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Andressa Bueno

MARCADORES DE ESTRESSE OXIDATIVO NO TESTÍCULO, CÓRTEX E HIPOCAMPO E ALTERAÇÕES COMPORTAMENTAIS DE RATOS SUBMETIDOS A DIFERENTES PROTOCOLOS DE UTILIZAÇÃO DE ANABOLIZANTES

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Tese apresentada ao curso de Pós-Graduação em Medicina Veterinária, da, Universidade Federal de Santa Maria (UFSM,RS), como requisito parcial para obtenção do título de **Doutor em Medicina Veterinária**

Orientadora: Profa. Dra. Cinthia Melazzo de Andrade

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Aprovada em 04 de março de 2016:

_____
Cinthia Melazzo de Andrade, Dra. (UFSM)
(Presidente/Orientadora)

_____
Jessié Martins Gutierres, Dr. (Co-Orientador- UFSM)

_____
Maria Rosa Chitolina Schetinger, Dra. (UFSM)

_____
Vera Maria Melchiors Morsch, Dra. (UFSM)

_____
Roselia Maria Spanevello, Dra. (UFPel)

_____
Patricia Wolkmer, Dra. (UNICRUZ)

Santa Maria, RS
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DEDICATÓRIA

A todos que acreditavam.
A todos que duvidavam.

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"As pessoas estão sempre culpando as circunstâncias pelo que elas são. Eu não acredito em circunstâncias. As pessoas que progridem nesse mundo são aquelas que levantam e procuram as circunstâncias que elas querem, e, se não as encontram, as fazem."

(George Bernard Shaw)

RESUMO

MARCADORES DE ESTRESSE OXIDATIVO NO TESTÍCULO, CÓRTEX E HIPOCAMPO E ALTERAÇÕES COMPORTAMENTAIS DE RATOS SUBMETIDOS A DIFERENTES PROTOCOLOS DE UTILIZAÇÃO DE ANABOLIZANTES

AUTOR: Andressa Bueno
ORIENTADORA: Profa Dra Cinthia Melazzo de Andrade

Na presente tese verificamos os efeitos de diferentes protocolos de adiministração de esteróides androgênicos anabolizantes (EAA) utilizando duas diferentes moléculas frequentemente eleitas pelos usuários, a Boldenona (BOL) e o Estanazolol (ES). Dentre os protocolos utilizados, o protocolo I, representa os usuários que desejam acelerar os efeitos anabólicos usando doses superiores às recomendadas, em um intervalo de tempo mais curto, o protocolo II representa grupos de usuários de esteróides anabolizantes, que elegem uma dose moderada e tempo intermediário e protocolo III, que representa usuários que reduzem a dose e prolongam o tempo de exposição com o objetivo de diminuir os efeitos androgênicos.Nossos dados sugerem fortemente que o tratamento com BOL e ES afetam o comportamento de ansiedade, dominância e agressividade em ratos de forma dependente da dose e do tempo de exposição. Além disso, destaca-se que a perda da memória induzida pela BOL correlaciona-se com o aumento da atividade da acetilcolinesterase (AChE) no córtex cerebral e no hipocampo, e que essa disfunção do sistema colinérgico poderia estar envolvido no mecanismo dessa alteração, somado ainda ao dano associado ao estresse oxidativo também descrito em nossos resultados. Ao mesmo tempo, avaliou-se o impacto desses tratamentos sobre a morfologia e os parâmetros de estresse oxidativo dos testiculos de ratos. Foi evidenciado a elevação dos marcadores de estresse oxidativo e nitrosativo assim como alterações significativas na morfologia normal do testiculo, que provavelmente está associado a presença dessas espécies reativas. Ainda que não tenhamos aprofundados os mecanismos pelos quais os EAA interferem com a fisiologia testicular e as alterações comportamentais, está claro que há um envolvimento das espécies reativas de oxigênio (ERO) e espécies reativas de nitrogênio (ERN) no desenvolvimento das mesmas. Nossos dados levantam a preocupação de que os EAA, mesmo em pequenas doses, possam provocar consequências neuropatológicas levando a alterações comportamentais distintas de acordo com o protocolo utilizado. Da mesma forma, no testiculo, não houve protocolo seguro que descartasse a possibilidade de alterações reprodutivas que poderiam a longo prazo levar a esterilidade.

Palavras chaves: Boldenona. Estanazolol. Comportamento. Testículo.

ABSTRACT

OXIDATIVE STRESS MARKERS IN TESTIS, CORTEX AND HIPPOCAMPUS AND BEHAVIORAL CHANGES OF RATS SUBMITTED TO DIFFERENT PROTOCOLS OF ANABOLIC ANDROGENIC STEOIRDS

AUTHOR: Andressa Bueno
ADVISOR: Prof Dr Cinthia Melazzo de Andrade

In this research we observed the effects of different anabolic androgenic steroid administration for protocols (EAA), selecting two molecules, Boldenone (BOL) and Stanozolol (ST).Among the used protocols, Protocol I, represents users who want to accelerate the anabolic effects using higher than recommended doses, in a shorter period of time, the Protocol II represents groups of anabolic steroid users, who elect a moderate dose and time intermediate and protocol III, which represents users that reduce the dose and prolong the exposure time for the purpose of decreasing androgenic effects. Our findings strongly suggest that the treatment with BOL and ST affect the anxiety, dominance and aggression behavior in mice dose and time of exposuredependent. Furthermore, it is emphasized that the memory impairment induced by boldenone is correlated with the increase of acetyl cholinesterase (AChE) activity in cerebral cortex and hippocampussuggesting that a dysfunction of the cholinergic system may be involved in the mechanism of this alteration, along with the damage associated with oxidative stress as well described in our results. Simultaneously, we evaluated the impact of these treatments on the morphology and oxidative stress parameters in the testes of rats. The increased of oxidative and nitrosative stress markers was evident, as well as significant changes in the normal morphology of the testicle, which is probably associated with the presence of these reactive species.The mechanisms by which the EAA interfere with testicular physiology and behavioral changes have not been deeply studied by our group, but there is strong evidence of the involvement of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in development. Our data shows that EAA, even at low doses, might result in a neuropathological consequences. Similarly, in the testis, no protocol proved to be safe in removing the possibility of reproductive disturbances that could lead to long-term sterility.

Key words: Boldenone. Stanazolol. Behavior. Testis.

LISTA DE ABREVIATURAS E SIGLAS

AChE	Acetilcolinesterase
ACh	Acetilcolina
BOL	Undecilenato de boldenona
CAT	Catalase
DNA	Ácido desoxirribonucleico
DHT	Diidrotestosterona
EAA	Esteróide androgênico anabolizante
ES	Estanazolol
EO	Estresse oxidativo
ERRO	Espécie reativa de oxigênio
ERN	Espécie reativa de nitrogênio
FSH	Hormônio folículo estimulante
H_2O_2	Peróxido de hidrogênio
HOCL	Ácido hipocloroso
$HRO_2^{\bullet}$	Hidroperoxil
LH	Hormônio luteinizante
LPO	Lipoperoxidação
MDA	Malondialdeído
MPO	Mieloperoxidase
ND	Nandrolona
NAGase	N-acetyl-β-D-glucosaminidase
O_2	Oxigênio
$O_2^{\bullet-}$	Ânion superóxido
$OH^{\bullet}$	Radical hidroxila
RA	Receptores andrógenos
RE	Receptor estrogênico
$O^{2-\bullet}$	Peroxil
SNC	Sistema nervoso central
SH	Grupo sulfidrila
SOD	Superóxido dismutase
TBARS	Substâncias reativas ao ácido tiobarbitúrico
NO	Óxido nítrico
NOS	Óxido nítrico sintase
i-NOS/ NOS-2	Óxido nítrico sintase induzível
ONOO	Peroxinitrito

SUMÁRIO

1. INTRODUÇÃO

Os registros de uso de substâncias que aumentam a capacidade física e proporcionam vantagem competitiva ao usuário, o que conhecemos por dopagem, data do século 19, quando ciclistas europeus utilizaram estimulantes como a heroína, cocaína e anfetamina nos jogos olímpicos. Somente em meados de 1935 é que a estrutura química da molécula de testosterona (T) foi isolada, e a partir daí disponibilizada em forma oral e injetável para uso terapêutico em humanos. Na década de 40 estudos com equinos evidenciaram melhora no desempenho dos animais que faziam uso dessa molécula. Na próxima década a utilização se estendeu aos atletas olímpicos americanos, soviéticos e alemães (KOCH, 2002). O uso abusivo dos esteróides androgênicos anabolizantes (EAA) por atletas de elite se difundiu entre 1950 e 1960 nos países do leste europeu, coincidindo com o término da Segunda Guerra Mundial e com o crescimento da indústria farmacêutica (HANDELSMAN e HEATHER, 2008).

Em razão desse contexto, o Comitê Olímpico Internacional (COI) determinou que se estabelecessem testes que comprovassem a administração dessas substâncias por parte dos competidores, e seu uso passou a ser rotina a partir dos jogos de Munich em 1972, passando a ser conhecido como exame antidopagem. Foi somente em 1976 que esses testes passaram a detectar a presença de EAA e começaram a integrar o exame anti dopagem em Seul no ano de 1988 (BOWERS, 2002).

Passadas algumas décadas, os EAA deixaram de ser consumidos somente pela elite desportiva e passaram a ser utilizados pela população em geral (EVANS-BROWN e MCVEIGH,2008). Atualmente, o aumento ao redor de 30% do comércio destes fármacos para fins não terapêuticos, tornou-se um problema de saúde pública (EVANS, 2004). Em resposta ao aumento do consumo destas substâncias, à fabricação clandestina e comércio ilegal, em 1991 os EAA foram classificados como substâncias de uso controlado pelo congresso norte americano (SHAHIDI, 2001; SJÖQVIST, GARLE e RANE 2008). Também no Brasil o uso de EAA é considerado dopagem desde 1985 pelo decreto de uma portaria do Ministério da Educação e sua comercialização sem receituário médico é considerado tráfico de entorpecentes e é crime segundo o Código Civil.

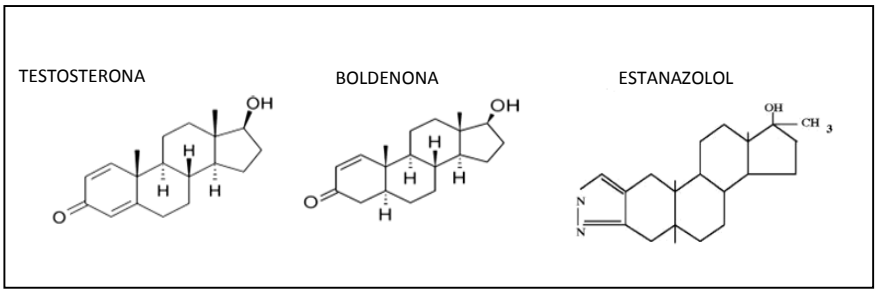
A testosterona pertence aos hormônios sexuais que interagem com receptores andrógenos (RA). No sexo masculino, ela é naturalmente sintetizada a partir do colesterol principalmente nas células testiculares de Leydig e em quantidade bastante inferior nas glândulas supra-renais (DURDIAKOVA, OSTATNIKOVA e CELEC, 2011). EAA referem-se a moléculas sintéticas derivadas do hormônio T, modificadas quimicamente para aumentar a meia vida do princípio e também o seu o efeito anabólico, e minimizar o efeito androgênico (SHAHIDI, 2001).

Existem três principais classes de EAA e elas determinam o anabolismo e androgenicidade dos fármacos que as compõe (CLARK e HENDERSON, 2003). A primeira classe é representada por compostos injetáveis (propionato e cipionato de testosterona), onde a esterificação retarda a degradação do composto e prolonga o tempo de ação devido à liberação sistêmica mais lenta, além de possuir maior afinidade pelos RA do que a T (SHAHIDI, 2001; BASARIA, WAHLSTROM e DOBS, 2001; HALL e HALL, 2005). A segunda classe é também de compostos injetáveis, como o decanoato de nandrolona (ND) e o undecilenato de boldenona (BOL), que sofreram um alongamento de sua cadeia, conferindo uma atividade androgênica reduzida resultante de uma redução da afinidade da molécula aos RA (WINTERS, 1990; SHAHIDI, 2001). Na terceira classe as moléculas, que inclui o estanazolol (ES), sofreram um processo de alquilação, que dificulta a metabolização hepática, e torna possível a administração via oral e redução drástica do potencial androgênico (BASARIA, WAHLSTROM e DOBS, 2001).

A BOL é classificada como um hormônio EAA de cadeia extensa (1,4-androstadiene-17β-ol-3-one), comercialmente disponível em veículo oleoso a base de óleo de gergelim, o que confere a esta molécula uma meia vida longa e a impossibilidade de ser administrado com outros fármacos simultaneamente. Difere da testosterona apenas por uma ligação dupla em seu primeiro anel (PLUMB, 2005; STOLKER et al., 2007). Já o ES, possui um anel de pirazol ligado ao anel heterocíclico externo (SCHANZER, OPFERMANN e DONIKE, 1990) (Figura1).

A Agência Internacional de Pesquisa sobre Câncer (IARC) classifica a BOL como medicamento de 2ª classe (promotor de crescimento), com potencial carcinogênico, mais elevado que outros andrógenos como o ES, ND e T, sendo, portanto uma substância de uso banido (IARC, 1987). O ES é considerado 50 vezes mais anabólico do que a BOL, com o agravante dessa última molécula possuir um considerável efeito androgênico (PLUMB, 2005).

Figura 1- Fórmula química da Testosterona, Boldenona e Estanazolol.



Fonte:KALININI, 2011.

A T atua nas células alvo através de um metabólito ativo, a diidrotestosterona (DHT), a qual é convertida localmente por uma enzima. A T e a DHT se ligam ao receptor andrógeno RA. Outro metabólito hormonal metabolicamente ativo da T é o estradiol que se liga ao receptor estrogênico RE. Por seu caráter lipofílico, a T e os EAA, dentre outros esteróides, atravessam as membranas biológicas muito facilmente. No ambiente intracelular há formação do complexo hormônio-receptor, através de uma ligação específica, que depende das características químicas de cada esteróide. Os andrógenos ligam-se aos RA citoplasmáticos, e tal ligação é acompanhada da interação dos complexos resultantes com sítios nucleares e efeitos genômicos subsequentes. Estas ações caracterizam a via denominada “via clássica” de ação dos esteroides. Existe ainda outra via de sinalização destes hormônios denominada “via rápida”, que pode ocorrer independentemente da ligação aos RA. O efeito rápido dos hormônios esteróides consiste basicamente na alteração da fluidez da membrana plasmática (FALKENSTEIN et al., 2000; CATO, NESTL e MINK, 2002; KICMAN, 2008).

EAA são usados para melhorar a força e resistência atlética, em caninos, equinos e em atletas humanos, através do aumento da massa muscular através da produção de proteínas, por um equilíbrio positivo no balanço de nitrogênio, que estimula sua produção e diminui a destruição proteica (KICMAN, 2008). O número de adolescentes que usam EAA tem crescido significativamente ao longo dos últimos 10 anos, com estimativas de que varie entre 4-12% de usuários entre os adolescentes.

Essas moléculas também são utilizados por atletas ou não-atletas para fins atléticos ou estéticos, sendo que a dose utilizada supera em 10 a 100 vezes a dose terapêutica e esse abuso acarreta muitos efeitos adversos (YERSALIS e BAHRKE, 1995; MCGINNIS, 2004; Lumia e MCGINNIS, 2010). Quase todos os principais tecidos do corpo possuem RA, e por essa razão são alvos dos EAA, e por consequência sofrem os efeitos de seu abuso (KARILA, HOVATTA e SEPPÄLÄ, 2004).

As indicações terapêuticas clássicas para o uso de EAA incluem principalmente o hipogonadismo, múltiplas causas de falência da medula óssea, deficiências na mineralização óssea e algumas desordens que provocam perda muscular (SHAHIDI, 2001). Os efeitos deletérios do abuso dessas substâncias incluem insuficiência hepática, acne, diminuição da lipoproteína de alta densidade (HDL), adenoma hepático, alterações de humor, comportamento agressivo e tendência ao suicídio, bem como déficits de aprendizagem e memória espacial (CLARK, HARROLD e FAST, 1997 BOYADJIEV et al., 2000; TANEHKAR et al., 2013).

Diversos grupos de pesquisadores estudaram o papel dos EAA como promotores de crescimento testicular; glândula bulbouretral e próstata (GROOT e BIOLATTI 2004; CANNIZZO et al., 2007), na função reprodutiva de garanhões (SQUIRES et al., 1982; SQUIRES & MCKINNON,1987; GARCIA et al., 1987) e no desempenho reprodutivo de coelhos (THABET et al., 2010). Em homens que fizeram uso abusivo de EAA, há uma notável redução nos níveis de testosterona sérica, bem como de hormônio folículo estimulante (FSH) e hormônio luteinizante (LH). Estudos afirmaram que o hipogadismo hipogonadotrófico está relacionado a essa redução de testosterona sérica e morfologia anormal do testículo. (JAROW e LIPSHULTZ, 1990, TOUSSON et al., 2012). Além disso, tem sido associada ao uso de anabolizantes, com redução da qualidade espermática e que pode estar relacionadas com a infertilidade (TORRES-CALLEJA et al., 2001).

A capacidade dos anabolizantes alterar a histopatologia testicular levando a lesões que dependem tanto da molécula administrada como também da dose já está descrita em diversas literaturas que relataram detalhadamente essas alterações, que em via de regra diminuem o epitélio germinativo e, por consequência, os espermatozoides viáveis culminando com a oligospermia e/ou azoospermia e consequente infertilidade (GROOT e BIOLATTI 2004; ODA e EL-ASHMAWY, 2012; TOUSSON et al., 2012; TOUSSON et. al., 2013; TOUSSON, 2013).

Pouco se sabe sobre o envolvimento do estresse oxidativo no desenvolvimento dessas lesões testiculares. Estudos apresentados por diversos autores mostraram que a administração de testosterona sintética pode influenciar potencialmente a produção de espécies reativas de oxigênio (ERO) no testículo (PELTOLA et al., 1996; CHAINY et al.,1997; TURNER e LYSIAK, 2008; TÓTHOVA et al.,2013; FRANKENFELD, et al., 2014; MOHAMED e MOHAMED, 2014).

Outro estudo revelou que a administração de testosterona conduziu a um aumento significativo no estresse oxidativo em testículos de coelhos (AYDILEKet al., 2004). Somado a esses resultados, o tratamento intra testicular ou sistêmico com EAA diminui a expressão das enzimas SOD, CAT e Gpx nos testículos (CHAINY, SAMANTARAY e SAMANTA, 1997; AYDILEK, AKSAKAL e KARAKILCIK, 2004; AITKEN e SHAUN, 2008, MOHAMED e MOHAMED, 2014).

Em situações fisiológicas, a formação de ERO ocorre normalmente no organismo, especialmente em razão do metabolismo mitocondrial. Em torno de 1 a 2% do O_2 escapa da redução para sua neutralização, resultando na formação de ânion superóxido ($O^{2\bullet-}$), peroxil ($RO^{2\bullet}$), hidroperoxil ($HRO^{2\bullet}$), e o radical hidroxila ($OH^{\bullet}$); e as espécies não radicalares como o peróxido de hidrogênio (H_2O_2) e o ácido hidrocloroso (HOCL) (MOLINA, 2003; HALLIWELL et al., 2000;HALLIWELL e GUTTERIDGE, 2008). Em proporções controladas pelos mecanismos defensivos celulares, conhecidos como sistemas antioxidantes eles terminam por serem neutralizados.

As células possuem um sistema de proteção antioxidante enzimático e não enzimático, que tem a importante função de inibir os efeitos deletérios das ERO através do equilíbrio entre agentes pró-oxidantes e antioxidantes (CHIHUAILAF et al., 2002; HALLIWELL e GUTTERIDGE, 1999). Esse sistema antioxidante enzimático é composto pela superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx), que constituem a primeira linha de defesa endógena de neutralização das ERO. Através destas enzimas as células mantêm as concentrações do radical superóxido e de peróxidos de hidrogênio baixas, evitando assim, a formação do radical hidroxila (HALLIWELL e GUTTERIDGE, 1999).

A SOD é uma metaloenzima que participa do processo de detoxificação dos radicais livres, ela é específica na remoção do radical superóxido, catalisando a sua dismutação a peróxido de hidrogênio, através da reação que transforma dois ânions de radical superóxido ($O_2^{\bullet}$) em um peróxido de hidrogênio menos reativo que o

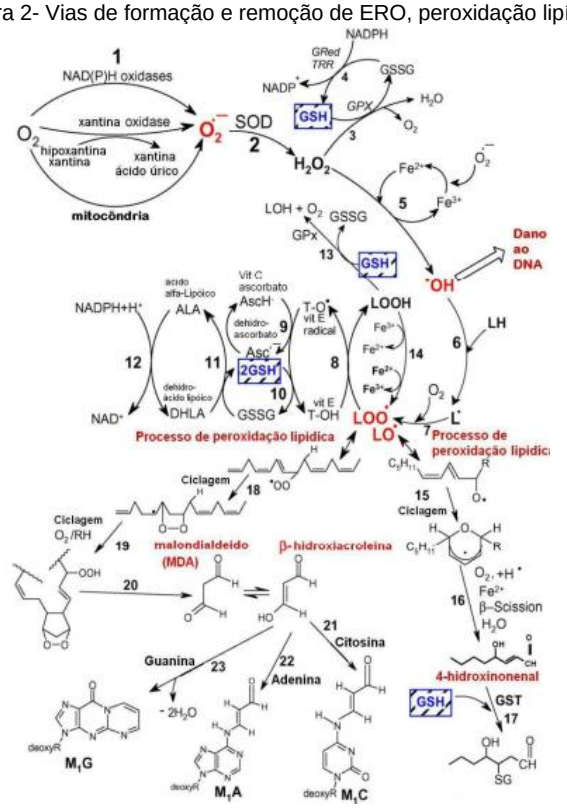
anterior. O peróxido de hidrogênio formado é degradado pela ação da CAT ou da GPx, resultando em H_2O e O_2. (ANDERSON, 1996).

Dentre os antioxidantes não enzimáticos pode-se destacar a vitamina C, a vitamina E os compostos orgânicos contendo grupos sulfidrila (SH) denominados tióis. A vitamina C apresenta propriedades antioxidantes protegendo várias moléculas contra o dano causado pelas ERO (HALIWELL et al., 2000). Além de sua ação direta contra radicais livres, o ácido ascórbico ou vitamina C afeta indiretamente o balanço entre antioxidantes e oxidantes, já que promove a regeneração do alfa tocoferol um importante agente lipossolúvel (LIU et al., 1998). Os tocoferóis ou vitamina E são varredores de radicais peroxil sendo, portanto os inibidores mais importantes da peroxidação lipídica em animais. Em adição, a glutationa reduzida (GSH) é um tiol de baixo peso molecular que se encontra no interior celular, sendo importante na proteção contra o dano oxidativo (FERREIRA e MATSUBARA, 1997).

Em situações patológicas, a produção de ERO pode aumentar substancialmente, exaurindo esse sistema antioxidante, resultando em dano a diversas moléculas, o que se denomina estresse oxidativo (SALVADOR e HENRIQUES, 2004; BURTON e JAUNIAUX, 2011). As macromoléculas, como o DNA, as proteínas e os lipídeos, são sensíveis à presença de ERO. A peroxidação lipídica inicia quando estas alteram as ligações dos ácidos graxos da membrana celular alterando sua coesão e permeabilidade alterando as funções metabólicas celulares (CHIHUAILAF et al., 2002). Este processo resulta na formação de hidrocarbonetos e aldeídos, como o malondialdeído (MDA), que são mensurados para a determinação do dano a essa estrutura através das substâncias reativas ao ácido tiobarbitúrico (TBARS) (BEHULIAK et al., 2009). Membranas testiculares são altamente vulneráveis ao estresse oxidativo devido ao seu elevado teor de ácido graxo poli-insaturado; por conseguinte, maiores níveis de MDA poderiam ser esperados em danos provocados pelas ERO neste tecido (Figura 2).

Apesar da intensa atividade mitótica e o alto metabolismo local, o testículo possui uma circulação pobre que provoca uma baixa tensão de O_2 local sendo este, mesmo que indiretamente, um mecanismo adaptativo de defesa do órgão contra o dano oxidativo (PELTOLA et al., 1994, KODAMA et al., 1996; SIKKA, 2004).

Figura 2- Vias de formação e remoção de ERO, peroxidação lipídica e ação antioxidante.



Fonte: VALKO- 2007 (adaptado)

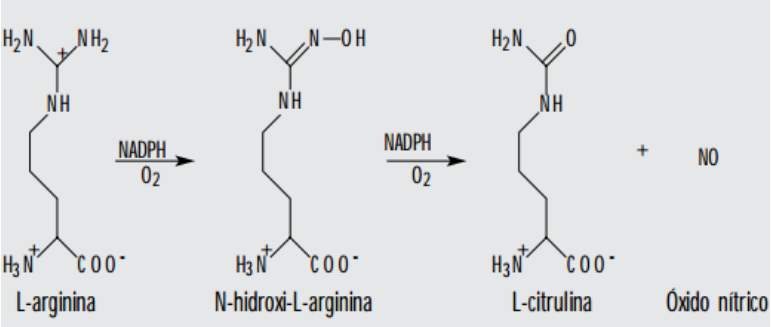
Além disso, tem sido demonstrado que o testículo possui um sistema antioxidante muito eficiente, rico em GSH, SOD e GPx e que esse sistema possui um importante envolvimento na proliferação e diferenciação espermática promovendo uma proteção dessas células germinativas ao ataque das ERO (TEAF et al., 1985).

O testículo possui além das formas convencionais da SOD, citosólica e mitocondrial, uma SOD extracelular (SOD-ex) produzida pelas células de Sertoli e germinativas (MRUK et al. 2002). Esse sistema é de suma importância, pois tem sido relatadas evidências do envolvimento do aumento do estresse oxidativo provocado por xenobióticos, hormônios, cigarro, etanol, e criptorquidismo na disfunção espermática, revelando a íntima relação do aumento das ERO com a patologia

espermática (ROSENBLUM et al., 1985; KOJIMA et al., 1990; AHOTUPA e HUHTANIEMI, 1992; PELTOLA et al., 1994; CHAINY et al., 1997).

Nesse contexto de produção espécies radicalares que interagem moléculas orgânicas via de regra lesionando a célula, está inserido também o oxido nítrico (NO), que é uma espécie reativa de nitrogênio sintetizada pela enzima óxido nítrico sintase (NOs). Esta enzima catalisa a oxidação da arginina que tem como produto final a citrulina e uma molécula de NO (Figura 3) (FILHO e ZILBERSTEIN, 2000).

Figura 3- Síntese de óxido nítrico a partir da L-arginina.



Fonte: DUSSE, VIEIRA E CARVALHO, 2003.

O NO constitui um dos mais importantes mediadores de processos intra e extracelulares. Pesquisas vêm demonstrando que ele está envolvido no controle da pressão sanguínea, neurotransmissão, inibição das plaquetas e aderência de neutrófilos (FILHO e ZILBERSTEIN, 2000; DUSSE et al., 2003). A isoforma óxido nítrico sintase induzível (i-NOs), atualmente denominada NOs-2 é produzida por macrófagos e outras células, quando ativadas por citocinas pró-inflamatórias (FILHO e ZILBERSTEIN, 2000; JANJIC et al., 2012). O NO resultante da ativação de NOs-2 possui ação citotóxica e citostática, propiciando a eliminação de microorganismos, parasitas e células tumorais, estando envolvido na via de sinalização apoptótica (JANJIC et al., 2012).

Porém nessa ação, dentro do fagolisossomo, o NO reage com o ânion superóxido ($O^{2\bullet-}$), produzindo peroxinitrito ($ONOO^-$), um radical altamente reativo e capaz de lesar a proteína, o DNA e a membrana lipídica (COTRAN, 2000; ABBAS et al., 2012). Essa elevação de NO resulta no denominado estresse nitrosativo que pode estar associado a infertilidade e ainda, ao dano testicular induzido por EAA que induz por apoptose das células de Leydig (O'BRYAN et al., 2000; CHUNG et al., 2001; DOSHI et al., 2012; JANJIC et al., 2012).

Aquimiotaxia, que é a atração de células inflamatórias pela elevação do NO, propicia para o local da lesão, um influxo leucocitário que provavelmente está associado ao estresse oxidativo, uma vez que o metabolismo celular aumenta o acúmulo de ERO. Nesse sentido, a mieloperoxidase (MPO) e a N-acetyl-β-D-glucosaminidase (NAGase) sinalizam essa infiltração celular de neutrófilos e macrófagos respectivamente (WINTERBOURN, VISSERS, e KETTLE, 2000; SIMOJOKI et al., 2009; WINTERBOURN, e KETTLE, 2013). O aumento desses marcadores de infiltração celular já está descrito em diversos processos patológicos como doenças do sistema nervoso central (SNC), na mastite bovina, no líquido sinovial de articulações com degeneração e também a sua elevação induzida pelo exercício (ART et al. 2006; SIMOJOK et al., 2009; RAYNER, LOVE e HAWKINS, 2014; CARVALHO et al., 2015). Porém nosso grupo de pesquisa desconhece algum relato de elevação desses marcadores inflamatórios no testículo, induzidos pelo uso de EAA.

Afora os efeitos descritos, que se referem de forma geral ao contexto reprodutivo masculino, os EAA interagem da mesma forma com RA no SNC. Dessa interação resultam uma série de alterações comportamentais que são detectadas em tarefas específicas. Ainda que exista alguma relação entre ambos os sistemas, como por exemplo, os níveis séricos de testosterona que podem estar relacionado ao comportamento agressivo, de forma geral a fisiopatologia parece ser inter-relacionada. Os RA presentes no SNC tornam esses tecidos alvos das mesmas espécies reativas que atuam no testículo, e consequentemente passíveis de alterações.

Inúmeros trabalhos descrevem a interferência dos EAA no comportamento agressivo de machos. Essa associação é bastante contraditória, pois a análise dos resultados é afetada por diversas variáveis, como a combinação de EAA, dose, tempo de tratamento, idade dos animais e a metodologia (CLARK e HENDERSON 2003; MCGINNIS, 2004; SALAS-RAMIREZ, MONTALTO e SISK, 2010).

Enquanto alguns autores relataram que o uso de T aumenta significativamente a agressividade, outros pesquisadores atestam que a ND não altera esse parâmetro e ainda, que o ES diminui o comportamento agressivo quando comparado ao grupo controle. Essa discordância demonstra claramente a dependência dos fatores descritos anteriormente para o desenvolvimento do padrão comportamental (BREUER, et al., 2001; MCGINNIS, et al., 2002). Os efeitos paradoxais do ES destacam a disparidade de ação de diferentes EAA e a necessidade de compreender o mecanismo molecular de cada composto no cérebro. Em humanos, o uso de EAA também está fortemente associado com comportamento agressivo e violento. A agressividade é geralmente empregada para a defesa do território e acesso às fêmeas enquanto a violência é uma expressão exagerada da agressividade (MCGINNIS et al., 2004). Um grupo de pesquisa descreve ainda que o comportamento agressivo aumenta quando ocorre a privação do princípio após o seu uso, e que isso acontece de forma mais marcada em animais jovens, enquanto os adultos apresentaram um padrão de ansiedade mais acentuado na fase de privação (MORRISON, RICCI e MELLONI, 2015).

Pouco se sabe sobre os efeitos dos EAA sobre a ansiedade. O primeiro estudo destes efeitos dos EAA foi realizado por Bitran et al., 1993 no qual demonstraram que altas doses de propionato de T induziu um efeito ansiolítico aos 6 dias de tratamento. Porém com 14 dias de tratamento, os animais não foram diferentes do grupo controle. Estes resultados indicam um caráter ansiolítico transitório do tratamento. De uma maneira geral os poucos estudos que abordaram EAA e ansiedade apresentam resultados controversos. Mais especificamente, o tratamento com DN em ratos apresentou tanto efeitos ansiolíticos como ansiogênicos (BITRAN, KELLOGG e HILVERS, 1993; CLARK e HENDERSON, 2003). Existe alguma especulação sobre a adaptação de receptores gabaérgicos e a modulação da ansiedade, mas esse fato não foi cientificamente comprovado, sendo que os mecanismos dessa interação entre ansiedade e EAA ainda não está demonstrado. Um grupo de pesquisa testou o efeito ansiolítico através de um teste de privação de água e oferta seguida ou não de choque. Os animais tratados com EAA aceitavam mais número de choques para a obtenção de água quando comparado aos animais controle que rapidamente abandonavam a tentativa de obtenção hídrica (CARLSON et al, 2001).

Resultados de diversos autores tem demonstrado uma ação ansiolítica da T (BING et al., 1998; AIKEY et al., 2002). Porém assim como no comportamento agressivo, há contradições com relação ao efeito dos EAA sobre a ansiedade, uma vez que Minkin, Meyer e Van-Haaren, (1993), demonstraram que a ND aumentou o comportamento ansioso.

Altas concentrações de RA estão localizados no córtex e no hipocampo, indicando o possível envolvimento dessas estruturas em uma variedade de funções das células neurais que incluem influências na memória e aprendizado (SIMERLY et al., 1990). Inesperadamente, os resultados de estudos sobre os efeitos de EAA sobre o aprendizado e a memória são inconsistentes. Alguns autores afirmaram que o uso de anabolizantes andrógenos não induzem deficiências de aprendizagem e memória (CLARK, MITRE e BRINCK-JOHNSEN, 1995; SMITH, STACKMAN e CLARK, 1996). Em contrapartida, outros relataram prejuízos na memória espacial induzidas pelo uso de ND (KOUVELAS et al., 2008; MAGNUSSON et al., 2009; TANEKHAR et al., 2013).

Os mecanismos da deficiência de aprendizagem e diminuição da memória não estão bem definidos. Estudos demonstram que os EAA provocam um aumento na imuno reatividade dos RA e que o mecanismo da diminuição da memória está diretamente ligado a reatividade desses receptores (MENARD e HARLAN, 1993; KOUVELAS et al., 2008).

O sistema colinérgico tem um papel fundamental em várias funções vitais, como o aprendizado, a memória e a organização cortical do movimento, o que faz este sistema ser atual alvo de inúmeras pesquisas (MESULAM et al., 2002). A interrupção da transmissão sináptica pela hidrólise do neurotransmissor é uma característica substancial das sinapses colinérgicas, sendo a acetilcolinesterase (AChE) a encarregada de executar a quebra do neurotransmissor acetilcolina (ACh), uma molécula chave na sinalização colinérgica (ZIMMERMAN e SOREQ, 2006). A taxa de secreção da AChE é modulada pela estimulação neuronal, quantidade do neurotransmissor na sinapse e tratamento com fármacos (DESCARRIES, GISIGER e STERIADE, 1997). Martins et al. (2010) descreveram o aumento da atividade dessa enzima induzida por nandrolona. Kaufer et al. (1998) relataram que indivíduos que apresentaram sinais clínicos neuropsiquiátricos similares aos provocados pelo uso abusivo de EAA tiveram alterações nos níveis de AChE, o que sugere que a atividade desta enzima também estava alterada.

Não está descrito, até o momento, a elevação da atividade dessa enzima induzida pela BOL ou pelo ES, tampouco sua correlação com a perda de memória nesse contexto. Esse padrão contraditório dos achados dos grupos de pesquisa acerca do impacto dos EAA sobre o comportamento reflete a dependência de parâmetros como agressividade da molécula, dose utilizada, tempo de exposição ao princípio e até mesmo a tarefa empregada para avaliar o efeito.

A BOL e o ST são as drogas mais frequentemente utilizadas na medicina veterinária e estão entre os quatro anabolizantes mais usados em humanos. Diante desse quadro o objetivo desta tese é elucidar de que forma esses dois anabolizantes, interferem com os padrões comportamentais, através da realização de testes comportamentais; e também com como interferem na lesão testicular, através da avaliação de marcadores de estresse oxidativo e inflamatórios, afim de determinar qual dos dois compostos é mais deletério, e ainda se há nesse contexto influência da dose e do tempo de exposição, já que poucos trabalhos relatam os efeitos da BOL e raros descrevem a influência do uso do ES nesses parâmetros. Com os resultados, o grupo espera poder contribuir para uma redução do uso abusivo de EAA.

2. ARTIGOS CIENTÍFICOS

Os resultados desta tese estão sob a forma de dois artigos científicos. Os itens materiais e métodos, resultados, discussão e referências bibliográficas encontram-se nos artigos.

Artigo 1

Este manuscrito foi submetido para publicação no periódico PLOS ONE ISSN 1932-6203, Qualis A1 na Medicina Veterinária com fator de impacto 3,23.

Federal University of Santa Maria
Center of Rural Sciences
Veterinary Hospital
97105-900/Santa Maria, RS, Brazil

January, 2016

To the Editors:

I would like to submit a research article for publication in PLOS One, titled "Impact of dose and duration of androgenic anabolic steroid exposure on behavior in rats" (word count: 6,380; figures 9; references 71). The paper was co-authored by Andressa Bueno, Fabiano B. Carvalho, Jessié M. Gutierres, and Cibele L. Lhamas.

The main contribution of our paper is that outlines different outcomes of androgenic anabolic steroid (AAS) abuse depending on the dose and duration of exposure on behavioral parameters in rats. We believe that this contribution is theoretically and practically relevant because the pathological effects of AAS are understudied, despite its widespread abuse in both athletes and non-athletes alike.

Our research is of particular interest and use to toxicologists and primary care physicians who may encounter individuals demonstrating aggressive behavior or other abnormal patterns of behavior due to AAS abuse.

We have read and understood your journal's policies on copyright, ethics, etc., and believe that neither the manuscript nor the study violates any of these.

Thank you for your consideration. We hope our manuscript is suitable for publication in your journal.

Sincerely,

Cinthia M. Andrade
Programa de Pós Graduação em Ciências Biológicas: Bioquímica Toxicológica,
Setor de Bioquímica e Biologia Molecular do Laboratório de Terapia Celular,
Centro de Ciências Rurais,
Universidade Federal de Santa Maria,
Santa Maria/RS 97105-900, Brasil.
Tel./fax: + 55-55 3220 8460
cmelazzomazzanti@gmail.com

Jessié Martins Gutierres
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Departamento de Bioquímica e Biologia Molecular
Centro de Ciências Naturais e Exatas
Universidade Federal de Santa Maria
Santa Maria/RS 97105-900, Brasil.
Tel./fax: + 55-55 3220 8460
E-mail address: jessiegutierres@hotmail.com

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TITLE OF THE PAPER

Impact of dose and duration of androgenic anabolic steroid exposure on behavior in rats

AUTHORS

Andressa Bueno

JOB CODE

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Impact of dose and duration of anabolic androgenic steroid exposure on oxidative stress markers in the central nervous system, acetylcholinesterase activity and behavior in rats

Andressa Bueno[1], Fabiano B. Carvalho[2], Jessié M. Gutierres[2], Cibele L. Lhamas[1], Cinthia M. de Andrade[1,2#]

[1]Programa de Pós Graduação em Medicina Veterinária, Hospital Veterinário, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Santa Maria/RS, 97105-900, Brasil.
[2]Programa de Pós Graduação em Ciências Biológicas: Bioquímica Toxicológica, Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Santa Maria, Santa Maria/RS 97105-900, Brasil.

 # Corresponding Authors

Cinthia Melazzo de Andrade: Programa de Pós Graduação em Medicina Veterinária, Hospital Veterinário Universitário, Laboratório de Bioquímica e Estresse Oxidativo, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Santa Maria/RS 97105-900, Brasil. Tel./fax: + 55-55 3220 8460
E-mail address: cmelazzomazzanti@gmail.com

Jessié Martins Gutierres: Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Centro de Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria/RS 97105-900, Brasil. Tel./fax: + 55-55 3220 9557.
E-mail address: jessiegutierres@hotmail.com

Abstract

In the present study, we compared the supra-physiological effects of two androgenic anabolic steroids (AAS), boldenone (BOL) and stanazolol (ST), using three different treatment protocols. Protocol I used higher than recommended doses administered over a shorter time interval. Protocol II used a moderate dose administered over an intermediate period of exposure. Protocol III used a reduced dose administered over an extended period of exposure. Our data strongly suggest that treatment with BOL and ST affects emotional, dominance, and aggressive behaviors in a dose- and time-dependent manner. Moreover, BOL treatment using Protocol III (a reduced dose over an extended period of exposure) impaired memory and increased acetylcholinesterase (AChE) activity in the cerebral cortex and hippocampus, suggesting dysfunction of the cholinergic system associated with oxidative status. Our data raise the concern that long-term use of steroids, even in small doses, neuropathological consequences can cause behavioral changes depending to the protocol used.

Key-words: boldenone; stanazolol; behavior; acetylcholinesterase; oxidative stress.

1. Introduction

Anabolic androgenic steroids (AAS) are a large class of synthetic androgens that mimic the effects of male sex hormones such as testosterone and dihydrotestosterone [1,2],]. In fact, data from the use of ASS in this last decade brings a considerable number of teenagers using drugs either for young athletes or by non-athletes for a cosmetic proposal or physical performance, the estimates are that these users administer the AAS about 10 -100 times the physiological doses [3,4]

AAS in supraphysiological doses affect several central nervous system-related behaviors such as memory, aggression, anxiety, and depression [3-7]. In this context, studies investigating the mechanisms of AAS demonstrated effects on neurotransmission in the CNS by directly affecting the cellular membrane, modulating synthesis and degradation of neurotransmitters, and altering neurotransmitter metabolism [8-11]. In addition, androgenic receptors can be found in brain structures such as the hippocampus, amygdala, and cerebral cortex. Reports indicate that the use of AAS interferes with important signaling and neurotransmission systems that modulate animal behavior, such as glutamatergic [12-14], cholinergic [15-17], and opioid systems [18-20].

Behavioral responses to AAS depend on several factors, including the chemical structure of the steroid administered, whether a single compound or cocktail is administered, the recipient's age, and duration of treatment [21]. While many controlled studies have described the behavioral changes induced by AAS, these studies primarily utilize hormone cocktails in the experimental designs. Therefore, the aim of this study was to comparatively and separately assess the effects of boldenone (BOL) and stanazolol (ST) on behavioral tasks to determine if one or both of them alter learning, memory, anxiolytic-like behavior, and dominant or submissive social behavior. In addition, in an attempt to simulate different user groups, we analyzed three different protocols that varied in dose and time factors to determine their impact on user outcomes.

2. Methods

2.1 Animals

Sixty six Male *Wistar* rats (45 days of age) weighing +/-200g were used in the study. The animals were maintained in the Central Animal House of the Federal

University of Santa Maria in colony cages at an ambient temperature of 23± 2°C and relative humidity 45–55% with 12 h light/dark cycles. The animals had free access to a standard rodent pellet diet and water *ad libitum*. All procedures were carried out according to the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care. This work was approved by the ethical committee of the Federal University of Santa Maria (protocol number 032/2014).

2.2 Experimental protocol

Experimental protocols for stanazolol (ST, Estrombol™, Fundación Lab, Argentina) and boldenone undecylenate (BOL, Equipoise™, Fort Dodge Lab, USA) were divided into I, II and III. In the three protocols to administration of vehicle (olive oil) or AAS intramuscular (total volume 0.2 ml) once a week. Protocol I (n=33): 5mg/kg ST or BOL for 4 weeks. Protocol II (n=33): 2.5 mg/kg ST or BOL for 8 weeks. Protocol III (n=33): 1:25 mg/kg ST or BOL for 12 weeks. A representative scheme can be found in Figure 1 Experimental Protocol.

2.3 Behavioral Tests

2.3.1 Elevated plus maze task

On the first day of behavioral parameters evaluation, the anxiolytic-like behavior was evaluated using the task of the elevated plus maze (DAY 1), as previously described [22]. The apparatus consists of a wooden structure raised to 50 cm from the floor. This apparatus is composed of 4 arms of the same size, with two closed-arms (walls 40 cm) and two open arms. Initially, the animals were placed on the central platform of the maze in front an open arm. The animal had 5 min to explore the apparatus, and the time spent and the number of entries in open and closed-arms were recorded. The apparatus was thoroughly cleaned with 30% ethanol between each session.

2.3.2 Open Field

Locomotor and exploratory activities were evaluated in the apparatus used for object recognition task as previously described [23]. The crossing and rearing numbers was achieved on the first day of exposure to the apparatus (habituation day, DAY 2).

The animals were transferred to an apparatus with 100cm x 100cm (floor) x 50cm (walls) open field, with the floor divided into 16 squares (6,25cm²). During the 5-min open field session, the crossing and rearing numbers was recorded.

2.3.3 Object Recognition Test

The object recognition task takes advantage of the rats' spontaneous tendency to explore the environment and does not require punishment or reward [24]. It consists of three sessions: habituation (DAY 2), training (DAY 3) and retention (DAY 3) and was conducted as previously describe [25]. In brief, rats were left to freely explore during 10 min a square arena (100 cm X 100 cm length X 50 cm height) in the absence of objects. Twenty-four hours after habituation, training was conducted by placing individual rat during 10 min into the arena, in which two identical objects (objects A1 and A2) were positioned in two adjacent sides. During training, the animals should explore between 40 and 60 % of the time each object or be excluded from the experiments. In the retention session (test day), carried out 24 h after training, the rats were allowed to explore for 10 min the arena, in which one of the familiar objects used during training was replaced by a novel object (object B). Objects were made of odourless plastic and similar in size. Between each trial, the objects and the field were cleaned with a 30 % ethanol solution. The total time spent sniffing or touching each object with the nose and/or forepaws and the number of rearing

2.3.4 Agonistic behavior tests

The animals tested for agonistic behavior using the resident-intruder paradigm previously described by Salas-Ramires et al. (2007) [21]. After a 5-min acclimation period, an age- and weight-matched male intruder was placed in the Vehicle, BOL and ST home cage. Next, with a camera was recorded the episodes or scores to determine the dominant behavior over the intruder (contact team and Offensive posturing), as well as determine the dominant behavior over the territory (by number of Flank marks). The aggressive behavior has verified through attacks on the intruder and number of bites (the subject male bit the intruder), since the submissive behavior was defined by defensive posturing, tail-walking up and escape dashes. The intruder was used for more than one behavioral test, and all subjects Were tested During the first 4 h of the dark cycle under dim red illumination to control for circadian influences on behavioral responding [27].

2.4 Sample preparation for biochemical parameters

The animals were anesthetized and then euthanized. The brain was removed, and the cerebral cortex and hippocampus were separated. Next, the brain structures were placed in a solution of 10 mM Tris–HCl and 0.1 mM EDTA, pH 7.4, on ice. The brain structure was homogenized in a glass potter in Tris–HCl solution [27]. An aliquot of the homogenate was separated. After centrifugation of 1.500 g at 4ºC for 15 min, aliquots of the supernatant were stored at −80 °C until the biochemical analyses.

2.4.1 Measurement of intracellular reactive oxygen species (ROS) production

2′-7′-Dichlorofluorescein (DCF) levels were determined as an index of the reactive species production by the cellular components. Aliquots (50 µl) of cerebral cortex and hippocampus supernatants were added to a medium containing Tris–HCl buffer (10 mM; pH 7.4) and 2′-7′-dichlorofluorescein diacetate DCFH-DA (1 mM). After DCFH-DA addition, the medium was incubated in the dark for 1 h until fluorescence measurement procedure (excitation at 488 nm and emission at 525 nm, and both slit widths used were at 1.5 nm). DCF levels were determined using a standard curve of DCF, and results were corrected by the protein content [28].

2.4.2 Malondialdehyde (MDA) levels

MDA levels in the cerebral cortex and hippocampus homogenate were measured by thiobarbituric acid reactive species (TBARS) method as previously describe [29] with a few modifications [30]. In short, the reaction mixture contained 200 µl of homogenate or standard (MDA 0.03 mM), 200 µl of 8.1% sodium dodecylsulfate (SDS), 750 µl of acetic acid solution (2.5 M HCl, pH 3.5) and 750 µl of 0.8% TBA. The mixtures were heated at 95ºC for 90 min. After centrifugation at 1700 g for 5 min, the absorbance was measured at 532 ηm. The MDA tissue levels were expressed as µmol MDA/ mg of protein.

2.4.3 Glutathione reduced (GSH) levels

GSH was determined in cerebral cortex and hippocampus as previously described [31]. Aliquots of the supernatant adjusted to 1 mg/ml of protein content (100 µl) were added to a phosphate buffer 300 mM (85 µl), pH 7.4, and the reaction was

read at 412 ηm after the addition of 10 mM DTNB (50 µl). The results were expressed as µmol of GSH/mg of protein.

2.4.4 Non-protein thiols (NPSH) levels

Tissue NPSH were determined in cerebral cortex and hippocampus as previously described [31]. Briefly, the supernatant was diluted (1:1) with 10% TCA, homogenized and centrifuged at 2,000 g for 10 min. Subsequently, the supernatant was incubated with 10 mM DTNB in a final volume of 2 ml, and the absorbance was read at 412 ηm. A cysteine solution was used as the reference standard. NPSH were expressed as µmol SH/mg of tissue.

2.5 Determination of AChE activity in brain

The AChE enzymatic assay was determined as previously described [32], with a modification of the spectrophotometric method as previously described [33]. The reaction mixture (2 ml final volume) contained 100 mM K^+-phosphate buffer, pH 7.5 and 1 mM 5,5′-dithiobisnitrobenzoic acid (DTNB). The method is based on the formation of the yellow anion, 5,5′-dithio-bis-acid-nitrobenzoic, measured by absorbance at 412 nm during 2 min incubation at 25°C. The enzyme (40–50 µg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh). All samples were run in triplicate and the enzyme activity was expressed in µmolAcSCh/ h/ mg of protein. Protein was determined previously in a strip that varied for each structure: cerebral cortex 0.7 mg/ml and hippocampus 0.8 mg/ml.

2.6 Protein determination

Protein was measured by the Coomassie Blue method [34], using bovine serum albumin as the standard.

2.5 Statistical analysis

Statistical analysis tests was carried out by One-way ANOVA followed by Student-Newman-Keuls test. $P<0.05$ was considered to represent a significant difference in all experiments. All data were expressed as mean ± SEM.

3. Results

3.1 Anxiolytic-like behavior in rats treated with BOL and ST according to Protocol I, II, and III.

Figure 2 shows the anxiolytic-like behavior of rats in the elevated plus maze. In rats receiving BOL and ST according to Protocol I, there was a decrease in time spent in the open-arms [$F_{(2,17)}$ = 30.53, p<0.001, graph A]. In rats receiving BOL and ST according to Protocols II and III, there were no differences observed in the time spent in the open-arms [$F_{(2,17)}$ = 0.301, p>0.05, graph B; and $F_{(2,17)}$ = 0.027, p> 0.05, graph C, respectively]. In rats receiving BOL according to Protocol I, there was a decrease in the number of entries to the open-arms [$F_{(2,17)}$ = 14.11, p<0.001, graph D]. However, in rats receiving ST according to Protocol I, there were no changes in the number of entries to the open arms. There were no effects on the number of entries to the open arms observed in rats receiving BOL and ST according to Protocol II [$F_{(2,17)}$ = 0.006, p>0.05, graph E] and III [$F_{(2,17)}$ = 0.571, p>0.05, graph F]. Rats receiving BOL according to Protocol I spent more time in the closed-arm [$F_{(2,17)}$ = 5.729, p<0.05, graph G]. There were no effects on the time spent in the closed arm in rats receiving BOL and ST according to Protocol II [$F_{(2,17)}$ = 0.057, p>0.05, graph H] and III [$F_{(2,17)}$ = 0.557, p>0.05, graph I]. Treatment with BOL and ST did not affect crossing numbers in Protocol I, II, or III [$F_{(2,17)}$ = 1.855, p>0.05, graph J; $F_{(2,17)}$ = 0.824, p>0.05, graph K; and $F_{(2,17)}$ = 0.025, p>0.05, graph L, respectively].

3.2 Dominant behavior over an intruder in rats treated with BOL and ST according to Protocols I, II, and III.

Figure 3 shows the time (A, B, and C) and events (D, E, and F) of dominant behavior over an intruder induced by treatment with BOL or ST according to Protocol I, II and III. In rats treated with BOL and ST according to Protocol I, II, and III, an increased time of dominant behavior was observed [$F_{(2,17)}$ = 14.74, p<0.01, graph A; $F_{(2,17)}$ = 36.22, p<0.001, graph B; and $F_{(2,17)}$ = 9.623, p<0.01, graph C, respectively]. Similarly, in rats treated with BOL and ST according to Protocol I, II, and III, an increase in dominant behavior events were observed [$F_{(2,17)}$ = 10.04, p<0.05, graph D; $F_{(2,17)}$ = 25.49, p<0.001, graph E; $F_{(2,17)}$ = 8.263, p<0.05, graph F, respectively].

3.3 Dominant behavior over territory in rats treated with BOL and ST according to Protocol I, II, and III.

Figure 4 shows the time spent to mark territory (A, B, and C) and the number of these events (D, E, and F) induced by treatment with BOL or ST according to Protocol I, II and III. Rats treated with ST according to Protocol I, II, and III spent an increased amount of time marking territory [$F_{(2,17)}$ = 8.815, $p<0.05$, graph A; $F_{(2,17)}$ = 20.38, $p<0.001$, graph B; and $F_{(2,17)}$ = 14.73, $p<0.01$, graph C, respectively]. In rats treated with BOL according to Protocol I, there were no observed increases in time spent marking territory [$p>0.05$, graph A]; however, rats treated according to Protocol II and III spent significantly more time marking territory [$F_{(2,17)}$ = 20.38, $p<0.001$, graph B; and $F_{(2,17)}$ = 14.73, $p<0.01$, graph C, respectively].

Rats treated with ST according to Protocol I, II, and III had a significant increase in the number of territorial marking events [$F_{(2,17)}$ = 16.37, $p<0.001$, graph D; $F_{(2,17)}$ = 74.68, $p<0.001$, graph E; and $F_{(2,17)}$ = 42.82, $p<0.001$, graph F, respectively]. Rats treated with BOL according to Protocol I had no significant increases in the number of territorial marking events [$p>0.05$, graph D]; however, these numbers significantly increased in rats receiving BOL according to Protocol II and III [$F_{(2,17)}$ = 74.68, $p<0.001$, graph E; and $F_{(2,17)}$ = 42.82, $p<0.001$, graph F, respectively].

3.4 Submissive behavior of rats treated with BOL and ST according to Protocol I, II, and III.

Figure 5 shows the time (A, B, and C) and events (D, E, and F) of submissive behavior induced by treatment with BOL or ST according to Protocol I, II and III. In rats treated with ST according to Protocol I, there were no changes in submissive behavior time [$p>0.05$, graphs A, B, and C). Treatment with BOL decreased submissive behavior time only when administered according to Protocol II [$F_{(2,17)}$ = 5.146, $p<0.05$, graph C].

Treatment with BOL and ST reduced the number of submissive behavior events only when administered according to Protocol I [$F_{(2,17)}$ = 5.054, $p<0.05$, graph B].

3.5 Aggressive behavior of rats treated with BOL and ST according to Protocol I, II, and III.

Figure 6 shows aggressive behavior events after treatment with BOL or ST according to Protocol I, II, and III. In rats receiving ST according to Protocol I, II, and III, there were no changes in aggressive behavior ($p>0.05$, graph A; $p>0.05$, graph B; and $p>0.05$, graph C, respectively). However, an increase in aggressive behavior was

observed in rats treated with BOL according to Protocol II and III [$F_{(2,17)}$ = 23.38, p<0.001, graph B; and $F_{(2,17)}$ = 21.92, p<0.001, graph C, respectively].

3.6 Object recognition task indices and acetylcholinesterase (AChE) activity in the brain of rats treated with BOL and ST according to Protocol I, II, and III.

Figure 7 shows the index value of rats in the object recognition task as well as acetylcholinesterase (AChE) activity in the cerebral cortex and hippocampus of rats treated with BOL and ST according to Protocol I, II and III. Rats treated with BOL and ST according to Protocol I and II showed no significant differences in the object recognition task index or AChE activity in the cerebral cortex and hippocampus [p>0.05, graphs A and B; D and E; and G and H, respectively). However, rats treated with BOL according to Protocol III had reduced index scores in the object recognition task [$F_{(2,29)}$ = 3.901, p<0.05, graph C] and increased AChE activity in the cerebral cortex [$F_{(2,29)}$ = 8.321, p<0.01, graph F] and hippocampus [$F_{(2,29)}$ = 4.896, p<0.05, graph I].

3.7 Markers of oxidative stress in cerebral cortex of rats treated with BOL or ST according to Protocol I, II, and III.

Figure 8 shows markers of oxidative stress in cerebral cortex. Increased levels of reactive oxygen species (ROS) were observed in rats treated with BOL according to Protocol I [$F_{(2,29)}$ = 7.547, p<0.01, graph A] and III [$F_{(2,29)}$ = 6.743, p<0.01, graph C]. In contrast, treatment with ST resulted in increased ROS levels only when administered according to Protocol III [$F_{(2,29)}$ = 6.743, p<0.01, graph C]. There were no significant differences observed in rats receiving BOL and ST according to Protocol II [$F_{(2,29)}$ = 1.550, p>0.05, graph B].

In rats treated with BOL according to Protocol III, significant differences were observed in malondialdehyde (MDA) levels [$F_{(2,29)}$ = 6.561, p<0.01, graph F]. No significant differences were observed for MDA in rats treated with BOL and ST according to Protocol I [$F_{(2,29)}$ = 0.490, p>0.05, graph D] and II [$F_{(2,29)}$ = 1.106, p>0.05, graph E].

Reduced glutathione (GSH) levels were only observed in rats treated with ST according to Protocol I [$F_{(2,29)}$ = 6.495, p<0.01, graph G]. No significant differences were observed in the GSH levels of rats receiving BOL and ST according to Protocol II [$F_{(2,29)}$ = 1.830, p>0.05, graph H] and III [$F_{(2,29)}$ = 1.372, p>0.05, graph I].

Non-protein thiols (NPSH) decreased in rats receiving BOL and ST according to Protocol I [$F_{(2,29)}$ = 10.09, p<0.001, graph J]. No significant differences were observed in rats receiving the drugs according to Protocol II [$F_{(2,29)}$ = 0.968, p>0.05, graph K] and III [$F_{(2,29)}$ = 1.550, p>0.05, graph L].

3.8 Markers of oxidative stress in the hippocampus of rats treated with BOL or ST according to Protocol I, II, and III.

Figure 9 shows markers of oxidative stress in hippocampus. ROS levels increased in rats treated with BOL according to Protocol I [$F_{(2,29)}$ = 20.28, p<0.001, graph A] and III [$F_{(2,29)}$ = 12.67, p<0.001, graph C]. In contrast, ROS levels increased in rats treated with ST according to Protocol III only [$F_{(2,29)}$ = 12.67, p<0.001, graph C]. No significant differences in ROS levels were observed in rats treated with ST according to Protocol II [$F_{(2,29)}$ = 0.175, p>0.05, graph B].

In rats treated with ST according to Protocol I, increased levels of MDA were observed [$F_{(2,29)}$ = 6.610, p<0.01, graph D]. There were no significant differences in MDA levels in rats treated with BOL and ST according to Protocol II [$F_{(2,29)}$ = 1.113, p>0.05, graph E] and III [$F_{(2,29)}$ = 1.973, p>0.05, graph F].

GSH levels were unchanged in rats treated with BOL and ST according to Protocol I [$F_{(2,29)}$ = 2.095, p>0.05, graph G], II [$F_{(2,29)}$ = 2.617, p>0.05, graph H], and III [$F_{(2,29)}$ = 0.675, p>0.05, graph I].

NPSH levels were decreased in rats treated with BOL and ST according to Protocol I [$F_{(2,29)}$ = 5.226, p<0.05, graph J] and III [$F_{(2,29)}$ = 3.987, p<0.05, graph L]. No significant differences in NPSH were observed in rats treated with BOL and ST according to Protocol II [$F_{(2,29)}$ = 1.202, p>0.05, graph K].

4. Discussion

AAS abuse has been linked to several medical complications such as sterility, gynecomastia, and increased risk of cardiovascular and hepatic disease [35]. In spite of these well-characterized metabolic and cardiovascular adverse effects, few studies have examined parameters of the brain cholinergic pathway and oxidative status along with the relation between timing and dose in cases of AAS abuse. Brain cholinergic pathway and oxidative stress are known to contribute to abnormalities in behavioral locomotion, memory, anxiety, and aggressiveness.

In the present study, we measured the supra-physiological effects of BOL and ST using three different protocols for administering the drugs. Protocol I used higher than recommended doses administered over a shorter time interval to accelerate the anabolic effects. Protocol II used a moderate dose administered over an intermediate period of exposure. Protocol III used a reduced dose administered over an extended period of exposure to minimize the androgenic effects (see protocol schematic in Fig 1).

Our research focused on comparing the behavioral changes in rats receiving BOL and ST according to the three protocols described. Parameters as the locomotion were unchanged in rats receiving BOL and ST, regardless of the treatment protocol used (Fig 2). However, anxiety levels significantly increased in animals receiving BOL and ST according to Protocol I (4 weeks, 5 mg/kg BOL and ST) (Fig 2. A, D, and G); furthermore, treatment with BOL reduced the number of entries and time spent in the open arms of the elevated plus maze.

In addition to measuring the effects of BOL and ST on emotional and locomotors parameters, it was crucial to assess patterns of dominance, submission, and aggressive behaviors of animals in respect to intruders when treated according to Protocol I, II, and III. Remarkably, animals treated with ST according to Protocol I (i.e., characterized by accelerating the anabolic effects with a high dose) had an increase in the number of dominant behavior events and time (sec) in comparison to animals treated with BOL (Fig 3, Protocol I) during 30 days of treatment. However, the aggressive profile in these animals was similar to previous reports of BOL treatment [36], wherein BOL (1 mg/kg) increased the aggressive scores in adolescent or adult rats after 4 weeks of treatment.

A moderate dose of BOL and ST (2.5 mg/kg) administered for 60 days (Protocol II) significantly increased the number of dominant behavior events and time (sec) in animals treated with BOL compared to that observed in those treated with ST, showing an inverse effect that was dose- and time-dependent when comparing these two anabolic steroids (Fig 3, Protocol II). Interestingly, reducing the dose and increasing the exposure time resulted in significant differences between control animals and animals receiving AAS treatments, but no significant differences were observed between animals receiving BOL and ST (Fig 3, Protocol III).

We observed similar results in dominant territorial marking behavior in rats receiving BOL and ST according to Protocol II and III (Fig 4). However, in rats receiving ST according to Protocol I, there was an increase in the number of events compared with controls and BOL groups (Fig 4, Protocol I). In rats receiving ST according to Protocol II, the number of territorial markings increased, but the time spent marking territory did not when compared to rats receiving BOL; however, both steroids significantly increased the dominant territorial marking parameters in relation to control group (Fig 4, Protocol II). The results for Protocol III were similar to Protocol I (Fig 4, Protocol III). Additionally, it is important to note that the number of submissive behavior events was decreased significantly in rats receiving BOL and ST according to Protocol I in relation to control group; submissive behavior time was significantly decreased in rats treated with BOL according to Protocol II when compared to control and ST-treated rats (Fig 5, Protocol I and II). Finally, when we observed aggressive behavior (i.e. number of bites), treatment with BOL significantly increased the number of bites in rats treated according to Protocol II and II when compared to that observed in control and ST-treated rats using both Protocol II and III (Fig 6); no significant differences were observed in rats treated with ST according to Protocol I, II, and III when compared to control (Fig 6).

In accordance with Kalinine et al., (2014) [12], a neural circuit composed of several regions including the prefrontal cortex, amygdala, hippocampus, hypothalamus, anterior cingulated cortex, and other interconnected structures has been implicated in the regulation of emotions. Furthermore, it is plausible that functional or structural abnormalities in these regions can increase the susceptibility for impulsive aggression and violence [37]. AAS also affect aggressive behaviors in rodents [38-40]. Single compounds such as testosterone and testosterone propionate increase aggression in intact adult male rats and mice [41,42]. Adolescent male Syrian hamsters exposed to a cocktail of AAS for 2 or 4 weeks showed increased aggressive behavior [21,36,43,44]. Consequently, the choice of a specific protocol for drug administration (both time and dose dependent), as well as the type of AAS to be used can affect the brain differently, resulting in a greater or lesser degree of aggressiveness.

The array of physiological and behavioral effects of these chemically disparate drugs is vastly compounded not only by complexity in the patterns of self-administration, but also by the heterogeneity of the subjects that take them [45,46].

Symptoms resulting from the chronic use of supra-therapeutic doses of ASS include mania, increased anxiety, irritability, extreme mood swings, and abnormal levels of aggression, depression, and even suicide. In particular, individuals administering the highest doses of AAS have been shown to have elevated scores on the “Symptom Check List-90”, a self-report system that includes a number of different dimensions of anxiety [47]. These findings support our data clearly showing different behavioral responses in rats treated with BOL and ST depending on the length of exposure and the dose chosen. However, to investigate the effect of BOL and ST on cognitive processes, additional experimental techniques could be used. Since gonadal hormones are known to play a crucial role in cognitive processes such as spatial learning performance [48-50] and extinction responses in a passive avoidance task [51,52], it is conceivable that BOL and ST also influence cognitive functions. In particular, current literature shows many controversial data regarding the occurrence of cognitive disorders in animals treated with AAS [53,54], but more recent studies have clearly shown that individuals abusing AAS have a high risk for developing cognitive disorders and important morphological changes in the amygdala, hippocampus, and forebrain [55,56]. Thus, this study evaluated the memory of the animals treated with BOL and ST in object recognition task. We note that only in the rats receiving BOL according to Protocol III was memory significantly affected compared to the control and ST groups. This clearly demonstrates that the use of BOL at a reduced dose but for a long time can have a greater impact on learning and memory of animals compared to the ST treatment (Fig 7C).

The underlying biochemical mechanisms of AAS are still poorly understood. The mesocorticolimbic dopaminergic pathway is considered to play an important role in the reinforcement circuitry of the brain [57-59], and a connection between AAS and central dopaminergic and serotoninergic activity has been reported in animal studies [60-62]. However, very little information has clarified the role of the cholinergic system and acetylcholine, both recognized for their important roles in memory formation and learning, in the changes observed according to the type, time, or dose of AAS used. Because acetylcholine coordinates the neuronal network response, modulation of the cholinergic system is an essential mechanism underlying complex behaviors; stimulation of n-acetylcholine receptors can increase the release of glutamate, γ-aminobutyric acid (GABA), dopamine, acetylcholine, norepinephrine, and serotonin [63-65].

AChE is an important enzyme that regulates the concentration of acetylcholine in the synaptic cleft. We observed increases in AChE activity in the cerebral cortex of animals treated with BOL and ST according to Protocol III only when compared to control and ST-treated rats (Fig 7F). However, in the hippocampus, we only observed significant differences between BOL and control groups (Fig 7I). These data suggest that memory deficits accompanied by elevated AChE activity and decreased acetylcholine levels, over the time and reduced dose of used in Protocol III, were more pronounced for BOL than for rats treated with ST, since memory and AChE activity remained unaffected. In support of these results, studies have shown that the AAS, methandrostenolone, changes the expression of neuronal growth factor and its receptors while reducing the protein levels of choline acetyltransferase activity. Methandrostenolone also results in the synthesis of acetylcholine in the basal forebrain and impairs behavioral performance in the Morris water maze task [56]. Experimental studies in animals strongly suggest that apoptotic mechanisms are at least in part involved in AAS-induced neurotoxicity (see review [66]). Furthermore, a great body of evidence is emerging to suggest that increased susceptibility to cellular oxidative stress plays a pivotal role in the pathogenesis of many neurodegenerative disorders and cognitive impairment [23,67,68]. As in other drug-induced encephalopathies, the key mechanisms involved in AAS–induced neuropathology could represent a target for future neuroprotective strategies.

An increase in ROS was observed in the cerebral cortex of rats receiving BOL according to Protocol I, along with a significant reduction in NPSH content (Fig 8 A and J, respectively). Furthermore, rats receiving BOL according to Protocol III had a significant increase in ROS and MDA levels, while rats receiving ST had an increase in ROS levels (Fig 8 C and F). Similarly, an increase in ROS levels and reduction in NPSH levels were observed in the hippocampus of rats receiving BOL according to Protocol I; rats receiving ST had a significant increase in MDA levels and reduction in NPSH content (Fig 9 A, D, and J). A significant increase in ROS levels and reduction in NPSH content was observed in rats receiving both BOL and ST according to Protocol III (Fig 9 C and L). However, there were no significant changes in the cerebral cortex and hippocampus in rats receiving BOL and ST according to Protocol II. Holmes and colleagues [69] suggested that androgens are neuroprotective when oxidative stress levels are minimal, but when oxidative stress levels are elevated, androgens exacerbate oxidative stress damage [69]. Similar results were reported by

Cunningham and colleagues [70] who demonstrated that testosterone appears to have negative consequences on brain function under conditions of elevated oxidative stress in the Caucasian race. The same group demonstrated that in a preexisting oxidative stress environment, androgens could further exacerbate oxidative stress damage [71]. Our data support these ideas since animals treated with BOL and ST had an increase in oxidative stress parameters. In addition, it is interesting to note that when the animals were exposed to different behavioral parameters, such as the addition of an intruder animal, the oxidative effects were exacerbated.

In conclusion, our data strongly suggest that treatment with BOL and ST affects emotional, dominant, and aggressive behaviors differently according to the dose and time of exposure. Moreover, we highlight that BOL induced memory impairment and reduced acetylcholine levels with a reduced dose and extended time--suggesting dysfunction of the cholinergic system associated with oxidative status. In this way, the use of these substances should be avoided provided they do not have a very strong therapeutic reason.

Conflicts of Interest statement

There are no conflicts of interest.

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Legends

Figure 1. Experimental Protocols: **Protocol I:** Intramuscular injection of vehicle (olive oil, 0.2 ml), stanazolol (ST, 5 mg/kg), or boldenone (BOL, 5 mg/kg) once a week for 4 weeks. **Protocol II:** Intramuscular injection of vehicle (olive oil, 0.2 ml), stanazolol (ST, 2.5 mg/kg), or boldenone (BOL, 2.5 mg/kg) once a week for 8 weeks. **Protocol III:** Intramuscular injection of vehicle (olive oil, 0.2 ml), stanazolol (ST, 1.25 mg/kg), or boldenone (BOL, 1.25 mg/kg) once a week during 12 weeks. Day 1: elevated plus maze task (EPM). Day 2: Olpen field task and habituation in the object recognition task. Day 3: training in the object recognition task. Day 4: test in the object recognition task. Day 5: agonist behavior (resident–intruder paradigm).

Figure 2. Anxiolytic-like behavior of rats treated with vehicle (olive oil, 0.2 ml), boldenone (BOL), or stanazolol (ST) once a week (intramuscular) according to Protocol I (4 weeks, 5 mg/kg), Protocol II (8 weeks, 2.5 mg/kg), or Protocol III (12 weeks, 1.25 mg/kg) then submitted to elevated plus maze task. Time spent in the open-arms (A, B, and C), entry to open-arms (D, E, and F), closed-arms (G, H, and I), and crossing numbers (J). *Denotes significant difference from the vehicle group. #Denotes significant difference between BOL and ST. One-way ANOVA followed by Student-Newman-Keuls test. All data are expressed as mean ± SEM (*,# p<0.05, **,## p<0.01, ***,### p<0.001).

Figure 3. Dominant behavior over the intruder of rats treated with vehicle (olive oil, 0.2 ml), boldenone (BOL), or stanazolol (ST) once a week (intramuscular) according to Protocol I (4 weeks, 5 mg/kg), Protocol II (8 weeks, 2.5 mg/kg), or Protocol III (12 weeks, 1.25 mg/kg). Dominant behavior time for Protocol I (A), II (B), and III (C) and dominant behavior events for Protocol I (D), II (E), and III (F). *Denotes significant difference from the vehicle group. #Denotes significant difference between BOL and ST. One-way ANOVA followed by Student-Newman-Keuls test. All data are expressed as mean ± SEM (*,# p<0.05, **,## p<0.01, ***,### p<0.001).

Figure 4. Dominant behavior over territory in rats treated with vehicle (olive oil, 0.2 ml), boldenone (BOL), or stanazolol (ST) once a week (intramuscular) according to Protocol I (4 weeks, 5 mg/kg), Protocol II (8 weeks, 2.5 mg/kg), or Protocol III (12 weeks, 1.25 mg/kg). Time spent marking territory for Protocol I (A), II (B), and III (C) and territory marking events for Protocol I (D), II (E), and III (F). *Denotes significant difference from the vehicle group. #Denotes significant difference between BOL and ST. One-way ANOVA followed by Student-Newman-Keuls test. All data are expressed as mean ± SEM (*,# p<0.05, **,## p<0.01, ***,### p<0.001).

Figure 5. Submissive behavior of rats treated with vehicle (olive oil, 0.2 ml), boldenone (BOL), or stanazolol (ST) once a week (intramuscular) according to Protocol I (4 weeks, 5 mg/kg), Protocol II (8 weeks, 2.5 mg/kg), or Protocol III (12 weeks, 1.25 mg/kg). Submissive behavior time for Protocol I (A), II (B), and III (C) and submissive behavior events for Protocol I (D), II (E), and III (F). *Denotes significant difference from the vehicle group. #Denotes significant difference between BOL and ST. One-way ANOVA followed by Student-Newman-Keuls test. All data are expressed as mean ± SEM (*,# p<0.05, **,## p<0.01, ***,### p<0.001).

Figure 6. Aggressive behavior of rats treated with vehicle (olive oil, 0.2 ml), boldenone (BOL), or stanazolol (ST) once a week (intramuscular) according to Protocol I (4 weeks, 5 mg/kg, graph A), Protocol II (8 weeks, 2.5 mg/kg, graph B), or Protocol III (12 weeks, 1.25 mg/kg, graph C). *Denotes significant difference from the vehicle group. #Denotes significant difference between BOL and ST. One-way ANOVA followed by Student-Newman-Keuls test. All data are expressed as mean ± SEM (*,# p<0.05, **,## p<0.01, ***,### p<0.001).

Figure 7. Object recognition task index and acetylcholinesterase (AChE) activity in the brain of rats treated with vehicle (olive oil, 0.2 ml), boldenone (BOL), or stanazolol (ST) once a week

(intramuscular) according to Protocol I (4 weeks, 5 mg/kg, graph A), Protocol II (8 weeks, 2.5 mg/kg, graph B), or Protocol III (12 weeks, 1.25 mg/kg, graph C). Object recognition task index for Protocol I (A), II (B), and III (C). AChE activity in the cerebral cortex for Protocol I (D), II (E), and III (F) and hippocampus for Protocol I (G), II (H), and III (I). #Denotes significant difference between BOL and ST. One-way ANOVA followed by Student-Newman-Keuls test. All data are expressed as mean ± SEM (*,# p<0.05, **,## p<0.01, ***,### p<0.001).

Figure 8. Parameters of oxidative stress in cerebral cortex of rats treated with vehicle (olive oil, 0.2 ml), boldenone (BOL), or stanazolol (ST) once a week (intramuscular) according to Protocol I (4 weeks, 5 mg/kg, graph A), Protocol II (8 weeks, 2.5 mg/kg, graph B), or Protocol III (12 weeks, 1.25 mg/kg, graph C). Reactive oxygen species (ROS) production for Protocol I (A), II (B), and II (C); malondialdehyde (MDA) levels for Protocol I (D), II (E), and III (F); reduced glutathione (GSH) levels for Protocol I (G), II (H), and III (I); non-protein thiol (NPSH) levels for Protocol I (J), II (K), and III (L). #Denotes significant difference between BOL and ST. One-way ANOVA followed by Student-Newman-Keuls test. All data are expressed as mean ± SEM (*,# p<0.05, **,## p<0.01, ***,### p<0.001).

Figure 9. Parameters of oxidative stress in hippocampus of rats treated with vehicle (olive oil, 0.2 ml), boldenone (BOL), or stanazolol (ST) once a week (intramuscular) according to Protocol I (4 weeks, 5 mg/kg, graph A), Protocol II (8 weeks, 2.5 mg/kg, graph B), or Protocol III (12 weeks, 1.25 mg/kg, graph C). Reactive oxygen species (ROS) production for Protocol I (A), II (B), and II (C); malondialdehyde (MDA) levels for Protocol I (D), II (E), and III (F); reduced glutathione (GSH) levels for Protocol I (G), II (H), and III (I); non-protein thiol (NPSH) levels for Protocol I (J), II (K), and III (L). #Denotes significant difference between BOL and ST. One-way ANOVA followed by Student-Newman-Keuls test. All data are expressed as mean ± SEM (*,# p<0.05, **,## p<0.01, ***,### p<0.001).

Figure 1

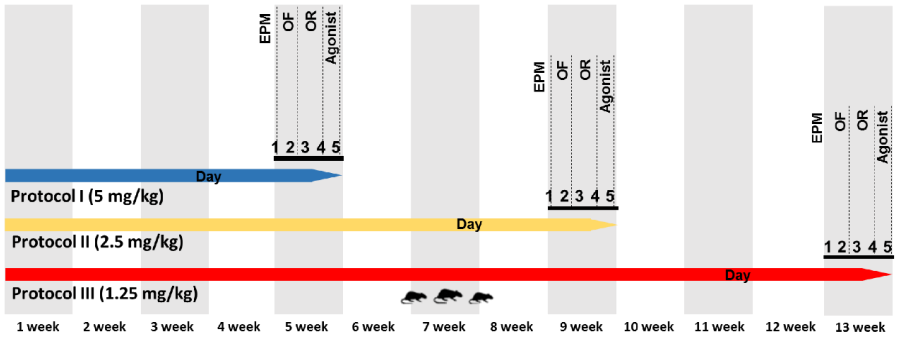
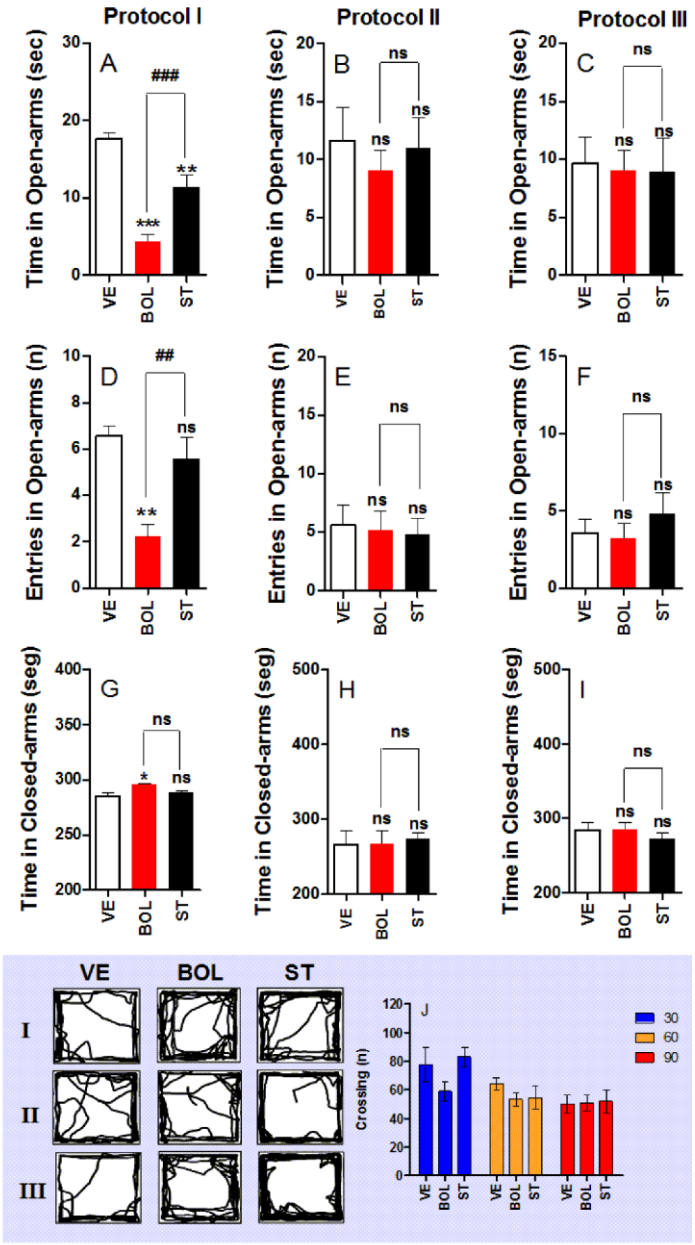

EPM
OF
OR
Agonist
1 2 3 4 5
Day
Protocol I (5 mg/kg)
Protocol II (2.5 mg/kg)
Protocol III (1.25 mg/kg)
1 week
2 week
3 week
4 week
5 week
6 week
7 week
8 week
9 week
10 week
11 week
12 week
13 week

Figure 2


Protocol I
Protocol II
Protocol III
Time in Open-arms (sec)
Entries in Open-arms (n)
Time in Closed-arms (seg)
A
B
C
D
E
F
G
H
I
VE
BOL
ST
###
**
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##
*
ns
I
II
III
Crossing (n)
30
60
90
J

Figure 3

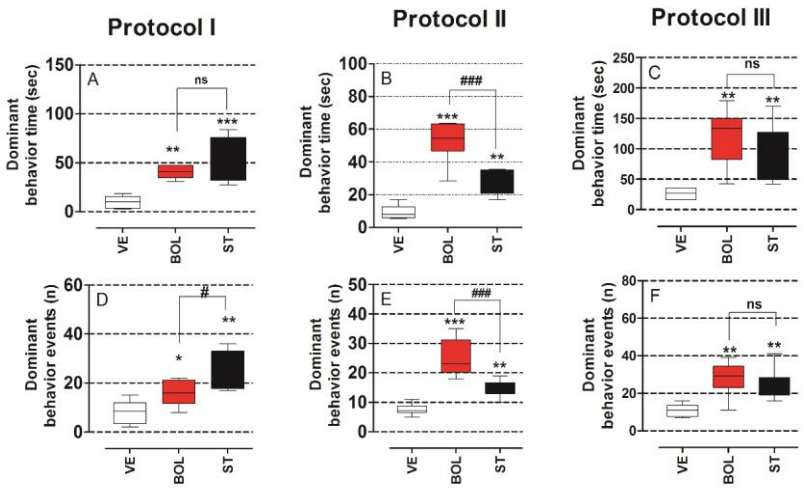
Protocol I
Protocol II
Protocol III
A
B
C
D
E
F
Dominant behavior time (sec)
Dominant behavior events (n)
VE
BOL
ST
ns
###
#
*
**
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Figure 4

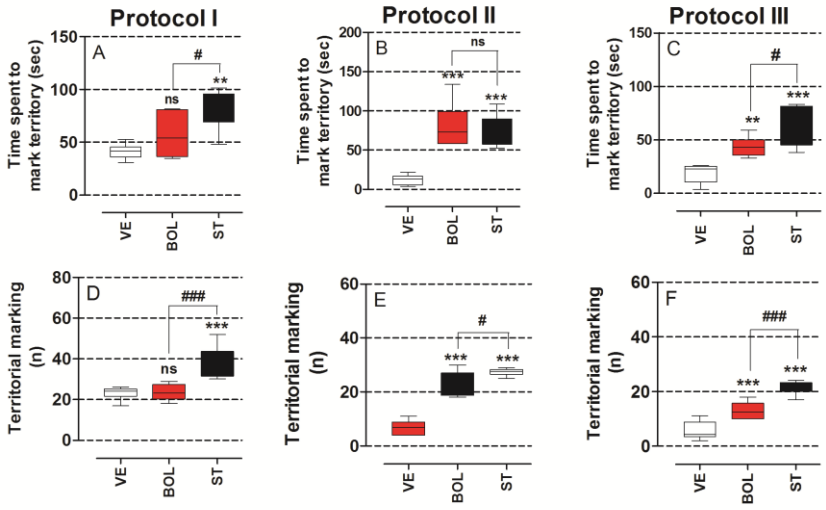

Protocol I
Protocol II
Protocol III
A
B
C
D
E
F
Time spent to mark territory (sec)
Territorial marking (n)
VE
BOL
ST
ns
#
**
***
###

Figure 5

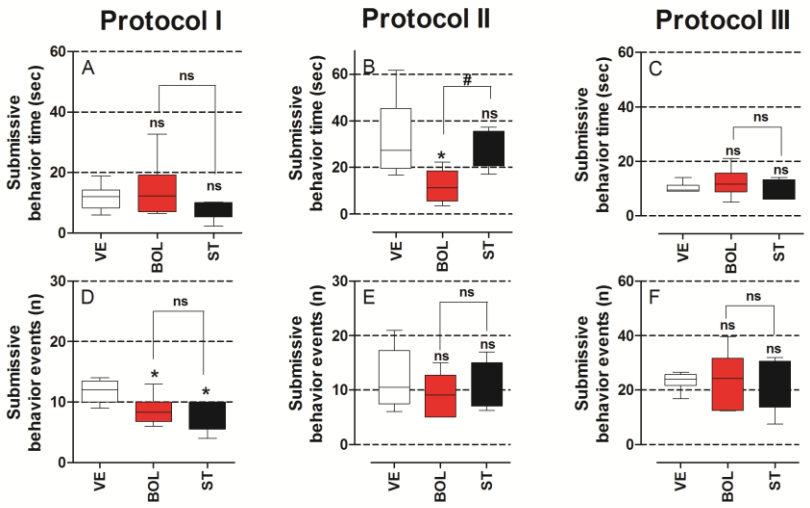

Protocol I
Protocol II
Protocol III
A
B
C
D
E
F
Submissive behavior time (sec)
Submissive behavior events (n)
VE
BOL
ST
ns
*
#

Figure 6

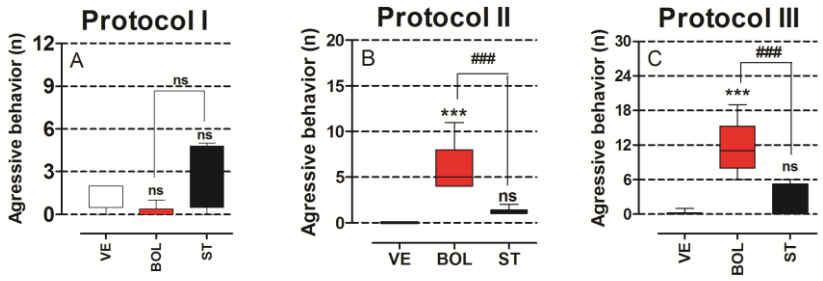

Protocol I
A
Agressive behavior (n)
12
9
6
3
0
ns
ns
ns
VE
BOL
ST
Protocol II
B
Agressive behavior (n)
20
15
10
5
0
###
***
ns
VE
BOL
ST
Protocol III
C
Agressive behavior (n)
30
24
18
12
6
0
###
***
ns
VE
BOL
ST

Figure 7

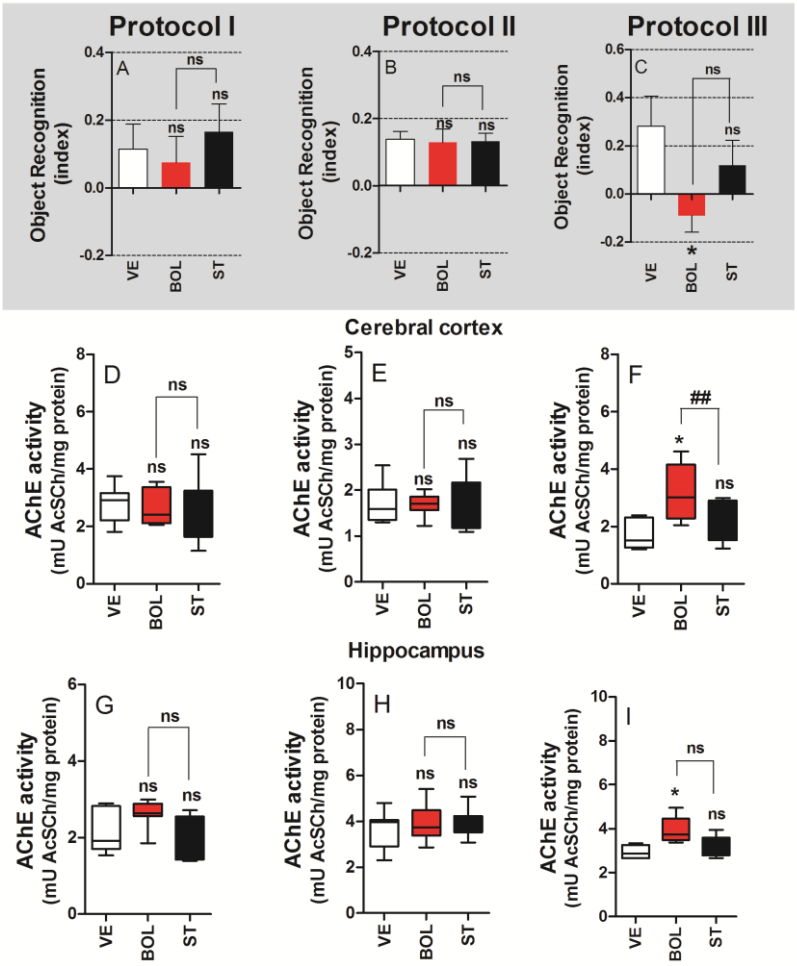

Protocol I
Protocol II
Protocol III
Object Recognition (index)
A
B
C
ns
*
VE
BOL
ST
Cerebral cortex
Hippocampus
AChE activity (mU AcSCh/mg protein)
D
E
F
G
H
I
##

Figure 8

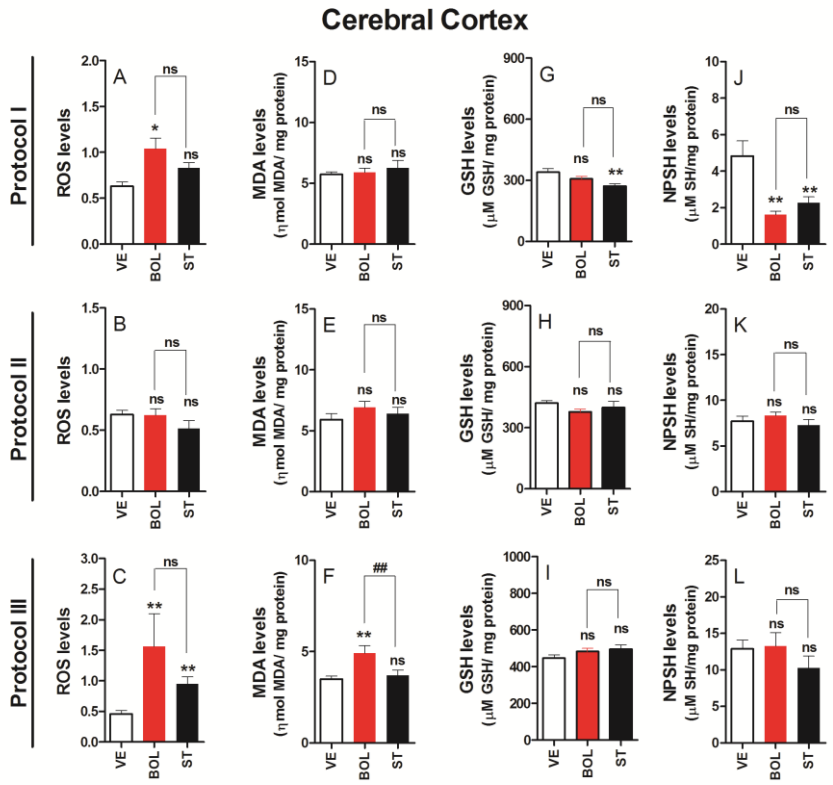
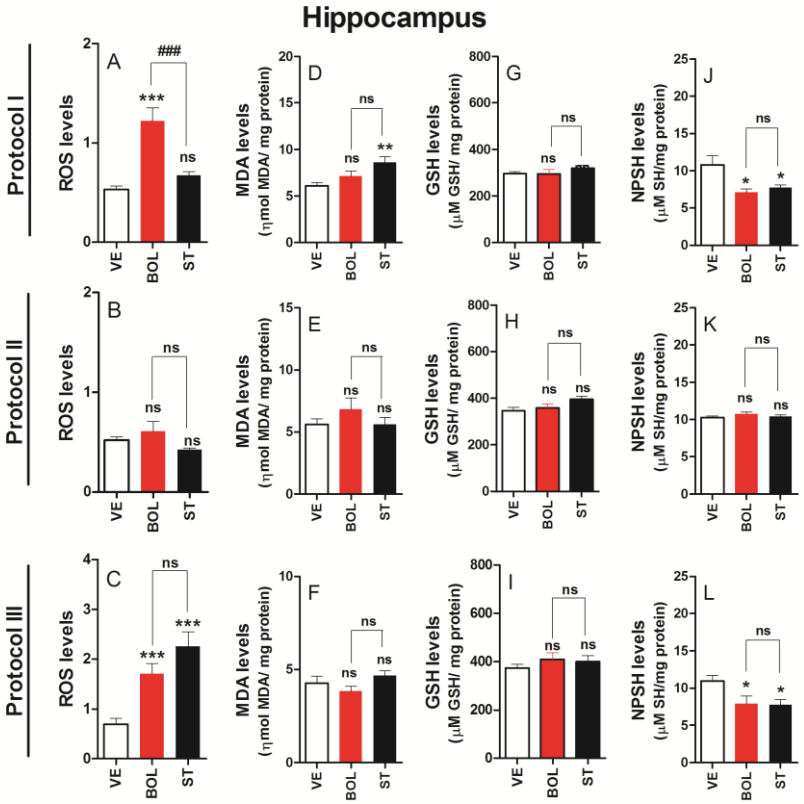

Cerebral Cortex
Protocol I
Protocol II
Protocol III
ROS levels
MDA levels
(ηmol MDA/ mg protein)
GSH levels
(μM GSH/ mg protein)
NPSH levels
(μM SH/mg protein)
A
B
C
D
E
F
G
H
I
J
K
L
VE
BOL
ST
ns
*
**
##

Figure 9


Hippocampus
Protocol I
Protocol II
Protocol III
ROS levels
MDA levels (nmol MDA/ mg protein)
GSH levels (µM GSH/ mg protein)
NPSH levels (µM SH/mg protein)
A
B
C
D
E
F
G
H
I
J
K
L
VE
BOL
ST
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Artigo 2

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Impacts of dose and time of boldenone and stanazolol exposure in inflammation markers, oxidative and nitrosative stress and histopathological changes in the rat testis.

Andressa Bueno[1], Fabiano B Carvalho[2], Jessié M Gutierres[2#], Cibele L Lhamas[1], Guilherme Dorneles[1], Juliana Sorraila[2], Indiara Brusco[2], Sara M Oliveira[2], Marta G Amaral[3], Cinthia M de Andrade[1,2#].

[1] Programa de Pós-Graduação em Medicina Veterinária, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Santa Maria/RS, 97105-900, Brasil.
[2] Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Centro de Ciências Naturais e Exatas; Universidade Federal de Santa Maria, Santa Maria/RS 97105-900, Brasil.
[3] Programa de Pós-Graduação em Biotecnologia, Unidade de Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Campus Universitário, Capão do Leão, Pelotas/RS 96010-900, Brasil.

Corresponding authors.

Cinthia Melazzo de Andrade: Programa de Pós-Graduação em Medicina Veterinária, Hospital Veterinário, Laboratório de Bioquímica e Estresse Oxidativo, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Santa Maria/RS 97105-900, Brasil. Tel./fax: + 55-55 3220 9557.
E-mail address: cmelazzomazzanti@gmail.com

Jessié Martins Gutierres: Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Centro de Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria/RS 97105-900, Brasil. Tel./fax: + 55-55 3220 9557.
E-mail address: jessiegutierres@hotmail.com

Abstract

The present study was conducted to analyze the adverse effects of the anabolic steroids boldenone (BOL) and stanazolol (ST) in the reproductive function of male rats. These molecules were administered using three different protocols. In Protocol I, BOL and ST were used in a higher dose than what is recommended but for a short period. In Protocol II, a moderate dose of these compounds was applied for an intermediate period, whereas in Protocol III a reduced dose was administered but for an extended period. Notably, Protocols I and III resulted in increased levels of reactive oxygen specimens (ROS) and nitric oxide (NOx), respectively, whereas non-protein thiols (NPSH) levels were decreased only after Protocol III. Myeloperoxidase activity was significantly increased after treatment with BOL than with ST in protocols I and III. Boldenone and ST also caused a significant up-regulation in the levels of serum testosterone when protocols I and II were performed. There were also histopathological alterations in the testis induced by treatment with BOL, namely degenerative changes primarily characterized by a decrease in the germinal epithelium. Together, these results suggest that steroid administration has a significant detrimental effect of damage to the testis and none of the protocols was able to reduce the deleterious effects of androgenic testicular tissue.

Keywords: boldenone, stanazolol, myeloperoxidase, N-agase, oxidative stress, testis.

1 Introduction

The deleterious effects that arise from the prolonged usage of high doses of anabolic androgenic steroids (AAS) has been widely documented and include liver and cardiac failure, acne, a decrease in the levels of the high-density lipoprotein (HDL), hepatic adenomas, mood fluctuation, aggressive behavior, violence, suicide attempts and even deficits in spatial learning and memory [1-5].

Although these molecules can have therapeutic advantages, given their structural and functional similarity to testosterone, aesthetic and competitive reasons often lead users to exceed by up to one hundred times the recommended dose, in order to improve muscle size and strength, thus enhancing athletic performance. Furthermore, a cocktail are used in cycles of 4–12 weeks to maximize steroid receptor binding and increase the desired effect [6, 7].

Several AAS molecules are available in the market and differ essentially in their anabolic potential, route of administration and prescription. For instance, boldenone (BOL) has exclusive veterinary prescription. These anabolic effects of AAS are observed primarily in muscle growth, due to the promotion of a positive nitrogen balance through stimulation of protein production and reduction of protein degradation. Also, AAS contributes to the retention of body water, nitrogen, sodium, potassium and calcium ions [8, 9]. It is also known that high concentrations of AAS compete against glucocorticoids for their receptors, which are responsible for protein catabolism, leading to a decrease in this process [10, 11].

In addition, exogenous AAS, as BOL and stanazolol (ST), may also have an androgenic effect resulting in negative side effects. These deleterious effects markedly occur in the male reproductive system leading to infertility, as these include apoptosis of testicular germ cells, severe reduction of proliferating nuclear antigen-positive cell spermatogonia, reduction in serum testosterone levels, seminal volume, sperm motility and sperm count, and a significant increase in the levels of the luteinizing hormone and follicle-stimulating hormone [12-15]. Previous results also show that AAS increase the levels of reactive oxygen species (ROS) and decrease the antioxidant capacity in several organs, such as the liver and the kidney. The close relationship between increased levels of ROS and negative consequences in sperm morphology has been previously described, and in particular, the antioxidant status of the semen was found to be significantly lower compared to normal semen [16-18]. Specifically in the testicle, several studies have reported histopathological changes caused by anabolic steroids

as well as changes in testosterone levels, which controls spermatogenesis [19, 20]. In theory, exposure to AAS induces changes in sperm due to the high oxidative damage that is triggered by excessive amounts of these molecules in the testicular tissue.

Many controlled studies describe changes in the testis induced by AAS used in a cocktail of hormones or use potentially androgenic molecules. Thus, the aim of this study was to evaluate and compare the individual effects of BOL and ST administered to rats in different doses and for different time periods, in order to determine whether they contribute or not for alterations in oxidative damage and antioxidant status, as well as for morphological and histological parameters in the rat testis.

2 Materials and methods

2.1 Animals

Sixty six male *Wistar* rats (±45 days) weighting approximately 250 g were used in this study. The animals were kept in the Central Animal House of the Federal University of Santa Maria in colony cages at an ambient temperature of 23 ±2°C and a relative humidity of 45–55%, with light/dark cycles of 12 h. The animals had free access to a standard rodent pellet diet and water *ad libitum*. All procedures were carried out according to the recommendations for animal care from CONCEA. This work was approved by the ethical committee of the Federal University of Santa Maria (protocol number 032/2014).

2.2 Administration of BOL and ST

The treatment with stanazolol (ST, Estrombol™, Fundación Lab, Argentina) and boldenone undecylenate (BOL, Equipoise™, Fort Dodge Lab, USA) was carried out in three different protocols (I, II and III). In all the protocols the administration of the vehicle (olive oil) or the intramuscular injection of the AAS (total volume 0.2 ml) were made once a week. In protocol I (n=33), 5mg/kg ST or BOL were administered for 4 weeks; in protocol II (n=33), 2.5 mg/kg ST or BOL were administered for 8 weeks and in protocol III (n=33) 1:25 mg/kg ST or BOL were administered for 12 weeks. A representative scheme of the protocols can be found in figure Experimental protocol-Figure 1.

2.3 Morphological assessment of testis

After dissection the testes were removed, weighed, and the volume of water displaced by the organ was measured by placing it in a graduated cylinder with 2 ml of ringer lactate solution.

2.4 Histopathological assessment

The testis were fixed in 10% paraformaldehyde, embedded with paraffin and sections with approximately 5 µm thickness were excised, followed by hematoxylin and eosin (H&E) [21].

2.5 Oxidative stress parameters

2.5.1 Measurement of reactive oxygen species (ROS) production

2′-7′-Dichlorofluorescein (DCF) levels were determined as an index of the production of ROS in the cells [22]. Aliquots (50 µl) of testis supernatants were added to a medium containing Tris–HCl buffer (10 mM; pH 7.4) and 2′-7′-dichlorofluorescein diacetate DCFH-DA (1 mM). After the addition of DCFH-DA, the medium was incubated in the dark for 1 h until the fluorescence measurement procedure. The excitation was made at 488 nm and the emission at 525 nm, with both slit widths at 1.5 nm. Cellular DCF levels were determined using a standard curve of DCF and results were normalized by the protein content.

2.5.2 Measuremente of testis malondialdehyde (MDA)

MDA levels were obtained from the testis homogenate as previously described [23] but with a few modifications [24]. In short, the reaction mixture contained 200 µl of testis homogenate or standard (MDA 0.03 mM), 200 µl of 8.1% sodium dodecylsulfate (SDS), 750 µl of acetic acid solution (2.5 M HCl, pH 3.5) and 750 µl of 0.8% TBA. The mixtures were heated at 95ºC for 90 min. After centrifugation at 1700 g for 5 min, the absorbance was measured at 532 ηm. The levels of tissue MDA were expressed as µmol MDA/ mg of protein.

2.5.3 Assay of NOx (NO_2 *plus* NO_3)

For NOx determination, an aliquot was homogenized (1:1) in 200 mM Zn_2SO_4 and acetonitrile. The testis homogenate was then centrifuged at 16,000 g for 30 min at

4ºC and the supernatant was separated for the analysis of NOx content as previously described [25]. Nitrite and nitrate solutions were used as the reference standards. NOx concentrations were determined by the absorbance at 570 ηm and were expressed as µmol/mg of protein.

2.5.4 Non-protein thiols (NPSH)

Tissue NPSH levels were determined as previously described [26]. Briefly, the supernatant was diluted (1:1) in 10% TCA, homogenized and centrifuged at 2,000 g for 10 min. Subsequently, the supernatant was incubated with 10 mM DTNB in a final volume of 1 ml, and the absorbance was read at 412 ηm. A cysteine solution was used as the reference standard. NPSH levels were expressed as µmol SH/mg of tissue.

2.5.5 Glutathione reduced levels (GSH)

GSH levels were determined in the testis supernatant as previously described [26]. Aliquots of the supernatant adjusted to 1 mg/ml of protein content (0.1 ml) were added to a phosphate buffer 300 mM (0.85 ml), pH 7.4, and the reaction was read at 412 ηm after the addition of 10 mM DTNB (0.05 ml). The results were expressed as µmol of GSH/mg of protein.

2.6 Serum testosterone and cholesterol

Serum cholesterol levels were measured using a semi-automatic analyzer (TP Analyzer Plus®, Thermoplate, China) and commercial kits (Labtest® Diagnóstica S.A., Lagoa Santa, MG, Brazil). All tests were carried out in triplicates. The assay for serum testosterone be carried out with a commercial kit (Coat-A-Count Total assay T, Diagnostic Products Corp., Los Angeles, CA) with a lower limit of detection 0,14nmol/L (Coviello et al., 2005).

2.7 Leukocyte infiltration markers

To estimate the infiltration of inflammatory cells in the testis, samples were collected and the activities of myeloperoxidase (MPO) and N-acetyl-β-D-glucosaminidase (NAGase), which are markers of neutrophil and macrophage infiltration, respectively, were determined [27-29]. First, the samples were homogenized in Tris-HCl buffer (10 mM, pH 7.4) and EDTA (0.1 mM) containing 0.5% Hexadecyltrimethyl-ammonium bromide and centrifuged at 16,000 g at 4ºC for 20 min;

the supernatant was then collected. For the measurement of MPO activity, 10 µL of supernatant were added to 200 µL of acetate buffer (200 mM, pH 5.4) and 20 µL of 3,3',5,5'-Tetramethylbenzidine (18.4 mM) in a 96-well plate and incubated at 37ºC for 3 min, in duplicates. To stop the reaction, the microplates were incubated in an ice bath, and 30 µL of acetic acid was added. The color formed was assessed at 630 ηm.

For the measurement of NAGase activity, 25 µL of the supernatant were incubated with 25 µL of 4-nitrophenyl-N-acetyl-β-D-glucosaminide (2.24 nM) and 100 µL of citrate buffer (50 mM, pH 4.5) at 37ºC for 1 h. After incubation, 100 µL of glycine buffer (0.2 mM, pH 10.4) were added to stop the reaction and to allow for the development of color; it was measured at 405 ηm. The absorbance of all reactions was measured in a Fisher Biotech Microkinetics Reader BT 2000 microplate reader. The values are expressed as the optical densities corrected for the protein content.

2.8 Protein determination

Protein concentration was measured using the Coomassie Blue method [30], with bovine serum albumin as standard.

2.9 Statistical analysis

Results are presented as means ± S.E.M. of the number of experiments indicated. Statistical significance was assessed by one-way analysis of variance followed by Student-Newman-Keuls test. $P<0.05$ was considered to represent a significant difference in all experiments. The statistical analyses were performed using the software package GraphPad Prism 5.

3 Results

3.1 Assessment of testicular parameters

Figure 2 shows the testicular parameters analyzed, as well as weight, displaced water volume and weight index. BOL and ST administration did not alter the weight of the testis after any of the protocols used (I [$F_{(2,28)}= 0.616$, $p>0.05$, graph A], II [$F_{(2,29)}= 0.058$, $p> 0.05$, graph A] and III [$F_{(2,29)}= 2.877$, $p> 0.05$, graph A]), nor the displaced water volume (I [$F_{(2,28)}= 0.077$, $p>0.05$, graph B], II [$F_{(2,29)}= 0.307$, $p> 0.05$, graph B] and III $F_{(2,29)}= 2.807$, $p> 0.05$, graph B]). However, BOL treatment induced a decrease in the weight index after protocol III [$F_{(2,29)}= 5.268$, $p<0.05$, graph C], but not after

protocol I [$F_{(2,28)}$= 0.218, p>0.05, graph C] or protocol II [$F_{(2,29)}$= 1.601, p> 0.05, graph C]. Administration of ST also induced no significant differences when ST was applied in the three protocols (I [$F_{(2,28)}$= 0.218, p>0.05, graph C], II [$F_{(2,29)}$= 1.601,p> 0.05, graph C] and III [$F_{(2,29)}$= 5.268, p<0.05, graph C]).

3.2 Serum testosterone and cholesterol

Figure 3 shows the levels of serum testosterone and cholesterol. In protocols I [$F_{(2,11)}$= 5.616, p<0.05, graph A] and II [$F_{(2,11)}$= 14.55, p< 0.01, graph B] BOL and ST lead to an increase in the levels of serum testosterone, whereas when applied in Protocol III, no significant effect was observed [$F_{(2,11)}$= 0.658, p>0.05, graph C]. Also, BOL was able to induce an increase in the levels of serum cholesterol after protocol I [$F_{(2,26)}$= 4.415, p<0.05, graph D], but when administered in protocols II [$F_{(2,28)}$= 4.115, p>0.05, graph E] and III [$F_{(2,28)}$= 1.513, p>0.05, graph F] no effect was observed. Stanazolol did not induce changes in the levels of serum cholesterol after any of the three protocols.

3.3 ROS, MDA and NOx levels

Figure 4 shows the levels of ROS, malondialdehyde (MDA) and NOx in the testis. Boldenone and ST lead to a change in the concentration of testis ROS after protocols I [$F_{(2,28)}$= 20.10, p<0.01, graph A] and III [$F_{(2,29)}$= 8.529, p< 0.001, graph C], but not after protocol II [$F_{(2,29)}$= 1.296, p>0.05, graph B]. When applied in protocols I [$F_{(2,28)}$= 2.047, p>0.05, graph D] and II [$F_{(2,29)}$= 2.451, p>0.05, graph E], BOL and ST induced no significant differences in the levels of MDA in the testis. Boldenone and ST increased MDA levels in testis in protocol III [$F_{(2,29)}$= 4.771, p<0.05, graph F] II [$F_{(2,29)}$= 0.307, p> 0.05, graph E] and III [$F_{(2,29)}$= 2.807, p> 0.05, graph F]. Treatment with BOL lead to an increase in measured NOx after all three protocols (I [$F_{(2,28)}$= 7.268, p<0.01, graph G], II [$F_{(2,29)}$= 6.942,p< 0.01, graph H] and III [$F_{(2,29)}$= 5.553, p<0.05, graph I]). Also, ST induced an increase in measured NOx after protocol II, but not when administered in the other two protocols.

3.4 Non-protein thiols (NPSH) and glutathione reduced (GSH) levels

Figure 5 shows NPSH and GSH levels in the testis. Boldenone and ST induced no changes in the levels of GSH after the different protocols (I [$F_{(2,28)}$= 0.1751, p>0.05, graph A], II [$F_{(2,29)}$= 0.114,p>0,05, graph B] and III [$F_{(2,29)}$= 1.651, p>0.05, graph C]).

However, BOL was able to induce a decrease in the levels of NPSH after protocol III only [$F_{(2,29)}$= 5.260, p<0.01, graph F], whereas in the other protocols no change was detected (I [$F_{(2,28)}$= 0.0151, p>0.05, graph D], II [$F_{(2,29)}$= 0.9041, p>0.05, graph E]).

3.5 Leukocyte infiltration markers and histopathological changes

Figure 6 shows the analysis of myeloperoxidase (MPO) and N-acetyl-β-D-glucosaminidase (NAGase) activity in the testis. When BOL and ST were administered in protocol I there was no statistical difference in MPO activity when compared to the control [$F_{(2,28)}$= 1.855, p>0.05, graph A]. When applied in protocol II, however, BOL lead to an increase in MPO activity [$F_{(2,29)}$= 7.401, p<0.01, graph A]. Both BOL and ST induced increased levels of MPO activity after protocol III [$F_{(2,29)}$= 5.381, p>0,05, graph A]. NAGase levels were not affected by treatment with either ST or BOL after any of the three protocols (I [$F_{(2,28)}$= 1.008, p>0.05, graph B], II [$F_{(2,29)}$= 1.413,p> 0.05, graph B] and III [$F_{(2,29)}$= 2.674, p>0.05, graph B]).

While sections of the testis of control animals present the different stages of spermatogenesis with normal aspect, testicular sections of animals treated with ST showed incomplete spermatogenesis (arrow) and absence of spermatozoa (SZ) in the lumen (*). In the testicular sections of rats treated with boldenone, severe degenerative changes were observed, mainly characterized by a reduction in the germinal epithelium. These changes were observable after protocol II but were more evident when BOL was administered for a prolonged period of time, *i.e.* in the animals treated with protocol III (Figure 6, painel C).

4 Discussion

In the present study, we observed the effect of the over-physiological usage of BOL or ST by the administration of these molecules in three different protocols. In protocol I, the objective was to reproduce the consequences of using higher doses of AAS than recommended, during a short time interval. Protocol II aimed at representing users who use a moderate dose of AAS for an intermediate time period. Finally, protocol III meant to represent users who are concerned about the androgenic effects of AAS and thus reduce the dose, but prolong the exposure time. After the administration of these different protocols, we observed that BOL and ST induced changes in the testis, such as the oxidative status and testicular morphology and

histology. In fact, previous studies have shown that testosterone administration may potentially influence the production of ROS in the testis [16, 31].

Here we demonstrated that when the AAS were administered in a high-dose for a short period of time (protocol I) there was an increase in the levels of ROS, and that BOL is capable of a more aggressive effect than ST (Fig. 4A). In a moderate dose for a moderate time period (protocol II), both BOL and ST failed to induce increased levels of ROS, possibly due to the effective removal of ROS by the antioxidant system (Fig. 4B). However, when the exposure time was increased, despite the reduced dose administered, both BOL and ST lead to increased levels of ROS (Figure 4C), suggesting that in these conditions the antioxidant system is not effective in the removal of these deleterious molecules. It is possible that both the high dose and the long exposure time to AAS represent harmful conditions and that the combination of these two factors result in a more severe injury.

Our findings are in agreement with several other studies that used different androgenic molecules, with potential anabolic, and different doses. An increase in the levels of ROS can have detrimental effects in testicular tissue, resulting in various pathological processes, given that ROS are potentially important mediators of the testis physiology and toxicology [19, 32, 33]. Exogenous testosterone seems to increase oxidative stress in the testis by a decrease in intra-testicular endogenous testosterone and an increase in corticosterone and oxidative stress in the epididymis [34-36].

Testicular membranes are highly vulnerable to oxidative stress due to their high content of polyunsaturated fatty acids. Therefore, higher thiobarbituric acid reactive substances (TBARS) would be expected to contribute to ROS-induced damage [37-39]. TBARS are products of lipid peroxidation, and hence they reflect the damage occurring in phospholipids, especially those in the plasmatic membrane [40]. Mohamed and Mohamed (2014) [41] demonstrated the ability of nandrolone to increase the levels of testicular MDA. Another study analyzing the possible effects of testosterone in the rabbit testes revealed that the administration of testosterone led to a significant increase in oxidative stress [42-44]. Our findings show that BOL and ST were only able to increase MDA levels when administered for a prolonged time (protocol III), after which more pronounced histological changes were observed when compared to protocol II (Fig. 7). The changes described previously in the histopathological study agree with various authors who used different anabolic molecules in varying doses and exposure times [13, 15}.

We believe ST is a less deleterious effect, as it was shown to be less effective in the development of aberrant conditions that invariably result in infertility [13, 19, 41]. Although other authors have not used the same anabolic molecules that we used, it is known that all testosterone analogues have a similar biological activity, differing only in their anabolic and androgenic potentials. The two molecules used in our study apparently also differ in the capacity to trigger oxidative stress, since boldenone had a more aggressive effect than ST.

Boldenone also triggered a significant increase in nitric oxide (NO) after all three protocols. Stanazolol was also able to increase NO levels but only in protocol II (Fig. 4 G, H and I). These results clearly demonstrate that, for this parameter, BOL can be more deleterious than ST. Nitric oxide is one of the most important intracellular and extracellular mediators and is involved in the regulation of testicular vasculature, Leydig cell steroidogenesis and spermatogenesis in the normal testis [45-48]. Macrophages and other cells, when activated by pro-inflammatory cytokines induced by immunological or inflammatory stimuli produce the isoform NOS-2, increased NOx. Our findings are in accordance to BATAGLIA et al. (2000) [49] and JANJIC et al. (2012) [50] that describe that the abusive intake of AAS increase testicular NOx and nitrosative stress, with subsequent testicular damage.

In our study, BOL and ST did not induce a significant decrease in the testicular levels of GSH (Fig. 5 A e B), although there was a slight tendency to a decrease when the exposure time was prolonged (protocol III) (Fig. 5C). However, in animals treated with BOL using protocol III we detected a statistically significant reduction in the testicular antioxidant capacity; it is possible that this decline does not occur due to a decrease in the content of glutathione (Fig. 5 D, E and F).

Different results were reported by Thótová and colleagues (2013) [19], who observed a decrease in the antioxidant defense of the testes, as well as increased oxidative damage, induced by AAS. Similarly, CHAINY et al. (1997) [16] and MOHAMAD and MOHAMAD (2015) [51] reported that enzyme activity related with the metabolism of superoxide radical (SOD) and hydrogen peroxide (CAT and GPx) of the testis was found to be significantly decreased after administration of testosterone. On the other hand, the study from AYDILEK et al. (2004) [43] is in accordance with our findings and report that administration of testosterone did not influence the levels of vitamin C and GSH. Once more, we believe that the discrepancy between the different studies is related with the aggressiveness of the anabolic molecules used, and depend

on the dose and exposure times of administration. It is fair to assume that a molecule with greater anabolic potential more easily saturates the antioxidant capacity of the tissue as it induces higher levels of ROS, thus leading to increased cell damage.

Several studies support the idea that higher doses or prolonged treatments easily saturate the antioxidant system [34, 43, 51] Inflammatory infiltration markers such as MPO and NAGase have been shown to be increased in various pathologies involving different tissues in the central nervous system and the synovial fluid of joints, with evidence of degeneration, bovine mastitis and elevation exercise-induced [52-57]. NAGase is a lysosomal enzyme and reflects udder tissue damage due to inflammation. Myeloperoxidase (MPO) is released extracellularly and phagosomal into compartments by neutrophils [54, 56]. As far as we know, no previous study showed increase of testicular MPO levels induced by the use of ASS. However, our findings strongly suggest that this is an accurate marker for testicular injury, since MPO levels increased significantly after BOL treatment in protocols II and III, and after ST administration in protocol III. These results suggest that inflammatory infiltration is characterized by the presence of neutrophils. The activation of leukocytes is a key event that triggers the release of a range of enzymes from intracellular granules and the production of superoxide ($O2^{\bullet -}$) and the release of reactive species can contribute to the site increased oxidative stress, damage to biological molecules [56]. Indeed, these inflammatory cells are the best indicators of oxidative and nitrosative stresses, and the observed increase in the levels of ROS and NOx at the same time reinforces our hypothesis. Therefore, it is possible to trace the pathophysiological cycle of the lesion, although we were not able to clearly demonstrate the presence of neutrophils in the histopathological testicular sections.

In conclusion, testicular damage was triggered by treatment of BOL and ST, and was directly related with the dose and exposure time of administration, as was clearly shown in this report. Furthermore, our results suggest that there is no secure way to use BOL and ST without increasing the risk of developing infertility, since both molecules induced testicular damage, even when administered in a low dose and for a reduced period of time. For this reason, and the fact that the use of steroids has historically increased, it is of the utmost importance to study the mechanisms of action of these steroids. It would also be interesting to evaluate whether the concomitant use of antioxidants can possibly reduce the impact of AAS by counterbalancing their negative side effects.

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Legends

Figure 1 - Experimental protocols used in this study. Protocol I: Intramuscular injection of vehicle (olive oil, 0.2 ml), stanazolol (ST, 5 mg/kg) or boldenone (BOL, 5 mg/kg) once a week for 4 weeks. Protocol II: Intramuscular injection of vehicle (olive oil, 0.2 ml), stanazolol (ST, 2.5 mg/kg) or boldenone (BOL, 2.5 mg/kg) once a week for 8 weeks. Protocol III: Intramuscular injection of vehicle (olive oil, 0.2 ml), stanazolol (ST, 1.25 mg/kg) or boldenone (BOL, 1.25 mg/kg) once a week during 12 weeks.

Figure 2 - Testicular parameters for weight (graph A), displaced water volume (graph B) and weight index (graph C) of rats treated with vehicle (olive oil, 0.2 ml), boldenone (BOL) or stanazolol (ST) once a week (intramuscular) following Protocol I (4 weeks, 5 mg/kg,), Protocol II (8 weeks, 2.5 mg/kg) or Protocol III (12 weeks, 1.25 mg/kg,). # indicates significant difference between BOL and ST. One-way ANOVA followed by Student-Newman-Keuls test. All data are expressed as mean ± SEM (*,# p<0.05, **,## p<0.01, ***,### p<0.001).

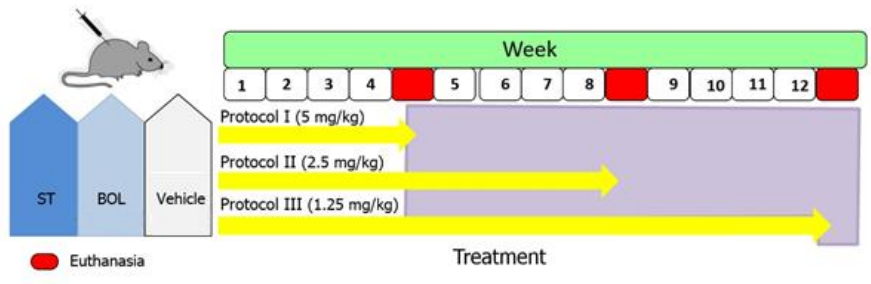
Figure 3 - Serum testosterone and cholesterol levels of rats treated with vehicle (olive oil, 0.2 ml), boldenone (BOL) or stanazolol (ST) once a week (intramuscular) following Protocol I (4 weeks, 5 mg/kg, graph A), Protocol II (8 weeks, 2.5 mg/kg, graph B), or Protocol III (12 weeks, 1.25 mg/kg, graph C). # indicates significant difference between BOL and ST. One-way ANOVA followed by Student-Newman-Keuls test. All data are expressed as mean ± SEM (*,# p<0.05, **,## p<0.01, ***,### p<0.001).

Figure 4 - Reactive oxygen species (ROS), malondialdehyde (MDA) and NOx levels in testis of rats treated with vehicle (olive oil, 0.2 ml), boldenone (BOL) or stanazolol (ST) once a week (intramuscular) following Protocol I (4 weeks, 5 mg/kg, graph A), Protocol II (8 weeks, 2.5 mg/kg, graph B) or Protocol III (12 weeks, 1.25 mg/kg, graph C). # indicates significant difference between BOL and ST. One-way ANOVA followed by Student-Newman-Keuls test. All data are expressed as mean ± SEM (*,# p<0.05, **,## p<0.01, ***,### p<0.001).

Figure 5 - NPSH and GSH levels in testis of rats treated with vehicle (olive oil, 0.2 ml), boldenone (BOL), or stanazolol (ST) once a week (intramuscular) following Protocol I (4 weeks, 5 mg/kg, graph A), Protocol II (8 weeks, 2.5 mg/kg, graph B) or Protocol III (12 weeks, 1.25 mg/kg, graph C). # indicates significant difference between BOL and ST. One-way ANOVA followed by Student-Newman-Keuls test. All data are expressed as mean ± SEM (*,# p<0.05, **,## p<0.01, ***,### p<0.001).

Figure 6 - Myeloperoxidase (MPO) and N-acetyl-β-D-glucosaminidase (NAGase) activity and histopathological assessment of testis of rats treated with vehicle (olive oil, 0.2 ml), boldenone (BOL) or stanazolol (ST) once a week (intramuscular) following Protocol I (4 weeks, 5 mg/kg, graph A), Protocol II (8 weeks, 2.5 mg/kg, graph B), or Protocol III (12 weeks, 1.25 mg/kg, graph C). # indicates significant difference between BOL and ST. One-way ANOVA followed by Student-Newman-Keuls test. All data are expressed as mean ± SEM (*,# $p<0.05$, **,## $p<0.01$, ***,### $p<0.001$). Histophatologial changes.

Figure 1



Comentado [C1]: N é 33 e não 10

Figure 2

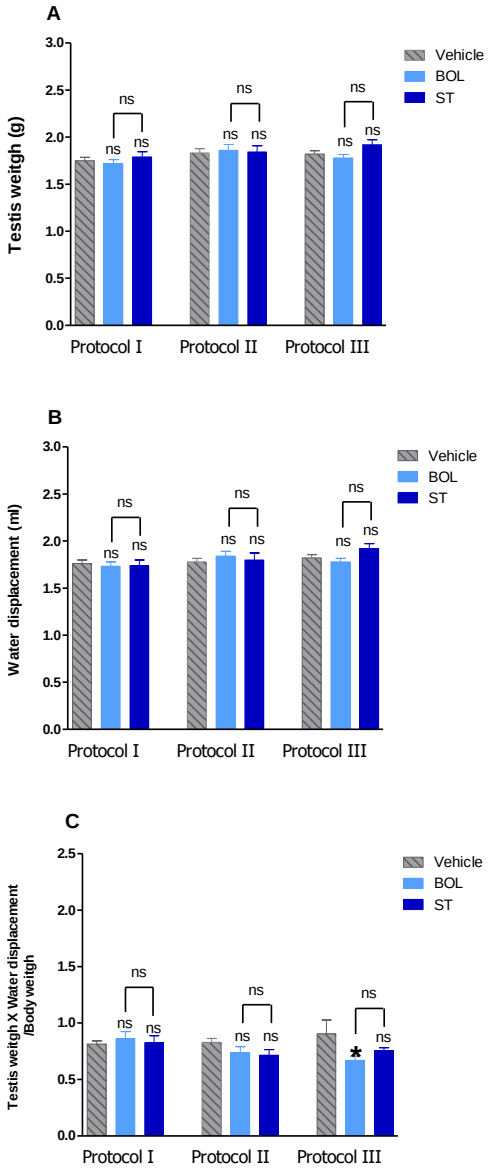

A
Testis weitgh (g)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ns
Vehicle
BOL
ST
Protocol I
Protocol II
Protocol III
B
Water displacement (ml)
C
Testis weitgh X Water displacement /Body weitgh
*

Figure 3

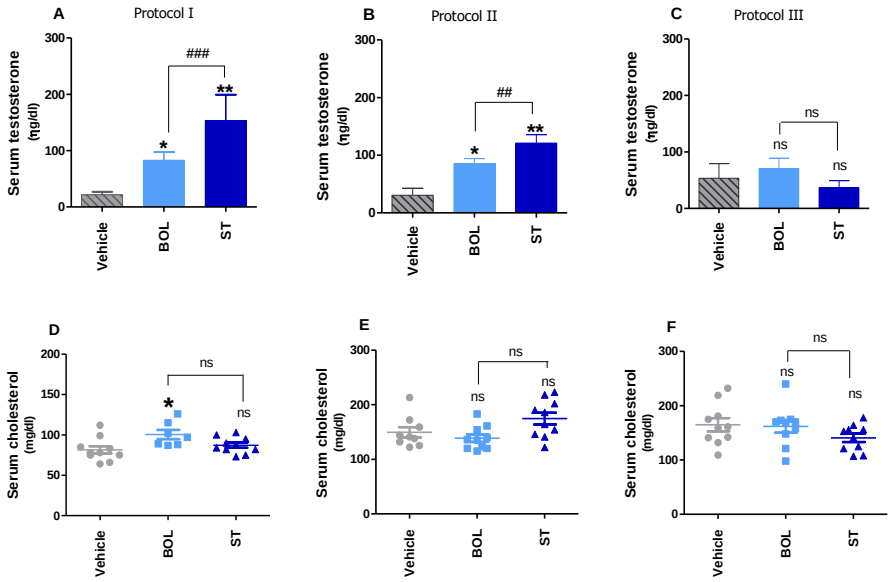

A
Protocol I
Serum testosterone (ng/dl)
300
200
100
0
###
**
*
Vehicle
BOL
ST
B
Protocol II
Serum testosterone (ng/dl)
300
200
100
0
##
**
*
Vehicle
BOL
ST
C
Protocol III
Serum testosterone (ng/dl)
300
200
100
0
ns
ns
ns
Vehicle
BOL
ST
D
Serum cholesterol (mg/dl)
200
150
100
50
0
ns
*
ns
Vehicle
BOL
ST
E
Serum cholesterol (mg/dl)
300
200
100
0
ns
ns
ns
Vehicle
BOL
ST
F
Serum cholesterol (mg/dl)
300
200
100
0
ns
ns
ns
Vehicle
BOL
ST

Figure 4

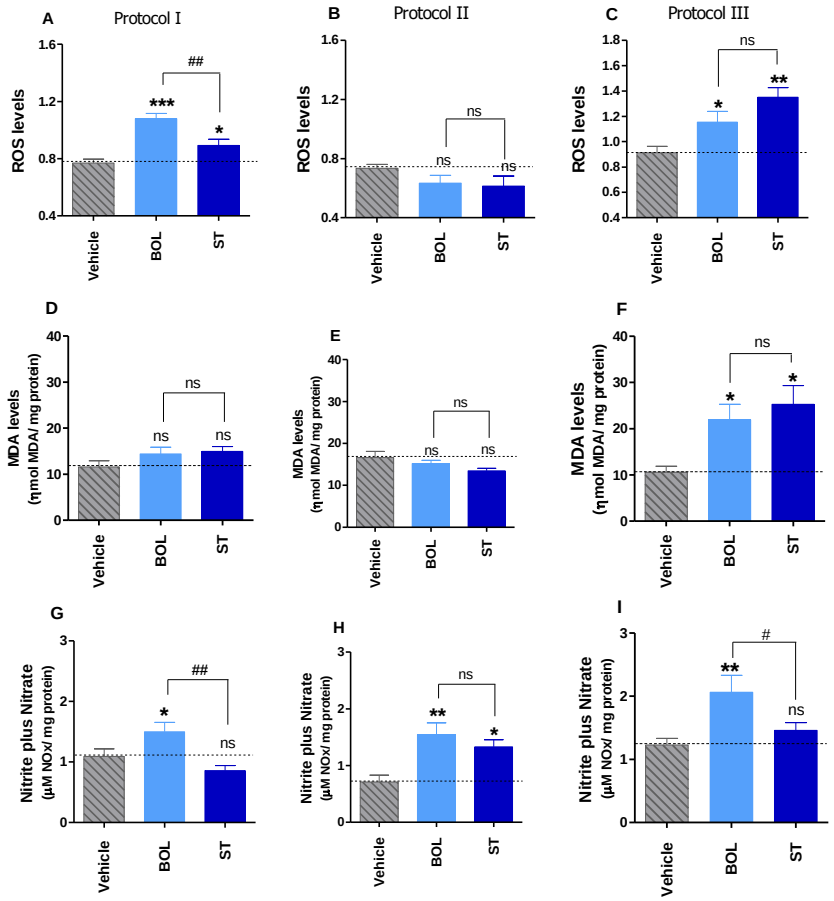

A
Protocol I
ROS levels
B
Protocol II
ROS levels
C
Protocol III
ROS levels
D
MDA levels (ηmol MDA/ mg protein)
E
MDA levels (ηmol MDA/ mg protein)
F
MDA levels (ηmol MDA/ mg protein)
G
Nitrite plus Nitrate (µM NOx/ mg protein)
H
Nitrite plus Nitrate (µM NOx/ mg protein)
I
Nitrite plus Nitrate (µM NOx/ mg protein)
Vehicle
BOL
ST
ns
*
**
***
#
##

Figure 5

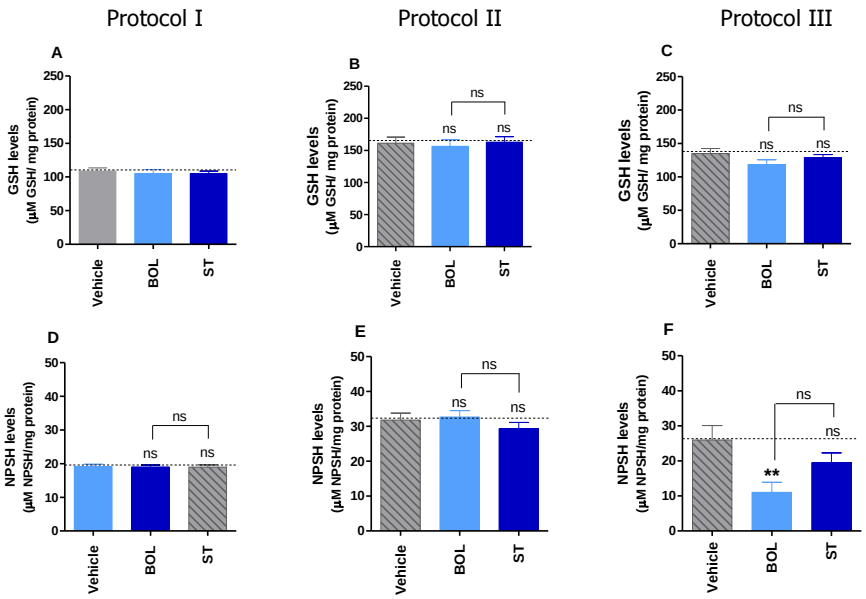
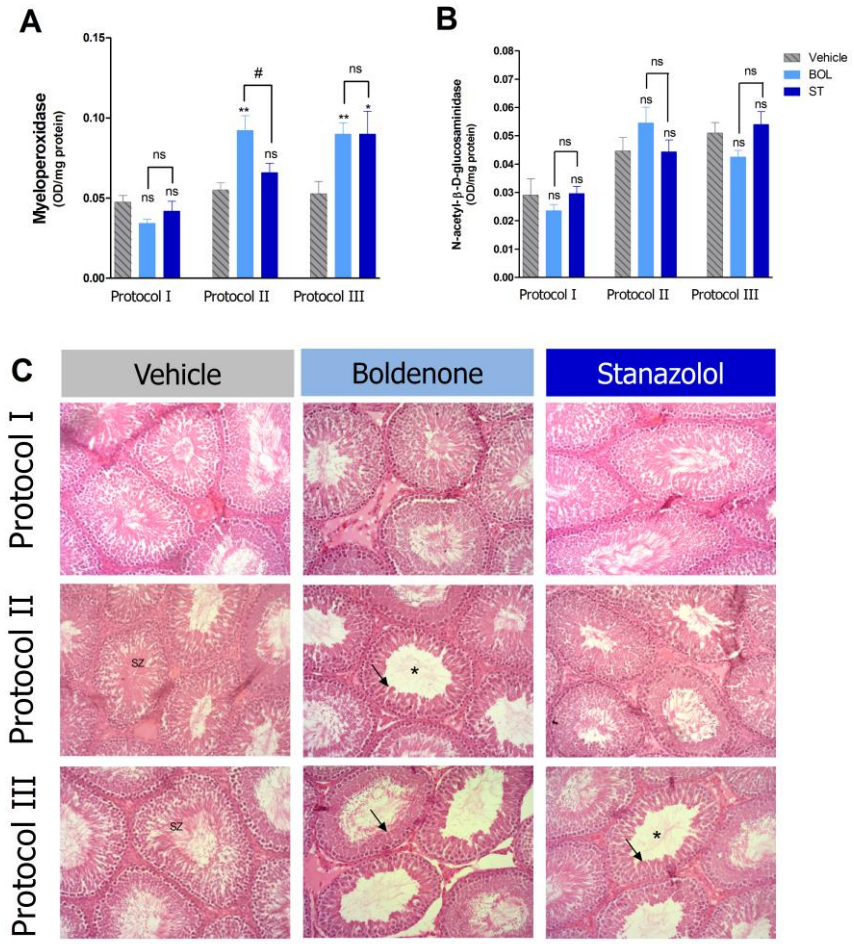

Protocol I
Protocol II
Protocol III
A
B
C
D
E
F
GSH levels (µM GSH/ mg protein)
NPSH levels (µM NPSH/mg protein)
Vehicle
BOL
ST
ns
**

Figure 6


A
Myeloperoxidase
(OD/mg protein)
0.15
0.10
0.05
0.00
ns
ns
ns
#
**
ns
**
*
Protocol I
Protocol II
Protocol III
B
N-acetyl-β-D-glucosaminidase
(OD/mg protein)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Vehicle
BOL
ST
Protocol I
Protocol II
Protocol III
C
Vehicle
Boldenone
Stanazolol
Protocol I
Protocol II
Protocol III
SZ
SZ
*
*

3. DISCUSSÃO

A presente tese investigou o impacto de três diferentes protocolos de exposição a esteróides androgênicos anabolizantes (EAA) variando dose e tempo. O propósito destes protocolos visaram avaliar se seria mais deletério acelerar o efeito anabólico aumentando a dose administrada e reduzindo o tempo de exposição ao fármaco (protocolo I), seguir a terapia convencional indicada a qual preservou a dose e tempo recomendado (protocolo II) ou reduzir os efeitos androgênicos das altas doses reduzindo a mesma e aumentando o tempo de exposição (protocolo III). De fato, foi observado um perfil diferenciado nos marcadores testiculares, cerebrais e comportamentais investigados neste estudo.

No primeiro manuscrito verificamos que o aumento da dose e a redução do período de tratamento foi capaz de aumentar o comportamento tipo ansioso em roedores, bem como o perfil dominante e agressivo destes animais. Em adição, a boldenona (BOL) mostrou um efeito mais pronunciado que o estanazolol (ES), o que evidencia o caráter mais androgênico daquela molécula.

Em contrapartida, o aumento do período de exposição no protocolo III resultou numa piora da capacidade cognitiva desses roedores, que pode estar associada a um aumento na atividade da acetilcolinesterase (AChE) no córtex cerebral e hipocampo. Essa enzima tem um importante papel na manutenção dos níveis de acetilcolina (ACh) na fenda sináptica, um importante neurotransmissor associado com os processos de aprendizado e memória, o que poderia explicar os resultados encontrados neste estudo. Já nos protocolos I e II, não foram observadas nenhuma alteração na atividade da AChE.

Em relação aos níveis elevados de testosterona, estes estão associados com o aumento do comportamento dominante e agressivo. Verificamos no manuscrito 2 um aumento dos níveis séricos de testosterona nos protocolos I e II, da mesma forma que houve uma alteração significativa no comportamento dominante e agressivo, que se tornou mais elevado. Embora no protocolo III, tenha sido observadas alterações neste tipo de comportamento, porém não acompanhado da elevação nos níveis séricos de testosterona. Uma vez que os animais estavam recebendo EAA exógeno, a ausência de um aumento significativo de testosterona sérica no protocolo III pode estar associado a uma redução dos níveis endógenos de testosterona. Associado a este fato, foi observado uma hipotrofia testicular neste mesmo protocolo, o que poderia estar relacionado a essa queda na produção de testosterona, que em suma

resulta na interrupção da espermatogênese. Em adição, esta hipótese pode ser confirmada através dos dados obtidos pela histologia. Foi notada uma redução do epitélio germinativo testicular como uma consequência da redução nos níveis desse hormônio. Para além desse fato, a baixa dose utilizada no protocolo III por um período de tempo mais prolongado, poderia ter dado ao organismo tempo hábil para organizar o *feed back* do eixo hipotálamo hipofisário gonadal, representando menos impacto para esse parâmetro.

Neste estudo, também foi observado um aumento na atividade da mieloperoxidase (MPO), indicando uma possível infiltração de neutrófilos no testículo, que não pôde ser confirmada pela histologia. Da mesma maneira houve uma elevação na produção de NOx, produtos finais da síntese de óxido nítrico (NO). O óxido nítrico tem um importante papel na inflamação, aumentando a vasodilatação e favorecendo a infiltração de células periféricas para os tecidos. Além disso, o NO quando produzido pela i-NOS também ativa as vias de sinalização que culminam na produção de citocinas pro inflamatórias e prostaglandinas. O aumento da MPO e do NOx poderia acusar um processo inflamatório local no tecido testicular. Em adição, a elevação dos marcadores de estresse oxidativo reforçam esse contexto uma vez que essas células infiltradas são deflagradoras do processo oxidativo.

Além disso, estudos tem evidenciado que doses supra fisiológicas de EAA levam a um aumento na produção de espécies reativas favorecendo o estabelecimento de estresse oxidativo em diferentes tecidos. Em ambos os trabalhos, vimos um aumento nos marcadores de estresse oxidativo tanto nos tecidos cerebrais quanto no testicular. Outro achado importante é que tanto o protocolo I quanto o protocolo III tiveram um impacto mais deletério, sendo o segundo protocolo o menos agressivo. Destacamos também um efeito mais agressivo da boldenona em relação ao estanazolol, reafirmando o caráter mais androgênico dessa molécula.

Diante dos resultados deste estudo, pode-se concluir que acelerar os efeitos do EAA encurtando o período de exposição e aumentando a dose visando o efeito anabólico precoce, tem um maior impacto no aumento da ansiedade. Porém prolongar o período de exposição, mesmo que em uma dose reduzida, causa um comprometimento nos processos cognitivos e na sinalização colinérgica. Em adição, o tempo prolongado de exposição também piora a lesão testicular causando hipotrofia testicular e redução do epitélio germinativo, que clinicamente resulta em oligospermia e em caso mais grave azoospermia e infertilidade. Esse evento é acompanhado do

aumentando dos marcadores de estresse oxidativo e inflamatório, resultado da lesão testicular provocada pelo aumento das ERO. Estes dados confirmam os anteriormente relatados na literatura e ampliam o conhecimento da ação específica das moléculas de BOL e ES sobre os marcadores de estresse oxidativo testicular e do SNC, além do impacto dessas moléculas sobre o comportamento perante a diferentes protocolos de utilização.

4. **CONCLUSÃO**

Após as análises dos dados obtidos a partir do experimento realizado, conclui-se que:

1. Os efeitos do uso da BOL e do ES estão relacionados com a dose e o tempo de exposição.
2. Os diferentes protocolos de utilização de BOL e ES influenciam o comportamento de diferentes maneiras.
3. Acredita-se que o aumento na atividade da acetilcolinesterase induzido pelo BOL possa estar associado ao déficit cognitivo demonstrado.
4. Sugere-se que o estresse oxidativo no SNC induzido pela BOL e o ES está associado às alterações comportamentais demonstradas.
5. Sugere-se que o estresse oxidativo no testículo esteja associado ao processo inflamatório induzido pelo uso de BOL ou ES.
6. A atividade da MPO pode ser utilizado como um marcador de inflamação testicular.

Dessa forma, entende-se que os EAA são deletérios tanto ao SNC quanto ao sistema reprodutor masculino, não havendo segurança em nenhum dos protocolos utilizados no que se refere aos parâmetros analisados. Assim o grupo sugere que o uso de EAA deva ser totalmente evitado afora sob alguma indicação clínica que sobreponha os efeitos colaterais descritos.

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