Universidade Estadual Paulista
"Júlio de Mesquita Filho"
Campus de Botucatu

INSTITUTO DE BIOCIÊNCIAS

Departamento de Farmacologia

Tese de Doutorado

"Efeito do exercício físico resistido e/ou do decanoato de nandrolona sobre aspectos sociais e reprodutivos, bem como sobre a resistência mecânica do fêmur, em ratos machos."

Tese apresentada ao Departamento de Farmacologia do Instituto de Biociências de Botucatu, como parte dos requisitos para a obtenção do título de Doutor em Ciências Biológicas (AC:Farmacologia).

Leandro Barile Agati

Orientador: Prof. Titular Oduvaldo Câmara Marques Pereira

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"Most scientists that I know work for recognition by other scientists. We have been trained this way. As students, we had to impress our professors."
(Dr. Young)

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"A man's friendships are one of the best measures in the world."

(Charles Darwin)

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Resumo

Efeito do exercício físico resistido e/ou do decanoato de nandrolona sobre aspectos sociais e reprodutivos, bem como sobre a resistência mecânica do fêmur, em ratos machos.

Resumo

Considerando-se a importância do exercício físico para a prevenção de problemas na saúde de indivíduos adultos e idosos associada à procura pela melhor forma de se exercitar, tem aumentado a busca por ergogênicos nos dias de hoje. Assim, os ergogênicos têm sido apontados como as principais substâncias utilizadas em tratamentos para o retardo da velhice e prevenção de doenças como enfarte e osteoporose. Porém pouco se sabe sobre o melhor método para estes tratamentos. Logo, o objetivo do presente trabalho foi determinar os efeitos produzidos pelo treinamento resistido de alta intensidade e/ou tratamento com decanoato de nandrolona (anabolizante esteróide) sobre os aspectos sociais e reprodutivos, e à resistência mecânica do fêmur em ratos machos. Os ratos foram divididos em seis grupos experimentais: controle não treinado, controle treinado, anabolizante treinado, anabolizante não treinado, controle treinado pirâmide e anabolizante treinado pirâmide. O programa de exercício foi realizado três dias por semana, durante 8 semanas. Concomitantemente, o tratamento com o anabolizante esteróide - decanoato de nandrolona - foi realizado duas vezes por semana e ao final deste treinamento os ratos foram submetidos a um teste de agressividade (comportamento social) e a seguir eutanasiados. Foram realizadas análises ósseas (cabeça do fêmur), atividade da creatina kinase (CK), contagem espermática e viabilidade dos espermatozóides ao final das 8 semanas, com exceção a mensuração da CK que foi realizada ao longo do treinamento pirâmide. Os grupos submetidos ao exercício foram subdivididos para a realização de um protocolo de treinamento chamado “pirâmide”. Neste treinamento, os ratos foram submetidos a uma série de repetições máximas, na qual foi testada sua resistência à fadiga, de grande importância para avaliarmos o dano ou fortalecimento ósseo, assim como outras possíveis alterações. Pela análise destes resultados pudemos observar que os ratos tratados com o decanoato apresentaram comportamento agressivo após 4 semanas de tratamento/isolamento, mas após 8 semanas do isolamento, não apresentaram diferença em relação ao grupo controle. Os níveis de testosterona plasmática no grupo pirâmide e

controle não treinado mostraram serem significantemente maiores em relação aos outros grupos, enquanto que o grupo pirâmide tratado com o anabolizante mostrou o mais alto valor de corticosterona plasmática em relação a todos os demais grupos. A massa corporal e o ganho de peso ao longo das 8 semanas foi também menor neste grupo. Em adição, o mesmo grupo pirâmide/anabolizante apresentou os parâmetros seminais mais danificados ao final do tratamento/treinamento, com alterações significantes para a viabilidade e contagem espermática. O anabolizante que por sua vez levou a uma hipertrofia na vesícula seminal comprometeu a resposta contrátil do órgão, reduzindo o número de receptores alfa1, bem como sua resposta contrátil à noradrenalina. Por outro lado, o anabolizante confirmou também seu papel já reportado na literatura como causador de distúrbios sociais violentos e agressivos. A associação treino tradicional/anabolizante mostrou ser benéfica à resistência mecânica do fêmur deste grupo em relação ao grupo tratado somente com anabolizante. Já o exercício tradicional mostrou aumento significante na capacidade de deformação do osso. Por outro lado, os níveis de CK não apresentaram diferenças significantes entre os grupos.

Portanto, o exercício pirâmide apresentou um papel importante como modulador endócrino, apresentando-se como uma ótima alternativa de treinamento para melhores resultados. Porém sua associação à nandrolona acarretou vários prejuízos, comprometendo até mesmo a fertilidade. Sendo assim, pudemos observar que o tratamento crônico com o anabolizante esteróide precisa ser minuciosamente avaliado pelo médico quando indicado à determinada patologia, até mesmo porque com base nos resultados do presente estudo, o esteróide isolado não mostrou nenhum benefício tanto ao osso, quanto à fertilidade, uma vez que os prejuízos trazidos pelos efeitos colaterais do anabolizante esteróide envolveram aspectos sociais, endócrinos e reprodutivos. Demonstramos também que muitas vezes o benefício à saúde pode ser adquirido de forma natural, como quando do uso de um protocolo que inclua unicamente um exercício físico específico, ressaltando a importância da escolha da atividade física mais apropriada. E ainda, interessantemente podemos apresentar pela primeira vez na literatura dados científicos de um protocolo muito promissor, o treinamento pirâmide.

Palavras-chaves: Decanoato de nandrolona; Exercício fisico; Parâmetros Seminais; Osso; Agressividade; Ratos machos.

Abstract

Abstract

Considering the importance of physical exercise in the prevention of several health diseases on adults and elderly, searching the best way of exercise, the sought for ergogenics has increased nowadays. On this sense, ergogenics have been pointed as one of the most utilized substances on the treatment of aging, prevention of heart attack and osteoporosis. However, little is known about the best method to these treatments. Therefore, the aim of the present study was to determine the effects produced by the high intensity resistance exercise and/or treatment with nandrolone (anabolic steroid) on social and reproductive aspects, as well as, the bone mechanical resistance on adult male rats. The rats were divided into six experimental groups: Untrained/Vehicle, Untrained/Anabolic, Trained/Vehicle, Trained/Anabolic, Pyramid/Vehicle and Pyramid Anabolic. The exercise protocol was performed three times a week, one day a part, during 8 weeks. The treatment with the anabolic steroid was realized within the eight weeks and at the end of the treatment/training, the animals were euthanized and the following analyses were assessed: femur mechanical resistance, creatine kinase (CK) activity, sperm count and seminal parameters, hormonal levels and the aggressive behavior. For the aggressive behavior the animals were isolated, and only the treatment was performed. The CK activity was the only measurement assessed during the 8 weeks. The groups submitted to the exercise were subdivided on the sixth week to accomplish another exercise protocol, known as pyramid training. This training protocol associates increasing working loads with low repetitions during the program, in which we tested the fatigue resistance, extremely important to evaluate the damage on bone strength, as well as other alterations. Through this analysis we could observe that the rats treated with decanoate presented aggressive behavior after 4

weeks of treatment/isolation, but after 8 weeks of isolation there was not any more difference in relation to control group. The pyramid and untrained/vehicle groups showed the highest testosterone levels (statistically significant) in relation to all the other groups, while the pyramid/anabolic presented the highest significant plasmatic corticosterone level in relation to the other experimental groups. The body mass and the body mass gain throughout the 8 weeks was the smallest in this group. In addition, this latter showed the most damage seminal parameters at the end of the treatment/training, with significant alterations in the viability and sperm count. The steroid *per se* altered the seminal vesicle wet weight, causing a hypertrophy, compromised the contractile pattern, lowered the number of α_1-adrenoceptors, as well as the contractile response to norepinephrine. On the other hand, the steroid also confirmed its role already reported in the literature as presenting aggression-enhancing properties, such as violent and aggressive social disturbs. The association of the traditional training and the steroid showed to be beneficial to the femur mechanical resistance in relation to the group treated only with the steroid. Whereas, the traditional training presented significant increase in the bone maximum force of deformation. In contrast, there was not any difference in the CK levels among the groups. The pyramid training, tested experimentally for the first time, showed an important role as an endocrine modulator, being a good alternative of physical training to achieve better results. However, its association with the decanoate caused several side effects, even compromising the fertility. Therefore, we could conclude that the chronic treatment with the anabolic steroid needs to be carefully analyzed by a physician when prescribed for a disease, because as we could observe in our results the steroid isolated did not any improve not even for the bone, nor for the fertility, since the present study showed the damages on social, endocrine and reproductive aspects brought by the nandrolone decanoate side

effects. Interestingly, our data demonstrates that the improvements for the health can be achieved in a natural form, such as the use of a specific protocol of physical exercise, highlighting the importance of designing an appropriate training protocol. Moreover, we confirmed for the first time with scientific data the excellence of pyramid protocol, very popular among athletes, which improved the long term effect for the endocrine system in male rats; and contributed significantly as for the present risk of a inappropriate choose of training program, which can damage not just the acute response, but instead of that, a long term response brought by the stress from the physical activity, modulating negatively the testosterone and corticosterone levels, that later can lead to harmful alterations for the individual .

Keywords: Nandrolone decanoate; Physical exercise; Seminal Parameters; Bone; Aggressiveness; Male rats.

Prólogo

Prólogo

Durante todo o período do curso de Doutorado no Programa de Pós-graduação em Ciências Biológicas- Área de Farmacologia houve a possibilidade de participação em diferentes atividades extracurriculares além de outros trabalhos de pesquisa desenvolvidos no laboratório. Estes trabalhos possibilitaram a divulgação de resultados em Congressos Nacionais e Internacionais e envio de trabalhos científicos para periódicos indexados. E ainda, a premiação em Congressos Internacionais originou o convite para a realização do meu Doutorado Sanduíche (PDEE-Capes) na University of Illinois at Chicago, aonde pude realizar um complemento ao meu projeto e adquirir uma valiosa experiência acadêmica.

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Formação Complementar

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2. Curso de curta duração em V Curso de Verão: Manipulação de Ácidos Nucleicos:2008. Universidade Estadual Paulista Júlio de Mesquita Filho, UNESP, Sao Paulo, Brasil

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Produção bibliográfica

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Organização de evento

1. AGATI, Leandro Barile

XI Workshop da Pós-Graduação e Genética, 2010. (Outro, Organização de evento)

2. Conferencista no **X Workshop de Genética**, 2010. (Congresso)

Mini-curso: Programação Fetal e diferenciação sexual do cérebro.

Estágio no exterior

1. The Psychiatric Institute, University of Illinois at Chicago, Chicago, IL.
   Periodo: Setembro à Dezembro de 2011.
   Orientador: PhD. MD. Graziano Pinna (Projeto e resultados segue no Apêndice)

Parecer de artigos científicos

1. Revisor de artigo para a revista General and Comparative Endocrinology, Elsevier: http://ees.elsevier.com/gce/ **2010/2011**
2. Revisor de artigo para a revista Experimental Physiology http://ep.physoc.org/ **2008/2009**

Prêmios e títulos

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2011 Bolsa de doutorado sanduiche- Capes PDEE 2533-11-1

2010 Young Investigator Travel Award, INF - recebido para apresentação de trabalho: **Could resistance exercise in neonatal androgenized rats affect body/organs development and bone resistance?** The 7th International Congress of Neuroendocrinology, 2010, Rouen. Young Investigator Travel Award.

2008 Menção Honrosa, Fesbe

Sumário

Sumário

Introdução

Introdução

1.1 Considerações gerais

O ritmo de vida acelerado da população, o estresse e o aumento no número de doenças causadas por fatores estressogênicos, assim como enfartes, disritmias e outras fizeram com que a população do século XXI se preocupasse mais com a manutenção da saúde.

Assim, o estresse oxidativo é um importante fator de risco contribuinte para doenças crônicas (Rao & Rao, 2007). Dietas à base de frutas e vegetais têm sido também apontadas como grande atuante na prevenção de doenças e manutenção da saúde. Ainda, substâncias como ergogênicos estão sendo utilizadas em larga escala, principalmente por freqüentadores de academias na busca de um corpo "sarado". Verificamos então ser muito comum hoje em dia, academias estarem lotadas com pessoas de todas as idades, sendo que uma grande parcela procura por substâncias que facilitem a aquisição de bons resultados com o exercício físico. Neste sentido, utilizações de recursos ergogênicos - apesar dos efeitos colaterais - são claramente aceitos como coadjuvantes capazes de aprimorar o desempenho e a segurança do atleta ou de indivíduos que visam o fisicoculturismo. Elementos como treinamento, condicionamento, uso adequado de água, melhores equipamentos, sobrecarga de carboidratos, suplementos vitamínicos e de ferro, técnicas de aquecimento, entre outros estão dentro do espírito competitivo e podem ser considerados ergogênicos (Foss & Keteyian, 2000). Assim, a eleição pelo estudo dos ergogênicos farmacológicos torna-se primordial, uma vez que os anabolizantes esteróides estão classificados como o grupo de substâncias ergogênicas mais utilizadas no processo de doping (Parssinen & Seppala, 2002) e por atletas que não se dirigem a competições.

Já entre os diversos tipos de protocolo de treinamento mais populares, entre fisicoculturistas, sobressai-se o treinamento "pirâmide". Este objetiva o princípio de aquecer o músculo progressivamente antes de utilizar a carga máxima para um determinado exercício e assim evitar riscos de ruptura de tecido mole, sendo excelente para preparação psicológica para os sets mais pesados. Estimula unidades motoras de diferentes potenciais de excitação, durante o treinamento, aumenta a força dinâmica e a força pura (Vianna, 2002).

1.2 Definição dos esteróides androgênicos-anabólico (EEAs)

Esteróides androgênicos-anabólicos ou anabolizantes androgênicos são derivados sintéticos da testosterona e foram desenvolvidos com o objetivo de minimizar seus efeitos masculinizantes (androgênico), maximizando assim os efeitos sobre a síntese protéica e o crescimento muscular (anabólico) (HAUPT & ROVERE, 1984).

O uso destas substâncias está relacionado a diversos efeitos colaterais para a saúde humana. Estes efeitos podem acontecer de forma reversível ou irreversível e podem incluir problemas hepáticos, endócrinos, cardiovasculares, esqueléticos e subjetivos, como alterações de comportamento, agressividade (KIBBLE & POSS, 1987; WILSON,1988; LUKAS, 1993) assim como alterações na função reprodutiva de atletas do sexo masculino (De Arce et al., 1994; Ayers et al., 1985).

A preocupação principal da sociedade atual quanto aos EAAs está mais relacionada ao seu uso por atletas e *bodybuilders*. Muito embora, já existam também relatos que indicam o seu abuso disseminado entre adolescentes e jovens não ligados a esportes (Kimdlundh et. al, 1999; Sjoqvist et al., 2008). O uso de EEAs com o propósito de aumento do desempenho físico não é um fenômeno recente: a testosterona, assim como com os seus derivados, tem sido usada neste intuito desde seu isolamento e caracterização como hormônio masculino. Como ocorre freqüentemente com substancias de abuso, as intenções originais eram de encontrar substâncias farmacêuticas que poderiam tratar diferentes doenças e melhorar a saúde. Ainda assim, o uso de EEAs na clínica tem certa importância como para o tratamento de hipogonadismo, impotência, osteoporose e anemia. Porém sua utilização clínica é insignificante se comparado ao seu uso ilegal.

1.3 Aspectos sociais

Alguns dos efeitos psicológicos mais proeminentes advindos do uso de EAAs são estados de manias, definidas como: irritabilidade, agressividade, euforia, hiperatividade e comportamento perigoso (Pope and Katz, 1994; Perry et. al, 2003). Estes efeitos podem variar da depressão para a psicose dependendo da variação individual, estado e padrão de administração da droga, dose, e especificidade do composto EAAs utilizado (Clark and Henderson, 2003). A agressividade, particularmente em resposta a provocação, é

comumente reportada como um dos efeitos colaterais após o uso de EAAs. (Bahrke et al., 2000).

Contudo, pouco se sabe sobre os mecanismos pelo quais os EAAs levam a comportamentos agressivos e variações de humor, causando desvios sociais. Muitos trabalhos já mostraram esta associação, mas Trainor et. al (2009) detalharam em vários experimentos que as relações entre os androgênios esteróides, como a testosterona, e a agressividade são complexas. Em quadros clínicos de variações humorais, como transtorno bipolar, uma proteína extracelular essencial para a migração neuronal e posicionamento durante o desenvolvimento, a Reelina chamou atenção por sua ligação a esta doença. Este link é também reportado em outros trabalhos que mostram níveis reduzidos de Reelina em pacientes com transtornos bipolar e esquizofrenia (Fatemi et al, 2000; Torrey et al, 2005), e também após um estresse crônico (Lussier et al, 2009). Devido ao alto índice de comorbidade patogênica destas doenças (Altamura et al, 2011; Kessler et al, 2005) a investigação pela ligação dos níveis de Reelina em regiões do cérebro (Figuras 3,4,5) e sua ligação com possíveis desvios comportamentais, como a agressividade, pode elucidar uma das vias atuantes por este comportamento característicos de usuários de EAAs.

1.4 Aspectos reprodutivos

Os efeitos dos compostos EAAs nos tecidos da reprodução masculina são controversos. Pode-se observar que a espermatogênese não é interrompida após a administração de EAAs;(Johnson et al., 2007; Knuth et al., 1989), enquanto outros pesquisadores mostram que EAA diminui a densidade, mobilidade e morfologia do esperma de homens, sendo os responsáveis pela infertilidade(Holma et al.1977; Ludwig 1950). Isto ocorre porque a testosterona exógena e seus derivados semi-sintéticos causa a retroalimentação negativa no eixo hipotalamo-pituitária-gônada, impedindo assim a espermatogênese (Ludwig 1950). Poucos estudos identificaram que o EEAs causam atrofia testicular e de órgãos da reprodução e ainda redução na produção espermática (Clark et al., 1997). Uma baixa contagem espermática acompanhada de redução significante no número de espermas com uma morfologia normal foram também observados em usuários que abusavam de EAAs (*bodybuilders*). Torres-Calleja, et al., Assim, alteração na contagem e qualidade

espermáticas podem ocasionar vários efeitos para a fertilidade masculina. E ainda, investigações recentes mostram que não somente os EAAs podem causar danos à fertilidade masculina, mas apontam também o volume de exercício físico como a variável que mais afeta a reprodução (Dohle et al., 2003; Ferry et al., 2000). Outros autores já sugeriram que a intensidade do exercício é igualmente deletéria, ou até mais prejudicial que o volume para a função reprodutiva. (Gayan-Ramirez et al., 2000; Joumma and Leoty, 2001) Sendo assim, a preocupação pela atividade física mais adequada torna-se extremamente importante para a obtenção de bons resultados à saúde e à redução do estresse diário. Outra preocupação seria também o aparecimento de problemas na ejaculação ocasionados por efeitos da atividade física extenuante e dos EAAs sobre os receptores alfa1 do trato urinário. É descrito na literatura a presença de adrenoceptores alfa1 (subtipos alfa1a, alfa1b e alfa1d) em órgãos do tecido reprodutor masculino, como na vesícula seminal, responsáveis pela ejaculação, e que estes são modulados pela presença de andrógenos (Shima, 1993 and Silva et al., 1999). Sabendo-se que o EAAs em doses suprafisiológicas suprime a produção endógena de testosterona, a conseqüente redução/ausência deste hormônio pode levar a redução no número de adrenoceptores no tecido reprodutor masculino. Sendo assim, redução no nível plasmático de testosterona circulante ocasionado pela prática do exercício físico e/ou uso de EAAs pode ser um fator determinante para comprometer a fertilidade masculina.

A possibilidade de ocorrência de efeitos colaterais na fertilidade advindos do tratamento (anabolizante/exercício) foi estudada através da análise de desenvolvimento de órgãos, parâmetros seminais e estudos funcionais de órgãos dos indivíduos tratados.

1.5 Influência sobre a estrutura óssea

Por outro lado, devido ao alto número de "artifícios" oferecidos no mercado atualmente, muitas doenças, como a osteoporose, estão sendo tratadas e prevenidas com o uso de ergogênicos. A osteoporose é uma doença complexa, caracterizada pela perda de massa óssea, resultando em fraqueza óssea e em aumento da susceptibilidade a fraturas (Culhan et al., 1994 e Mitchel et al., 1998). Essa doença é atribuída à idade, deficiência na pós-menopausa estrogênica e problemas clínicos, e tem uma imensa significância

socioeconômica, sendo reconhecida como um dos maiores problemas de saúde pública (Culhan et al., 1994). Embora a osteoporose seja considerada uma doença de idosos, sua predisposição começa na infância e adolescência. É extremamente importante obter a quantidade máxima de osso na maturidade esquelética (massa óssea de pico), que ocorre tipicamente ao final da adolescência, embora aumentos discretos na massa óssea possam ocorrer após esse período, em ambos os sexos. O principal determinante da massa óssea de pico é a genética, contribuindo com até 70% a 80% da variabilidade. Fatores ambientais incluindo nutrição e exercício são importantes para os 20% a 30% restantes (Oglouian, M.J.G., 2003).

Muitos tratamentos têm sido desenvolvidos com o objetivo de prevenir a perda de massa óssea e promover o seu aumento, incluindo a reposição hormonal, de compostos bifósforos e programas de atividade física (Kehoe, 2006).

Daly et al. (2004) reportaram que muito embora tenha sido documentado que atletas jovens e adultos tenham ambos grande massa, tanto óssea, como muscular, não existem dados conclusivos para provar que o estímulo criado pela contração muscular tenha levado a um incremento proporcional na massa óssea. Sabe-se, porém, que o tratamento da osteoporose com ergogênicos farmacológicos, como o decanoato de nandrolona, pode apresentar efeitos indesejados ao comportamento, como agressividade, irritabilidade e hostilidade (Hartgens & Kuipers, 2004) entre outros, e que até mesmo em doses baixas podem estar presentes.

Foi demonstrado então que um programa de exercício físico moderado foi capaz de evitar a perda de massa óssea depois de ovariotectomia em ratas (Peng et al., 1994 e Barengolts et al., 1996). Além disso, a força óssea e conteúdo ósseo mineral em ratas jovens e ovariotectomizadas aumentou durante um protocolo de natação (Hart et al., 2001).

Muitos autores sugerem que exercícios dinâmicos de alto-impacto e treinamento de resistência são mais benéficos para o tecido ósseo, produzindo maior desgaste do que a caminhada, pois estes exercícios estimulam os osteoblastos a aumentarem a densidade óssea, tornando o osso mais resistente ao estresse causado (Notomi et al., 2000 e Honda et al., 2003). Notomi et al. (2000) reportaram que o treinamento de resistência com pulo aumentou a formação de massa óssea, devido ao acréscimo de osteoblastos e o decréscimo de reabsorção óssea e no número de osteoclastos. Enquanto que, Tamaki et al (2001)

avaliaram que a severidade da lesão induzida pelo exercício de levantamento de peso está associada ao aumento no nível de atividade de Creatina Kinase (CK) plasmática após uma série de exercício físico.

Um dos objetivos do presente estudo foi então avaliar este protocolo de exercício físico pirâmide, muito popular no meio esportivo por aumentar desempenho e rendimento do atleta, e também comparar os seus resultados aos do protocolo de exercício físico de salto freqüentemente utilizado em nosso laboratório (Figuras 1 e 2).

Portanto, os efeitos do exercício físico no tecido ósseo variam muito de acordo com o tipo de exercício, intensidade, duração e freqüência (Honda et al., 2003). Alguns autores já até reportaram que exercício físico extenuante tem efeitos inibitórios sobre a massa e força óssea (Nordsletten et al., 1994). Pereira et al. (2007a) reportaram também que o exercício resistido de alta intensidade levou a redução na testosterona plasmática de ratos machos treinados em relação aos não-treinados.

Sugere-se que o tecido ósseo responda melhor a exercícios físicos dinâmicos, assim como saltos, ao invés de cargas estáticas (Robling et al., 2002 e Turner & Robling, 2003). Desta forma, presume-se que o osso acomoda-se melhor ao exercício de cargas estáticas, alcançando saturação mecano-sensitiva, a qual não provoca mais aumento na massa e força óssea, a não ser que a magnitude da carga seja aumentada (Robling et al., 2002 e Honda et al., 2003). Em nosso laboratório, dados preliminares mostraram que o treinamento resistido de alta intensidade e o anabolizante esteróide quando aplicados isoladamente foram capazes de levar o osso a resistir a uma carga maior (maior resistência óssea). Quando da associação do treinamento resistido de alta intensidade com o anabolizante esteróide essa vantagem não foi significativa, provavelmente devido a uma menor capacidade de deformação do osso quando dessa associação (Pereira et al., 2007b).

Desta forma, acreditamos que um programa de treinamento de carga progressiva possa ter um efeito estimulante no músculo, sobrecarregando o tecido ósseo e produzindo um aumento na massa óssea. Neste sentido, buscou se determinar os efeitos de um programa de exercício físico resistido de alta intensidade no tecido ósseo e na fadiga muscular (níveis de CK) em ratos machos tratados ou não com um anabolizante esteróide. Adicionando ao protocolo de exercício tradicionalmente utilizado foi investigado também um protocolo de exercício pirâmide.

Sendo assim, o presente estudo objetivou avaliar o efeito de duas variáveis isoladas e também de sua associação, o tratamento com o decanoato de nandrolona e a prática do treinamento físico em ratos machos adultos. Foram investigados assim os prejuízos e benefícios trazidos aos diferentes grupos experimentais. Desta forma, procurou-se elucidar mecanismos envolvidos em possíveis alterações sociais, reprodutivas e ósseas. Adicionalmente, procurou-se ainda salientar a importância de ambas as variáveis, para uma possível futura indicação terapêutica a partir dos resultados encontrados.

Figura 1 – Sequência do exercício de salto

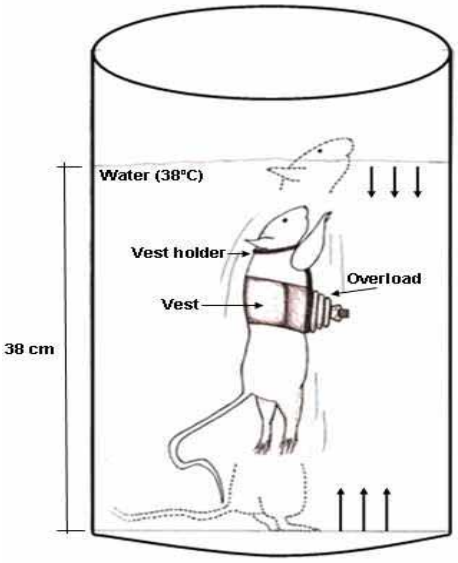

Figura 2 – Ilustração do exercício de salto

Figura 3 – Capacete com cânulas em camundongo

Figura 4 – Aparelho de infusão

Figura 5 – Infusão de Reelina em camundongo

Capítulo 01

Nandrolone decanoate induces aggressive behavior in male adult rats exposed to an isolated condition only for a short period

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Abstract
This study examines the effect of chronic administration of the anabolic androgenic steroid nandrolone decanoate on adult male rats in an isolated condition. The isolation condition was assessed for behavior test. The animals received 5 mg/Kg of nandrolone decanoate or vehicle, twice a week/8 weeks. Throughout the study the rats were isolated and in the middle of the treatment (4^{th} week) they were analyzed to establish a comparison. Hormone profiles, alterations in testes and body weights were also evaluated. The study showed that 4 weeks of nandrolone decanoate treatment induced aggressive behavior; however 8 weeks of the same AAS did not enhance the aggressiveness. The nandrolone treatment also decreased significantly the weight gain, the testes weight and the testosterone profile. The main finding is that during the treatment with nandrolone decanoate only on the 4^{th} week the animals showed more numbers of aggressive behaviors (attacks, threats, etc), but on the 8^{th} week (end of treatment) the aggressiveness was not different from control, even though the treatment had brought the complete blockade of HPG (hypothalamus-pituitary-gonad) axis, decreasing significantly testosterone level and testis weight. Further studies will be performed to elucidate the lack of aggressiveness from the Nandrolone group during the 8 weeks of treatment.
Keywords
Aggressive behavior; Nandrolone decanoate; Hormone profiles; HPG (hypothalamus-pituitary-gonad) axis; Isolation rearing.

1. Introduction

AAS (anabolic androgenic steroid) is current been used by a massive portion of the human population, who practice some kind of sport, in order to obtain fast improving results; however it has been described that its use is linked to increased aggression and violence [1,2,3,4,5,6,7].

The psychiatric side effects of AAS range from depression to psychosis depending on the individual taking the steroids, the stage and pattern of drug intake, dose, and the specific AAS compound that is used. For example, increased energy and libido, as well as delusions and manic episodes, are often associated with AAS [8]. Nevertheless, aggression, particularly in response to provocation, is one of the most commonly reported psychiatric side effects after use of AAS [9].

The aggression-enhancing properties of AAS have been confirmed in several studies in rodents [10,11,12,13,14,15,16,17,18,19]. Many of the rodent studies of AAS effects on aggression have focused on inter-male aggression, a form of aggressive behavior that is dependent of the presence of androgens [20].

Steroid hormones have long been focus of investigators studying the neuroendocrine bases of aggressive behavior. Although testosterone is generally a key hormone regulating aggression, either directly or by serving as a prohormone for 5a-dihydrotestosterone (DHT) and estradiol, detailed experiments have demonstrated that the relationships between steroid hormones, such as testosterone, and aggression, are complex [21].

Hormones are closely associated with the regulation of aggressive behavior across species. Sex steroid hormones interact on several levels to influence the likelihood of aggression. Acute hormonal responses to the environment can have different effects on

behavior than the baseline hormonal state [22] and isolation increase the likelihood of attack behaviors [23].

As many studies on this matter are controversial, and fail to identify at what point does AAS treatment disturbs the social behavior, the aim of the present study was to investigate how nandrolone decanoate affects social behavior (with focus on aggression) in an isolated condition. The rats were analyzed in the middle and at the end of the treatment (8 weeks) to investigate possible behavioral changes and its interaction with hormonal levels (endocrine response).

2. Material and Methods

2.1. Animals and Treatment

Male wistar rats were kept in a controlled environment with temperature at 25±1 ºC; humidity of 55±5%; 12 h light/dark cycle (lights on at 6:00 a.m.) and had free access to regular lab chow and tap water. At post-natal day (P) 90 the animals were treated with the anabolic steroid nandrolone decanoate (Deca-Durabolin®) 5mg/kg) or vehicle-control (ground-nut oil + propylene glycol), i.m., twice a week, for eight weeks. The employed high dose of nandrolone decanoate corresponded to that frequently used by athletes [7]. During this period the animals were isolated, but they were able to see, smell and hear conspecifics in the shared colony room but were without touch and the affiliative context of group housing. Animals were left in these conditions until P 146 when the behavioral effects of chronic social isolation were measured using well-established behavioral assessments. The duration of social isolation was based on previous studies of isolation rearing [24,25]. At the end of behavioral testing, animals were sacrificed and hormone profiles and testes weight were obtained. Thus, these animals were divided into: Control group and Nandrolone group (n=8 animals per group). Animals were weighed daily to determine body weight gain.

The animals used in the present study were maintained in accord with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and approved by Institute of Biosciences /UNESP-Botucatu Ethical Committee for Animal Research (Protocol number 034/05).

2.2. Testes

At the end of the experiment, the right testis of all animals was weighed.

2.3. Behavioral Testing

Isolated rats received their corresponding treatment schedules twice a week and aggression tests were conducted on the 4th week and at the end of the experimental period (8th week). We performed a pre-test in the middle of the experiment (4th week), in order to compare the AAS development effect. Aggression tests were conducted in the isolated rat's cage except that the wire-top was replaced by a transparent polypropylene cover (the same size as the cage but perforated all over the surface for proper ventilation). Such a transparent polypropylene cover was employed in order to facilitate video recording of upright offensive postures. The aggression test consisted of, first assessment of the locomotor activity of

isolated rat alone for 5 minutes and followed by a subsequent aggressive confrontation for another additional 10 minutes. The latter involved that the non-drugged social intruder be introduced or placed into the home cage of an isolated rat. The isolated rat was distinguished by coloring or darkening the fur of the isolated rat with markers. The overall observations (15 minutes) were made in an enclosed arena without the experimenter being present and recorded remotely using a video camera connected to a standard videocassette recorder. Finally, the videotapes were analyzed offline [26]. The following aggressive behaviors were recorded: threats, confrontation, attacks, boxing, fighting, avoidance, persecution–fleeing and dominance–submission [27].This facilitated measuring of: 1) latency for the first bite, 2) total number of threats, 3) duration of threats (total time).

2.4. Testosterone and Corticosterone quantification

At about 48 hours after the last behavior test, the animals were anesthetized and blood samples were collected through the abdominal aorta into heparin-coated syringes (always between 8:30 and 9:30 a.m.) from rats of both experimental groups. Blood was centrifuged (2,500 rpm for 20 min at 2º C) and plasma testosterone was determined by chemotropic assay, while corticosterone concentration determined by competitive immunoassay using the Coat-a-Count® Rat Corticosterone Test (Diagnostic Products Corporation, USA). The antibody used was highly specific for testosterone and the test had an analytical sensitivity of 0.10 ng/ml. The intra-assay coefficient of variation was 4.8%.

2.5. Data analysis

The Student t test was used to compare the means of pairs of samples. Two-tailed levels of significance were used in all statistical calculations. Statistical significance level for group differences was set at $P \leq 0.05$. All data are expressed as mean $\pm$ standard error of the mean (SEM).

3. Results

3.1. Body weights (Figure 1)

There were not any differences in body weights between control group and nandrolone group prior treatment, nor at the end of the treatment. Changes in body weights were only seen when we analyzed the weight gain at the end of the treatment. This difference was extremely significant, which shows that the nandrolone group gained less weight than the control, after 8 weeks of treatment with nandrolone decanoate, (Control 101.51$\pm$15.81, Nandrolone 61.25$\pm$18.52).

3.2. Testis weights (Table 1)

Testis from nandrolone group is significantly reduced in relation to control group.

3.3. Hormonal levels (Table 1)

Testosterone level of nandrolone group decreased significantly in relation to control. However the corticosterone level remained the same between groups, without statical significance.

3.4. Aggressive behavior (Table 2)

According to the protocol, an adaption was made in order to evaluate the evolution effect of the steroid treatment during the 8 weeks. Therefore a pre-test was performed within 4 weeks of isolation and the second test in the last week of isolation, 8th week. In the pre-test there was a significant difference in the total number of threats between groups. Nandrolone group performed more number of threats than the control. The other significant difference seen was in the same control group, comparing the pre-test and the test; they also showed higher number of threats at the end of the experimental period. No more significant difference was seen between both groups in the other variables.

4. Discussion

The present study examined the impact of a high dosage of nandrolone decanoate treatment in adult male rats on social behavior aspects and its relation to testosterone and corticosterone profiles, body weight gain and testis weight. We also analyzed the effects of the AAS during half of the treatment in order to check the behavior changes of these rats, paired and unpaired analysis.

The mean body weight of each group did not have any significant difference. However, as observed in previous studies [13,28,29] the Nandrolone treated rats did not gain as much weight as control rats throughout the treatment. Whether this reduction in weight gain is due to decreased food intake [30] or increased metabolism was not determined. A pair fed study, with daily i.m. injections of Nandrolone doses, supports the hypothesis of decreased food intake [31]. The significant decreased body weight gain from the Nandrolone group was only seen after 8 weeks of treatment, but the loss in the weight gain started on the third week of treatment (Figure 1).

The AAS treatment also showed to be effective in the blockade of HPG (hypothalamus-pituitary-gonadal) axis, reducing the testis weight and suppressing testosterone production significantly (Table 1). This result corresponds to others reported in the literature [32, 33]; and confirms that our AAS dosage was indeed effective.

Nevertheless, the aggressive behavior observed in the present study was only seen in the pre-test by the Nandrolone group with significant higher number of threats. Whereas in the test, we did not observe any differences between groups, only in the control group using a paired Student t test analysis, which was already expected due to the 8 weeks period of isolation. The significant higher number of threats performed by the Nandrolone group after 4 weeks of treatment is according to the aggressiveness already reported by the AAS compounds in the literature [11,13,14, 18]. Although several studies, including the present, have reported increased aggression after chronic nandrolone treatment [11,14,18,35] this was not supported by some other studies [34,16,17]. There could be several reasons for these divergent results, such as the route of administration [35], doses, length of treatment, different behavioral models to measure aggression, and the time point of behavioral testing. It has also been suggested that the experience of aggression or lack thereof, prior to AAS treatment, may affect the aggressive behavioral response [8]. We believe that the aggressiveness showed only in the 4th week might be related to a combination of facts: a probably elevated level of androgens (exogenous and endogenous), lack of corticosterone level and an initial period of isolation.

Although most studies correlate lower levels of cortisol to aggressive behavior [36,37], in this study normal level of corticosterone was seen at the end of the treatment, which indicates that during the experiment the animals were not suffering from stress condition. Nevertheless, links between cortisol and aggression have not been completely consistent [38]. Interestingly, the aggressive behavior showed in the pre-test by the Nandrolone group matches with the beginning of weight loss by this group (Figure 1), so we hypothesize that this point of the treatment with nandrolone decanoate may be the threshold for a complete blockade of HPG axis, however we did not quantify the testosterone level at this point of the treatment. Further studies will be performed in order to confirm this hypothesis.

On this sense the present study showed that 4 weeks of nandrolone decanoate treatment can induce aggressive behavior in male rats with the used dosage, in an isolated condition. However 8 weeks of the same AAS does not enhance the aggressiveness, in fact, it reduces the social response; even though the treatment had brought the complete blockade of HPG axis, decreasing significantly testosterone level, weight gain and testis

weight. Further studies will be performed to elucidate the lack of aggressiveness from the Nandrolone group at the end of the experiment.

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Tables

Table 1

Testis weights and concentration of gonadal hormones for each group

Group	Testis (g)	Testosterone (ng/dl)	Corticosterone (ng/dl)
Control	1.680±0.06	228.61±69.46	96.05±25.61
Nandrolone	1.473±0.06 *	3.67±0.66 **	136.71±38.50

Results are means± SE

Significantly different than Control group, ANOVA, *p<0.05, ** p<0.0001.

Table 2

Total behavioral activity (total frequency count of all behaviors recorded during social pre-test and test sessions over the course of the study)

	Pretest			**Test**		
Group	Latency for the first bite (s)	Total number of threats (n)	Total time of threats (s)	Latency for the first bite (s)	Total number of threats (n)	Total time of threats (s)
Control	118.38±29.15	21.38± 2.51	140.00±23.54	77.38±9.74	32.50±3.30&	185.88±23.92
Nandrolone	121.13± 48.52	32.75± 3.21*	175.88± 21.34	105.13±23.37	38.13±3.46	158.88±20.42

Results are means± SE

*Significantly different than Control group, ANOVA, $p<0.05$.

&Significantly different than Control group in the pre-test, ANOVA, $p<0.05$.

Figures

Figure 1

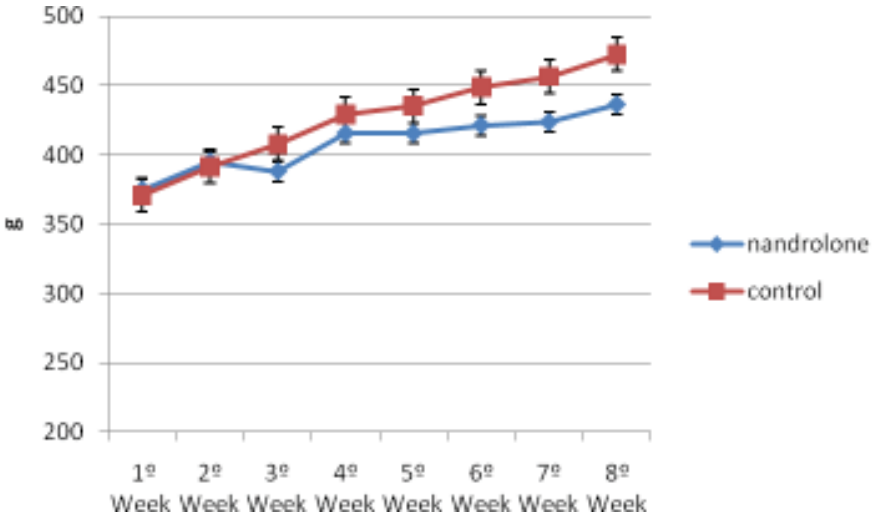

Fig. 1. Change in body weight during the experimental period of 8 weeks.
Values are means± SE (n = 8/group for control and nandrolone).
There is not any significant difference between them.

Capítulo 02

Pyramid Training and Nandrolone decanoate decrease sperm concentration and viability in rats

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Running Title: Exercise and AAs action in sperm markers

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Abstract

Objective: To investigate the effect of two different training protocols on various markers of semen quality and hormonal levels.

Design: Experimental cohort study.

Setting: Laboratory of receptors and transduction mechanisms and pharmacology of reproduction.

Patient(s): Adult Wistar rats.

Intervention(s): Seventy five-day-old rats (6 groups): received nandrolone decanoate i.m. or vehicle, and were submitted or not to strenuous exercise.

Main Outcome Measure(s): Sperm parameters (volume, vitality, sperm count, motility, and morphology) and testosterone and corticosterone levels.

Result(s): Sperm concentration; vitality; and type ‘‘b’’ velocity were significantly different among the rats of the different training protocols ($P < 0.05$). Furthermore, significantly lower testosterone and corticosterone levels were only present in pyramid (strenuous exercise) anabolic group. On the other hand, pyramid vehicle group showed the highest significant level of testosterone among the groups.

Conclusion(s): There are differences in the seminal profiles among the exercised groups. The differences are more marked as intensity of exercise increases (pyramid groups) and associated with the use of steroid, especially for testosterone and corticosterone levels. These results clearly show that corticosterone plays a key role maintaining the balance of seminal parameters, and also indicate that the intensity of the exercised ought to be carefully analyzed and taken into account when designing a training protocol, and that the use of steroids concomitant can be even more harmful to the reproductive function.

Key Words: Fertility damages; hormonal profile; pyramid exercise; anabolic androgenic steroids.

Introduction

Physical exercise is promoted as a panacea for fitness, health, stress reduction, and life quality improvement, a matter of great importance in today's society (1). Despite this increased interest, not having an adequate knowledge of how to perform these activities might, on occasion, lead to the appearance of negative side effects (e.g., lesions, pathologies) (2).

Exercise training has shown to influence a lot on male infertility and it has been proposed secondary to changes observed in the reproductive hormone and semen profile of some endurance trained male athletes. Evidence exists that a subset of endurance trained men, particularly runners, present with subclinical changes in their reproductive hormone profile (3). Likewise repeated strenuous treadmill exercise in stallion can negatively influence semen quality (4). Moreover, short-term uses of anabolic androgenic steroids (AAs) to improve physical attributes have shown to disturb the male fertility, affecting on sperm counts, blocking the spermatogenesis and lowering testosterone level, but whether such usage has any significant impact of an irreversible nature, is unknown (5).

AAs are often combined with physical activity due to their capacity of fast recovering of skeletal muscle, increased metabolism and strength (6, 7). Therefore its use is even growing among recreational athletes who claim for fast results.

More recently, different types of training, such as pyramid training has become famous among athletes in order to improve performance. This training protocol associates increasing working loads with low repetitions during the program. However there is no scientific data showing any real results of such improve in performance.

Due to the difficulty of performing clinical trials, sample size, controlled variables of the study and in addition to that, the fact that studies from animal experiments have also resulted in conflicting conclusions, the main aim of the present study was to investigate whether two different protocols of exercise and AAS can have distinguish effects on semen parameters and hormonal levels by using male rats.

Material and Methods

Animals and experimental groups

Wistar rats were kept in a controlled environment with temperature at 25+1 ºC; humidity of 55+5%; 12 h light/dark cycle (lights on at 6:00 a.m.) and had free access to regular lab chow and tap water. At PND75 the animals received the anabolic steroid nandrolone decanoate (Deca-Durabolin®) 5mg/kg or vehicle (ground-nut oil + propylene glycol), i.m., twice a week and/or submitted to high-intensity resistance exercise protocol, both for eight weeks. The employed dose of nandrolone decanoate corresponded to that frequently used by athletes (8). Thus, these animals were subdivided into: Untrained/Vehicle, Untrained/Anabolic, Trained/Vehicle, Trained/Anabolic, Pyramid/Vehicle and Pyramid Anabolic.

The animals used in the present study were maintained in accord with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and approved by Institute of Biosciences /UNESP-Botucatu Ethical Committee for Animal Research (Protocol number 034/05).

Nandrolone Treatment

Nandrolone decanoate (Deca-Durabolin®) ampoules containing 25 mg were obtained from Organon, São Paulo, Brazil. Nandrole ampoule was administered intra muscular i.m., via a needle. Eighteen male rats received 5mg nandrolone/kg twice a week (group N), and

control male rats received vehicle ground nut oil (group V). Administrations were given between 10:00 AM and 12:00 PM for a period of 8 weeks, and after that sperm analysis was performed.

Training protocol

The animals of the trained experimental groups were submitted individually to jump sessions in a PVC cylinder of 50cm in height and 25cm in diameter, containing water at 30°C to a depth of 38cm. The training protocol was according to the literature's protocol (Figure 1) (9, 10). A weight overload was housed on the thorax of the animal. In the first week, an initial adaptation to the liquid medium was accomplished, with overload of weight equivalent to 50% of the body mass of the animal, from the 1st to the 3rd day. The traditional training protocol consisted of 24 jump sessions, in liquid medium with weight overload, 3 days a week, between 1 p.m. and 3 p.m., and in each session 4 series of 10 jumps were accomplished (Table 1). On the 6th and 7th week of training some trained rats were submitted to a different type of training, a pyramid training (VP and AP). The pyramid training consisted of only 8 reps with an overload of weight equivalent to the animal's total body mass. Each jump was considered complete if the animal had put its nose out of the water. Between series was a 30-second interval, while during the exercise the animal was alone in the water and then maintained at rest. At about 48 hours after the end of the treatment/training, the animals from all experimental groups were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and blood samples were collected

Plasma testosterone and corticosterone quantification

At about 48 hours after the last training blood samples were collected through the abdominal aorta into heparin-coated syringes (always between 8:30 and 9:30 a.m.) from

rats of the experimental groups that did not receive treatment with nandrolone decanoate. Blood was centrifuged (2,500 rpm for 20 min at 2º C) and plasma testosterone was determined by chemotropic assay, while corticosterone concentration determined by competitive immunoassay using the Coat-a-Count® Rat Corticosterone Test (Diagnostic Products Corporation, USA). The antibody used was highly specific for testosterone and the test had an analytical sensitivity of 0.10 ng/ml. The intra-assay coefficient of variation was 4.8%.

Analyses

Sperm parameters assessment

To collect semen and assess semen parameters we used the method described by Seed, et al.(11). The diffusion method was used for sperm collection (11,12). A small piece (10 mm) of the vas deferens was placed in a petri dish containing five mL Hank's balanced salt solution (HBSS) and the sperms were allowed to diffuse into the buffer. After five min, the vas deferens was removed and the suspension was gently shaken to homogenize and spread the sperms. Sperm motility was classified as: a) fast progressive (a) when sperms moved rapidly in linear directions; b) slow progressive (b) when sperms moved slowly in linear directions; c) non-progressive (c) when sperms moved in circular directions; and d) immotile (d) when sperms had neither linear nor circular movements (11). To count the sperms, we used a hemocytometer as was recommended by Seed, et al (11). Four replicate counts were used for each subject. An aliquot of sperm suspension was placed on a slide and air dried and then stained with Eosin. To assess morphology, the sperms were classified into two main categories: a)normal and b) abnormal, which included sperms with amorphous head, bicephal, fused body, bicudate and normally-shaped head with separated flagellum (11).

Statistical methods

The means + S.E.M or medians (IQ1-IQ3) had been calculated and compared previously by analysis of variance. The Tukey-Kramer test or Dunn test was employed for further comparisons between pairs of means or medians respectively, with comparisons selected between groups that presented only one difference from the three analyzed parameters. The Student´s t test was used to compare only two groups. Statistical significance level was $p<0.05$.

Results

Testosterone and Corticosterone levels

At the end of treatment/training, there was a significant alteration on the testosterone level (ng/dL), (UV 228.61±69.46, UN 3.67±0.66, TV 6.0±4.0, TN 8.48±2.96, PV 333.51±22.01, PN 3.95±0.95, $p<0.001$ between UV and PV in relation to the other groups), and on corticosterone level (ng/dL) (UV 96.05±25.61, UA 136.71±38.5, TV 80.67±9.07, TN 143.16±25.52, PV 138.69±45.04, PN 364.46±113.58, $p<0.05$ between PN in relation to the other groups) (Figure 2).

Semen characteristics

Significant changes were noticed for the following parameters that are shown in Table 2: sperm concentration, MOTILITY b and vitality.

It can be noted that the values of all parameters showed a trend toward lower values in the pyramid groups and higher for the trained vehicles and untrained anabolic, reaching statistical significance and, in some cases, even clinical significance as in the case of morphology. Thus, the sperm concentration was higher in the untrained anabolic group (80.2± 2.92/mL) followed by trained vehicle group (78.8 ± 5.47/mL) and lower in the pyramid anabolic group (58.7 ± 1.52/mL); the pyramid anabolic group also showed a

decrease in the viability parameter, whereas the trained group showed the highest value (Table 2). In addition the trained group showed a significant increase on the percentage of sperm type “b” velocity in relation to pyramid groups.

Discussion

The results showed that the training combined with the nandrolone decreased the testosterone level of the rats and increased the corticosterone level in pyramid protocol; however the pyramid training isolated increased the testosterone level significantly in relation to the traditional protocol. This finding is surprisely new, and indicates that not only the testosterone concentration is elevated directly following heavy resistance exercise (13), but also remains elevated after 48 hours.

The main finding of this study was that even a slight difference applied in the protocol of resistance training (pyramid groups) produced significant changes in the experimental animals’ sperm markers and endocrine system. Even though the study is experimental, it also corroborates with the literature which reports different sperm markers among different sports modalities (2).

Moreover, the traditional exercise group (TV) showed a trend for higher value of type “a” and “b” velocity forms (not significant). Despite the fact that type “a” in untrained groups were below 25%, they recovered their level of motility with type “b”, however pyramid groups showed significant lower levels of type “b”, thereby suggesting an impairment of motility. These results seem to agree with Arce et al. (14), who also found statistical difference for endurance training.

The adverse effect of physical stress combined with the nandrolone decanoate in the pyramid anabolic group might be the result of elevated corticosterone levels. Direct or indirect action of glucocorticoids on spermatogenesis has been observed in bulls [15], as well as blocking endogenous testosterone (T) stimulation of the seminiferous tubule resulted in retention of mature elongated spermatids, which correlated with a reduced ejaculated sperm concentration in primates (16). These observations align closely with those seen in humans (17), where it was demonstrated a reduced sperm concentration after

a few weeks of gonadotropin suppression (18) as evidence of a reduced spermiation. Because FSH and T affect spermatogenesis acting on receptors on Sertoli cells (19), a reduced spermiation resulting from withdrawal of FSH and of endogenous T stimulation of the seminiferous tubule probably should be the result of a Sertoli cell dysfunction.

In the present study we showed that T level changed, the decreased levels of T were suggestive for a reduced Leydig cell function after traditional physical exercise. A decrease of T levels were reported just after a strenuous physical exercise at high altitudes (HA) (20, 21), but not 24 hours later (21, 22), suggesting that a reduced T level is transiently observed immediately after physical exercise at HA, but a fast normalization is probably fostered by the increased level of LH. Therefore the present study demonstrates a modulation of the endocrine system after pyramid exercise, providing for the first time scientific data validating the improvement on performance by this protocol of exercise.

Unexpectedly, we showed a significant increased level of T upon only pyramid training, and this was probably related to a compensatory aspect brought by the protocol. It is interesting to note that the corticosterone level was only affected with the association of pyramid training and AAs. Therefore the high corticosterone level presented in the pyramid anabolic group was key determinant to damage some seminal parameters, which indicates that this association may have compromised the male fertility of this group. The lack of a fertility test unable us to confirm whether the animals from pyramid anabolic group had their fertility indeed compromised, even though their sperm parameters, such as concentration and viability, are significantly low and indicate to us a clear damage.

In conclusion, somehow the association of steroids with both types of resistance exercise had different response on semen parameters as well as testosterone and corticosterone levels. Our results clearly demonstrate that only the association of pyramid exercise

(strenuous training) and anabolic steroid in rats can damage semen quantity and quality, as well as testosterone and corticosterone profile. This was the first time that the pyramid training was utilized and scientific data was brought; more analyses are being conducted to shed light on this protocol of training and to explain the real role of corticosterone on seminal parameters.

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Tables

Table 1 - Training protocol

Training week(s)	Training day(s)	Training sequence	Overload in relation to body mass
1st week (Adaptation)	**1st**	3 X 5 jumps	**50%**
	2nd	3 X 7 jumps	**50%**
	3rd	3 X 9 jumps	**50%**
2nd and 3rd weeks	**4th until 9th**	4 X 10 jumps	**50%**
4th and 5th weeks	**10th until 15th**	4 X 10 jumps	**60%**
6th and 7th weeks	**16th until 21st**	4 X 10 jumps or	**70%**
		Pyramid 2 X 10 jumps	**70%**
		Pyramid 2 X 8 jumps	**100%**
8th week	**22nd until 24th**	4 X 10 jumps	**80%**

Groups	Viability (VITALITY) (%)	Normal sperm (%)	concentration (x106/ml)	% Type "a" velocity	% Type "b" velocity	% Type "c" velocity	% Type "d" velocity
UV (5)	98.8±0.49	97±0.32	76.80±6.00	11.6±2.4	32.20±3.40	31.0±2.55	25.20±3.18
UN (6)	99±0.32	97.2±0.37	80.2±2.92	11.4±1.63	33.8±2.50	24.60±1.08	28.20±1.86
TV (5)	99.4±0.25	96.4±0.81	78.8±5.47	15.4±6.69	35.2±3.87#	23.80±4.04	25.60±5.16
TN (5)	98.4±0.25	96.2±0.49	63.13±4.07	14.8±4.04	26.80±2.32	27.60±4.63	30.80±1.77
PV (5)	98.75±0.37	97.75±0.49	65.42±1.34	15.6±0.87	22.2±1.80	31.0±1.67	31.0±1.45
PN (5)	97.0±0.32**	97.25±0.19	57.58±1.52*	14±2.51	22.0±2.63	35.20±2.35	28.60±3.12

Table 2- Semen parameters from rats from the six different groups.

Note: Values given as mean± SEM

Number of animals in parentheses.

* Significant difference in relation UN group, $p<0.05$.

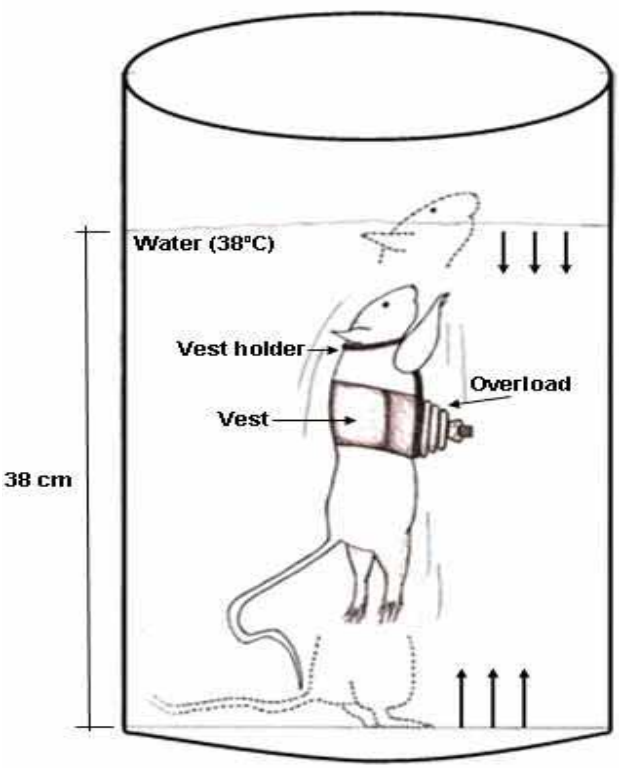
** Significant difference in relation TV group, $p<0.05$.

Significant difference in relation PV and PN group, $p<0.05$.

Figures

Figure 1 - Sketch of the resistance training apparatus.

Imaged given by Andreo Fernando Aguiar.



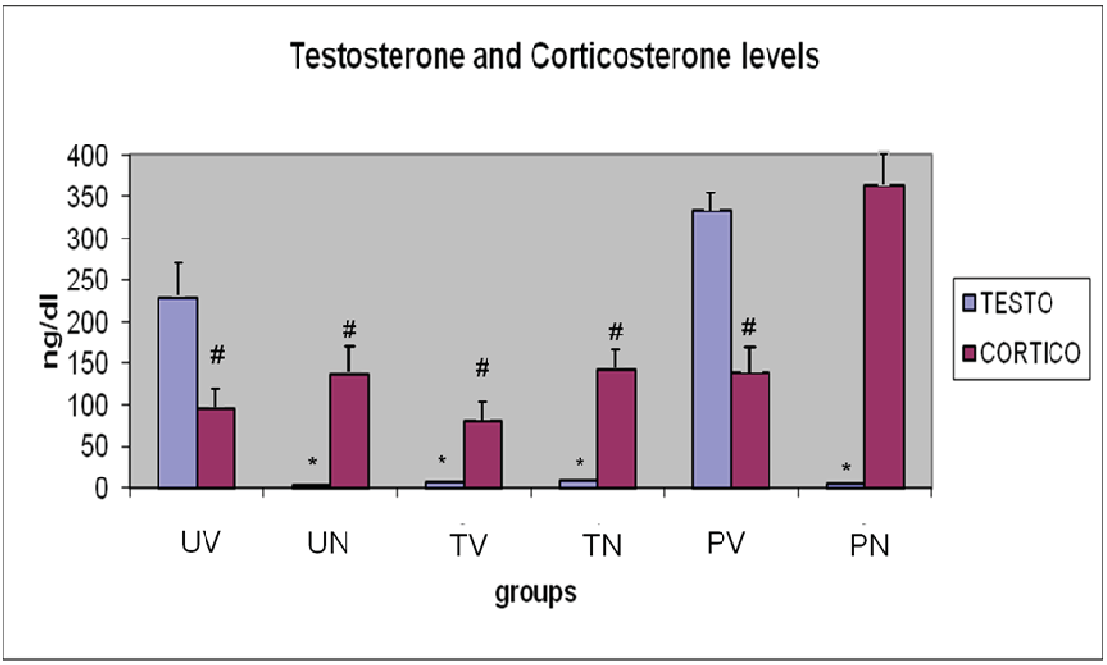


Figure 2 - Testosterone and Corticosterone levels at the end of experimental period (133 days).

Note: Column given as mean ± SEM. Number of animals per group= 6.

* Significant difference in relation to UV and PV groups, $p<0.05$.

\# Significant difference in relation to PN group, $p<0.05$.

Capítulo 03

Endocrine and bone response to resistance exercise and/or nandrolone decanoate

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We declare no conflict of interest.

Abstract
Little is known about high intensity exercise training influence and its association with steroids on endocrine system and bone formation. In addition to that, another form of resistance exercise has become popular among athletes and recreational individuals, it is called pyramid training. This training consists in increase the load and decrease the number of reps progressively, and it is believed to improve performance. We therefore conducted two protocols of training (pyramid and traditional) and treatment with androgenic anabolic steroid (AAS) in order to check the endocrine and bone response, as well as bring scientific data for the pyramid protocol. The animals received 5 mg/Kg of nandrolone decanoate or vehicle, twice a week for 8 weeks and/or were exposed to traditional resistance exercise or pyramid training. The training protocol consisted of 8 weeks of training, with progressive workload. Body weight variation, femoral mechanical test, Serum Creatine Kinase (CK) activity, Testosterone and Corticosterone plasma levels were measured. For analysis of the mechanical behavior of the bone, force was applied on the femoral head until failure. Results demonstrated that, physical exercise within AAS had significantly less body weight gain; traditional training with nandrolone improved femur resistance; CK activity was higher post-exercise in pyramid group with nandrolone; lower testosterone and higher corticosterone levels were only present in pyramid anabolic group. Interestingly, pyramid vehicle group showed the highest significant level of testosterone among the groups. Our results clearly demonstrate that only pyramid exercise in rats can modulate testosterone and corticosterone profiles, and steroid isolated treatment does not improve bone resistance. These results indicate that the intensity of the exercise ought to be carefully analyzed and taken into account when designing a training protocol; otherwise the damages for the endocrine system can be even more harmful than the use of steroids. Nonetheless, the use of steroid concomitant can also increase the risk of a negative endocrine modulation.

Key Words: Hormonal profile, resistance exercise, pyramid training; nandrolone decanoate; bone resistance.

Introduction

The use of steroid is now worldwide popular among young people who are performing a physical activity, especially in weightlifting and other sports that requires strength. The results brought by them are quick, but little is known at what point these results are better than the ones solely brought by a specific training program. In addition to that, the list of side effects is extent [1, 2] and unfortunately its use continues to grow. Besides the illicit use of these substances, there are still some clinical uses such as: hypogonadism, impotence, osteoporosis and anemia.

Resistance training programs have long been utilized as a significant component of a comprehensive fitness program for healthy adults of all ages [3]. Even though many works

describe its benefits, not much is known about some major nuances that it might present for the individual. Resistance exercise, such as weightlifting, appropriately induces muscle hypertrophy and is commonly associated with muscle damage and increased levels of serum CK in humans [4, 5, 6].

Many works from the literature [7, 8, 9, 10] have already described that physical exercise influences the endocrine system. Moreover, hormonal responses play a significant role in tissue growth as well as in the regulation of energy substrate metabolism [11] during the recovery period after a training session. In addition, training studies have also shown that acute responses of hGH or changes in resting concentrations of testosterone and cortisol, correlate well with changes in muscle size and strength [10, 12, 13].

An important factor in designing a daily or a long-term resistance-training program is the volume of exercise defined as the total amount of work performed during each session. The appropriate amount of total work would be the one that would induce the highest anabolic hormonal responses, or an optimum combination of anabolic and catabolic hormonal responses, creating the most favorable environment for neuromuscular adaptations. A simple way to modify total work is to alter the number of sets performed at each exercise. Previous research has shown that the number of sets affects hormonal concentrations. More specifically, the performance of three sets at each exercise resulted in higher testosterone, hGH, and cortisol responses as compared with the performance of one set [14, 16]. However, it is unknown whether a higher number of sets would have caused higher hormonal concentrations as well or if there is a point above which an increase in the number of sets does not induce higher hormonal responses. Similarly, it is believed that weight-bearing exercise can contribute to increased bone mass in young individuals and preservation and potentially accretion of bone in adults. Data generated from animal and theoretical work suggest that for loading to incur an increase in bone mass, the regimen must be of sufficient magnitude to exceed the minimum effective strain [16] and should be applied in an intermittent/ dynamic fashion and in a pattern that is different from ‘‘normal’’ [17].

Therefore, our laboratory tested two different protocols of resistance training programs in rats, the traditional one as described previously in the literature [18], only with a day of resting period; and an adaption of this protocol, that we called pyramid training [Tab. 1].

The Pyramid training consisted of only 2 sets of 8 reps with an overload of weight equivalent to the animal's total body mass, 100% body weight load during the 6th and 7th week. The Pyramid training is a very popular protocol among bodybuilders' training programs, and even for recreational athletes who utilize it to increment maximum strength. It is believed that reducing the number of reps and increasing the amount of load in a set, it produces greater results. However there is no scientific data to prove this hypothesis. In addition to the exercise, the influence of an anabolic androgenic steroid (nandrolone decanoate) was also tested during the 8 weeks of training (Fig.1).

The purpose of the present study was a) to check whether there were significant changes in the endocrine system in relation to the two main variables- exercise and/or AAS – among the groups and also related to the control group; b) to show the effects of steroids and/or physical exercise on bone mechanical resistance; c) to provide scientific data, confirming if pyramid training is indeed more effective as a physical exercise program, bringing the benefits for individual performance; d) to elucidate the long-term effects of both resistance training programs (after 48h from the last training session), not the acute response of testosterone and corticosterone .

Material and Methods

Animals and experimental groups

Wistar rats were kept in a controlled environment with temperature at 25+1 ºC; humidity of 55+5%; 12 h light/dark cycle (lights on at 6:00 a.m.) and had free access to regular lab chow and tap water. At PND75 the animals were treated with the anabolic steroid nandrolone decanoate (Deca-Durabolin®) 5mg/kg) or vehicle (ground-nut oil + propylene glycol), i.m., twice a week and/or submitted to high-intensity resistance exercise protocol, both for six weeks. The employed dose of nandrolone decanoate corresponded to that frequently used by athletes [19]. Animals were weighed daily to determine body weight gain. Thus, these animals were subdivided into: Untrained/Vehicle (UV), Untrained/Anabolic (UA), Trained/Vehicle (TV), Trained/Anabolic(TA), Pyramid/Vehicle (PV) and Pyramid/Anabolic (PA).

The animals used in the present study were maintained in accord with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and

approved by Institute of Biosciences /UNESP-Botucatu Ethical Committee for Animal Research (Protocol number 034/05).

Training protocol

The animals of the trained experimental groups were submitted individually to jump sessions in a PVC cylinder of 50cm in height and 25cm in diameter, containing water at 30°C to a depth of 38cm. The training protocol was according to the literature's protocol [18, 20, 21]. A weight overload was housed on the thorax of the animal. In the first week, an initial adaptation to the liquid medium was accomplished, with overload of weight equivalent to 50% of the body mass of the animal, from the 1st to the 3rd day of training. The traditional training protocol consisted of 24 jump sessions, in liquid medium with weight overload, 3 days a week, between 1 p.m. and 3 p.m., and in each session 4 series of 10 jumps were accomplished (Tab 1). On the 6^{th} and 7^{th} week of training some trained rats were submitted to a different type of training, a pyramid training (PV and PA). The pyramid training consisted of 2 sets of only 8 reps with an overload of weight equivalent to the animal's total body mass. Each jump was considered complete if the animal had put its nose out of the water. Between series was a 30-second interval, while during the exercise the animal was alone in the water and then maintained at rest. At about 48 hours after the end of the treatment/training, the animals from all experimental groups were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and blood samples were collected

Bone mechanical test

The flexion-compression tests were performed in a EMIC®-10000N universal testing machine. A load cell with maximum capacity of 50kgf was used for obtainment of the forces exerted, and the deformations were captured by the internal displacement sensors of the machine. The acrylic-bone set was fixed in a vice coupled to the base of the universal testing machine. Vertical force was applied with an accessory measuring 2 mm in diameter on the femoral head until fracture occurred. The speed of application of the force was 0.1mm/min. Force x deformation graphs were obtained from the tests, based on which it was possible to obtain stiffness and maximum force as mechanical properties.

Measurement of serum creatine kinase activity

Blood samples (0.2 ml) were obtained from the caudal vein 30 min before and 30 min after the exercise session in both the Pyramid vehicle (n=6) and Pyramid Anabolic (n=6) groups. Serum creatine kinase (CK) activity was measured using a standard kit (EnzyChromTM

Creatine Kinase Assay Kit (ECPK-100) and was used to estimate exercise-induced muscle damage. The activities were expressed in international units (IU)/ml.

Plasma testosterone and corticosterone quantification

At about 48 hours after the last training blood samples were collected through the abdominal aorta into heparin-coated syringes (always between 8:30 and 9:30 a.m.) from rats of the experimental groups that did not receive treatment with nandrolone decanoate. Blood was centrifuged (2,500 rpm for 20 min at 2º C) and plasma testosterone was determined by chemotropic assay, while corticostrone concentration determined by competitive immunoassay using the Coat-a-Count® Rat Corticosterone Test (Diagnostic Products Corporation, USA). The antibody used was highly specific for testosterone and the test had an analytical sensitivity of 0.10 ng/ml. The intra-assay coefficient of variation was 4.8%.

Statistical methods

The means + S.E.M or medians (IQ1-IQ3) had been calculated and compared previously by analysis of variance. The Tukey-Kramer test or Dunn test was employed for further comparisons between pairs of means or medians respectively, with comparisons selected between groups that presented only one difference from the two analyzed parameters. Statistical significance level was $p<0.05$.

Results

Body weights

The body mass gain at the end of the program (8 weeks) was significantly lower in both groups of resistance training within the administration of nandrolone decanoate, TN and PN groups (Fig 2). There were also alterations presented in the other groups, but they were not statistically significant.

Femur mechanical resistance

The resistance training traditional utilized (TA and TV groups) promoted an increase in maximum force (Fmax) of the femur. However only the TA group increase was statistically significant (Fig 3). As

for the maximum force deformation (DFmax) no differences were observed among the groups (Tab 2), even though TV group presented a slight increase in this index.

Serum CK activity

The mean pre-exercise serum CK levels were different between the groups, PA group level was significantly lower (Fig 4). PV group did not present any difference after exercise session. However PA group showed a significant higher value post-exercise.

Testosterone and Corticosterone levels

At the end of treatment/training, there was a significant alteration on the testosterone level (ng/dL), (UV 228.61±69.46, UA 3.67±0.66, TV 6.0±4.0, TA 8.48±2.96, PV 333.51±22.01, PA 3.95±0.95, $p<0.001$ between UV and PV in relation to the other groups), and on corticosterone level (ng/dL) (UV 96.05±25.61, UA 136.71±38.5, TV 80.67±9.07, TA 143.16±25.52, PV 138.69±45.04, PA 364.46±113.58, $p<0.05$ between PA in relation to the other groups) (Figure 5).

Discussion

This is the first study to characterize the chronic effect of two different resistance exercise programs in rodents and to give scientific data for the pyramid training. The results suggest that levels of testosterone fall dramatically after 8 weeks of resistance exercise (training program) and/or AAS treatment. However, the same result was not seen in the pyramid training solely, on the contrary. The increase in the corticosterone levels was amazingly high only in the pyramid anabolic (PA) group.

The training program within the anabolic steroid also decreased the body mass gain of the rats, in both protocols of training (Fig. 2). Whether this reduction in weight gain is due to decreased food intake [22], or increased metabolism was not determined. A pair fed study, with daily i.m. injections of Nandrolone doses, supports the hypothesis of decreased food intake [23]. Nonetheless, probably a greater fat metabolism was also caused by the physical exercise.

In the present study, the serum CK activities were the same in both pyramid groups post-exercise. However, Tamaki et al. 2001 reported that serum CK activities were lower in the

Steroid than in the Control group 30–60 min after the exercise bout, whereas the total amount of weight lifted was higher in the Steroid group. Similarly, a diminished CK response in humans using AAS has been reported after a single bout of heavy-resistance exercise [24]. Evidence suggests that AAS agents may have a membrane-stabilizing effect [25, 26] and that this may blunt the rise in serum CK efflux after muscle damage. Probably the evidence that AAS prevents rise in CK level is only based in an acute response, whereas our results are based in a chronic response to training and AAS treatment, resulting in this controversial data. Interestingly, the rise in CK activity level post-exercise in the PA group is correlated to its higher corticosterone level. Therefore this latter data suggests that although AAS is known to prevent rises in CK level, the corticosterone within the CK level from pyramid nandrolone group may indicate a higher muscle damage degree from this association, AAS and resistance exercise, in a chronic manner.

The data from mechanical femur test showed us some benefits brought only by the traditional resistance exercise protocol, however with the association of nandolone (TA) the group showed statistical significance. Furthermore it is already known that IGF-1 levels increase after physical exercise. And also, IGF-1 has been identified as an important regulator of osteoblast activity [27, 28, 29]. Consequently, higher levels of IGF-1 could further contribute to increased bone formation. Interestingly and in line with this notion, reduced bone mineral density has been observed in men with decreased IGF-1 levels [30]. Therefore we suggest that the traditional resistance exercise is key determinant to augment bone mechanical resistance via IGF-1 increase, and that nandrolone decanoate isolated does not produce the same effect, on the contrary, it may induce the opposite response. Moreover, the different responses in Fmax and Defmax presented by our two protocols of training are in agreement with what was previously described by our laboratory, Aguiar et al. [31] showed that different training modalities caused distinct results to the bone, since the chronic adaptations of the bone tissue present peculiar characteristics in response to the stimulus applied during the activity

As expected, we observed that the steroid blocked the Hypothalamic Pituitary Gonad (HPG)-axis, as reported already in the literature [32, 33, 34], and confirms that our AAS dosage was indeed effective, causing the negative feedback. In addition, the exercise within the nandrolone brought about some stress for the rats only in the PN group, increasing the

level of corticosterone. All these finding corroborate with other works [35, 36]. However, some results were surprisingly new for us. The pyramid training showed a different pattern of response in relation to the other training protocol and to the control group. Interestingly, the resistance exercise elevated the level of corticosterone due to the stress caused, and decreased the testosterone level as usually expected for some resistance exercise programs [37], but these alterations were not seen in the pyramid vehicle group. On the contrary, the resistance exercise from the pyramid protocol program elevated the testosterone and decreased the corticosterone level significantly (fig. 2). Therefore, the pyramid association with the steroid changed dramatically all the response produced by the exercise. And what is astonishing about this result is the fact that, this response was evaluated after 48 hours of the last session of exercise/treatment, indicating a tremendous modulation of endocrine system imposed by the pyramid exercise. What is also noteworthy, the steroid *per se* was as harmful for the endocrine system as the traditional resistance exercise (fig 2). This latter showed to be extremely harmful for the endocrine system, as observed in the TV group, which presented significant low testosterone level. In accordance with the latter result, our previous work Pereira et al. [38] also showed that rats submitted to the traditional resistance exercise for six weeks presented the same decrease in testosterone level, confirming that resistance exercise works as an endocrine disruptor.

Furthermore, we also observed that increasing the workload from 70% to 100% of total body mass caused higher testosterone and lower cortisol concentrations. Based on these hormonal responses, it could be hypothesized that the use of heavy workload with low number of reps (8) would be more effective for neuromuscular adaptations than the use moderate workload with high (10) number of reps.

In conclusion, the intensity of the exercise ought to be carefully analyzed and taken into account when designing a training protocol; otherwise the damages for the endocrine system can be even more harmful than the use of steroids. Nonetheless, the isolated use of steroid does not improve bone formation, on the other hand, it damages; whereas its association with physical exercise can increase the risk of a negative endocrine modulation.

The present study confirmed for the first time with scientific data the excellence of pyramid protocol, very popular among athletes, which improved the long term effect for the endocrine system in male rats; and contributed significantly as for the present risk of a inappropriate choice of training program, which can damage not just the acute response, but instead of that, a long term response brought by the stress from the physical activity, modulating negatively the testosterone and corticosterone levels.

Acknowledgments

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Figures and Tables:

Table 1 - Training protocol

Training week(s)	Training day(s)	Training sequence	Overload in relation to bod mass
1st week (Adaptation)	**1st**	3 X 5 jumps	**50%**
	2nd	3 X 7 jumps	**50%**
	3rd	3 X 9 jumps	**50%**
2nd and 3rd weeks	**4th until 9th**	4 X 10 jumps	**50%**
4th and 5th weeks	**10th until 15th**	4 X 10 jumps	**60%**
6th and 7th weeks	**16th until 21st**	4 X 10 jumps or	**70%**
		Pyramid 2 X 10 jumps	**70%**
		Pyramid 2 X 8 jumps	**100%**
8th week	**22nd until 24th**	4 X 10 jumps	**80%**

Table 2- Analysis of femoral mechanical deformation (defFmax) from experimental groups at the end of treatment/training.

Groups	UV	UA	TV	TA	PV	PA
Deformation Fmax (mm)	1,39±0,21	1,33±0,14	1,57±0,15	1,10±0,06	1,36±0,14	1,39±0,07

Values expressed as mean ±SEM.
There is no significant difference among the groups, p>0,05.
N= 8 rats per group.

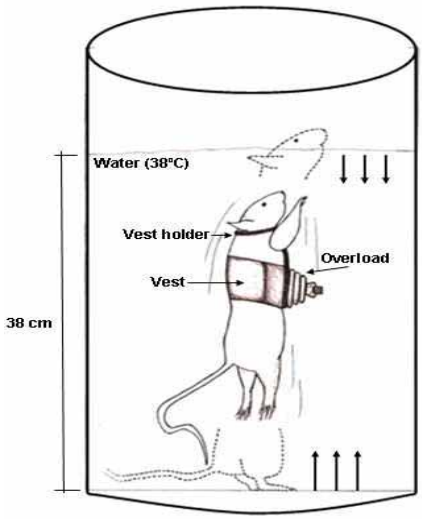

Figure 1

Sketch of the exercise performed by the animals

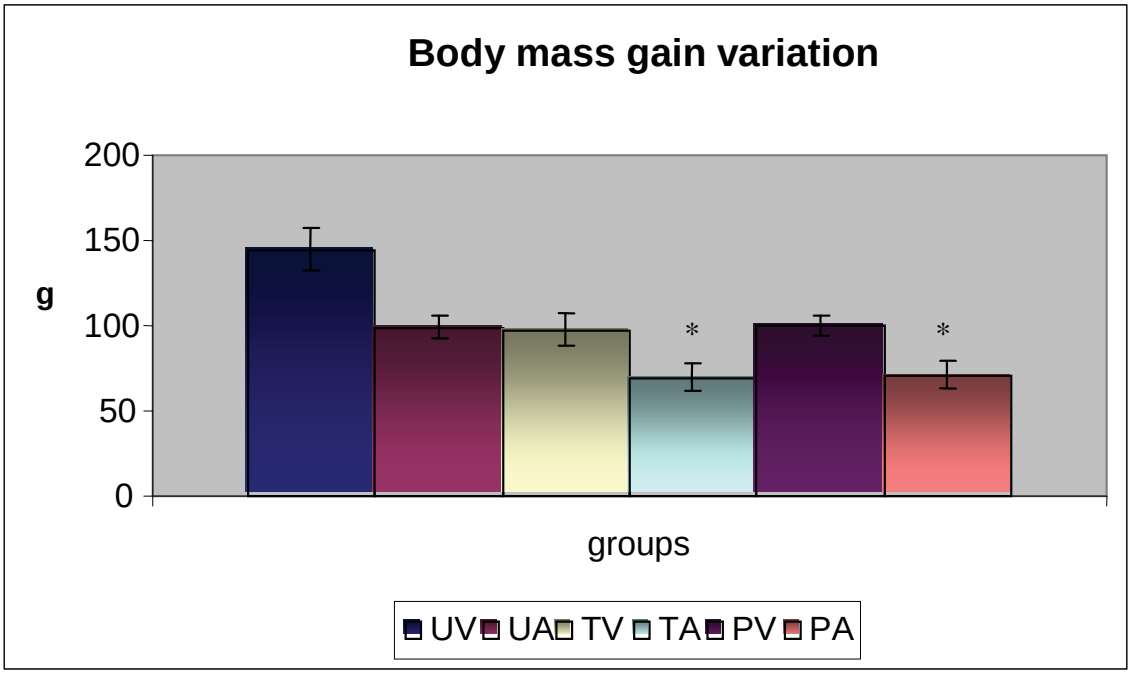

Figure 2
Body weight gain variation during experimental period (75 to 133 days old).
* different in relation to UV group, p<0.001.
N=8 rats per group.

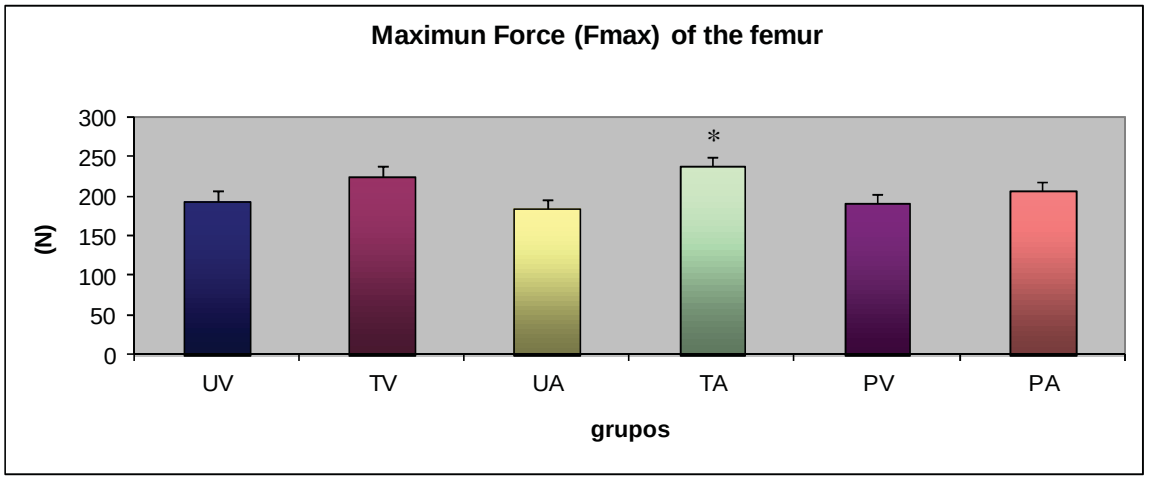

Figure 3- Maximum force (Fmax) analysis of the femur from experimental groups.

Values expressed in mean±SEM.
* $p<0.05$ compared with the UA group.
N= 8 rats per group.

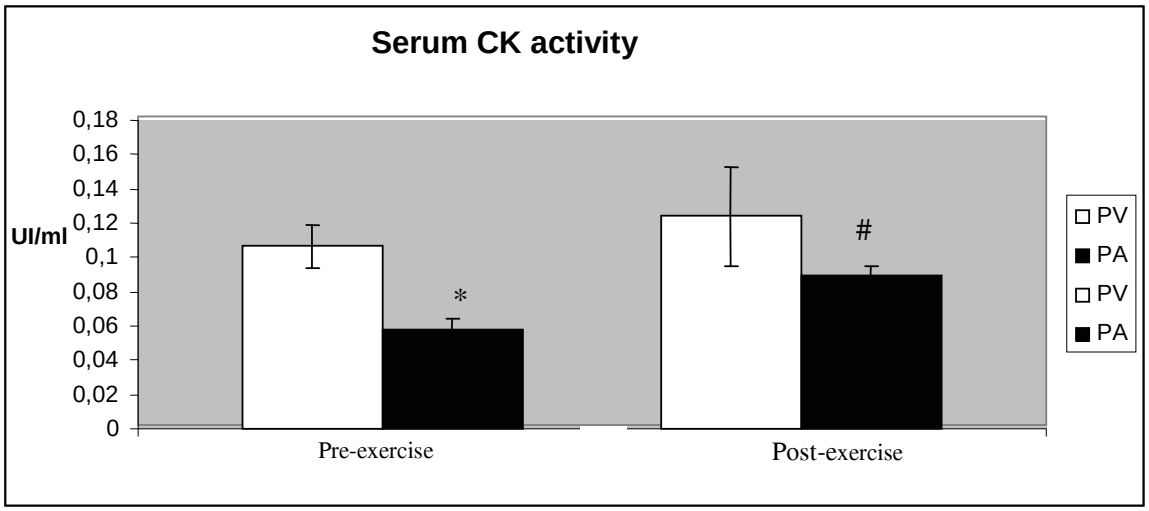

Figure 4- Mean serum creatine kinase (CK) activity at rest (pre-exercise) and 30 min after (post-exercise) pyramid training.

Values expressed in mean±SEM. IU, international unit.
N=5 rats per group;
* different in relation to PV group at the same period measure, p<0,001.
# different (increase of CK activity) in relation to PA pre-exercise level, p<0,001.

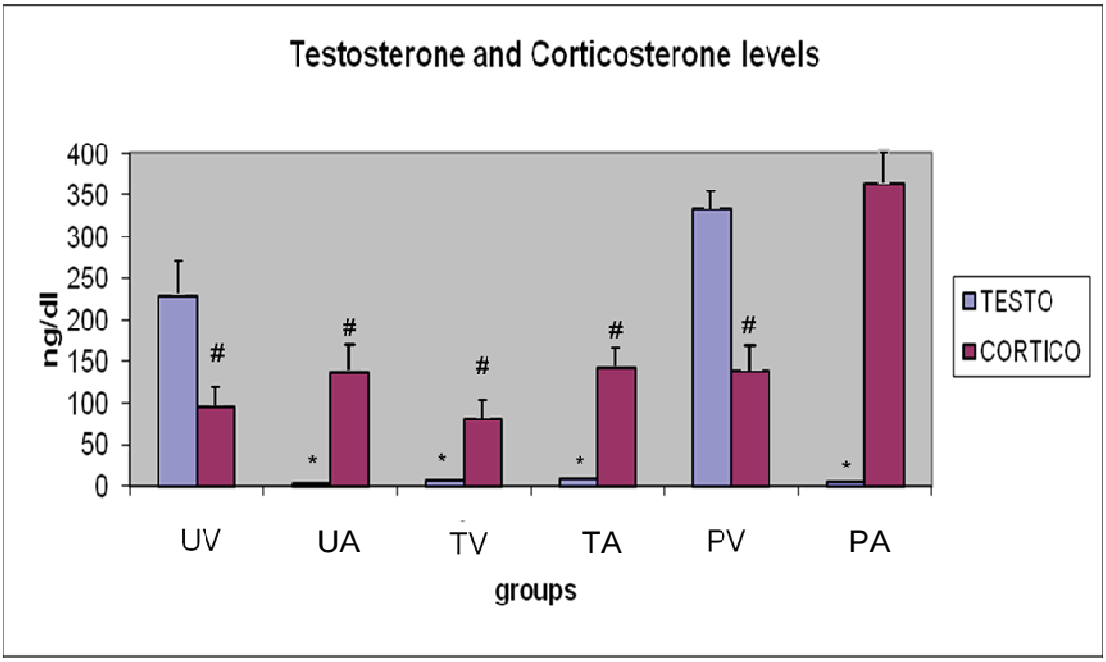

Figure 5
Testosterone and Corticosterone levels at the end of experimental period (133 days). N=7 rats per group;
* Significant difference in relation to UV and PV groups, $p<0.05$.
# Significant difference in relation to PN group, $p<0.05$.

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Capítulo 04

Effects of two protocols of resistance exercise and nandrolone decanoate on α1-adrenergic responsiveness of rat seminal vesicle

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We declare no conflict of interest.

Abstract

Resistance exercise and anabolic androgenic steroid (AAS) are often associated; however there are still several side effects especially to men that were not reported. This study aimed to investigate possible changes during two protocols of resistance exercise and nandrolone decanoate in the expression and pharmacological characteristics of α1-adrenoceptor in adult rat seminal vesicle. The animals received 5 mg/Kg of nandrolone decanoate or vehicle, twice a week for 8 weeks and/or were exposed to traditional resistance exercise or pyramid training. The training protocol consisted of 8 weeks of training, with progressive workload. The characteristics of α1-adrenoceptor in the seminal vesicle were determined using an isolated muscle bath, radioligand receptor binding and real-time reverse transcription-polymerase chain reaction techniques. Functional studies showed a lower sensitivity in the seminal vesicle contractile response from animals treated with nandrolone associated or not to resistance training, and the maximum contractile response was also altered by them. Both variables (resistance exercise/AAS) reduced the density of α1-adrenoceptor in these experimental groups, although mRNA expression of all 3 α1-adrenoceptor subtypes was not altered among them. Taken together, the results suggest that the effects of nadrolone decanoate with or without the association of resistance exercise affect contractile response of the seminal vesicle, and probably indicate an ejaculatory dysfunction in individuals who make use of them. Nevertheless, pyramid training did not show the same pattern of response from the traditional resistance exercise to norepinephrine when combined with nandrolone, indicating a protective aspect of the protocol, maintaining the sensitivity to the agonist.

1. Introduction

Resistance exercise is now in evidence by the media and became popular among those individuals who claim for physical performance. Unfortunately, this physical activity is often combined with the use of anabolic androgenic steroids (AAS) in order to obtain fast results. Moreover, altered androgenic levels have been found following administration of anabolic steroids as well as during prolonged physical activity (Opstad 1992; Hackney et al. 2003). Changes in testosterone levels can also modulate adrenergic neurotransmission in some reproductive organs, including seminal vesicle (MacDonald and McGrath 1980).

The seminal vesicle is a contractile organ that responds to α1-adrenoceptor stimulation (Porto et al., 1988). Radioligand binding assays (Shima, 1993) and functional studies, using selective α1A- and α1D- adrenoceptor antagonists (Silva et al., 1999), indicated that α1A-adrenoceptor subtype predominates in the rat seminal vesicle, although α1a-, α1b- and α1d-adrenoceptor subtypes have been detected in this tissue (Silva et al., 1999). α1-Adrenoceptors are widely distributed in all the organs participating in the emission phase of ejaculation, such as seminal vesicle and vas deferens (Markus and de Avellar, 1997; Silva et al., 1999; Mendes et al., 2004; Hisasue et al., 2006), suggesting that α1- adrenoceptors may play an important role in the emission phase.

Therefore, in this study using an integrated approach by correlating functional and contractile changes with biochemical, pharmacological and molecular events we tested the hypothesis that resistance exercise (traditional and pyramid) and/or steroid may cause alterations in the properties of α1- adrenoceptors in the rat seminal vesicle, thus, potentially affecting the function of these organs in which α1- adrenoceptors play a physiologic role.

2. Materials and methods

2.1. *Animals and experimental groups*

Wistar rats were kept in a controlled environment with temperature at 25+1 ºC; humidity of 55+5%; 12 h light/dark cycle (lights on at 6:00 a.m.) and had free access to

regular lab chow and tap water. At PND75 the animals received the anabolic steroid nandrolone decanoate (Deca-Durabolin®) 5mg/kg or vehicle (ground-nut oil + propylene glycol), i.m., twice a week and/or submitted to high-intensity resistance exercise protocol, both for eight weeks. The employed dose of nandrolone decanoate corresponded to that frequently used by athletes (Pope et al., 1988). Thus, these animals were subdivided into: Untrained/Vehicle (UV), Untrained/Anabolic (UA), Trained/Vehicle (TV), Trained/Anabolic (TA), Pyramid/Vehicle (PV) and Pyramid Anabolic (PA). At about 48 hours after the end of the treatment/training, the animals from all experimental groups were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and blood serum, the seminal vesicle was harvested. Dissected tissues were placed in ice-cold Krebs–Henseleit solution (in mM, NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, NaHCO3 24.9, KH2PO4 1.2, D-glucose 5.6, Na-pyruvate 2.0; pH 7.4 at 37 °C) and muscle strips were prepared. For biochemical and molecular studies, dissected tissues were frozen in liquid nitrogen and stored at −80 °C until assayed. Serum testosterone was determined with a chemotropic assay.

The animals used in the present study were maintained in accord with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and approved by Institute of Biosciences /UNESP-Botucatu Ethical Committee for Animal Research (Protocol number 034/05).

2.2 Training protocol

The animals of the trained experimental groups were submitted individually to jump sessions in a PVC cylinder of 50cm in height and 25cm in diameter, containing water at 30°C to a depth of 38cm. The training protocol was according to the literature's protocol (Cunha et al., 2005, Pereira et al., 2009, Aguiar et al., 2010). A weight overload was housed on the thorax of the animal. In the first week, an initial adaptation to the liquid medium was accomplished, with overload of weight equivalent to 50% of the body mass of the animal, from the 1st to the 3rd day. The traditional training protocol consisted of 24 jump sessions, in liquid medium with weight overload, 3 days a week, between 1 p.m. and 3 p.m., and in each session 4 series of 10 jumps were accomplished (Table 1). On the 6th and 7th week of training some trained rats were submitted to a different type of training, a pyramid training (PV and PA). The pyramid training consisted of only 8 reps with an overload of weight

equivalent to the animal's total body mass. Each jump was considered complete if the animal had put its nose out of the water. Between series was a 30-second interval, while during the exercise the animal was alone in the water and then maintained at rest.

2.3. Functional studies

Muscle strips were suspended in a 2 ml organ bath filled with Krebs–Henseleit solution (pH 7.4) at 37 °C and gassed with a 95% O_2 and 5% CO_2 mixture. Each strip was stretched until optimal force developed and then allowed to equilibrate for 90 min. Contractile responses to 80 mM KCl were achieved by equimolar replacement of NaCl by KCl in Krebs–Henseleit solution. Responses to 80mMKCl were assessed 2 or 3 times at 60-min intervals until responses were reproducible, and then contractile responses to norepinephrine were obtained. The contractile responses were expressed in terms of active force in g/mm^2 of cross-sectional area. Concentration–response curves to norepinephrine were obtained in a stepwise manner after the response to the previous concentration had reached a plateau. Concentration–response curves were analyzed by 4 parameters: the baseline response (bottom), the maximum response (top), the slope (Hill slope), and the drug concentration that provokes a response halfway between baseline and maximum (EC_{50}) (Yono et al., 2005). The EC_{50} value was expressed as negative logarithm (pD_2 value).

2.4. Radioligand receptor binding assay

The density of α_1-adrenoceptors was determined by saturation binding experiments of a pool of two samples using [125I]iodo-2-[β-(4-hydroxyphenyl)-ethylaminomethyl] tetralone ([125I]HEAT), as previously described (Nishi et al., 1998). Saturation binding data were analyzed using computer-assisted linear regression of bound/free vs bound to calculate the maximal number of binding sites, B_{max} values, and the equilibrium dissociation constant, *Kd* values.

2.5. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Using cDNA prepared by RT of RNA extracted from frozen tissues, the abundance of α_{1A}, α_{1B} and α_{1D}-adrenoceptor mRNA was quantified by real-time RT-PCR using SYBR Green I, as previously described (Yono et al., 2002) (see *Suplementary material). RT-PCR data were analyzed with the iCycler iQ real-time PCR detection system. In brief, an initial copy number of the target gene was calculated from its standard curve

and normalized against an initial copy number of the housekeeping gene, which was also calculated from its standard curve. The expression of *Gapdh* in mRNA was shown to be constant during development in each tissue (data not shown), suggesting that GAPDH mRNA is a suitable housekeeping gene for normalization of the target genes. Thus, the raw abundance values for target mRNA were normalized against *Gapdh* to correct for differences in RNA quantity and quality.

2.6. Data analyses

Data are presented as mean±SEM. Data sets were first compared by analysis of variance, and Tukey's test was used for post-hoc comparisons between pairs of means. The results were considered significant at $P<0.05$.

3. Results

3.1. General features

Table 2 shows rat body weight, seminal vesicle wet weight and serum testosterone level. Body weight was lower in PA group, however its seminal vesicle relative wet weight was increased as also in trained group associated with nandrolone (TA). Furthermore, pyramid vehicle (PV) and control (UV) rats showed an increase in serum testosterone.

3.2. Functional data

Maximum contractile responses to 80mMKCl expressed in g/mm^2 showed the rank order of seminal vesicle among the groups. There was no significant difference in KCl-induced maximum responses among the studied groups (Table 3).

Fig. 1 shows the concentration–response curves to norepinephrine in isolated seminal vesicle from each experimental group. Maximum contractile responses to 80mMKCl expressed in g/mm^2 presented by control group (UV) and the TA group were significant lower values in relation to the other nandrolone groups, UA and PA (Table 2). pD2 values for norepinephrine-induced contractions were significantly lower in UA and TA groups in relation to control, UV(Table 3).

3.3. Receptor binding data

Saturation binding data showed that the density of total α_1- adrenoceptors was significantly lower in all the experimental groups vs UV (control) (Fig. 2). Kd values for [125I]HEAT binding sites were similar in all groups (data not shown).

3.4. RT-PCR data

Fig. 3 shows the relative expression of α_{1A}, α_{1B} and α_{1D}-adrenoceptor mRNA normalized against *Gapdh* in isolated rat seminal vesicle. RT-PCR data indicated that all 3 α1-adrenoceptor subtypes were expressed in all groups studied. Although there was no significant treatment and/or training related difference in the expression of α_1- adrenoceptor subtype mRNA in the seminal vesicle.

4. Discussion

We provide evidence of physical training and steroid treatment dependent differences in the functional, biochemical and molecular properties of α1-adrenoceptors in the rat seminal vesicle. If similar receptor changes occur in the human seminal vesicle, men who make use of steroids and/or high resistance training may present ejaculatory dysfunction. These data may be related to ejaculatory dysfunction reported by Nojimoto et al., 2009, who observed alterations in the ejaculatory function depending on sibutramine doses. These findings support the contention that steroids and/or resistance exercise may be associated with a higher incidence of ejaculatory dysfunction in athletes, and even in recreational individuals.

As reported in a previous study from our laboratory (Pereira et al., 2009), resistance exercise promoted a decrease in body weight and the association with nandrolone augmented this response. This effect on body weight was certainly produced by an endocrine response, and as we could see testosterone levels from all groups, except pyramid vehicle, were different than control. Moreover, our binding data showed that the density of total α1- adrenoceptors was significantly lower in the all the experimental groups in relation to untrained/ vehicle group (UV). This corresponds with our functional data, which showed a reduced sensitivity in the response of the seminal vesicle to norepinephrine, although due to the number of groups, only UA and TA group were significantly different. In addition to that, Shima (1992) has already described that androgen regulates adrenergic receptor levels in membranes of the seminal vesicle, down-

regulating α1-adrenergic receptors. Greenberg et al., 1973 has also reported a decrease in the response of the seminal vesicle from testosterone-treated animals to epinephrine and norepinephrine with a decrease in the content of norepinephrine in this tissue.

The first phase of ejaculation is the emission phase, which involves secretion of seminal fluids from the accessory sex glands and contraction of the seminal tract from the epididymis to the prostate (Giuliano, 2006). Thus, it is conceivable that α1-adrenoceptor mediated contractions of the seminal vesicle may play an important role in the emission phase of ejaculation (Yono et al., 2008).

Therefore, to corroborate with results of the functional and biochemical properties from all the experimental groups, our RT-PCR data showed that all 3 α1-adrenoceptor subtypes were expressed in the rat seminal vesicle and that α1A-adrenoceptor mRNA was predominant among all experimental groups in this tissue. In the human seminal vesicle and vas deferens, the α1A-adrenoceptor is the predominant subtype expressed (Moriyama et al.,1997; Hisasue et al., 2006), and is the only α1-adrenoceptor subtype involved in the rat seminal contraction (Silva et al., 1999). However, no differences were seen in the abundance analysis from these receptors mRNA.

Contractile responses to KCl, a nonspecific contractile agent, were significantly different in UA and PA group, and although the nandrolone with or without resistance exercise decreases the sensibility to norepinephrine, it produced an increase in maximum contractile responses in the rat seminal vesicle. This result may be correlated with the fact that AAS causes hypertrophy in this organ due to its anabolic action. The lack of a normal androgen activity in this organ might explain the alterations on these groups.

In the current study, the nandrolone treatment and/or physical exercise (with exception for PV group) were related to decrease in serum testosterone and in the density of α1-adrenoceptors in the seminal vesicle adult rats. Exercise training frequently results in a decrease of serum testosterone (Eliakim and Nemet 2006, Vaamonde et al., 2006), whereas exogenous testosterone blocks basal production. Testosterone modulates α1-adrenoceptors by stimulation of transcription and/or translation resulting in new protein synthesis in genital tract smooth muscle cells (Phillippe et al., 1991). Thus, steroid and training related changes in the density of α1-adrenoceptors in these tissues might be explained, completely or in part, by the decline in serum testosterone with the blockage of HPG (hypothalamus-

pituitary- gonad) axis by nandrolone and training endocrine modulation. This finding is partly in agreement with the results from another study from our laboratory (Lydiane et al., 2008), which shows that nandrolone decanoate decreased sensitivity to norepinephrine in vas deferens, but forced swimming exercise increased the norepinephrine response pattern in this tissue. However, despite the fact that castration is associated with decreased serum testosterone, the density of α1-adrenoceptors in the rat seminal vesicle has been reported to increase with castration (Shima, 1992). Based on our data we can conclude that chronic treatment with nandrolone and/or resistance exercise provoked a down-regulation response in the density of α1- adrenoceptors in the rat seminal vesicle, suggesting that the anabolic effect of both variables (steroid and resistance exercise) might be the reason for the decreased sensitivity to norepinephrine, producing the rightward shifts of the concentration-response. These data are consistent with a previous study (Greenberg et al., 1973) showing that the norepinephrine response decreases after testosterone administration in seminal vesicle from guinea-pigs. Thus, the decreased values of pD2 in UA and TA groups in the seminal vesicle indicate an abnormal ejaculation.

Therefore we can conclude that the effects of nadrolone decanoate with or without the association of resistance exercise affect mainly α1A-adrenoceptor and probably indicate an ejaculatory dysfunction in individuals who make use of them. Nevertheless, pyramid training did not show the same pattern of response from the traditional resistance exercise to norepinephrine when combined with nandrolone, indicating a protective aspect of the protocol, maintaining the sensitivity to the agonist, even though it had altered the quantity of α_1- adrenoceptors. Furthermore, more studies are needed to explain the mechanism of how resistance exercise can compromise the organs from male reproduction and its nuances according to each protocol. These data together with the results of the present study support the effectiveness of androgens and traditional resistance training in modulating the autonomic-receptor response in smooth muscle of the reproductive tract

Acknowledgments

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Tables

Table 1 - Training protocol

Training week(s)	Training day(s)	Training sequence	Overload in relation to body mass
1st week (Adaptation)	**1st**	3 X 5 jumps	**50%**
	2nd	3 X 7 jumps	**50%**
	3rd	3 X 9 jumps	**50%**
2nd and 3rd weeks	**4th until 9th**	4 X 10 jumps	**50%**
4th and 5th weeks	**10th until 15th**	4 X 10 jumps	**60%**
6th and 7th weeks	**16th until 21st**	4 X 10 jumps or	**70%**
		Pyramid 2 X 10 jumps	**70%**
		Pyramid 2 X 8 jumps	**100%**
8th week	**22nd until 24th**	4 X 10 jumps	**80%**

Table 2

Body weight, seminal vesicle and serum testosterone of experimental animals

	Untrained		Trained		Pyramid	
	UV	**UA**	**TV**	**TA**	**PV**	**PA**
Body weight (g)	468.49±19.76	422.74±11.75	444.04±13.48	441.61±15.83	412.89±12.44	393.60±16.31*
Seminal Vesicle (VS/BWx100)	$1.36\pm9.17x10^{-2}$	$1.78\pm1.73x10^{-2}$	$1.57\pm9.09x10^{-2}$	$2.21\pm3.75x10^{-2*}$	$1.72\pm9.29x10^{-2}$	$2.14\pm1.68x10^{-2*}$
Testosterone (ng/ml)	228.61±69.46	3.67±0.66***	6.0±4.0***	8.48±2.96***	333.51±22.01	3.95±0.95***

Each value represents the mean±S.E.M. of the results from 7 rats.

* Significantly different in relation to UV.

*** Significantly different in relation to UV and PV.

Table 3

Pyramid

	Untrained		Trained			
	UV	**UA**	**TV**	**TA**	**PV**	**PA**
pD2	5.15+0.22	4.43+0.08*	4.63+0.16	4.39+0.13*	4.80+0.33	4.76+0.22
maximal contraction (g/mm2)	1.5±0.17	2.77±0.04*^	2.10±0.21	1.57±0.16	1.74±0.17	2.67±0.42*^

Each value represents the mean±SEM of the results from 3 rats.

* Significantly different in relation to UV rats.

^ Significantly different in relation to TA rats.

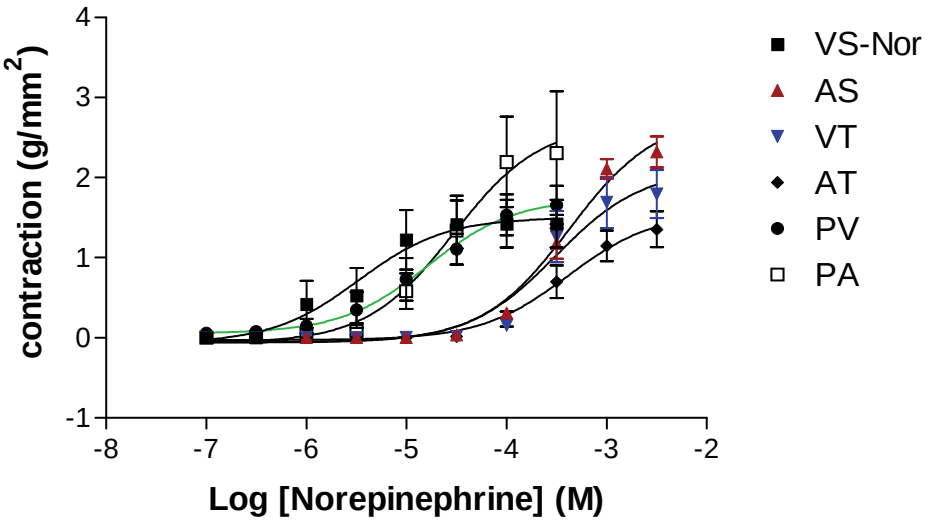

Fig.1 Concentration–response curves to norepinephrine in the rat seminal vesicle from all the 6 groups. Contractile responses are expressed in g/mm2. Each point represents the mean± S.E.M. of the results from 4 tissues.

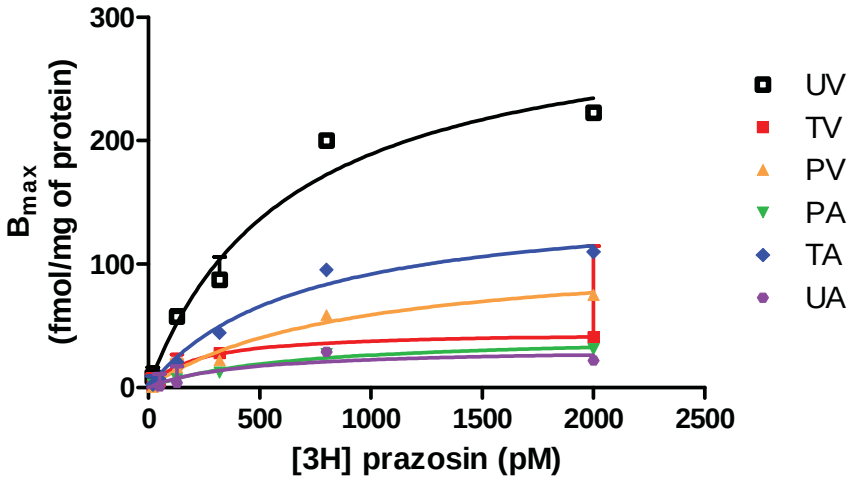

Fig 2. Saturation of [125I]HEAT binding in the rat seminal vesicle of all groups. Each point represents the mean±S.E.M. of the results from a Pool of all samples, repeated twice. Significantly different all groups in relation to UV (control).

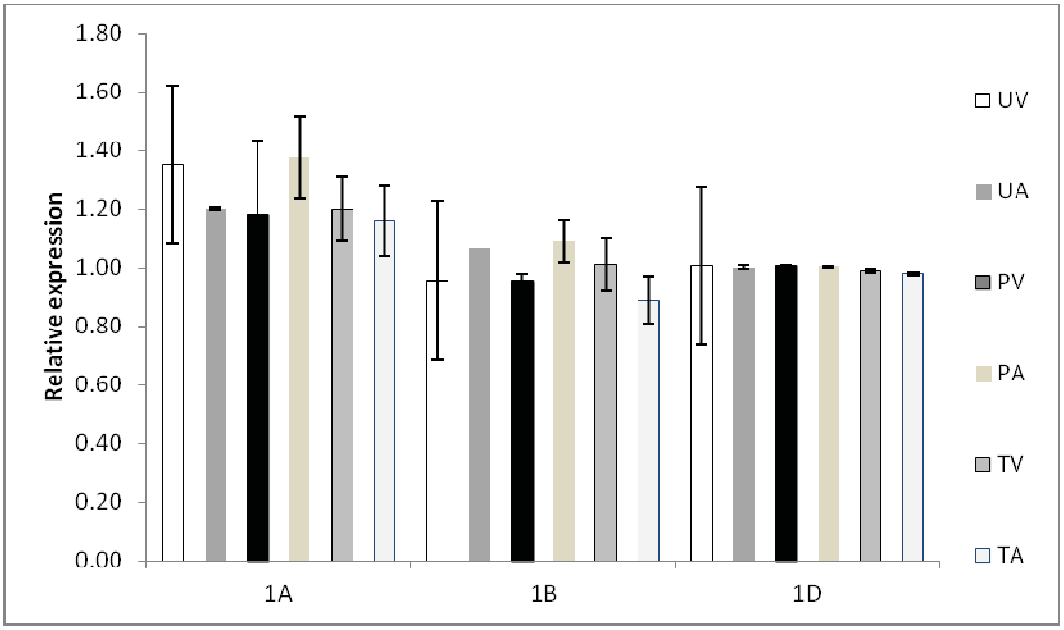

Fig. 3 Relative expression of α1A, α1B and α1D-adrenoceptor mRNA in the rat seminal vesicle, normalized against GAPDH. Each bar represents the mean±S.E.M. of the results from 3 tissues. No significant difference was observed among the groups.

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*Supplementary material

Primers used:

mRNA	Sequence (5′ → 3′)	Length (bp)	Accession no.[a]
α_{1A}-Adrenoceptor	Sense CGAGTCTACGTAGTAGCC Antisense GTCTTGGCAGCTTTCTTC	203	NM_017191
α_{1B}-Adrenoceptor	Sense ATCGTGGCCAAGAGGACC Antisense TTTGGCTGCTTTCTTTTC	201	NM_016991
α_{1D}-Adrenoceptor	Sense CGCGTGTACGTGGTCGCAC Antisense CTTGGCAGCCTTTTTC	219	NM_024483
Gapdh	Sense CTGACATGCCGCCTGGAGA Antisense ATGTAGGCCATGAGGTCCAC	260	AJ297631

[a]Genbank accession number of cDNA and corresponding gene, available at http:// www.ncbi.nlm.nih.gov/.

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Conclusão

VI. Conclusão:

- **Aspectos sociais:**

- O anabolizante esteróide, decanoato de nandrolona, levou ao aumento de agressividade tanto de ratos machos adultos, quanto de camundongos. Porém a latência deste comportamento agressivo é curta, sendo que após um determinado momento do isolamento ambos os animais, controle e tratado não apresentam mais diferenças entre si.
- A Reelina mostrou pela primeira vez seu papel no controle do comportamento agressivo, sendo que após uma hora de sua infusão (0.01 ng/nl) em camundongos agressivos tratados com esteróide, estes não apresentaram mais o comportamento agressivo. Porém seu efeito no controle da agressividade tem uma duração curta, somente de um dia. A confirmação da importância desta proteína foi feita através da quantificação por western blot, aonde vimos seus níveis reduzidos em determinadas regiões do cérebro de camundongos tratados com esteróide (proprionato de testosterona), como no hipocampo, e também através do uso de seu antagonista, a Rapamycin, no qual após a sua infusão (0.01ng/nl), pode-se ver o bloqueio dos efeitos da Reelina no grupo anabolizante, tratado com ambos (por infusão). Estes continuaram a apresentar o mesmo número e tempo de agressões, por bloqueio do efeito controlador da Reelina sobre agressividade.

- Aspectos reprodutivos:

-A associação pirâmide/anabolizante mostrou ser a que mais acarretou prejuízos a fertilidade, isto provavelmente tenha ocorrido devido a este grupo apresentar o nível mais alto de corticosterona plasmática e baixo nível de testosterona. Este estresse causado pela associação diminuiu a concentração espermática e viabilidade, o que provavelmente tenha causado uma infertilidade no grupo. Por outro lado, somente a variável pirâmide, não apresentou os prejuízos para a reprodução, pelo contrário, o presente protocolo de exercício agiu como um modulador endócrino, enquanto que o anabolizante agiu como um desregulador.

-A modulação negativa dos adrenoceptores alfa-1 na vesícula seminal ocorreu em todos os grupos tanto treinados e/ou tratados em relação ao grupo controle, porém a resposta contrátil à noradrenalina só foi afetada em dois grupos: anabolizante/não treinado e anabolizante/treinado, os quais tiveram valores reduzidos de pD2. Pode-se concluir que o anabolizante, assim como a sua associação (ao exercício tradicional) comprometeram a resposta contrátil da vesícula seminal, e possivelmente podem ser causadores de problemas ejaculatórios em indivíduos que fazem uso crônico dos anabolizantes ou de sua associação ao exercício.

- Muito embora o presente estudo tenha mostrado uma ausência de alteração na expressão de mRNA dos adrenoceptores alfa-1, os resultados dos estudos funcionais e de *binding* confirmaram esta alteração e corroboram com a literatura, mostrando que muitas vezes o nível de mRNA de receptores em um determinado tecido não corresponde com os níveis de proteína deste receptor.

- Aspectos ósseos

-O treino tradicional em associação com o anabolizante esteróide mostrou-se eficaz em aumentar a resistência do osso, ou seja, a força mecânica máxima (Fmáx) no grupo TA. Enquanto que do emprego do anabolizante isolado observou-se não ter efeito no fortalecimento ósseo, pelo contrário, até reduziu (porém não significativamente) a resistência do osso, mostrando que a sua ação está intrinsecamente relacionada ao estresse causado pela atividade física. Logo, pode-se sugerir que seu uso na terapêutica para obtenção de uma eficácia no fortalecimento ósseo deve estar sempre condicionado a um estresse físico.

- O treino tradicional modulou a estrutura óssea de forma a garantir uma melhor rigidez, porém diminuindo, apesar de não significantemente sua elasticidade. Pode-se supor que a estrutura óssea sofreu uma maior calcificação de trabéculas e muito de seu colágeno pode ter sido trocado. Mais estudos devem ser feitos para dar suporte a esta especulação.

- Conclui-se que somente o treino tradicional pareceu modular a estrutura óssea dos animais, tornando a mais resistente, provavelmente atuando na via IGF-1. E novamente podemos ver que o anabolizante isolado não apresenta qualquer efeito ergogênico para a estrutura óssea, pelo contrário, mostrou ter novamente um papel de desregulador endócrino.

- Creatina Kinase

-A atividade da Cretina Kinase foi significantemente maior no período de descanso nos animais pirâmides veículos em relação aos tratados com anabolizante. Interessantemente o tratamento com anabolizante influenciou a atividade da Creatina Kinase, reduzindo-a durante o repouso e aumentando significantemente sua atividade após trinta minutos do exercício. Sugere-se que o aumento observado no grupo pirâmide/anabolizante esteja relacionado ao estresse extenuante visto somente neste grupo (os níveis hormonais de testosterona e corticosterona confirmaram isto), e fora ocasionado da associação do anabolizante ao exercício pirâmide, provavelmente pelo bloqueio do eixo HPG (hipotálamo-pituitária-gonada).

Conclusões Gerais

Assim, os resultados deste estudo nos levam a ressaltar os benefícios que a atividade física pode trazer a um individuo como a perda de peso e a manutenção no nível de testosterona. E ainda, salientar a importância na escolha do tipo de atividade física, como observado neste estudo com o treinamento pirâmide. Portanto, sendo aplicada de maneira correta, a atividade física pode trazer benefícios para o individuo.

Podemos observar que a associação do anabolizante com o exercício pirâmide acarretou muitos prejuízos tanto a níveis hormonais, sociais, como em parâmetros seminais destes ratos, uma vez que com a associação temos a somatória de dois estresses causados ao individuo, um deles o físico, e o outro endócrino, sendo este último ocasionado pelo anabolizante.

Pode-se dizer que muitos mitos a cerca dos “efeitos milagrosos” do esteróide como ergogênico e incrementador de desempenho físico são falsos. Por outro lado a atividade física *per se* mostrou ser um ótimo “ergogênico”, aumentando o desempenho, potência física e funcionando como o regulador endócrino ideal para o indivíduo.

E para finalizar, os resultados obtidos nos grupos pirâmides são surpreendentes e inéditos, permitindo propor o presente protocolo de exercício pirâmide como um modulador endócrino e ótimo incremento ao vigor físico.

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Apêndice

INFLUÊNCIA DE ESTERÓIDES SOBRE ASPECTOS SOCIAIS E AOS NÍVEIS DE REELIN EM CAMUNDONGOS MACHOS.

Cronograma 2011 – UIC/Chicago (Projeto Capes PDEE)

Período de estágio: 4 meses
Setembro-

- Três primeiras semanas adaptação, reconhecimento do laboratório, treinamento das técnicas;
- Quarta semana: Inicio do tratamento dos animais com AAS (esteróide)

Outubro-

- Análise de comportamento dos animais tratados;

Novembro-

- Mensuração dos níveis de Reelin na região do hipocampo e córtex pré-frontal;

Dezembro-

- Análise dos resultados obtidos e correlação com os resultados no próprio projeto de doutorado (já analisados);

Background:

Reelin, intensively studied as an extracellular protein that regulates brain development, is also expressed in a variety of tissues [1,2]. Reelin has been implicated in at least 3 different human disorders: a) Humans with nonfunctional reelin genes exhibit lissencephaly and profound mental retardation [3]. b) Postmortem brains of patients with psychosis (schizophrenia and bipolar disorder) exhibit reelin mRNA and protein levels reduced by ~50% in every brain region examined [4,5]. In fact, the reelin deficit is the best replicated post-mortem neurochemical finding documented in schizophrenia [6]. c) Reelin is a candidate gene for autism based on its expression in brain regions affected in autism, its chromosomal localization, and its large size and complex regulation [7]. Fatemi et al. (2002) recently reported that plasma levels of reelin are reduced in both autistic patients and unaffected family members relative to unrelated adult controls [8].

In order to examine a possible role for Reelin, as an anti-aggressive, we isolated male adult mice, treated a group with Steroid, and checked for their aggressiviness. After 3

weeks, they were able to receive Reelin administration via cannula direct in the brain, and their behavior was monitored to confirm its effects.

Materials and method

Animals and tissue preparation

Adult male Swisse Webster mice (Harlan Breeders) (18-20 g body weight) maintained under a 12 h dark/light cycle with food and water ad libitum were used for all experiments. Mice were housed individually (socially isolated) in a cage of the same size for a time periods of four weeks preceding surgery, followed by one week of recovery before behavioral and biochemical studies (Pinna et al., 2003). The vivarium temperature was kept near 24 °C and the humidity near 65%. In a first series of experiments, 6 groups of rats (n=6 per group) were injected s.c. once a day for 28 days with vehicle (sesame oil) or testosterone propionate. These groups of animals were used for measurements of Reelin levels in the hippocampus and prefrontal cortex. Six additional groups of rats (n=6 per group) were treated once a day for 28 days with sesame oil, s.c. or testosterone propionate. These animals were used for behavioral test. All of the animal procedures used in our research were approved by the University of Illinois at Chicago Animal Care Committee.

Microinjections of Rapamycin and Reelin

Using a stereotaxic apparatus (Kopf Instruments, Tujunga, CA), mice were bilaterally implanted with 7 mm stainless-steel guide cannulae (26G; Plastics One) under sodium pentobarbital (50 mg/kg i.p.) anesthesia. Guides were directed toward the BLA (from bregma, AP - 1.4 mm; ML ± 3.1 mm; DV - 3.7 mm) or the striatum (from bregma AP + 1.3 mm; ML ± 1.8 mm; DV - 2.0 mm) (Franklin and Paxinos, 1997). The cannulae were affixed to the skull with dental acrylic and jeweler's screws. After 30 days of surgery, the mice received daily oil, or Testosterone Propionate (5 mg/kg), s.c., for 28 days. After that, they received infusion either Reelin (0.1 nmol/ul) or Rapamycin (0.1 nmol/ul) an antagonist of Reelin + Reelin (Aldrich) via a 33G stainless steel cannula attached to a 10 ml Hamilton syringe via FEP-tubing (CMA/Microdialysis AB, Stockholm, Sweden), which extended 1.0 mm beyond the tip of the guide cannula. Drug administration was controlled by a microinjection pump (CMA/100) that delivered 1 ml to the injection site at a rate of

0.2 ml/min. The internal cannula was left in place for 1 min after the end of infusion. The behavioral test was run 60 min after infusion.

Behavioral testing

Residente-intruder test

To test the aggressive behavior of resident male socially isolated mice, an intruder mouse of the same gender was placed in a resident home cage (24x17x12) and residente-intruder interactions were videotaped for 10 min. The aggressive behavior of resident socially isolated mice was characterized by an initial pattern of exploratory activity around the intruder, which was followed by rearing and tail rattle, accompanied in few seconds by wrestling and/or a violent biting attack (Pinna et al., 2003, 2005). The number of these attacks and/or amount of wrestling during the 10 min observation period was recorded.

Antibodies

The following primary antibodies were used for immunocytochemical studies and Western blot analyses: mouse monoclonal anti-Reelin G10 (dilution 1:1000; Millipore), rabbit polyclonal anti-βactin (1:3000, Sigma), anti-Mouse IgG (whole molecule)–Peroxidase antibody produced in goat (1: 3000; Sigma).

Preparation of protein extracts

Tissue pellets (-80°C) were incubated in ice-cold hypertonic lysis buffer [pH 7.6, 50 mM Tris-HCl, 150 mM NaCl, 5mM EDTA-Na2, 1%(v/v) Nonidet P-40, 1% Triton X-100, 0.5% (w/v) SDS, 0.25% (w/v) sodium deoxycholate] with 1% protease inhibitor (Sigma), and phosphatase inhibitor cocktails (Sigma). The tissue was lysed by repeated freezing in liquid nitrogen and thawing at 37°C (5 times). After the tissue was triturated with a pipette tip to homogenize larger pieces of tissue and sonicated for 5 min, the suspension was centrifuged at 20,000x g at 4°C for 30 min. The resulting crude supernatants were taken, and protein concentration was measured by using BCA Protein Assay Kit (Pierce). Aliquots of the supernatants were immediately used for Western blotting or were stored at -80°C until use.

Western blot analysis

Five to ten micrograms of each sample were diluted in ultrapure water containing 1x NuPAGE sample buffer (Invitrogen) and 1x NuPAGE reducing agent (Invitrogen) to

final volumes of 24 _l, boiled at 95°C for 5 min, and then immediately placed on ice. The samples were separated by NuPAGE Bis-Tris, pH 7.0, 4 – 12% PAGE (Invitrogen) with 1_ NuPAGE MES-SDS running buffer (Invitrogen) and transferred electrophoretically to Nytrocellulose membranes with 1xNuPAGE transfer buffer (Invitrogen). For blotting, membranes were blocked with 5% (w/v) nonfat milk in Tris-buffered saline (TBS) at room temperature (RT) for 20 min and finally incubated with primary antibodies in blocking solution at 4°C overnight. This was followed by detection with an alkaline phosphatase-conjugated secondary antibody (Western Breeze Chemiluminescent Immunodetection Kit, Invitrogen). The immunoreaction was visualized by enhanced chemiluminiscence. At least three Western blots were analyzed for each experiment.

Statistical analyses

Data are given as means ± SEMs unless otherwise indicated. Comparisons between the control group and each of the treatment groups were performed by one-way ANOVA followed by Dunnett's test, as indicated in the figure legend.

Results:

Reelin levels in hippocampus were significantly lower in Steroid group in relation to Control (Figure 01) after 3 weeks of treatment. No differences were observed in prefrontal cortex.

We can see on the first day before infusion, that TP animals are more aggressive than control (Figure 02). Aggression test in Day 1 (a) showed that after the Reelin infusion, TP+Reelin group reduced the time of aggression comparing their behavior before the infusion on the same day, although the effect of the protein only lasted for one day. Rapamycin+Reelin group continued presenting aggressive behavior, confirming that Rapamycin works as an antagonist, blocking the receptor of Reelin.

Conclusions:

We have shown that repeated injections of AASs at doses considered equivalent to those abused by humans induce aggressive behavior in isolated mice after 3 weeks. Based on our preliminary data, we could observe the importance of Reelin also working in anti-aggressive manner on mice treated with AAS, and also, this latter decreased Reelin level in hippocampus, indicating that it might be the trigger for the aggressive behavior.

Noteworthy, after infusion of Reelin, its anti-aggressive effects only last for one day, probably because this protein is fast metabolized. This was the first time that Reelin was tested as an anti-aggressive, and the preliminary results are very promising for therapy. More studies are being conducted to check the long term effects and the mechanism of action from this protein.

Figures

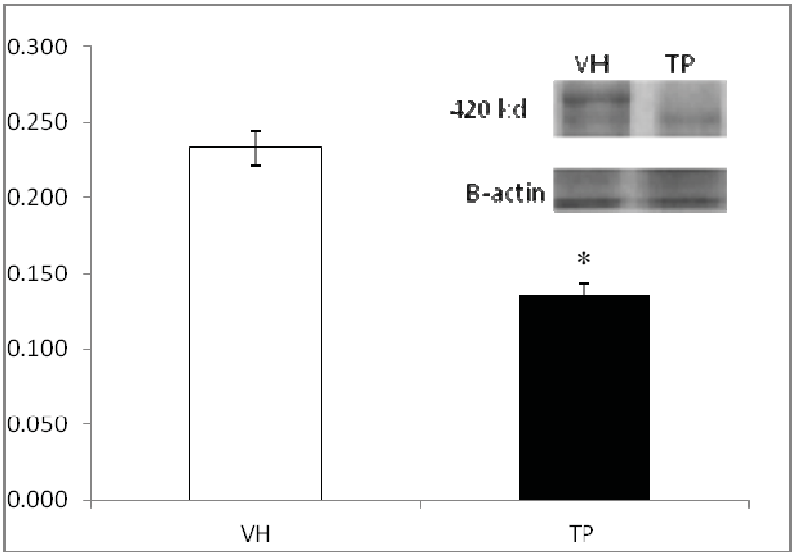

Figure 01- Densitometric analysis of reelin in tissue from control mice and treated with Testosterone Proprionate (TP); TP treatment down-regulates reelin in mice compared with untreated control tissue. β-actin was used as loading control.

*Statistically significant difference is indicated (unpaired *t* test, **p*_0.05)

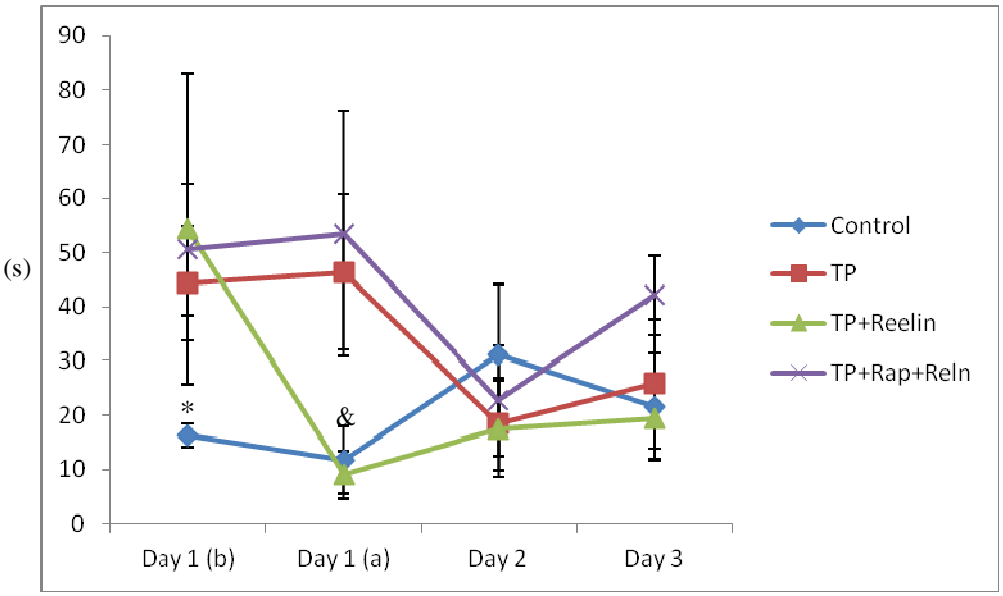

Figure 2- Aggressive behavior performed after 4 weeks of Testosterone Proprionate (TP) treatment, followed by microinfusions of Reelin (Reln) or Rapamycin (Rap) + Reln in socially isolated male mice that have been implanted bilaterally with guide cannulae into the BLA or striatum. Each value is the mean ±SEM of 4-6 mice. Drugs were microinfused 45±60 min before the test. *, &, $P < 0.05$; when control mice were compared with TP+Reelin; and & when TP+Reln was compared before and after infusion in Day 1, one-way ANOVA followed by Dunnett's test.

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Anexos