor Paul de Kruiff, *The Male Hormone* (*apud* BASARIA *et al.*, 2001). O uso de esteróides anabólicos se tornou popular entre os atletas em meados dos anos 50 (HAUPT & ROVERE, 1984). Os efeitos anabólicos e androgênicos desses agentes, quanto ingeridos ou injetados, em doses que produzem o aumento em tamanho e força, resultam em significativos efeitos adversos envolvendo a pele, fígado, sistemas cardiovascular, endócrino e reprodutivo (WARREN *et al.*, 1990). Os efeitos dos esteróides anabólicos sobre o comportamento dos usuários têm sido há muito tempo pesquisados. Estudos relacionaram o mau uso dessas drogas a mudanças súbitas de

temperamento e a síndromes comportamentais dentro e fora dos esportes (SILVA *et al.*, 2002).

A nandrolona ou 19-nortestosterona é um anabólico esteróide derivado da testosterona e foi sintetizada pela primeira vez por Birch em 1950 (*apud* LE BIZEC *et al.*, 1999). Inicialmente seu uso foi introduzido para o tratamento de anemia, osteoporose e câncer de mama (BASARIA *et al.*, 2001). Atualmente nandrolona se encontra disponível em muitos produtos farmacêuticos, mas o mais largamente encontrado é o Deca-durabolin<sup>®</sup> (AVOIS *et al.*, 2007).

A nandrolona tem seu nome na IUPAC como 17b-hydroxy-19-nor-4-andro-ster-3-one, e é um anabólico esteróide (químico construtor de músculos) que ocorre naturalmente no corpo humano, mas apenas em pequenas quantidades. É muito parecido em estrutura com o hormônio masculino testosterona, e tem muito dos mesmos efeitos em termos de aumento de massa muscular (WOOD, 2004). Segundo Baume *et al.* (2006) a nandrolona é um dos esteróides mais consumidos no mundo dos esportes e seu uso está proibido pela WADA (Word Anti-doping Agency).

Em geral, estrogênios não induzem mutações genéticas em testes mutagênicos clássicos com bactérias e mamíferos (ROY e LIEHR, 1999), no entanto, em nível de mutações cromossômicas, alguns estudos mostraram resultados positivos (WHEELER *et al.*, 1986; ECKERT e STOPPER, 1996; SCHULER *et al.*, 1998; FISHER *et al.*, 2001; TORRES-BUGARÍN *et al.*, 2007). Elevados níveis de estrógenos são também conhecidos por produzir efeitos adversos, tais quais embriotoxicidade, teratogenicidade e carcinogenicidade (IARC, 1979; HERTZ, 1985; MARCELOS e TOMATIS, 1992). Tendo em vista que o hormônio nandrolona nunca foi estudado *in vivo* pelo ponto de visto genotóxico, esse estudo visou avaliar o potencial clastogênico e/ou aneugênico do hormônio nandrolona em células da medula óssea através do teste do micronúcleo e o potencial genotóxico em leucócitos do sangue periférico de camundongos através do ensaio cometa.

## 2. CAPÍTULOS

### 2.1. Capítulo I: Artigo

#### CLASTOGENICITY AND/OR ANEUGENICITY OF NANDROLONE (DECA-DURABOLIN®) ANDROGENIC HORMONE IN MICE.

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RUNNING HEAD: Clastogenic/androgenic effects of nandrolone steroid hormone.

KEY WORDS: Nandrolone, deca-durabolin®, clastogenicity, micronucleus test; comet assay.

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## **Abstract**

**Objectives:** The aim of this study was to evaluate the genotoxic potential of nandrolone (deca-durabolin®) *in vivo* in bone marrow and peripheral blood cells of mice, using micronucleus and comet assays, respectively.

**Method:** The animals received intradermal injection of the 3 concentrations of the steroid (1.0, 2.5 and 5.0 mg/kg body weight). The cells were collected 24 h after the hormone-treatment for the micronucleus (clastogenicity endpoint) and comet assays (genotoxicity endpoint).

**Results:** Micronucleus test showed that the two higher tested-doses of the nandrolone induced statistically significant increase of the micronucleated cells and comet assay no evidenced significant increase in the DNA damage of the lymphocytes from peripheral blood.

**Conclusion:** Under our experimental conditions, the nandrolone steroid hormone showed clastogenic and/or aneugenic effects and, on the other hand, no genotoxic effects when administered intradermally to mice.

## 1. Introduction

Androgenic-anabolic steroids (AAS) are taken in large quantities by athletes and others to increase performance for several decades. These drugs include illicit substances as well as compounds that are marketed as nutritional supplements. AAS exert their effects in many parts of the body, including reproductive tissues, muscle, bone, hair follicles in the skin, the liver and kidneys, and the haematopoietic, immune and central nervous systems <sup>1</sup>. The androgenic effects of these hormones can be generally considered as those associated with masculinization and the anabolic effects as those associated with protein building in skeletal muscle and bone.

The drug known as nandrolone (also known commercially as Deca-Durabolin<sup>®</sup>) has the IUPAC name 17b-hydroxy-19-nor-4-andro-ster-3-one, and is an anabolic steroid (a muscle-building chemical) which occurs naturally in the human body, but only in tiny quantities. It is very similar in structure to the male hormone testosterone, and has many of the same effects in terms of increasing muscle mass, without some of the more unwanted side-effects such as increased body hair or aggressive behavior <sup>2</sup>.

In general, estrogens do not induce gene mutation in classical bacterial and mammalian mutation assays <sup>3</sup>, however, at the chromosomal level of mutation, some studies have shown positive results <sup>4-8</sup>. Elevated levels of estrogens also are known to produce adverse effects, such as embryotoxicity, teratogenicity and carcinogenicity <sup>9-11</sup>. Since the synthetic hormone nandrolone never was studied *in vivo* from the genetic toxicity point of view, this study aimed at evaluating the clastogenicity and/or aneugenicity of it in bone marrow cells and genotoxicity in peripheral blood cells of mice by micronucleus and comet assays.

## 2. Methods

### 2.1. Chemicals

The test substance, Nandrolone (Deca-durabolin<sup>®</sup>; Figure 1), was obtained from Organon do Brazil Laboratory. Cyclophosphamide (Sigma, CAS 6055-19-2, Lot:

108H0568) was used as the positive control substance due to their DNA damaging potential in the micronucleus and comet assays. It was dissolved in phosphate-buffer pH 6. Peanut oil (vehicle) was used as the negative control.

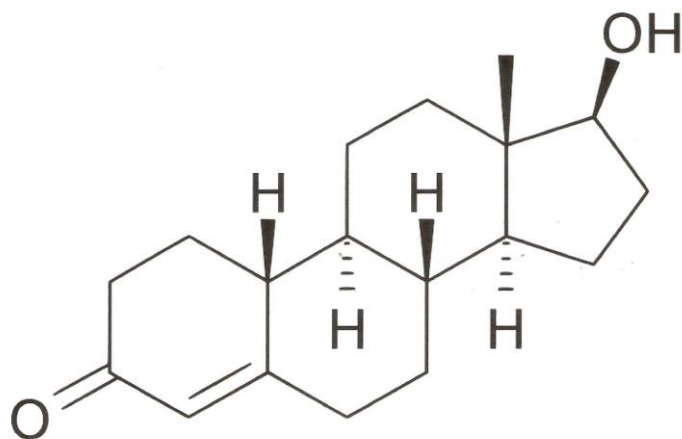


Fig. 1 – Nandrolone chemical structure.

## 2.2. Animals and dosing

Experiments were carried out on 10-week-old male Swiss albino mice (*Mus musculus*), weighing 25-30 g. The animals were acquired from the animal house of the Universidade de Campinas (UNICAMP), Campinas, São Paulo state, Brazil, and kept in polyethylene boxes (n= 11), in a climate-controlled environment ( $25 \pm 4^{\circ}\text{C}$ ,  $55 \pm 5\%$  relative humidity) with a 12-h light/dark cycle (7:00 am to 7:00 pm). Food (NUVILAB CR1 - NUVITAL) and water were available *ad libitum*. The mice were divided into experimental 5 groups of 10 animals. The nandrolone hormone was administered in a single dose of 0.5 mL by intradermal injection, at concentrations of 1,0; 2,5 e 5,0 mg/kg body weight, chosen on the basis of human use, which was 0,7 mg/kg. The negative control group received peanut oil. The positive control group received an intraperitoneal injection of cyclophosphamide 50 mg/kg. The animals used in this study were sacrificed by cervical dislocation. The Animal Bioethics Committee of the Faculdade de Medicina de Marília, CEP/FAMEMA, Marília, Brazil, approved the present study on September

28, 2006 (protocol number 47/06), in accordance with Brazilian regulations on animal care.

### 2.3. Cytogenetic Assays

#### 2.3.1. MN test

The assay was carried out following standard protocols as recommended by Schmid (1976)<sup>12</sup> and Krishna and Hayashi<sup>13</sup>. Ten male mice were used per group. The bone marrow from both femurs was flushed out using 2 mL of saline (0.9% NaCl) and centrifuged for 7 min. The supernatant was discarded and smears were made on slides. The slides were coded for a “blind” analysis, fixed with methanol and stained with Giemsa. For the analysis of the micronucleated cells, two thousand polychromatic erythrocytes (PCE) per animal were scored to determine the clastogenic property of the hormone. To detect possible cytotoxic effects, the PCE/NCE (normochromatic erythrocytes) ratio in 200 erythrocytes/animal was calculated<sup>14</sup>. The cells were blindly scored using a light microscope at 1000x magnification. The mean number of micronucleated polychromatic erythrocytes (MNPCE) in individual mice was used as the experimental unit, with variability (standard deviation) based on differences among animals within the same group.

#### 2.3.2. Comet Assay

The comet assay (SCGE) was carried out by the method described by Speit and Hartmann<sup>15</sup>, which is based on the original work of Singh<sup>16</sup> and includes modifications introduced by Klaude<sup>17</sup> as well as additional modifications. Peripheral blood samples were obtained from ten Swiss mice from each group at 24 h after treatment. An aliquot was removed from the peripheral blood cell suspension to determine cell viability. Cell counting was performed using a hemocytometer. Cell viability was determined by trypan blue dye exclusion. The number of trypan blue-negative cells was considered the number of viable cells and was greater than 85%. A 10- $\mu$ L aliquot of cells from each animal was mixed with 120  $\mu$ L of 0.5% low melting point agarose at 37°C, and rapidly spread onto two microscope slides per animal, pre-coated with 1.5% normal melting

point agarose. The slides were coverslipped and allowed to gel at 4°C for 20 min. The coverslips were gently removed and the slides were then immersed in cold, freshly prepared lysing solution consisting of 89 mL of a stock solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH set to 10.0 with ~8 g solid NaOH, 890 mL of distilled water and 1% sodium lauryl sarcosine), plus 1 mL of Triton X-100 (Merck) and 10 mL of dimethyl sulfoxide (Merck). The slides, which were protected from light, were allowed to stand at 4°C for 1 h and then placed in the gel box, positioned at the anode end, and left in a high pH (>13) electrophoresis buffer (300 mM NaOH-1 mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM, pH 10.0, EDTA) at 4°C for 20 min prior to electrophoresis, to allow DNA unwinding. The electrophoresis run was carried out in an ice bath (4°C) for 20 min at 300 mA and 25 V (0.722 V cm<sup>-1</sup>). The slides were then submerged in a neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 15 min, dried at room temperature and fixed in 100% ethanol for 10 min. The slides were dried and stored overnight or longer, before staining. For the staining process, the slides were briefly rinsed in distilled water, covered with 30 µL of 1x ethidium bromide staining solution prepared from a 10x stock (200µg/ml) and coverslipped. The material was evaluated immediately at 400x magnification, using a fluorescence microscope (Olympus BX 50) with a 515-560 nm excitation filter and a 590 nm barrier filter.

The extent and distribution of DNA damage indicated by the SCGE assay were evaluated by examining at least 100 randomly selected and non-overlapping cells on the slides (50 cells per slide), per animal, by an image analysis system (Comet Assay II – Perceptive Instruments, Haverhill, UK). Tail moment (the product of tail DNA/total DNA times the tail center of gravity, in arbitrary units) was the parameter used to score DNA damage. Comets with no heads and images with nearly all DNA in the tail, or with a very wide tail, were excluded from the evaluation because they probably represent dead cells<sup>18</sup>.

#### 2.4. Statistical analysis

Statistical analysis was performed to find out level of significance of the effect by employing one-way ANOVA with Kruskal-Wallis test for the comet assay and t-test for the MN assay. GraphPad InStat<sup>®</sup> software (version 3.01) was used. The results

obtained for test and the positive control groups were compared with the negative control group for significance. A difference of  $p < 0.05$  was considered statistically significant.

## **2. Results**

The results of micronucleus assay and PCE/NCE ratio determined after single oral administration of three different doses of nandrolone hormone to Swiss mice is shown in Table 1. Compared with its negative control, a dose-related increase in the mean number of MNPCEs in mouse groups was observed in the three hormone-tested groups, being statistically significant at the two higher doses ( $p < 0.05$  and  $p < 0.001$ , respectively). The majority of the micronucleus observed was big and very evident. The positive control group compared to the negative control was extremely significant ( $p < 0.001$ ). The estimated ratio of PCE/NCE in bone marrow preparations showed no statistically significant alterations in hematopoiesis as a result of nandrolone treatment, indicating no cytotoxic effects.

**Table 1.** Number of micronucleated polychromatic erythrocytes (MNPCE) observed in the bone marrow cells of male (M) Swiss mice treated with Nandrolone, and respective controls.

Treatments	Number of MNPCE per Animal										MNPCE Mean $\pm$ SD	PCE/NCE Mean $\pm$ SD
	M <sub>1</sub>	M <sub>2</sub>	M <sub>3</sub>	M <sub>4</sub>	M <sub>5</sub>	M <sub>6</sub>	M <sub>7</sub>	M <sub>8</sub>	M <sub>9</sub>	M <sub>10</sub>		
<b>Negative Control (peanut oil)</b>	6	4	3	2	2	2	4	0	2	0	<b>2.5 <math>\pm</math> 1.84</b>	<b>1.79 <math>\pm</math> 0.28<sup>NS</sup></b>
<b>Nandrolone (1.0 mg/kg)</b>	6	8	5	4	5	4	4	2	6	4	<b>4.8 <math>\pm</math> 1.62<sup>NS</sup></b>	<b>1.49 <math>\pm</math> 0.18<sup>NS</sup></b>
<b>Nandrolone (2.5 mg/kg)</b>	12	7	13	6	7	3	5	7	6	5	<b>7.1 <math>\pm</math> 3.11<sup>*</sup></b>	<b>1.52 <math>\pm</math> 0.22<sup>NS</sup></b>
<b>Nandrolone (5.0 mg/kg)</b>	11	11	13	12	7	8	13	14	6	15	<b>11.0 <math>\pm</math> 3.05<sup>**</sup></b>	<b>1.65 <math>\pm</math> 0.24<sup>NS</sup></b>
<b>Cyclophosphamide (50 mg/kg)</b>	36	32	38	26	25	35	40	24	36	40	<b>33.2 <math>\pm</math> 6.14<sup>***</sup></b>	<b>1.68 <math>\pm</math> 0.43<sup>NS</sup></b>

For each slides 2000 cells were analyzed. SD = standard deviation; NCE = normochromatic erythrocytes. \*Significantly different from negative control ( $p < 0.05$ ). \*\*Significantly different from negative control ( $p < 0.001$ ). \*\*\* Significantly different from negative control ( $p < 0.0001$ ).

The results of the comet assay in evaluating the nandrolone hormone, namely data on the tail moment (mean  $\pm$  SD) for mice treated with 1.0, 2.5 and 5.0 mg/kg, besides negative and positive control (50 mg/kg cyclophosphamide) are presented in Table 2. As expected, cyclophosphamide, the positive control induced a significant increase in tail moment in leukocytes ( $P < 0.001$ ). No statistically significant difference between treated and untreated animals was observed for nandrolone at all doses tested in leukocyte samples. The cell viability for leukocytes was greater than 85% using trypan blue staining, confirming the absence of cytotoxicity observed by the PCE/NCE ratio in the MN test.

**Table2.** DNA migration in the comet assay for the assessment of genotoxicity of Nandrolone in peripheral blood cells (collected 24 h after treatment) from male Swiss mice (M) *in vivo*.

Treatments	Tail Moment										Tail Moment Mean $\pm$ SD
	M <sub>1</sub>	M <sub>2</sub>	M <sub>3</sub>	M <sub>4</sub>	M <sub>5</sub>	M <sub>6</sub>	M <sub>7</sub>	M <sub>8</sub>	M <sub>9</sub>	M <sub>10</sub>	
<b>Negative Control (peanut oil)</b>	2.50	2.07	1.31	1.05	3.40	2.07	1.23	2.94	2.10	0.61	<b>1.93 <math>\pm</math>0.88</b>
<b>Nandrolone (1.0 mg/kg)</b>	1.27	0.42	0.81	0.68	0.24	0.91	1.09	1.40	0.37	0.89	<b>0.71 <math>\pm</math>0.34<sup>NS</sup></b>
<b>Nandrolone (2.5 mg/kg)</b>	2.87	0.20	0.13	0.19	0.71	0.49	0.58	0.54	0.45	2.53	<b>0.87 <math>\pm</math>0.99<sup>NS</sup></b>
<b>Nandrolone (5.0 mg/kg)</b>	0.95	0.94	1.98	1.50	1.66	0.83	1.34	1.23	1.24	1.36	<b>1.30 <math>\pm</math>0.35<sup>NS</sup></b>
<b>Cyclophosphamide (50 mg/kg)</b>	7.37	6.25	7.88	3.95	6.31	7.78	10.77	5.67	21.54	5.02	<b>8.25 <math>\pm</math>5.03*</b>

\* Extremely Significantly (p < 0.0001)

NS no significant

#### 4. Discussion

Due to the widespread use by the humans it is particularly relevant to study the genotoxic effect of the nandrolone hormone (deca-durabolin<sup>®</sup>) in *in vivo* mammalian system. Micronuclei are chromatin-containing bodies in the cytoplasm arising from acentric chromosomes fragments or from whole chromosome that was not incorporated in the daughter nuclei during the last stages of mitoses<sup>19</sup>. Chromosome fragment are associated with the clastogenic (chromosome breakage) activity of the tested substance whereas the presence of a whole chromosome indicates an adverse effect on the mitotic spindle apparatus (aneugenic effects). The difference in the size of micronuclei is an indicator of clastogenicity (small micronuclei) or aneugenicity (large micronuclei or whole chromosome)<sup>19</sup>. In this study, a dose-related increase in the mean number of MNPCEs in mouse bone marrow cells was observed in the three hormone-tested groups, being statistically significant at the 2.5 and 5.0 mg/kg doses. The sizes of micronuclei observed in most slides available suggest the aneugenic effect of the tested substance.

It is suggested that the testosterone and others similar AAS when administered at high doses may saturate the cellular receptors<sup>8</sup>. The testosterone derivatives could become aromatic and convert to 17 $\beta$ -estradiol, which induces various chromosomal and genetic lesions including aneuploidy, chromosomal aberrations, gene amplification, and microsatellite instability in cells in culture and/or *in vivo* and gene mutations in several cell test systems<sup>20</sup>. Some other sex hormones have shown capacity to produce alterations in chromosome number<sup>4,6</sup> and micronucleus induction by mitotic disturbances has been described<sup>5,7</sup>. From the present study it is evident that the nandrolone failed to induce genotoxic effects on peripheral blood cells of mice when tested using the comet assay. Liehr<sup>20</sup> suggest that 17 $\beta$ -estradiol is a weak carcinogen and weak mutagen capable of inducing genetic lesions with low frequency. Roy and Liehr<sup>3</sup> related that, in general, AAs do not induce gene mutation in classical bacterial and mammalian mutation assays, and at the chromosomal level, the majority of the studies have reported that this similar hormones do not induce chromosomal breaks or aberrations<sup>6,7,21</sup>. This can explain the non significant difference of tail moment on the nandrolone treated groups and the negative control, in the comet assay.

It is important to point out that nandrolone is a chemically modified testosterone hormone, which indicates that during the metabolic process some metabolites linked to the cancer process may be formed, together with high concentrations of 17 $\beta$ -estradiol<sup>8</sup>. Toxicity, mutagenicity, genotoxicity and cancerogenesis of sexual hormones are the result of a combination of genetic and epigenetic factors<sup>8-7</sup>

Moreover, the genotoxic activity of AAS is also due to metabolic activators and to an indirect process that takes place in the redox cycle, and the production of oxygen reactive types<sup>7</sup>. In this way, the metabolic activation of the testosterone derivatives leads to the formation of free radicals and, consequently, the formation of DNA adducts, which induce immediate alteration in this molecule<sup>22</sup>. Similarly, AAS can induce the activation of repair systems, indicating that some form of extensive DNA damage might be provoked<sup>22</sup>. The above-mentioned facts could explain the clastogenic results obtained for the presence of MN in bone marrow cells of mice after nandrolone administration.

In conclusion, the nandrolone decanoate hormone (deca-durabulin<sup>®</sup>) shows an clastogenic and/or aneugenic effects in bone marrow cells by applying the micronuclei

test and no mutagenic effects when investigated by the comet assay. This results are in agreement with other studies with testosterone derivate hormones when theirs genotoxic potentials were investigated<sup>8,7,20,22,23</sup>.

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### 3. CONCLUSÃO

Considerando os resultados obtidos no presente estudo, pode-se concluir que:

- A administração intradérmica do hormônio nandrolona acarretou efeitos clastogênicos e/ou aneugênicos em células da medula óssea de camundongos nas concentrações de 2,5 e 5,0 mg/kg. Como a grande maioria dos micronúcleos observados eram grandes e, face aos relatos na literatura de efeitos aneugênicos de outros hormônios esteróides similares, pode-se concluir também que a nandrolona apresentou efeitos aneugênicos nos eritrócitos policromáticos (EPC) da medula óssea.
- As doses testadas da nandrolona não causaram efeitos citotóxicos nos eritrócitos policromáticos da medula dos camundongos, como pôde ser evidenciado pela razão entre eritrócitos policromáticos (EPC) e eritrócitos normocromáticos (ENC).
- As três concentrações do hormônio não causaram efeitos genotóxicos no DNA dos linfócitos do sangue periférico dos camundongos.

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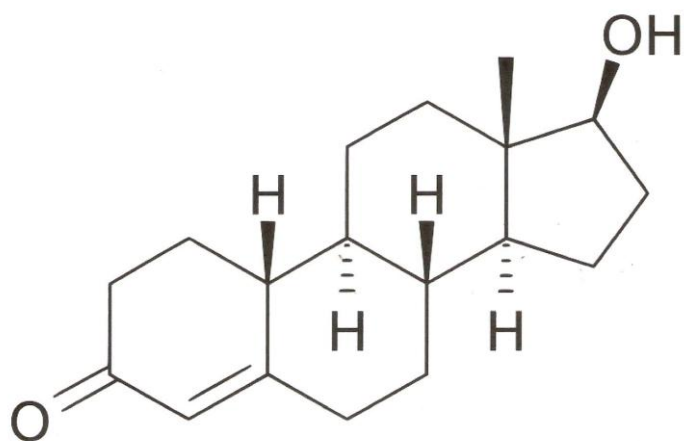
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## 5. APÊNDICES

Fig1. Nandrolone chemicals structure



**Table 1.** Number of micronucleated polychromatic erythrocytes (MNPCE) observed in the bone marrow cells of male (M) Swiss mice treated with Nandrolone, and respective controls.

Treatments	Number of MNPCE per Animal										MNPCE Mean $\pm$ SD	PCE/NCE Mean $\pm$ SD
	M <sub>1</sub>	M <sub>2</sub>	M <sub>3</sub>	M <sub>4</sub>	M <sub>5</sub>	M <sub>6</sub>	M <sub>7</sub>	M <sub>8</sub>	M <sub>9</sub>	M <sub>10</sub>		
<b>Negative Control (peanut oil)</b>	6	4	3	2	2	2	4	0	2	0	<b>2.5 <math>\pm</math> 1.84</b>	<b>1.79 <math>\pm</math> 0.28<sup>NS</sup></b>
<b>Nandrolone (1.0 mg/kg)</b>	6	8	5	4	5	4	4	2	6	4	<b>4.8 <math>\pm</math> 1.62<sup>NS</sup></b>	<b>1.49 <math>\pm</math> 0.18<sup>NS</sup></b>
<b>Nandrolone (2.5 mg/kg)</b>	12	7	13	6	7	3	5	7	6	5	<b>7.1 <math>\pm</math> 3.11<sup>*</sup></b>	<b>1.52 <math>\pm</math> 0.22<sup>NS</sup></b>
<b>Nandrolone (5.0 mg/kg)</b>	11	11	13	12	7	8	13	14	6	15	<b>11.0 <math>\pm</math> 3.05<sup>**</sup></b>	<b>1.65 <math>\pm</math> 0.24<sup>NS</sup></b>
<b>Cyclophosphamide (50 mg/kg)</b>	36	32	38	26	25	35	40	24	36	40	<b>33.2 <math>\pm</math> 6.14<sup>***</sup></b>	<b>1.68 <math>\pm</math> 0.43<sup>NS</sup></b>

For each slides 2000 cells were analyzed. SD = standard deviation; NCE = normochromatic erythrocytes.

\*Significantly different from negative control ( $p < 0.05$ ). \*\*Significantly different from negative control ( $p < 0.001$ ). \*\*\* Significantly different from negative control ( $p < 0.0001$ ).

**Table2.** DNA migration in the comet assay for the assessment of genotoxicity of Nandrolone in peripheral blood cells (collected 24 h after treatment) from male Swiss mice (M) *in vivo*.

Treatments	Tail Moment										Tail Moment Mean $\pm$ SD
	M <sub>1</sub>	M <sub>2</sub>	M <sub>3</sub>	M <sub>4</sub>	M <sub>5</sub>	M <sub>6</sub>	M <sub>7</sub>	M <sub>8</sub>	M <sub>9</sub>	M <sub>10</sub>	
<b>Negative Control (peanut oil)</b>	2.50	2.07	1.31	1.05	3.40	2.07	1.23	2.94	2.10	0.61	<b>1.93 <math>\pm</math> 0.88</b>
<b>Nandrolone (1.0 mg/kg)</b>	1.27	0.42	0.81	0.68	0.24	0.91	1.09	1.40	0.37	0.89	<b>0.71 <math>\pm</math> 0.34<sup>NS</sup></b>
<b>Nandrolone (2.5 mg/kg)</b>	2.87	0.20	0.13	0.19	0.71	0.49	0.58	0.54	0.45	2.53	<b>0.87 <math>\pm</math> 0.99<sup>NS</sup></b>
<b>Nandrolone (5.0 mg/kg)</b>	0.95	0.94	1.98	1.50	1.66	0.83	1.34	1.23	1.24	1.36	<b>1.30 <math>\pm</math> 0.35<sup>NS</sup></b>
<b>Cyclophosphamide (50 mg/kg)</b>	7.37	6.25	7.88	3.95	6.31	7.78	10.77	5.67	21.54	5.02	<b>8.25 <math>\pm</math> 5.03*</b>

\* Significantly different from the negative control ( $p < 0.0001$ ).

NS no significant