



**UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE
MESQUITA FILHO”**

FACULDADE DE MEDICINA

Carina Guidi Pinto

**Associação da suplementação de vitamina D₃ e do
alcoolismo experimental em ratos: efeitos morfológicos e
comportamentais**

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Câmpus de Botucatu, para obtenção do título de Mestre em Bases Gerais da Cirurgia.

Orientadora: Profa. Dra. Selma Maria Michelin Matheus

Botucatu

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(Salmos 23: 1-3)

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Lista de Abreviaturas

- 1,25(OH)₂D₃ - *Vitamin D3*/ Vitamina D₃ ativa
- 25(OH)D₃ - *Vitamin D3*/ Vitamina D₃ circulante
- Acb - *Nucleus Acumbens*/ Núcleo Acumbens
- ASR- *Acoustic Startle Reflex*/ Reflexo acústico de sobressalto
- Ca²⁺ - *Calcium*/Cálcio
- CAT – *Catalase*/ Catalase
- CNS - *Central Nervous System*/ Sistema Nervoso Central
- EDL – *Muscle extensor digitorum longus*/Músculo extensor longo dos dedos
- EROS - Espécies Reativas de Oxigênio
- GABA - *Gamma-aminobutyric acid*/ Ácido gama-aminobutírico
- GSH - *Reduced Glutathione*/Glutathione reduzida
- HE – Hematoxilina - Eosina
- O²⁻ - *Lucigenin*/ Lucigenina-Ânion superóxido
- OMS – Organização Mundial da Saúde
- P - *Phosphorus*/Fósforo
- PPI - *Evaluate Prepulse Inhibition*/ Inibição por estímulo prévio
- PTH – *Parathormone*/Paratormônio
- ROS – *Reactive Oxygen Species*
- SOD - *Superoxide Dismutase*/Superóxido Dismutase
- TBARS - *Thiobarbiturate Acid*/ Ácido Tiobarbitúrico
- UCh - *Chile University*/Universidade do Chile
- VDR - *Vitamin D₃ receptor*/ Receptores para Vitamina D₃
- VTA - *Ventral Tegmental Area*/ Área tegmental Ventral

Introdução

O uso de substâncias psicoativas é tão antigo quanto à humanidade. Entre elas, destacam-se as bebidas alcoólicas. O consumo de etanol e problemas relacionados a ele afetam amplamente todo o mundo, sendo um grande problema de saúde pública. E é considerado o terceiro maior fator de risco para doenças e incapacidades no mundo (Who, 2011).

O alcoolismo é definido pela Organização Mundial da Saúde (OMS) como uma doença que não constitui uma entidade nosológica definida, mas a totalidade dos problemas motivados pelo etanol, no indivíduo, vem causando perturbações da vida familiar, profissional e social, com repercussões econômicas, legais e morais (Mello et al., 2001). É considerada uma condição que se adquire pela repetida exposição ao etanol: quanto maior o consumo, maior o risco; tendo então diferentes causas entre elas a vulnerabilidade genética (Babor, 2009).

Em nosso país são observados mais dependentes de etanol do gênero masculino (Abreu et al., 2006; Mascarenhas et al., 2009), em número cerca de três vezes maior que do gênero feminino (Carlini et al., 2002), sendo em sua maioria jovens entre 21 a 30 anos de idade, com nível médio de escolaridade (Duailibi et al., 2007).

O uso indiscriminado do etanol está ligado a mais de 60 diferentes tipos de doenças e dados demográficos, também sugerem que aumento da compulsão por álcool está associado com uma razão de probabilidade mais elevada de mortalidade. O número de incapacitados provenientes do consumo de etanol é equivalente à soma dos casos de morte ou de doenças provocados pela hipertensão arterial e pelo fumo (Room et al., 2005; Courtne & Polich, 2009; Holahan et al., 2014).

O etanol é considerado uma substância tóxica, uma vez que tem efeitos diretos e indiretos sobre vários órgãos e sistemas (Babor, 2009; Jayasekara et al., 2014). O metabolismo do álcool resulta na formação de acetaldeído e de espécies reativas de oxigênio que danificam o tecido saudável (Jung et al., 2011).

No corpo humano o etanol alcança rapidamente a circulação sanguínea e os demais tecidos devido a sua alta lipossolubilidade, sendo que não há limites para a sua passagem pela barreira hematoencefálica, ativando mecanismos de lesão que poderão causar diferentes patologias. Alcançando livremente o sistema nervoso, que mesmo em baixas doses, causa a diminuição da coordenação motora e interferências na absorção e metabolismo dos nutrientes essenciais, minerais e

vitaminas A, B, C, D e E, assim podendo resultar em uma perda de massa corporal (Lieber, 2003; Burke et al., 2003; Breslow et al., 2006; Fernandes et al., 2010, Arceles et al., 2011).

Após a ingestão, os órgãos que acumulam maiores concentrações de etanol são: sangue, cérebro, rins, pulmões, coração, parede intestinal, fígado e músculo esquelético (Steiner & Lang, 2015).

Nas primeiras 3 horas após a administração de etanol foi observado nível significativamente superior de carbonos derivados de etanol no músculo esquelético quando comparado a outros tecidos, demonstrando que esse tecido está diretamente relacionado com seu metabolismo, atuando possivelmente na sua oxidação final (Cornier et al., 2000).

A sua ingestão crônica leva à alterações musculares severas (Lynch, 1969; Rubin et al., 1976; Levy et al., 1986; Levy, 1991; Torrejais et al., 2002). Características comuns observadas em alcoólicos crônicos que inclui fraqueza e dificuldades na marcha, com sintomas de redução da força muscular esquelética e perda de até 30% da massa muscular esquelética total (Wang et al., 2012; Jung et al., 2011).

Ekbohm et al. (1964) foram os primeiros a chamar a atenção para uma doença muscular crônica caracterizada por fraqueza e atrofia proximais que surgia como complicação do uso prolongado de etanol. Essa doença é comumente chamada miopatia alcoólica (Preedy et al., 2003).

A miopatia alcoólica crônica está presente em até 70% dos alcoólicos, sendo caracterizada histologicamente pela atrofia seletiva das fibras tipo II (glicolítica, contração rápida), especialmente fibras do tipo IIx enquanto que as fibras do tipo I geralmente não são afetadas (Preedy & Peters, 1990; Reilly et al., 2000). Além disso, aumento da apoptose das fibras musculares tem sido observado em alcoólatras (Fernández-Sola et al., 2003).

Alterações ultra-estruturais incluem edema intracelular, mitocôndrias alargadas e distorcidas, formação de “*type-grouping*” (grupamentos alterando a forma de mosaico das fibras musculares), dilatação do retículo sarcoplasmático e vacúolos, proliferação de tecido conjuntivo e infiltrado inflamatório, podendo ocorrer necrose (Levy, 1991; Sestoft et al., 1994; Koll et al., 2002; Adachi et al., 2003; Pereira & Conegero 2004; Ertem et al., 2009).

Vários mecanismos podem estar envolvidos na patogênese da miopatia alcoólica. Dados clínicos e experimentais justificam, que a desnutrição provavelmente contribui para a miopatia alcoólica crônica (Conde et al., 1992; Romero et al., 1994, Nicolas et al., 2003; Castellón et al., 2005). É mais comum do que outras doenças induzidas pelo álcool, como cirrose (15-20% dos alcoólatras crônicos), neuropatia periférica (15-20%), doença intestinal (30 -50%) ou cardiomiopatia (15-35%) (Preedy et al., 2003; Wang et al., 2012).

Além disso, em pacientes alcoólicos crônicos é observado neuropatia periférica, que é uma complicação potencialmente incapacitante, caracterizada por dor e disestesias (enfraquecimento ou alteração na sensibilidade dos sentidos) principalmente nas extremidades inferiores, e é pouco aliviadas por terapias disponíveis (Ratcliff, 1979; Koike et al., 2003). Com isso constantemente levam a dificuldade em caminhar e alterações na coordenação de movimentos (Juntunen et al, 1978; Hodges et al., 1986).

Os pacientes que apresentam a neuropatia alcoólica periférica, apresentam degeneração axonal das fibras nervosas sensoriais e motoras, no entanto, com maior envolvimento dos nervos sensoriais presentes nos membros inferiores e, redução na mielinização dessas fibras neurais (Ammendola et al., 2001).

Indivíduos que fazem o uso abusivo de etanol também tendem a consumir pequenas quantidades de nutrientes essenciais e vitaminas, e/ou possuem a absorção gastrointestinal prejudicada devido aos efeitos diretos do etanol (Mezey, 1980; Ryle & Thomson, 1984).

A etiologia da neuropatia induzida pelo etanol vem sendo debatida por mais de um século e, atualmente, considera-se que ela esteja associada a vários fatores de risco, tais como predisposição genética, má nutrição, doenças sistêmicas, deficiência de tiamina (vitamina B1) ou toxicidade dos metabólitos do etanol, como o acetaldeído que aumenta a concentração de espécies reativas de oxigênio (ERO), e histórico familiar de etilismo. Porém, ainda não está claro qual desses fatores possui papel fundamental na indução desta patologia (Mellion et al., 2010).

O alcoolismo crônico também tem influência negativa na regeneração do nervo periférico, com uma diminuição significativa no número de axônios e aumento da degeneração axonal (Aminoff, 2007; Ertem et al., 2009; Haes et al., 2010).

O alcoolismo, em seu consumo crônico, pode levar também a tendência de desequilíbrio alimentar. Estas relações podem fazer com que o alcoolismo crônico

seja um fator de risco para a miopatia e neuropatia decorrente da deficiência de vitaminas (Koike et al., 2003).

Devido ao consumo de etanol, o qual é uma substância tóxica, ocorre o aparecimento dos radicais livres ou espécies reativas de oxigênio (EROS), que contribuem para o aparecimento e estão presentes em diversas doenças, levando ao estresse oxidativo que é definido como um distúrbio no estado de equilíbrio, no sistema de pró-oxidantes e antioxidantes, nas células intactas (Adachi et al., 2000; Kotidis et al., 2012; Fernandez-Sola et al., 2007).

Vários estudos concluíram que o aumento dos níveis pró-oxidantes no músculo esquelético e reduções da capacidade antioxidante podem exacerbar os sintomas relacionados com a miopatia alcoólica (Fernandez-Sola et al, 2002; Adachi et al, 2000; Koo-Ng et al, 2000; Mansouri et al, 2001; Preedy et al., 2001).

O etanol é um potente inibidor da síntese de proteínas musculares (Preedy et al., 2001), o que também acontece em situações de desnutrição proteica (Svanberg et al., 2000).

A síntese de proteínas é intensamente diminuída em fibras do tipo II quando comparadas a fibras do tipo I. Em contraste, o efeito de etanol na degradação de proteínas é menos conhecido. No entanto, demonstrou-se que o etanol reduz o catabolismo proteico (Koll, et al., 2002), um efeito também observado na desnutrição crônica e proteica (Mitch & Goldberg, 1996).

O consumo de etanol gera desnutrição por vários mecanismos. Em primeiro lugar, substitui as calorias da dieta, mas suas calorias vazias, não parecem ser aproveitadas para o crescimento corporal e não são acompanhadas de vitaminas e sais minerais (Liber, 2003). Por outro lado a má nutrição assim gerada resulta em um prejuízo funcional gerando má absorção, má digestão e dano aos processos de detoxicação (Liber, 1991).

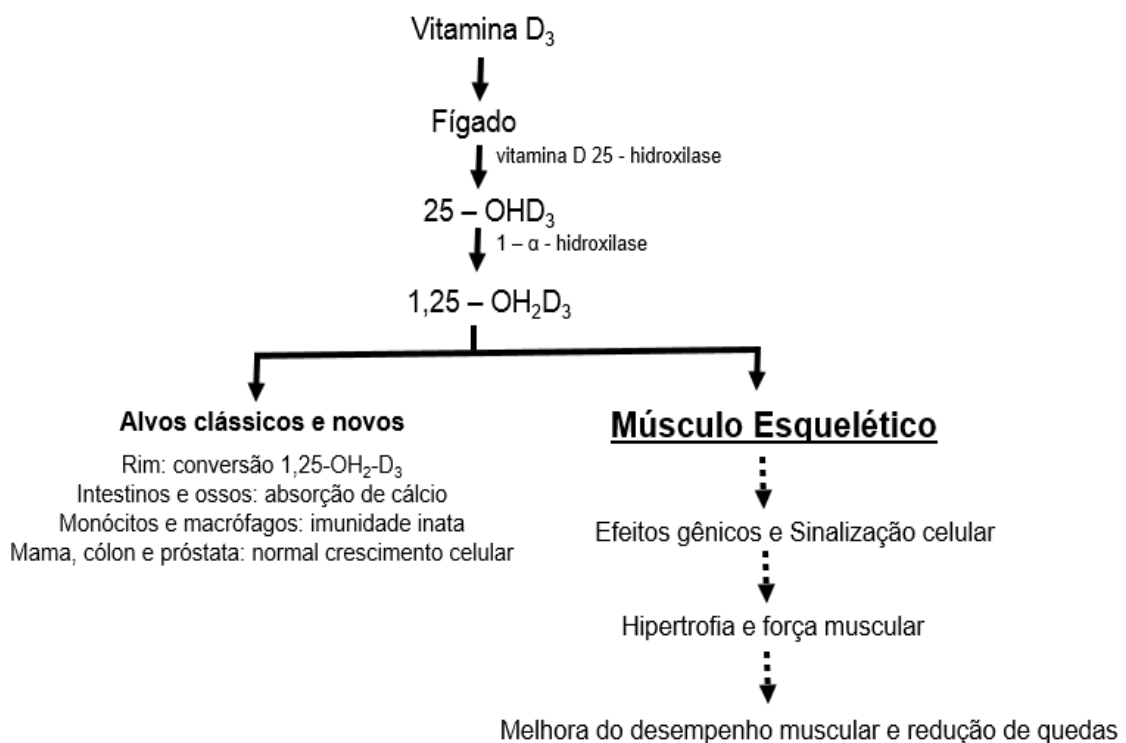
Muitos pesquisadores (Turner et al.,1988; Diamond et al., 1989; Laitinen & Välimäki, 1991; Lindholm et al., 1991; Santori et al., 2008), verificaram o efeito do etanol sobre tecido ósseo, e observara baixos níveis de vitamina D₃, em indivíduos alcoólicos e animais tratados com etanol.

Além dos possíveis efeitos sobre a ingestão, absorção ou síntese deficiente de vitamina D₃ (Manari et al., 2003), relacionado aos efeitos diretos e indiretos do etanol e ao estilo de vida peculiar do paciente alcoólico (Pits & Van Thiel, 1986), tem sido demonstrado que o etanol diminui a produção renal de vitamina D₃, afetando a

síntese metabólica da vitamina D₃ (Shankar et al., 2008). Este efeito está relacionado com o aumento do dano oxidativo induzido pelo etanol que acarreta redução do nível plasmático da vitamina D₃.

A vitamina D₃ possui ação em vários locais do organismo. Sendo que receptores para vitamina D₃ (VDR) são encontrados em vários tecidos (Demay, 2003; Projednic & Ceglia, 2014), incluindo o músculo esquelético (Bischoff et al., 2001). Há evidências do efeito da vitamina D₃ sobre o músculo esquelético na miopatia de deficiência de vitamina D₃, performance física e quedas, a qual é importante para crescimento e homeostase da musculatura esquelética. (Ceglia, 2008).

A vitamina D₃ é transportada para o fígado onde ele é hidroxilada a 25-hidroxivitamina D₃ (25(OH)D₃), a principal forma circulante de vitamina D₃. A 25(OH)D₃ é hidroxilada ainda mais pela, 1,25-di-hidroxivitamina D₃ (1,25(OH)₂D₃), a principal via de atividade da enzima 1- α -hidroxilase (Figura 1) (Projednic & Ceglia, 2014; Christakos et al., 2013).



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Figura 1. Alvos clássicos e novos para a vitamina D₃.

Camundongos “*knockout*” para VDR têm mostrado atrofia das fibras musculares. Em cultura de células, tem sido demonstrado que vitamina D₃ afeta a diferenciação de mioblastos quanto de miotubos (Dirks & Lennon-Edwards, 2011).

Baixos níveis de vitamina D₃ foram associados à redução da força de preensão manual e redução de massa muscular em um estudo realizado em 90 alcoólatras (González-Reimers et al., 2011).

Já a suplementação com vitamina D₃ reintegra o tecido muscular (Annweiler et al., 2010) e tem sido associada a um aumento de diâmetro médio e porcentagens de fibras de tipo II e também exerce efeitos antioxidantes (Chatterjee, 2001; Ceglia, 2009).

Fica evidente então que o consumo de etanol leva a uma diminuição nos níveis de vitamina D₃ e a deficiência de vitamina D₃ exerce um efeito prejudicial sobre o músculo, desse modo à deficiência de vitamina D₃ está diretamente relacionada com a miopatia alcoólica (González-Reimers et al., 2010).

Por outro lado, o etanol é considerado uma substância neurotóxica que afeta significativamente a estrutura e o metabolismo do sistema nervoso central (SNC) (Zimatkin & Phedina, 2015), e em roedores, apresenta – se com efeitos bifásicos sobre a atividade motora, mostrando-se estimulante ou depressor dependendo da dose e do tempo de uso da droga (Pohorecky, 1977; Little, 2000).

Estudo realizado por Izumi et al. (2015) constata que a intoxicação alcoólica é classificada como um fator de estresse em ratos, sendo que nessas condições há aumento nos níveis plasmáticos de corticosterona, resultando conseqüentemente em um aumento dos níveis desse glicocorticoide no cérebro (Chauveau et al., 2010). Atualmente os testes comportamentais para avaliação de substâncias tóxicas, como o etanol, que atuam no SNC, tem sido bastante utilizado em animais - ratos, entre eles destaca-se o teste de reflexo de sobressalto (Mejia-Toiber et al., 2014) e o teste de campo aberto (Teng et al., 2015).

O reflexo auditivo de sobressalto (ASR) é uma reação motora rápida e intensa que culmina na contração da musculatura estriada esquelética da face e do corpo como um todo em resposta a um estímulo acústico inesperado e de alta intensidade. O ASR é um reflexo acústico motor desencadeado por estruturas do tronco encefálico, que está presente em diversas espécies de mamíferos, inclusive no homem (Koch, 1999).

Em roedores, esse reflexo se manifesta com o encurtamento do comprimento total do animal e aumento da pressão arterial e frequência cardíaca, mediadas pelo sistema nervoso autônomo (Baudrie et al., 1997; Koch,1999). Ele possui um caráter defensivo frente a uma provável agressão ou de alerta perante a acontecimentos inesperados. As contrações musculares em conjunto levam o animal a adotar uma postura de defesa, protegendo partes vitais do corpo, como a face, pescoço e abdome (Figura 2).

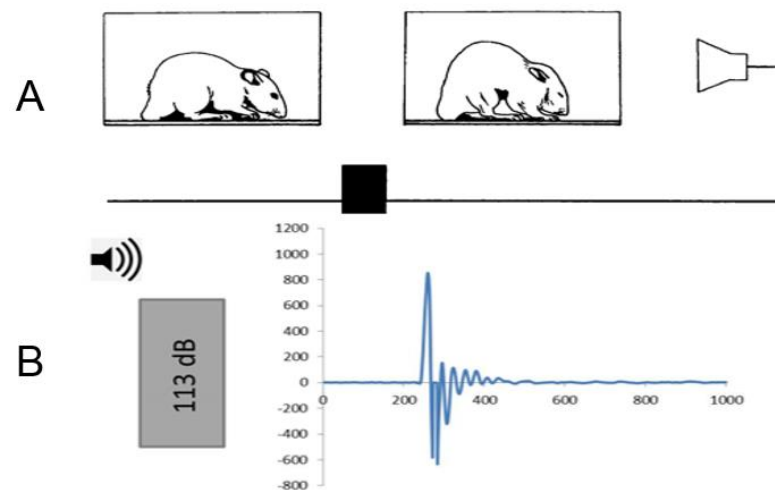


Figura 2. A -Esquema do reflexo auditivo de sobressalto no rato. Após ser estimulado por um som inesperado e de alta intensidade, o animal contrai a musculatura da face, pescoço e corpo para assumir uma postura defensiva. B- Gráfico da amplitude de resposta frente à apresentação de um estímulo sonoro de alta intensidade (113 dB). Modificado de Koch (1999).

Provas comportamentais envolvendo modulações do ASR têm despertado interesse em questões de diagnóstico clínico (Wilkins et al., 1986), tanto na clínica neurológica quanto psiquiátrica, por modificar-se diante de várias condições patológicas como doenças neurodegenerativas, esquizofrenia (Braff et al., 2001) depressão (Kaviani et al., 2004), transtorno do estresse pós traumático (Grillon et al., 1996), estresse (Andreski et al., 1998; Stam, 2007), ansiedade (Kaviani et al., 2004), medo (Anisman et al., 2000; Davis, 2006; Winslow et al., 2007), bem como frente a estados de dependência de drogas, como opiáceos (Mansbach et al., 1992; Borowski & Kokkinidis, 1994) e consumo de etanol (Grillon et al., 1994).

O etanol, reduz a amplitude do SAR (Grillon et al., 1994), como de se esperar por seu efeito depressor sobre o sistema nervoso central. Contudo, estudos realizados em roedores mostraram um aumento da amplitude, o que poderia ser

explicado pelo efeito bifásico da substância, que atua como estimulante inicialmente (Dudek et al., 1991), pela liberação de dopamina (Lewis & Gould, 2003), e, em seguida, como um depressor por ativação GABAérgica.

Entre as principais modulações do RAS, encontra-se a inibição por estímulo prévio (PPI), que é caracterizada pela diminuição ou completa abolição do reflexo quando o estímulo sonoro desencadeante é precedido por um estímulo sensorial (sonoro, visual ou tátil) de baixa intensidade, que isoladamente, não desencadearia o reflexo (RAS) (Figura 3).

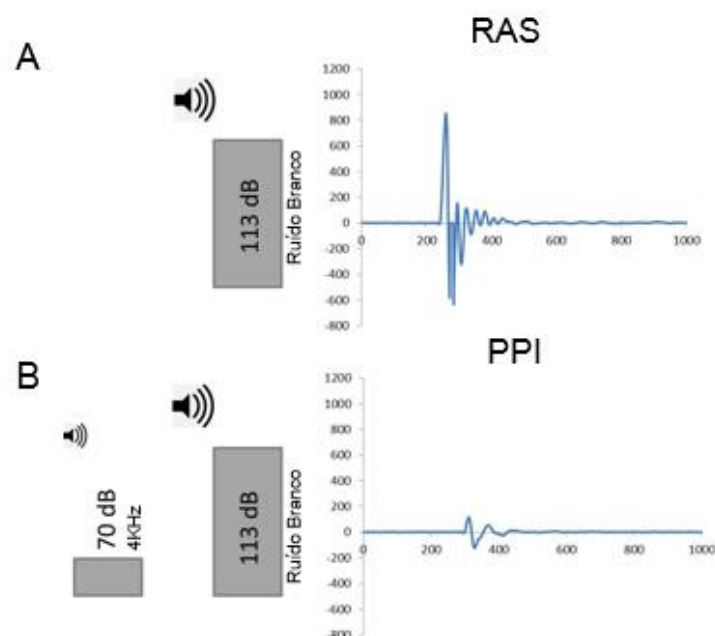


Figura 3. Registro da resposta do reflexo auditivo de sobressalto (RAS) e da resposta de sobressalto após a inibição por estímulo prévio (PPI). A - apresentação de um estímulo sonoro de alta intensidade (113 dB) que desencadeia uma resposta de sobressalto no animal de grande amplitude. B – apresentação de um estímulo sonoro precedido por um estímulo de menor intensidade (70dB) (PPI), o qual a amplitude é menor. Modificado de Koch (1999).

Como já citado acima, o teste do campo aberto é outro teste bem utilizado na avaliação comportamental e na utilização de fármacos e/ou drogas psicoestimulantes e ansiolíticas. Nos parâmetros avaliados, como por exemplo a locomoção do animal na arena, quando ocorre seu aumento desse resultado pode ser interpretado como um efeito do tipo ansiolítico/estimulante, bem como, quando

ocorre uma diminuição desta variável, está associada com efeitos ansiogénico/sedativo (Prut & Belzung, 2003).

Para este teste é utilizado uma arena circular dividida em 12 setores, onde é analisada a atividade locomotora dos roedores, ou seja, os deslocamentos entre um ponto a outro da arena e seu comportamento exploratório não locomotor observando movimentos que o animal pode realizar sem a necessidade de deslocamento (De Lima et al., 2005).

O teste de campo aberto têm sido utilizado para verificar os efeitos do etanol em diversas situações: injeções intraventriculares (Correa et al., 2003) e intraperitoneais (Pardo et al., 2013), sua abstinência (Karadayiana et al., 2013); sensibilização comportamental (Bellot et al., 1996; Araujo et al., 2005), sua relação com exercício físico (Sosa, et al., 2015) e seus efeitos agudo e crônico (Fukushiro et al., 2012).

No SNC há evidência de que a vitamina D₃ pode ser um importante modulador do desenvolvimento do cérebro, atuando nas desordens neurológicas e neuropsiquiátricas (Harms et al., 2011). Atua ainda como neuroprotetor, sendo que em níveis baixos pode predispor ao desenvolvimento de depressão e déficit cognitivo (Groves et al., 2013).

Modelo Experimental

Muitos são os modelos animais utilizados em pesquisas relacionadas ao consumo de etanol entre eles destacam-se os modelos de preferência por etanol. As variedades de ratos UChA (baixo consumo de etanol) e UChB (alto consumo de etanol) (UCh=Universidade do Chile) constituem modelos importantes desse tipo de animais consumidores de etanol e, por isso, essas variedades são importantes para o estudo de características que podem ser associadas ao alcoolismo humano. Eles derivam de uma colônia original de ratos Wistar que tem sido criada seletivamente na Universidade do Chile por mais de 70 gerações (Quintanilla et al., 2006). Denominou-se UChA e UChB as linhagens de baixo e alto consumo de etanol respectivamente. Eles diferem quanto à atividade cerebral da aldeído-desidrogenase e na capacidade de desenvolver tolerância aguda ao etanol (Tampier et al., 1984,1994).

Para que ocorra a manifestação da doença alcoolismo é necessário, predisposição genética para que o paciente ingira etanol em grandes quantidades

durante longo período, o que faz da linhagem UChB um adequado modelo experimental, pois possui predisposição genética para ingerir voluntariamente grandes quantidades de etanol a 10%. Há caminhos importantes para o enfoque dos estudos sobre o alcoolismo humano que podem ser investigados com o modelo animal UChB: a busca de marcadores genéticos que indiquem a predisposição ao alcoolismo, a busca de terapêuticas eficazes para o tratamento do alcoolismo e as alterações morfofuncionais provocados pela ingestão de etanol (Mardones, 1993; Martinez et al., 2000).

Considerando que o consumo crônico de etanol induz a miopatia (atrofia principalmente das fibras tipo II), neuropatia periférica e leva a uma diminuição nos níveis de vitamina D₃, que por sua vez desempenham um efeito prejudicial sobre o músculo esquelético e SNC.

O objetivo deste trabalho foi avaliar se a administração de vitamina D₃ durante o alcoolismo crônico pode minimizar os efeitos da miopatia e neuropatia alcóolica, bem como alterar aspectos comportamentais frente ao consumo de etanol.

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Capítulo 1

Behavior assessment of UChB and Wistar rats supplemented with vitamin D₃

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Physiology & Behavior, para o qual foi submetido (em anexo).

ABSTRACT

Ethanol intake compromises brain structure, presenting biphasic effects over motor activity, acting as a stimulant or depressor depending on the dose or duration of use. It interferes in vitamin D₃ absorption and metabolism, what correlates to some neurologic and neuropsychiatric disorders. There are reports on the association of ethanol with bone alterations, including low levels of vitamin D₃. Based on that, the objective of this study was to evaluate the effects in behavior tests of isolated administration of vitamin D₃ or its administration in association with ethanol, during chronic alcoholism. In order to achieve that, two experimental groups were used: male Wistar rats (n=20), and UChB lineage male rats (n=20) (volunteer ethanol drinkers). Both groups were divided in two subgroups: Vitamin D₃ – 12.5µg/kg/day (500 UI) of cholecalciferol (WV, n=10, and UV, n=10), and Control (WC, n=10, and UC, n=10), for a period of 75 days. Body weight analyses and behavior tests (acoustic startle reflex and open field) were conducted at 90 and 165 days of age. In addition to that, corticosterone plasma levels were measured at 165 days, with no statistical difference between the experimental groups. The Wistar group presented lower ASR values in the final moment (Control and supplemented), while the PPI percentages were higher in the initial group. In the UChB group there was no difference in PPI percentages with the pre-stimuli used. When the ASR responses were compared between groups (Wistar and UChB), the UChB group presented lower ASR amplitudes as compared to the animals of the Wistar group. In relation to PPI percentages in the three pre stimuli used in the UChB group, the percentages were higher as compared to the animals in the Wistar group, the ASR values been smaller in the UChB group. The presence of feces and urine was similar both in the initial and final moments, in the open field test, for all the experimental groups. Considering the other parameters of open field, decreased locomotion was noted in the UChB group in the vitamin D₃ subgroup, as well as in the final control group as compared to the initial control. When the Wistar and UChB groups were compared, the latter presented higher rates in locomotion. The results obtained are consonant to the depressive/sedative effect of ethanol over the CNS, reducing ASR amplitude, with a likely enhancement over the inhibitory circuits which mediate PPI, determining the increase in inhibition percentage in the UChB group, as well as an increase in locomotion presented by the UChB group in the open field test, characterized by a decrease in the aversive effect to the new environment. In addition we emphasize that the dose of vitamin D₃ used did not have effect over the behavior parameter analyzed, differences observed in this group seeming to be related to getting used to the gavage. This study describes for the first time in literature the differences in responses to the acoustic startle reflex test between Wistar and UChB rats, the latter genetically predisposed to ethanol intake, a lineage adequate to be used in experiments comprising analyses of behavior in face of ethanol intake.

Key words: ethanol, vitamin D₃ and behavior.

1.0 Introduction

Ethanol is among the most common substances of abuse in the world, leading to many problems. Ethanol is considered a neurotoxic substance that significantly affects the structure and metabolism of the central nervous system (CNS) [1].

In rodents CNS, ethanol presents biphasic effects over motor activity, stimulating or depressing it depending on the dose and duration of use [2;3].

In rats, alcohol intoxication is classified as a stress factor, and under that condition there are increased plasma levels of corticosterone [4], resulting in increased levels of that steroid in the brain [5].

Ethanol attenuates the co-release of gamma-aminobutyric acid (GABA), inhibiting its biosynthesis, a mechanism by which exposure to ethanol may modulate the activity of GABAergic synapses [6].

In order to evaluate the action of drugs over the CNS, among them ethanol, many behavioral assessments are conducted, including the acoustic startle reflex (ASR) [7;8;9;10;11;12]

ASR is an acoustic-motor reflex triggered by structures of the encephalic trunk, present in many mammal species, including man. It is a quick, intense motor reaction that culminates in the contraction of skeletal muscles from the face and body as a response to an unexpected and highly intense acoustic stimulus (Koch, 1999). This reflex may be altered under many physiological conditions such as fear [13;14;15], anxiety [16], and stress [17;18].

The effects of ethanol administration given intracerebroventricular [19] or intraperitoneal [20] have been studied in the open field test, as well as behavioral sensitization [21;22], relation to physical exercise [23], acute and chronic effects [24;25;26;27;28;29;30] and effects after abstinence [31].

Ethanol also interferes in the absorption and metabolism of essential nutrients [32;33;34;35;36].

Many researchers have analyzed the relation of ethanol with bone abnormalities, in which low levels of vitamin D₃ in alcoholics or animals treated with ethanol have been reported [37;38;39;40;41].

Ethanol has therefore proved to have a profound effect in calcium homeostasis and vitamin D₃ metabolism [42]. The impairment on Vitamin D₃ intake,

absorption, and synthesis refers to the direct or indirect effect of ethanol over the body, and may also occur as a consequence of life style in alcoholic subjects [43].

Besides the well-known role in calcium homeostasis, vitamin D₃ has been related to studies comprising the CNS [44].

There are evidences that vitamin D₃ may be an important modulator in brain development, acting in neurologic and neuropsychiatric disorders [45]. This vitamin also acts as a neuroprotective substance, and in low levels may lead to the development of depression and cognitive deficit [46].

Many are the animal models used in researches related to ethanol intake, among which stand out the models of ethanol preference as the UChA and UChB rats lineages (UCh = Chile University), which are important for translational studies [47;48].

ASR as well as the open field test are behavior tests sensitive to alcohol intoxication that alters calcium and vitamin D₃ metabolism, what in its turn under certain circumstances exerts neuroprotective effect over the CNS. The objective of this study was therefore to assess the effects of isolated administration or the association of vitamin D₃ and ethanol for chronic alcoholism and analyze the differences between Wistar rats and UChB front of behavioral tests.

2.0 Materials and Methods

2.1 Experimental groups

40 male adult 90-day-old rats were divided in two experimental groups: 20 UChB rats (voluntary ethanol drinkers, group U), originated from the animal research laboratory of the Anatomy Department of IBB/UNESP, and 20 Wistar rats (group W) originated from the animal research laboratory of the Biomedical Sciences Institute of USP São Paulo-SP). The animals were kept in polyethylene boxes (40x30x15cm), with solid floors, covered with wood shavings, under controlled conditions of luminosity (12h light, 12h dark), and temperature (20 to 25 °C), receiving filtered water and industrialized food (*Provence*[®]) *ad libitum*. All the experimental procedures were approved by the Committee on Animal Research and Ethics of the Biosciences Institute, UNESP, Botucatu (Protocol number 531).

In order to select the volunteer ethanol drinkers, after weaning at 21 days of age, the male UChB rats were divided in groups of two to four animals, receiving

water and industrialized food *ad libitum*. At 55 days of age they were isolated and, from 60 days on, filtered water and a 10% ethanol solution were offered, both periodically alternated, *ad libitum*. After 15 days evaluating the intake of the 10% ethanol solution, the selection and standardization of the UChB lineage was carried out [49]. The animals that had average intake higher than 2.0mL 10% ethanol/100g of body weight/day were selected for the UChB lineage. After selection and during the whole experiment 10% ethanol solution was offered to the UChB animals *ad libitum*.

Each experimental group (Wistar and UChB) was divided in two subgroups: Vitamin D₃ subgroup (n = 10 per lineage), which received 12.5 µg/kg (500 UI) of Cholecalciferol (C9756-Sigma) diluted in olive oil, given daily by gavage ; and Control subgroup (n = 10 per lineage), which received the vehicle (olive oil), with the same periodicity, both for 75 days.

Groups	Subgroups	Voluntary intake	Animals (n°)	Assessment moments
UChB	Control (UC)	Ethanol 10%	10	Initial - 90 days
				Final - 165 days
	Vitamin D ₃ (UV)	Ethanol 10%	10	Initial - 90 days
				Final - 165 days
Wistar	Control (WC)	Water	10	Initial - 90 days
				Final - 165 days
	Vitamin D ₃ (WV)	Water	10	Initial - 90 days
				Final - 165 days

2.2 Behavior assessment

The animals were individually weighted and the acoustic startle reflex and open field activity measured in two moments: initial, at 90 days (Wistar Initial and UChB Initial), and final, at 165 days of age (WC final; UC final; WV and UV). The initial behavior assessment comprised the animals of groups Wistar and UChB, the animals of the Vitamin D₃ subgroups, since they belonged initially to the groups of origin, were evaluated after the experimental period of Vitamin D₃ supplementation, at the final moment, at 165 days.

2.2.1 Measuring the Acoustic Startle Reflex

For the evaluation of the acoustic startle reflex, an assessment system made of an acoustically isolated box, a measuring platform, 9 cm from the loud-speakers, and a system that generated sound stimuli controlled by a software and connected to a computer (Insight Ltda.) was used. The ASR quantification was made by a sensor set under the test cage, which registered the animal's weight variation, during and after acoustic testing, considering that the response is a ballistic movement.

All the behavior tests were conducted between 1pm and 4pm. Each ASR session encompassed four different attempts: isolated sound stimulus, and sound stimulus preceded by a pre-stimulus of 65, 70 or 75 dB, in order to evaluate prepulse inhibition (PPI). During the whole session, the chambers had constant ventilation and background noise (white 60dB noise); the animals were monitored by means of a video camera. Every session began with a 5-minute acclimatization period. ASR/PPI measurements were then conducted using a sound stimulus, 113 dB SPL white noise, lasting for 20 ms. In the other attempts, a pre-stimulus of 4 KHz pure tone signal of 65dB SPL, 70dB SPL or 75 dB SP and lasting 20 ms preceded the stimulus in 50 ms. The interval between the attempts was 30 s with 33% random variation. ASR amplitudes and the PPI percentage, calculated as $PPI = [(reflex\ amplitude) - (reflex\ amplitude\ with\ pre\ stimulation)] \times 100 / (reflex\ amplitude)$ were evaluated in the attempts with isolated stimulus.

2.2.2 Open field test

Open field evaluation rendered possible the measurement of variables concerning the modifications in the exploratory activity and locomotion. The apparatus used was a round arena with a 90-centimeters diameter, with 12 radial divisions painted on the floor, and acrylic walls 50 cm high.

Each animal was placed in the center of the arena and its behavior was filmed during 5 min for evaluation. A researcher blinded to the lineage and treatment observed the following parameters: locomotion (number of entrances in any division with all four legs); immobility (how many times there was no movement); exploration (how many times the animal stood on the hind limbs); presence of feces, and presence of urine.

2.3 Euthanasia

After the 75 days of treatment with Vitamin D₃, on the day following the last session on behavior assessment, the animals were fasted for 12 hours, weighted, anesthetized by intraperitoneal injection of ketamine (Dopalen - 90 mg / kg) and Xylazine (Rompun - 10mg / kg). The blood was obtained by cardiac puncture and stored in a container with heparin. Plasma was then extracted centrifuging the blood at 2,000 RPM per 10 min and the corticosterone plasma concentration was determined by specific radioimmunoassay (RIA), after ethanol extraction as previously described [50]. RIA sensitivity and the intra and inter-assay variation of coefficients were 0.16 µg/dl and 5.1%, and 8.1%, respectively. The results were expressed as µg/dl.

3.0 Results

3.1 Body weight

Body weight at 165 days (final) was greater than at 90 days of age (initial) in both lineages ($p < 0.05$). When we evaluated animals of the same age, there were no significant differences between the subgroups (Figure 1).

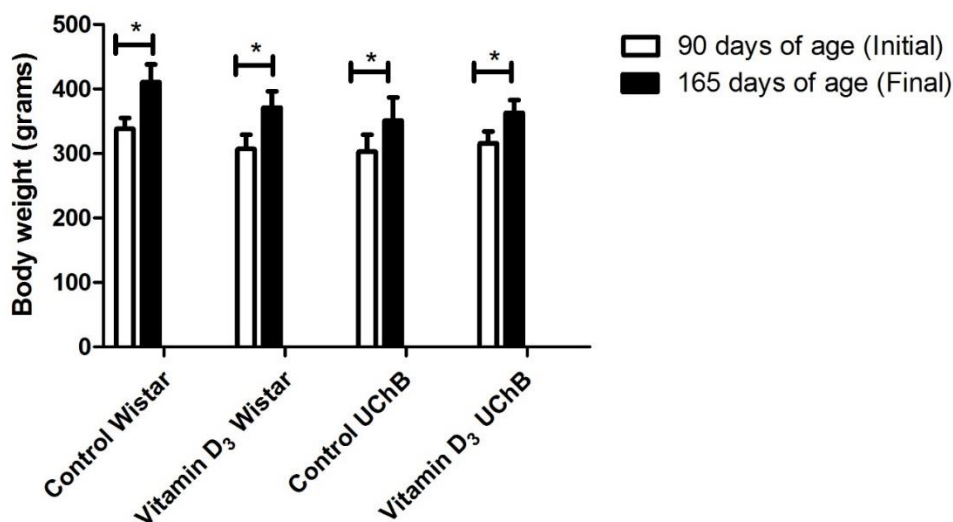


Figure 1. Graphic of initial (90 days) and final (165 days) body weight (g) of the experimental groups. The data are presented as averages and standard deviation and were analyzed by the ANOVA test for repeated measures followed by the Tukey-Kramer's post test ($p \leq 0.05$). * $p \leq 0.05$.

3.2 Behavior assessment

3.2.1 Measuring Acoustic Startle Reflex (ASR)

Considering that the amplitude of ASR is proportional to body weight [51], data concerning response amplitudes were normalized according to the animal's body weight, avoiding possible distortions in the results (ASR amplitude/body weight).

ASR amplitude in the Wistar group was smaller in the control-final subgroups (3.0 ± 0.4 ; $p < 0.001$), and in the group supplemented with vitamin D₃ (2.7 ± 0.5 ; $p < 0.001$) at 165 days, as compared to the subgroup control-initial (5.0 ± 0.3). There was no difference in the ASR amplitudes in any of the UChB lineage subgroups ($p > 0.05$) (Figure 2).

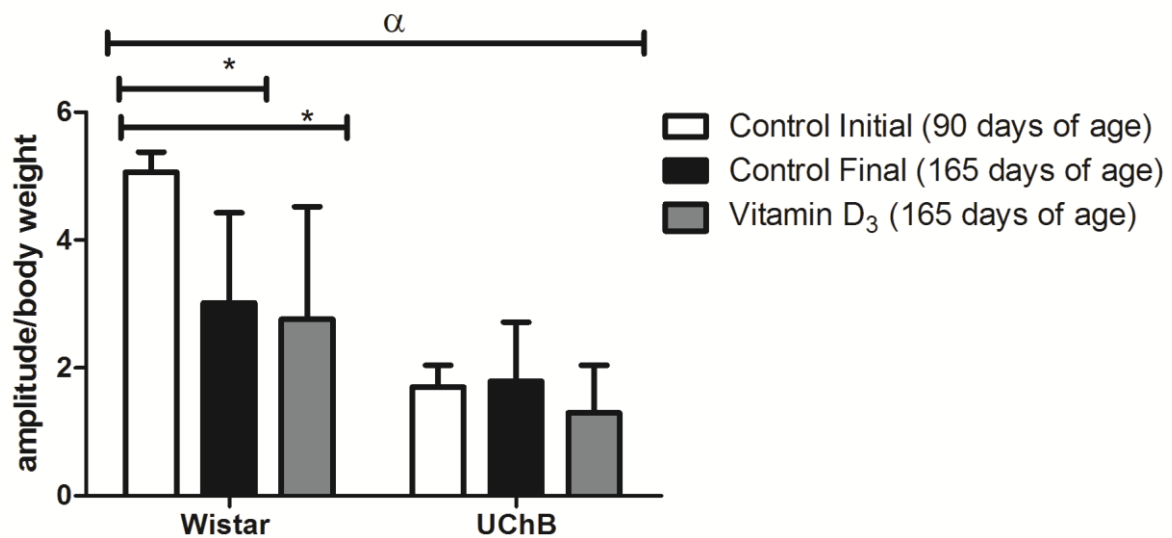


Figure 2. Graphic for normalized ASR amplitude. The Wistar final and Wistar Vitamin D₃ groups presented smaller startle amplitude as compared to the initial Wistar subgroup ($p < 0.001$). In regard to the comparison of lineages, the UChB group presents smaller troughs as compared to the Wistar group. The data are presented as average and standard error of the mean, and two consecutive factors were analyzed by ANOVA followed by Bonferroni's post test ($p < 0.05$) * = $p < 0.05$ and $\alpha = p < 0.0001$.

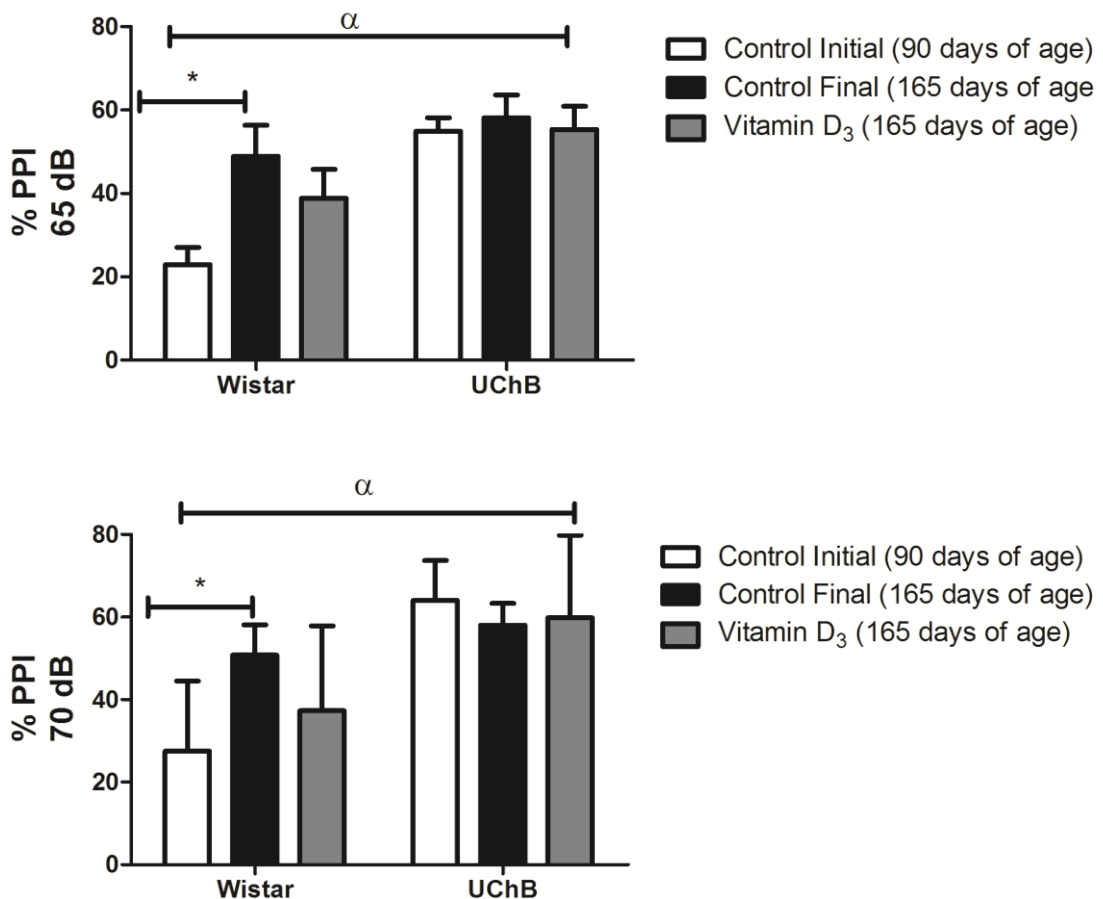
The three pre-stimuli intensities used in the sessions (65, 70, and 75dB) were efficient in triggering PPI in all experimental groups.

In the Wistar group, the control-Wistar subgroup had a higher percentage of PPI in the final moment, 65dB (48.9 ± 7.4 ; $p < 0.0001$), 70 dB (50.7 ± 7.3 ; $p < 0.005$) and 75 dB (53.7 ± 7.2 ; $p < 0.01$), as compared to the initial moment with the three

intensities of pre-stimulus used, 65dB (22.8 ± 4.1 ; $p < 0.0001$), 70 dB (27.5 ± 5.3 ; $p < 0.005$), and 75 dB (27.7 ± 5.6 ; $p < 0.01$) (Figure 3). In the subgroup supplemented with vitamin D₃ there was no significant difference ($p > 0.05$).

In the UChB group there was no difference in the PPI percentages between the initial and final moments, as well as between the three intensities of pre-stimulus used ($p > 0.05$) (Figure 3). This same behavior was sustained in the UChB group supplemented with vitamin D₃.

When the ASR responses between groups (Wistar and UChB) were compared, a significant difference was noted ($p < 0.0001$); the UChB group presented smaller ASR amplitudes as compared to the Wistar group ($p < 0.0001$). In relation to PPI percentages in the three pre-stimuli used: 65 dB ($p = 0.0002$), 70 and 75 dB ($p < 0.0001$), the UChB group presented higher percentages as compared to the Wistar group.



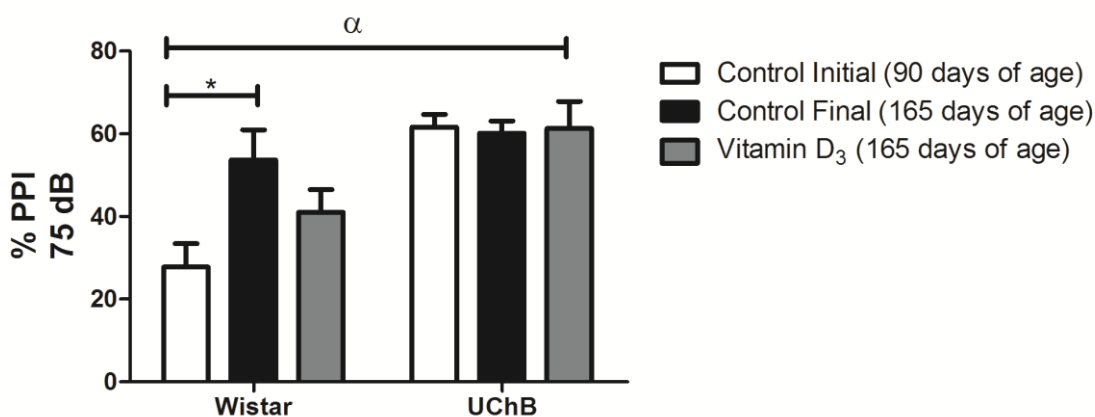


Figure 3. Graphic for the PPI percentage with the 65, 70 and 75 dB pre stimuli. The PPI percentages were smaller in the initial Wistar subgroup as compared to the final Wistar, regardless of the pre stimulus used: * = 65dB ($p < 0.0001$), 70 dB ($p < 0.005$) and 75 dB ($p < 0.01$). In relation to the statistical analyses of the lineages, in the UChB group the values were higher in all pre stimuli used: α = 65 dB ($p = 0.0002$), 70 and 75 dB ($p < 0.0001$). The data are presented as average and standard error of the mean, and two consecutive factors were analyzed by ANOVA followed by Bonferroni's post test ($p < 0.05$).

3.2.2 Open field test

During the open field tests, both in the initial and final moments there were similar amounts of feces and urine on the floor of the device for both groups, ($p > 0.05$) (Figure 4).

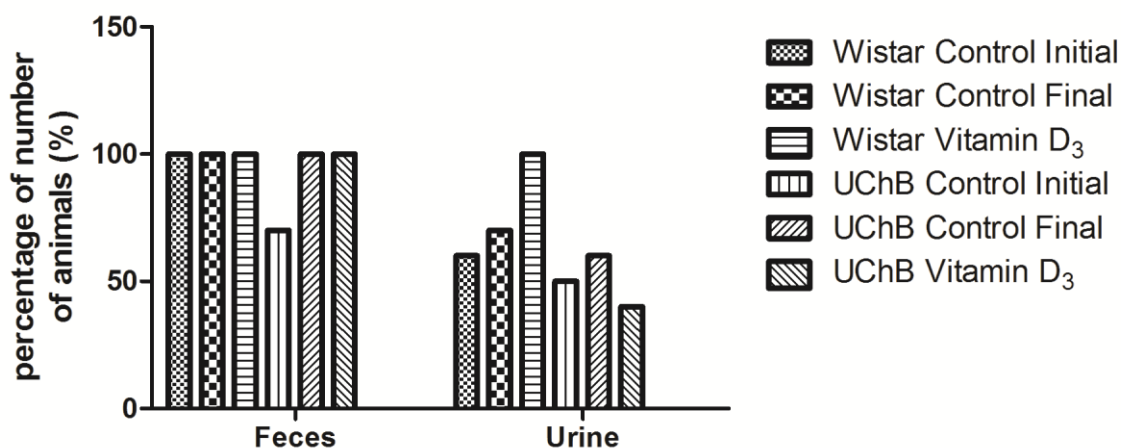


Figure 4. Graphic of the percentage of number of animals in the experimental groups where the presence of feces and urine were noted during the open field test. The data are presented in percentage, a statistical analyses was previously conducted by the Chi-square test for trend ($p < 0.05$).

The Wistar group was similar in relation to the behaviors analyzed in both moments, no difference being noted between the experimental groups ($p > 0.05$). In the UChB group there was a decrease in locomotion in the vitamin D₃ subgroup (57.8 ± 6.0 ; $p < 0.001$) and in the control-final subgroup (59.0 ± 5.2 ; $p < 0.01$) as compared to the control-initial subgroup (79.0 ± 5.4) (Figure 5).

When comparing the groups (Wistar and UChB) there was a significant difference between them in relation to the open field test ($p < 0.0001$).

Considering those differences observed, there was statistical difference only in locomotion, where the animals from the UChB group (Control-Initial: 79.0 ± 5.4 ; Control-Final: 59.0 ± 5.2 and Vitamin D₃: 57.8 ± 6.0) had higher locomotion pattern as compared to the animals of the Wistar group (Control-Initial: 43.6 ± 4.5 ; Control-Final: 46.4 ± 5.8 ; and Vitamin D₃: 35.0 ± 6.7) ($p < 0.001$) (Figure 5).

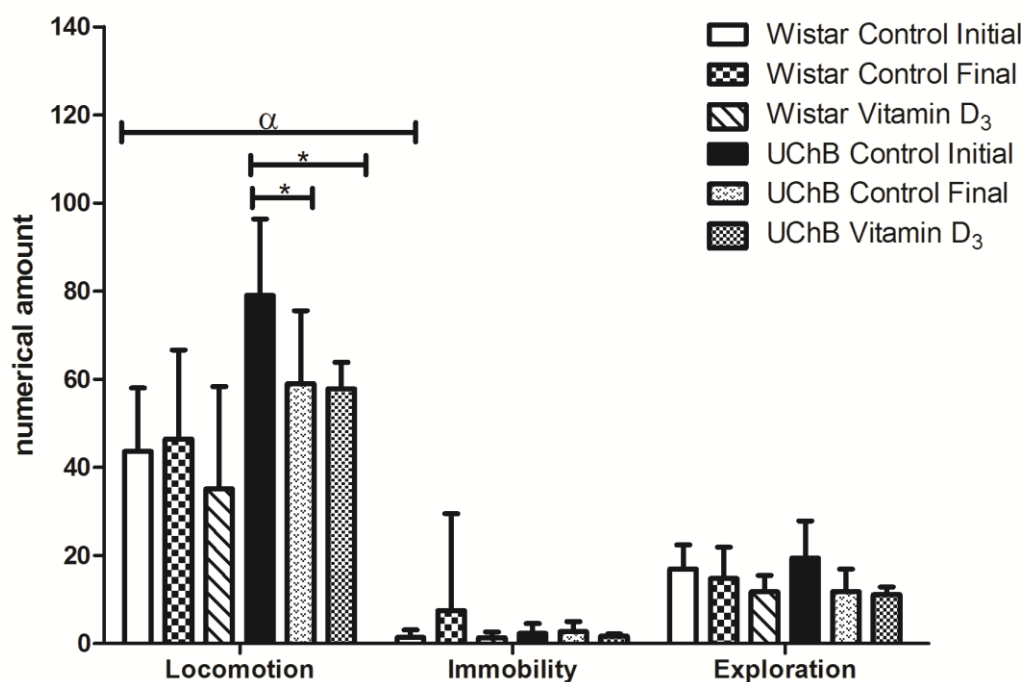


Figure 5. Graphic of the parameters evaluated in the open field test of the experimental groups, where the control animals of the UChB group and the Vitamin D₃ subgroup demonstrated decreased locomotion on the final moment at 165 days [($* = p < 0.01$) and ($* = p < 0.001$), respectively], in comparison to the initial moment. In relation to lineage they diverge ($\alpha = p < 0.0001$), and in the parameters analyses, there was statistical difference only in locomotion where the animals of the UChB group presented higher values as compared to the animals of the Wistar group ($\alpha = p < 0.001$). The data are presented as average and standard error of the mean, and were analyzed by ANOVA and Bonferroni's post test ($p < 0.05$) $* = p < 0.05$ and $\alpha = p < 0.0001$.

3.3 Biochemical analyses

3.3.1 Corticosterone plasma concentration

There was no statistical difference in cortisone plasma concentration ($\mu\text{g/dL}$) between the groups treated (WV= 6.06 ± 4.03 ; UV= 7.3 ± 3.5) and not treated with vitamin D₃ (WC= 8.3 ± 3.2 ; UC= 7.5 ± 4.8) ($p > 0.05$) (Figure 6).

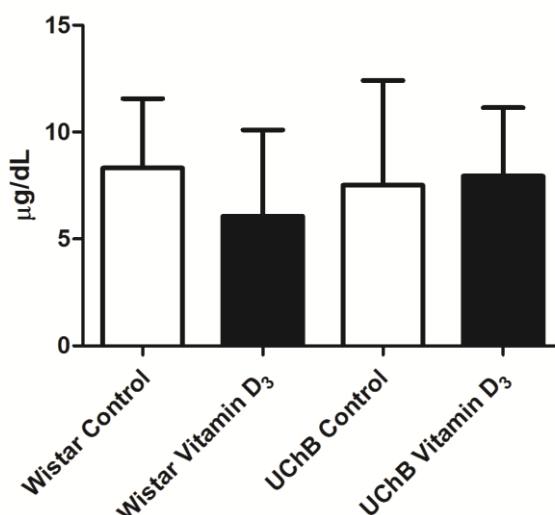


Figure 6. Graphic for the corticosterone plasma concentration ($\mu\text{g/dL}$) in the groups studied. Data are presented as average and standard deviation and were analyzed by the parametric Student's t-test ($p > 0.05$).

4.0 Discussion

Excessive use of alcohol may cause structural and functional abnormalities in the brain [52], besides damage to other organs, such as skeletal muscles [53].

The generation of reactive oxygen species (ROS), consequent of oxidative stress from acetaldehyde, radical that is a byproduct of ethanol metabolism [53;54;55;56] has been proposed as the cause for neuronal damage.

Behavior assessment contributes for the study of neural mechanisms associated to dependence, among them ethanol [11;12;29;30]

The measurement of acoustic startle reflex, is a widely employed assessment in the study of the sensory motor integration, both for basic as well as clinical research. ASR is measured with no invasive tests and has a high rate of phylogenetic homology between rodents and men [57;58].

All animals evaluated presented acoustic startle reflex after sound stimulus, consonant to previous reports [59]. The previous presentation of the pre-stimuli was also able to reduce the amplitude of response, following the PPI paradigm [59].

The control animals in the Wistar lineage at 165 days of age (final moment) had a smaller ASR amplitude and higher PPI percentage than the control animals at 90 days of age (initial moment). Vitamin D₃ supplementation did not change ASR amplitude or PPI percentage in any of the groups evaluated.

Studies correlate ASR amplitude to the level of anxiety in man [16;60] and rodents [61].

ASR amplitude may be increased in situations potentiated by fear [13;14;15], anxiety [16,62], and stress [17;18]. We therefore believe that the differences observed in ASR and PPI in the Wistar rats in the final moment are related to the decrease of anxiety in those animals. Anxious patients, for example, have larger PPI amplitude than subjects with low levels of anxiety [16].

Considering that the animals were submitted to daily gavage from 90 to 165 days of age, that being considered a stress factor for the animal [63], we assume that they got habituated to this stressor, what would lead to a reduction in anxiety levels.

No statistical difference was noted in ASR amplitude or PPI percentage in the animals of the UChB lineage, in any experimental subgroup.

ASR amplitude was smaller in the UChB as compared to the Wistar rats. Additionally, the UChB group presented higher PPI percentages. This result is in accordance to the depressor effect of ethanol over the CNS, in reducing ASR amplitude [8], likely stimulating the inhibitory circuits that mediate PPI, determining an increase in the percentage of the inhibition in UChB animals.

The deficiency in GABAergic transmission has been connected to the etiology of alcoholism and anxiety disorders, and has been proposed as being responsible for individual differences in ASR [64;65].

Many evidences demonstrate that drugs of abuse converge towards a common circuit in the limbic system. The main investigated pathway initiates in the ventral tegmental area (VTA) and sends dopaminergic projections to the *nucleus accumbens* (Acb). Activating GABA receptors, ethanol inhibits the GABAergic terminals, disinhibiting the dopaminergic neurons, as well as the glutamatergic terminals which innervate the Acb [66].

Our results are consonant to the findings of Jones et al. [8], who demonstrated a reduction in ASR amplitude in rats with preference for 10% ethanol solution (UChB).

Slawecki & Ehlers [9], studying the effect of ethanol in ASR and PPI in young and adult rats exposed to ethanol vapor, concluded that ASR amplitude was smaller, but the percentage of PPI increased significantly only in young rats.

On the other hand, the increase in ASR amplitude in rats with high preference for ethanol intake has also been reported [11;67], with no PPI alterations [11].

We believe that those differences in our results may be explained by the biphasic effect of ethanol [2], that in low doses induces a quick excitatory effect, acting as stimulant [68] as a consequence of the dopaminergic activation [69]. In high doses, however, or during chronic use, ethanol has a depressor effect over CNS, through GABAergic activation [70;71;72].

The open field test has been used in behavior trials on anxiety and for motor evaluation in rats, as well as in order to evaluate sedation or activation, providing indicators for neurologic alterations, for instance, after exposure to neurotoxic substances [27;31;73].

In relation to this test, four parameters were evaluated: locomotion, exploration, immobility, and presence of feces or urine. In animal models, the evaluation of these behavior parameters has been used in order to understand the effects of different psychostimulant and anxiolytic drugs [73], among others.

Our results demonstrated that in the UChB groups there was less locomotion when the final UChB subgroup was compared to the initial UChB subgroup. These data are in accordance to those found in ASR amplitude and PPI percentage, most likely due to the sedative/depressor effect of ethanol over the CNS.

Chronic ethanol intoxication during puberty lead to significant motor deficits in rats in the adult phase, with impairment in locomotion, coordination, and muscle strength, followed by neuronal death, increased levels of nitrite and of lipid peroxidation in the brain cortex [74;75].

The alterations in the open field activity may, therefore, indicate increased or reduced anxiety [76]. Karadayian et al. [31], for instance, consider that a decrease in locomotion and exploration concern behaviors which represent anxiety during ethanol abstinence. Fukushiro et al. [27], evaluating the acute and chronic effects of ethanol state that the increase in motor activity after acute administration of ethanol

characterizes a reduction in the aversive effect to the new environment, that is, an anxiolytic effect induced by ethanol.

Exploration, locomotion, and immobility may be affected by medications with action over the CNS. Passing feces may be modified by spasmolytic or spasmogenic drugs, as well as by sedation, fear or anxiety [77]. We therefore believe that the decrease in locomotion is also associated to the fact that the animals might have gotten accustomed to stressing stimuli, in this case the gavage protocol.

The data described above in relation to the animals supplemented with vitamin D₃, that is, the decrease in the UChB vitamin D₃ parameters as compared to initial UChB may be justified to this habituation to stimulus – gavage, as previously discussed, and not to the supplementation.

There were no differences in the plasma concentrations of corticosterone between the experimental groups, what may also reinforce the hypothesis that the animals were accustomed to stressing stimuli.

The data obtained in relation to locomotion, when the UChB group was compared to the Wistar, with higher locomotion rates, contradicts the results obtained in this group in relation to ASR and PPI, reinforcing the depressor/sedative effect of ethanol over the CNS, reducing ASR amplitude, with a probable reinforcement over the inhibitory circuits which mediate PPI, determining the increase in the percentage of inhibition in the UChB group. In the open field test, the depressor/sedative effect of ethanol over the CNS would promote a reduction of the aversive reflex to the new environment.

We also emphasize that the dose of vitamin D₃ used did not have effects over the behavior parameters analyzed.

Propensity to the compulsion of ethanol drinking may arise from a combination of cognitive, biologic, and social factors, what might contribute to the underlying “cause” of compulsion [78;79]. Among the possible biologic factors, genetics must have a significant role.

5.0 Conclusion

This study therefore describes for the first time in literature the differences in the responses to the acoustic startle reflex test and open field test between Wistar and UChB rats, the latter genetically predisposed to ethanol intake, a lineage adequate to be used in experiments that comprise behavior analyzes in the face of

ethanol intake and vitamin D₃ supplementation didn't affect the responses of behavioral tests.

Acknowledgements

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Capítulo 2

Effect of vitamin D₃ supplementation in alcoholic myopathy and neuropathy: experimental study in rats

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ABSTRACT

Excessive ethanol use is a global problem and represents the third greatest risk factor for disease and disability in the world. Ethanol is metabolized in the body to acetaldehyde by enzymes, generating reactive oxygen species (ROS). It thus injures healthy tissues causing diseases such as neuropathy and myopathy, as well as nutritional deficits including low levels of vitamin D₃. The objective of this study is therefore to verify whether supplementation with vitamin D₃ minimizes ethanol effects over alcoholic myopathy and neuropathy. Two experimental groups were used: male Wistar rats (n=20), and rats of UChB lineage (n=20) (volunteer ethanol drinkers). Both groups were divided in two subgroups: Vitamin D₃ – 12.5µg/kg/day (500 UI) cholecalciferol (WV, n=10 and UV, n=10), and Control (WC, n=10 and UC, n=10), for a period of 75 days. The volumes of consumed 10% ethanol solution and industrialized food, as well as changes in body weight were measured every week. Blood analyzes of vitamin D₃, calcium, phosphorus, and PTH were carried out. The EDL muscle of the right antimer was used for morphologic and morphometric assessment, and the EDL muscle from the left antimer, for evaluation of oxidative stress. The deep fibular nerve was harvested and used for morphologic and morphometric analyses. There was weight gain during the experiment. The caloric intake of feed did not demonstrate difference in relation to vitamin supplementation. Caloric intake from feed was not different in relation to vitamin supplementation. The feed and ethanol ingested were homogeneous in the UChB subsets. 25(OH)D₃ dosages were significantly different in the Wistar vitamin D₃ subset as compared to the control groups. Calcium, phosphorus, and PTH dosages were not different between the groups studied. In regard to biochemical dosages, the levels of GSH, SOD and CAT activities, O₂- release, and TBARS was not statistically different between the experimental groups, as well as in the animals that received vitamin supplementation. The morphologic analyzes of the deep fibular nerve showed a decrease in the diameters of the myelin sheaths and axons in the groups supplemented with vitamin D₃; the G rate did not vary. There was significant decrease in the amount of slow fibers in the subgroups supplemented with vitamin D₃. The area of fast fibers was significantly larger in the subgroups supplemented with vitamin D₃. The area of the slow fibers was significantly larger in the WV subset, and the UV subset had a significantly smaller area. The results obtained suggest that the ingestion of ethanol modulates the cellular and systemic anti-oxidant systems in a different way, and that the model used of chronic alcoholism did not cause myopathies and neuropathies, since vitamin D₃ supplementation modulated slow fibers into fast fibers in the EDL muscle.

Key-words: chronic alcoholism; myopathies; neuropathies and vitamin D₃.

1.0 Introduction

Excessive use of ethanol is a global problem and represents the third greatest risk factor in the world for diseases and disability (Rocco *et al.*, 2014).

After ingestion, ethanol is distributed throughout the body, rapidly crosses the blood-brain-barrier, and injures many tissues: liver, central and peripheral nervous system, cardiac and skeletal muscles (Chopra & Tiwari, 2011).

In the liver, alcohol is enzymatically metabolized into acetaldehyde. Those metabolic pathways generate reactive oxygen species (ROS) and lead to lipid peroxidation (Gao & Bataller, 2011; Tiwari & Chopra, 2012), what finally damages the healthy tissues (Jung *et al.*, 2011). Additionally, acetaldehyde itself is highly reactive, toxic, and immunogenic, affecting normal cell functions (Bootorabi *et al.*, 2008; Fritz *et al.*, 2013).

ROS are reactive chemical entities produced as intermediaries in the oxy-reduction reactions (redox). Imbalances in production and elimination of ROS by antioxidant systems result in oxidative stress and, presumably, in pathophysiologic alterations (Marchi *et al.*, 2014), decrease defense mechanisms, increase the oxidation of proteins, lipids, and DNA, leading to neural damage (Tiwari & Chopra, 2012).

Neuropathy is a frequent complication, its incidence varying from 32 to 76% in subjects that drink ethanol. This disease comprises axonal abnormalities with wallerian degeneration and reduction in fibers myelination (Yerdelen *et al.*, 2008), as well as axonal loss (Mellion *et al.*, 2013; Nguyen *et al.*, 2012).

Those abnormalities promote sensorial and motor deficits, affecting mainly the lower limbs (Vittadini *et al.*, 2001), what results in walking difficulties, as well as impaired motor coordination (Juntunen *et al.*, 1978; Hodges *et al.*, 1986).

Ertem *et al.*, (2009) noticed that chronic alcoholism impacts negatively in peripheral nerves regeneration associated to a significant decrease in the number of axons and increase in axonal degeneration.

As a consequence of the toxic effects of chronic ethanol use, already mentioned above, we also have to consider that chronic use of ethanol may lead to severe muscle problems (Lynch, 1969; Rubin *et al.*, 1976; Torrejais *et al.*, 2002; Adachi *et al.*, 2003), among which alcoholic myopathy has been described (Shankar *et al.*, 2008).

This disease affects from 40 to 60% of alcoholics, and it features mainly muscle weakness and atrophy (Preedy *et al*, 2003). Other typical common symptoms include gait anomalies, strength reduction, and loss of skeletal muscle mass (Jung *et al*, 2011; Wang *et al*, 2012).

In thesis, muscle atrophy may be a consequence of decreased protein synthesis or increased catabolism. Ethanol is a strong inhibitor of the protein synthesis in the muscles (Preedy *et al.*, 2001), type II fibers (Fast) being more affected than type I fibers (Slow) (Preedy & Peters, 1990; Reilly *et al.*, 2000; Adachi *et al.* 2003; Castellón *et al.*,2005; Otis *et al.*,2007; Otis & Guidot, 2009; González-Reimers *et al.*, 2010).

High concentrations of ethanol and its metabolites physiologically induce autophagy in skeletal muscles. Whether the autophagy is beneficial since it removes toxic protein aggregates or deleterious contributing to sarcopenia has yet to be established (Thapaliya *et al.*, 2014).

Many researchers have associated alcohol intake to bone alterations, where low levels of vitamin D₃ have been reported in alcoholic subjects or animals treated with ethanol (Turner *et al.*,1988; Diamond *et al.*, 1989; Laitinen & Välimäki, 1991; Lindholm *et al.*, 1991; Santori *et al.*, 2008; Fisher & Fisher, 2007; Miroliaee *et al.*, 2010).

It is therefore clear that ethanol has a profound effect in calcium homeostasis and vitamin D₃ metabolism (González-Reimers *et al.*, 2010); secondary hyperparathyroidism (Annweiler *et al.*, 2010), hypocalcemia and hypophosphatemia may also be present (Pfeifer *et al.*, 2002) as a consequence of vitamin D₃ deficiency.

Vitamin D₃ reintegrates muscle tissue (Annweiler *et al.*, 2010), increasing its strength, associated to a decrease in type II fibers atrophy (Sato *et al*, 2005), as well as to an increase in the average diameter and percentage of this type of fibers (Chatterjee, 2001; Ceglia, 2009).A relationship between vitamin D₃ and nervous regeneration has also been described (Chabas *et al.*, 2008), as well as myelination (Chabas *et al.*, 2013; Montava *et al.*, 2014).

Considering that daily doses recommended are difficult to obtain in regular diet due to the small amount found in food except for those enriched with this vitamin D₃, and that diet of alcoholics often is insufficient, thereby, the objective of this study was to evaluate the effects of administration isolated or vitamin D₃ and ethanol association for chronic alcoholism on the alcoholic neuropathy and myopathy.

2.0 Specific objectives:

- Assessment of alcoholic neuropathy and myopathy: morphologic and morphometric analyses of the muscle *extensor digitorum longus* (EDL – predominance of type II fibers) (Ceglia *et al.*, 2013) and of the deep fibular nerve, respectively.
- Assessment of oxidative stress (EDL muscle): levels of reduced glutathione (GSH); activities of superoxide dismutase (SOD), and of catalase (CAT), levels of O₂⁻ (lucigenin) and thiobarbituric acid (TBARS).
- Assessment of blood parameters: vitamin 25(OH)D₃; calcium (Ca²⁺); phosphorus (P); parathormone (PTH).

3.0 Material and Methods

Forty male adult 90-day-old rats were used, 20 UChB rats (from the Animal Research Laboratory Department of IBB/UNESP) and 20 Wistar rats (from the Central Animal Research Laboratory of USP São Paulo-SP). The rats were kept in polyethylene boxes (40x30x15cm), covered with wood shavings, under controlled conditions of luminosity (12h light, 12h dark), and temperature (20 to 25 C), receiving filtered water and industrialized food (*Provence*[®]) *ad libitum*. All the experimental procedures were approved by the Committee on Animal Research and Ethics of the Biosciences Institute, UNESP, Botucatu (Protocol number 531).

The animals were weaned at 21 days of age, and grouped in boxes containing from two to four animals, receiving water and industrialized food *ad libitum*. At 55 days of age they were individualized. At 60 days, the groups of animals that received alcohol had one bottle of water and one bottle containing 10% ethanol solution, periodically alternated, and both *ad libitum*. After 15 days evaluating the intake of the 10% ethanol solution, the lineage UChB was selected and standardized (MARDONES e SEGOVIA-RIQUELME, 1983). The animals that had average intake higher than 2.0mL 10% ethanol /100g of body weight/day were selected for the UChB lineage.

After selecting the UChB rats, 2 experimental groups were formed with 10 animals each. The groups (UChB and Wistar) were therefore subdivided in two subgroups: one subset (Vitamin D₃) that received 12.5ug/kg (500 UI) Cholecalciferol (C9756- Sigma) daily, diluted in olive oil given by gavage, and another subset (Control) that received the vehicle (olive oil) by gavage, both for 75 days.

3.1 Diet, energy balance, and biometric parameters

The volume of 10% ethanol solution consumed, the amount of food eaten, and the weight variation were evaluated every week.

3.2 Euthanasia and biological material harvesting

After the experimental period (75 days), the animals were fasted for 12 hours, weighted, and decapitated. Blood was collected and centrifuged at 2,000 rpm during 10 minutes, and the plasma/serum obtained kept at -80 C; Vitamin 25(OH)D₃; Calcium (Ca²⁺); Phosphorus (P); Parathormone (PTH) were measured. Those dosages were conducted in the *Laboratório de Análises VitaeLab* (analyses laboratory) in São Paulo- SP (vitamin D₃ –Chrom systems Instruments & Chemicals kit – HPLC, Ca²⁺ and P –Beckman Coulter reagent kits, and PTH - Access Intact PTH reagent kit by BeckmanCoulter).

After decapitation, the pelvic limbs were hair-shaved and the EDL muscles (*extensor digitorum longus*) of the right and left forelimbs were dissected (Figure 1), their motor points individualized, and the proximal third of the deep fibular nerve was harvested (Figure 2).

3.3 Oxidative Stress Analysis

The EDL muscle of the left foreleg was subdivided in 5 fragments in order to analyze the levels of reduced glutathione (GSH), superoxide dismutase activity (SOD), and catalase (CAT), as well as the levels of O²⁻ release (lucigenin), and of thiobarbiturate acid (TBARS).

3.3.1 Levels of GSH

GSH concentrations were evaluated as described in the work by Gonzaga *et al.* (2014). The results are expressed as µg de GSH/mg of protein.

3.3.2 Activity SOD

SOD activity was determined by a colorimetric method using a commercially available kit (Cayman Chemical, Ann Arbor, Michigan, EUA). It was subsequently evaluated using a commercially available kit (Sigma-Aldrich, St. Louis, MO, EUA). SOD was expressed as inhibition rate % / ml of plasma.

3.3.3 Activity CAT

CAT was determined as described in the work by Gonzaga *et al.*, (2014). One CAT unit was defined as the amount of enzyme necessary to break down 1 nmol of H₂O₂/min.

3.3.4 Levels of O²⁻

The levels of the anion superoxide were determined by the chemiluminescence lucigenin-enhanced assay (Leite *et al.*, 2013). The production of the anion superoxide is expressed as relative light units (RLU)/mg protein. In all the experiences, the Lowry test (Bio-Rad Laboratories, Hercules, CA, USA) was used in order to determine protein concentration.

3.3.5 TBARS

TBARS were determined by calorimetry using the commercially available kit (Cayman Chemical, Ann Arbor, MI, USA). The results were expressed as nmol/ mg of protein.

3.4 Morphologic and morphometric analyses of the deep fibular nerve

After individualization, the proximal third of the deep fibular nerve was fixed in a 1% osmium tetroxide solution, and processed according to the histologic routine. From the slides obtained (5µm cross-sectional slices), the number of axons, myelin thickness, fiber diameter, and the calculation of the G rate by means of the free software "Image J" were obtained.

3.5 Morphologic and morphometric analyses of the muscle fibers and immunohistochemistry analyses for the Fast and Slow fibers

The EDL muscle of the right antimer was used, reduced in its medium third. The fragments were covered with neutral powder and frozen in liquid nitrogen (stored in freezer at - 80° C).

Sequential histologic slices were subsequently obtained in cryostat Leica CM 1800 at -25° C, 8 µm thick, stained with HE and immunoperoxidase [Mouse Monoclonal Antibody MHC - slow (1: 180) and fast (1: 130) (NCL-MHCS; NCL and MHCf / Novacastra); H-Histofine Rat display system (Multi - Nichirei), revelation with chromogenic liquid DAB (1:50) and counter-staining with hematoxylin].

4.0 Statistical analysis

The results on body weight, ingestion of feed-derived calories (kcal), and ethanol were analyzed by the ANOVA test with repeated measures – Tukey-Kramer's multiple comparison test ($p \leq 0.05$).

For the biochemical results the parametric Student's t-test was used with Kruskal-Wallis normality test ($p \leq 0.05$).

The Vitamin D₃ dosage - 25(OH)D₃ was analyzed by the generalized linear model with gamma distribution. The analyses of the dosages of Calcium and phosphorus were done by Student's t-test with Kruskal-Wallis normality test ($p \leq 0.05$).

Morphometry of the fibular nerve was analyzed by generalized linear model with negative binomial distribution and the axonal count by the Student's t-test with Kruskal-Wallis normality test ($p \leq 0.05$).

In order to quantify the fast fibers, the generalized linear model with negative binomial distribution was used, and Poisson distribution for the slow fibers ($p < 0.005$).

The measurement of the fast fibers area was analyzed by generalized linear model with Gamma distribution, and the slow fibers were analyzed by the ANOVA test with repeated measures – Tukey-Kramer's multiple comparison test ($p \leq 0.05$).

5.0 Results

5.1 Body weight

Body weight analyzes were conducted at 90 and 165 days of age (figure 3). There was weight gain throughout the weeks ($p < 0.05$).

5.2 Feed and ethanol caloric intake (kcal) of the experimental groups

In Figure 4 we observe that the animals of the groups supplemented or not with vitamin D₃ had higher ethanol intake (ml) with no change in feed intake (g). From those values (figure 4) added to the values of feed intake of the Wistar group (figure 5) in weeks 1 (90 days of age) and 10 (165 days of age), the amount of calories consumed in feed (table 2) and ethanol (table 3) were calculated. 1g feed = 3.955 kcal; 1g ethanol = 7.1kcal.

In relation to vitamin D₃ supplementation in the UChB group as well as in the Wistar group, there was no difference in the intake of feed-derived kcal. A higher

quantity of feed kcal was eaten by the animals from the Wistar group (figure 5).

In the UChB group, vitamin D₃ supplementation did not change ethanol-derived kcal consumed. There was an increase in ethanol kcal consumed throughout the experiment in animals supplemented or not with vitamin D₃ (table 3), depicted in figure 4, which represents ethanol ingestion in ml.

5.4 Biochemical analysis

5.4.1 Dosage of enzymes involved in oxidative stress

All the biochemical analyses were conducted in the muscle tissue (EDL) of all the animals of the experimental groups. No statistical difference was found between the experimental groups studied in any of the parameters analyzed: GSH levels (Figure 6A), SOD activity (Figure 6B), CAT activity (Figure 6C), O²⁻ release determined by chemiluminescence lucigenin-enhanced assay (Figure 7A), and TBARS concentrations (Figure 7B).

5.5 Blood values

Circulating vitamin D₃ values 25(OH)D₃ in the Wistar group were higher ($p=0.0026$) in the WV subset. Calcium, phosphorus, and PTH were similar in this group ($p > 0.05$) (table 4).

In the UChB subgroup, the values for vitamin D₃, calcium, phosphorus, and PTH were similar ($p > 0.05$) (table 4).

5.6 Morphologic analyses of the deep fibular nerve

The general morphology analyses did not show any difference between the groups. Histology did not find morphology changes such as axon degeneration, endoneurial edema, or inflammatory infiltrate (Figure 8). Histologic features of the nerves of the Wistar and UChB groups were similar in relation to axon number, thickness of myelin sheath (Figure 9), and G rate (Figure 10) ($p > 0.05$).

In the groups supplemented with vitamin D₃ there was decrease in the average diameter of the nervous fibers (WC: 4.2 ± 0.2 ; WV: 3.7 ± 0.3 ($p = 0.0320$); and UC: 5.4 ± 0.6 ; UV: 4.2 ± 0.2 ($p = 0.0038$), and also of the axons (WC: 2.6 ± 0.13 ; WV: 2.2 ± 0.30 ($p = 0.0317$); and UC: 2.9 ± 0.3 ; UV: 2.1 ± 0.2 ($p = 0.0013$) (Figure 11).

5.7 HE and Immunohistochemistry of the muscle fibers

The morphologic analyses using HE found normal muscle fibers in all the experimental groups. The shape was of a polygon with peripheral nuclei and preservation of endomysium and perimysium (Figure 12A).

The quantification of fast and slow muscle fibers from the immunohistochemistry demarcation of the muscle fibers for MHC fast and slow in all the experimental groups demonstrated a mosaic pattern with predominance of fast fibers, compatible with the EDL muscle (Figure 12B and C).

The quantitative analysis of the fast fibers was constant in all the experimental groups. The number of slow fibers was decreased in the groups supplemented with vitamin D₃ (WC: 26.80± 8.17; WV: 19.40± 7.80, (p= 0.0154); and UC: 31.40± 9.40; UV: 23.60± 8.88, (p= 0.0191) (Figure 13).

The area of the fast fibers (type II) was significantly larger in the groups supplemented with vitamin D₃ (WC: 1990.07± 964.99 and WV: 2129.30± 870.18 UC: 1502.26± 807.65 and UV: 1743.15± 999.71) (p< 0.001) (Figure 14). In the Wistar group there was significant increase in the area of slow fibers (type I) in the subset WV as compared to the WC (WC: 872.35± 292.91; WV: 961.11± 285.43) (p = 0.0044). In the UChB group, in the UV subgroup there was a decrease in the area as compared to the subset UC (p= 0.0197, UC: 712.96± 185.23; UV: 644.96± 189.51).

6.0 Discussion

Acetaldehyde is a metabolite enzymatically generated from the ingestion of ethanol, leading to the production of reactive oxygen species (ROS) (Gao & Bataller, 2011; Tiwari & Chopra, 2012) that can cause many modifications in healthy tissues (Jung *et al.*, 2011), among them neuropathy (Yerdelen *et al.*, 2008; Nguyen *et al.*, 2012; Mellion *et al.*, 2013), alcoholic myopathy (Preedy *et al.*, 2003; Shankar *et al.*, 2008; Jung *et al.*, 2011; Wang *et al.*, 2012), as well as decreased levels of vitamin D₃ (Miroliaee *et al.*, 2010; González-Reimers *et al.*, 2010; Annweiler *et al.*, 2010), and atrophy of type II fibers (Preedy & Peters, 1990; Reilly *et al.*, 2000; Adachi *et al.*, 2003; Castellón *et al.*, 2005; Otis *et al.*, 2007; Otis & Guidot, 2009; González-Reimers *et al.*, 2010)

The intensity of such modifications is directly related to the amount and duration of alcohol intake. Ethanol intake is considered a source of energy since for each gram of ethanol metabolized 7.1 kcal are generated. This generation of calories

is known as “empty calories”, since in spite of the high energetic value, there are no essential nutrients such as proteins, vitamins, and other elements associated to them (Lieber, 2005).

In our study the animals that drank 10% ethanol solution increased body weight during the experiment. Many studies have reported increase in body weight with ethanol intake, related to growth/age of the animals during the period, as well as the feed formulation ingested concurrently with the ethanol (Mitchell & Herlong, 1986; Suter *et al.*, 1997).

Yeomans (2004) analyzed food intake in human beings one hour after drinking alcohol and suggested that, depending on metabolism or nutritional status, ethanol may either decrease satiety or act as an appetite stimulator.

Although we found body weight increase in the animals of this study, there was no significant increase in food intake; the weight gain may be related to an increase in adipose tissue as a consequence of ethanol use (Lukasiewicz *et al.*, 2005; Traversy & Chaput, 2015); the frequency of ethanol intake was more than seven times a week (Sayon-Orea *et al.*, 2011).

Ethanol intake increased during the period studied. Ethanol use leads to tolerance, so that increasingly higher doses are necessary in order to achieve drunkenness. Dependence is directly associated to excessive and compulsive use of ethanol (Kalsi *et al.*, 2009).

Vitamin D₃ supplementation did not influence the weight of the animals or the kcal ingested.

There are many reports on the effects of vitamin D₃ over the reintegration of muscle tissue (Annweiler *et al.*, 2010), associated to reduction in the atrophy of type II fibers (Chatterjee, 2001; Sato *et al.*, 2005; Ceglia, 2009), acting also in nervous regeneration (Chabas *et al.*, 2008), and myelination (Chabas *et al.*, 2013; Montava *et al.*, 2014).

In this study the vitamin D₃ used was 25(OH)D₃, which corresponds to its circulating form. The blood rates were statistically different only in the supplemented Wistar group. Calcium, phosphorus, and PTH were statistically similar in the experimental groups.

Vitamin D₃ is enzymatically converted into 25(OH)D₃ in the liver and then in the kidney to its active form; its conversion is regulated by the blood calcium and

phosphorus levels, under the influence of the parathyroid hormone (PTH) (Plum & Deluca, 2010).

Many researches have obtained different results in PTH levels in alcoholic people, varying from low to normal to high (Laitinen & Välimäki, 1991; Bikle *et al.*, 1993; García-Valdecasas-Campelo *et al.*, 2006; Santori *et al.*, 2008). High PTH levels are associated to muscle weakness, fatigue, and atrophy of type II muscle fibers (Patten *et al.*, 1974).

Although chronic ethanol users have low levels of vitamin D₃ (Turner, 2000; Wagnerberger *et al.*, 2012), our experimental groups did not present this abnormality. We believe that this finding may be justified by the fact that there is no standardization of the adequate methods and reference values for human beings (Lips, 2004) or for rodents.

Antioxidant unbalance, as well as increase in the lipid peroxidation and production of free radicals and ROS are also present in chronic alcoholism (Adachi *et al.*, 2000; Koo-Ng *et al.*, 2000; Mansouri *et al.*, 2001; Preedy *et al.*, 2001; Preedy *et al.*, 2001a; Fernandez-Sola *et al.*, 2002), with increased oxidative stress in muscle tissue (Fujita *et al.*, 2002).

Vitamin D₃ in physiologic concentrations acts protecting the cells against oxidative damage, (Codoñer-Franch *et al.*, 2012; Chandrashekar *et al.*, 2015; Bhat & Ismail, 2015).

The biochemical levels of GSH found in this study suggest that vitamin D₃ did not change the cellular non-enzymatic antioxidant capacity (Muniz *et al.*, 2015).

No alteration was found in the SOD, CAT, O²⁻ and TBARS activities in the Wistar animals or in the UChB, supplemented or not with vitamin D₃.

Muniz *et al.* (2015) and Hipólito *et al.* (2015) analyzed the oxidative stress in plasma of rats with chronic ethanol intake and found that SOD activity was not altered. Fernández-Sola *et al.*, 2002, found a decrease in muscle analyses for GSH, and increased SOD activity. Plasmatic CAT activity has been described as increased under the same conditions (Muniz *et al.*, 2015).

SOD is considered a transcriptional target of vitamin D₃, its expression tending to increase with vitamin increase (Zhong *et al.*, 2014).

Considering vitamin D₃ in the study by Bhat & Ismail, 2015, a decrease in the activity of CAT and SOD was observed in rats with vitamin D₃ deficiency.

Hipólito *et al.* (2015) observed an increase in the levels of plasma TBARS after ethanol intake, similar to what was found by Muniz *et al.* (2015), that analyzed rats cavernous tissue and did not find abnormalities in this parameter but found significant increase in O^{2-} production (Muniz *et al.*, 2015), also present in the aorta of rats with chronic ethanol intake (Passaglia *et al.*, 2015).

We believe that the discrepancy between the data obtained may be justified by the fact that most authors used plasma for their analyses, and our results were obtained from muscle tissue. One must also consider that muscles with different metabolism have different antioxidant defenses (Wolfson *et al.*, 1995). The muscles with a predominance of oxidative fibers – slow contraction (type I) have more antioxidant enzymes activity (Semba, *et al.*, 2007). The EDL muscle, target of this study, has predominance of glycolytic fibers – fast contraction (type II).

Axonal degeneration (Ertem *et al.*, 2009; Nguyen *et al.*, 2012; Mellion *et al.*, 2013), reduction both in the number of axons and in myelination (Yerdelen *et al.*, 2008) have been a common finding in chronic alcoholism. In our study no histologic abnormality or change in the number of axons was observed. In the subsets supplemented with vitamin D₃ there was a decrease in the diameter of the fibers and of the axons, although no significant change was found in G rate.

There are evidences that vitamin D₃ has positive effect over myelination (Chabas *et al.*, 2008; Bianco *et al.*, 2011; Chabas *et al.*, 2013; Montava *et al.*, 2014; Gueye *et al.*, 2015), vitamin D₃ receptor (VDR) being present both in the Schwann cells and in oligodendrocytes (Chabas *et al.*, 2013).

The G rate is a parameter related to the conduction velocity of the nervous impulse. According to Mendonça *et al.* (2003), low values (around 0.4) usually indicate axonal degeneration, while high values (around 0.7) indicate myelin degeneration or regeneration. The results found in this study indicate, therefore, that the velocity of nervous impulse in the experimental groups is within the normal ranges found in the literature.

Both groups had intact axons and myelin sheaths, with regular shape, therefore compatible with normal nerves, reinforcing those aspects.

The morphology analyses done with HE did not demonstrate changes in the muscle fibers in relation to any of the parameters analyzed. The fibers had polygonal shape, peripheral nuclei, and preservation of endomysium and perimysium.

Morphology studies describe that in alcoholic myopathy there is intracellular edema, enlarged and distorted mitochondria, type-grouping, vacuoles formation, proliferation of connective tissue, and inflammatory infiltrate, with the possibility of necrosis (Sestoft *et al.*, 1994; Koll *et al.*, 2002; Adachi *et al.*, 2003; Fernández-Sola *et al.*, 2002; Ertem *et al.*, 2009).

Immunohistochemistry for all types of muscle fibers demonstrated a mosaic pattern, with predominance of fast fibers (glycolytic and fast- contracting), compatible with the EDL muscle.

Most authors have reported an increase in type II fibers (fast) with vitamin D₃ supplementation (Sorensen *et al.*, 1979; Sato *et al.*, 2005; Pfeifer *et al.*, 2009; Ceglia *et al.*, 2013; Bhat *et al.*, 2015).

Our results therefore point towards vitamin D₃ contributing to enhance the glycolytic patterns of the EDL muscle. In the quantitative analyses of the fibers we found decreased numbers in the slow fibers in the subsets supplemented with vitamin D₃ (Wistar and UChB), data also found by other authors (Chatterjee, 2001; Sato *et al.*, 2005; Ceglia, 2009). The literature shows that ethanol promotes atrophy of type II (fast) muscle fibers (Preedy & Peters, 1990; Reilly *et al.*, 2000; Adachi *et al.*, 2003; Castellón *et al.*, 2005; Otis *et al.*, 2007; Otis & Guidot, 2009; González-Reimers *et al.*, 2010).

The area of slow muscle fibers (type I) is minimally affected in alcoholism; in some circumstances it may present compensatory hypertrophy, and in severe cases, atrophy (Takeda *et al.*, 2000; Preedy *et al.*, 2001).

There are few reports in the literature in relation to the area of slow muscle fibers (type I) and vitamin D₃. Girgis *et al.*, 2012, studying knockout rats for vitamin D₃ receptors observed atrophy in slow muscle fibers (type I) and fast fibers (type II).

In this study there was significant decrease in slow fibers (type I) in the UChB group supplemented with vitamin D₃. We therefore believe that these results may be justified by the fact that the dose of vitamin D₃ used was not enough to promote this type of change. In the Wistar group supplemented with vitamin D₃ there was an increase in the area of slow fibers, what suggests modulation of this type of muscle fiber into the fast type (Ceglia, 2009).

The use of vitamins and other antioxidants generally demands a dose definition, more studies been necessary on the mechanism of action before prescription (Witting *et al.*, 1997; Witting *et al.*, 1999; Drummond *et al.*, 2011).

The results obtained suggest that ethanol intake modulates the cellular and systemic antioxidant systems in a different way, and that the model of chronic alcoholism used did not cause myopathy or neuropathy, since vitamin D₃ supplementation promoted modulation of slow fibers into fast fibers in the EDL muscle.

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Figures

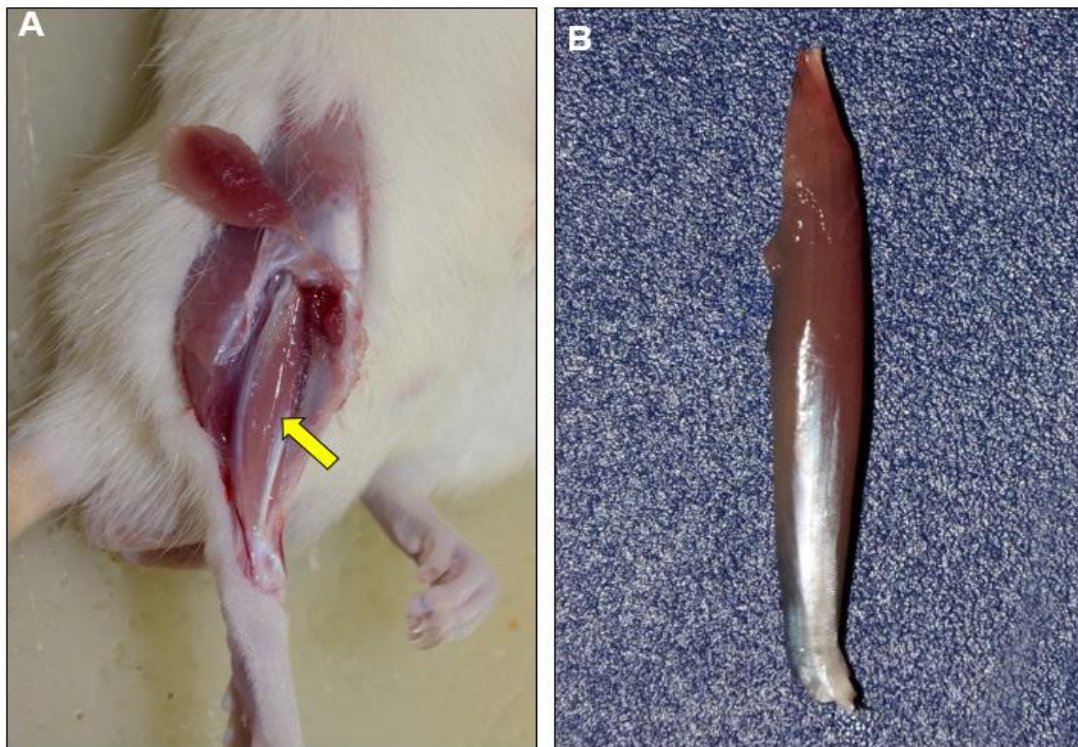


Figure 1. A: EDL muscle *in situ* and B: EDL isolated muscle



Figure 2. Deep fibular nerve *in situ*

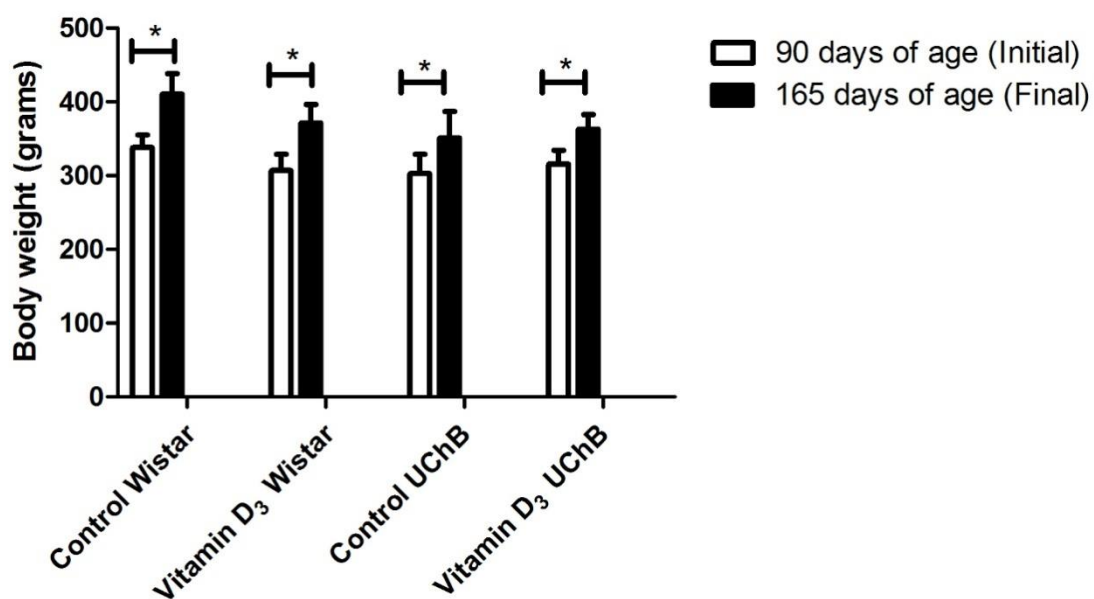


Figure 3. Graphic of initial (90 days) and final (165 days) body weight (g) of the experimental groups. Bars and vertical columns represent averages and standard deviations where * corresponds to $p \leq 0.05$.

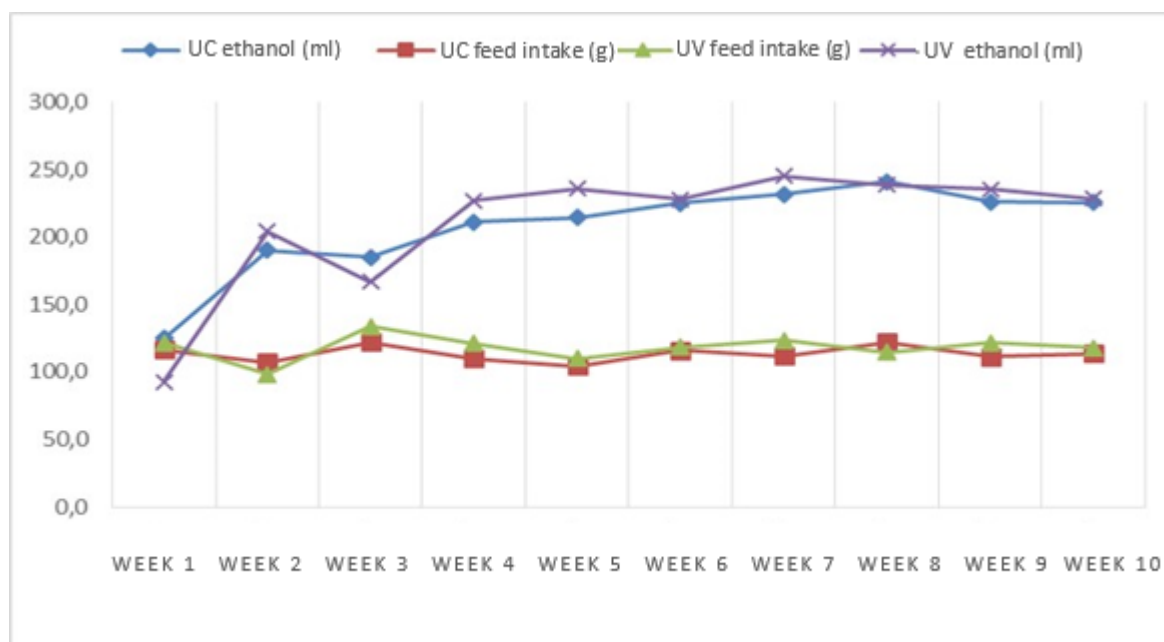


Figure 4. Schematic representation of feed intake (g) and ethanol intake (ml) rate of the experimental groups in weeks 1 and 10.

Table 2. Averages and standard deviations of food-derived calories ingested (Kcal/day) by the experimental groups. Data were analyzed by ANOVA test with repeated measures – Tukey-Kramer’s multiple comparison test ($p \leq 0.05$).

Feed-derived Calories (kcal)				
	WC	WV	UC	UV
Initial (90 days of age)	702.7 ± 58.35 aA	639.4± 44.8aA	460.8 ± 32.4bB	480.5± 67.3bB
Final (90 days of age)	669.1± 236.3aA	583.4± 42.6aA	441.0± 71.0bB	480.5 ± 38.5bB

Lower cases correspond to the groups comparison with the weeks fixed.

Upper cases correspond to the weeks comparison with the groups fixed.

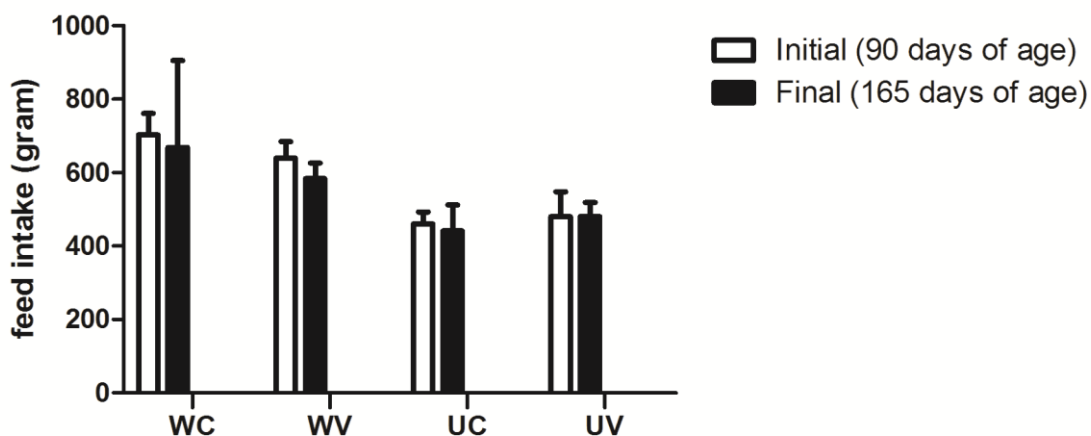


Figure 5. Graphic of the experimental groups feed intake (g) in initial and final. Bars and vertical columns represent averages and standard deviations.

Table 3. Averages and standard deviations of ethanol-derived calories (kcal/day) in groups UC and UV. Data were analyzed by ANOVA test with repeated measures – Tukey-Kramer’s multiple comparison test ($p \leq 0.05$).

Ethanol-derived calories (kcal/day)		
	UC	UV
Initial (90 days of age)	70.00±29.63 aA	51.80±5.64 aA
Final (90 days of age)	126.29±14.27 aB	127.98±17.09 aB

Lower cases correspond to the groups comparison with the weeks fixed.

Upper cases correspond to the weeks comparison with the groups fixed.

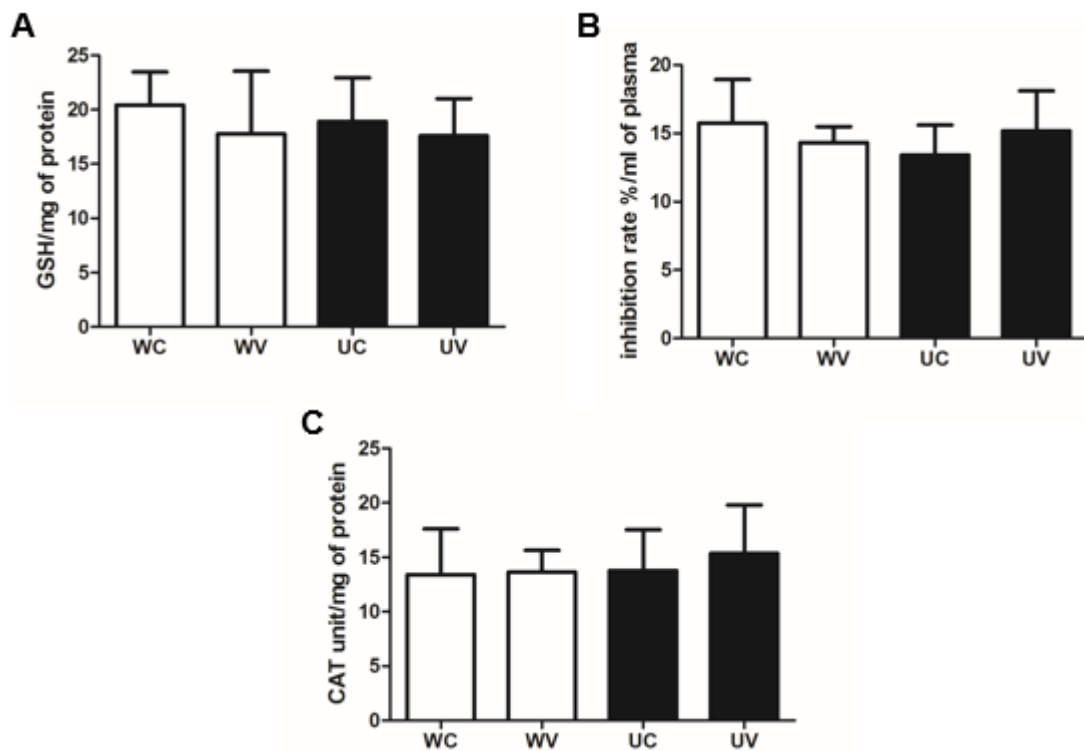


Figure 6. Graphic of averages and standard deviations of the effects of vitamin D₃ supplementation and ethanol in the analyses of oxidative stress in the EDL muscle of rats. A: GSH levels; B: SOD activity; and C: catalase activity ($p < 0.05$, parametric Student's t-test).

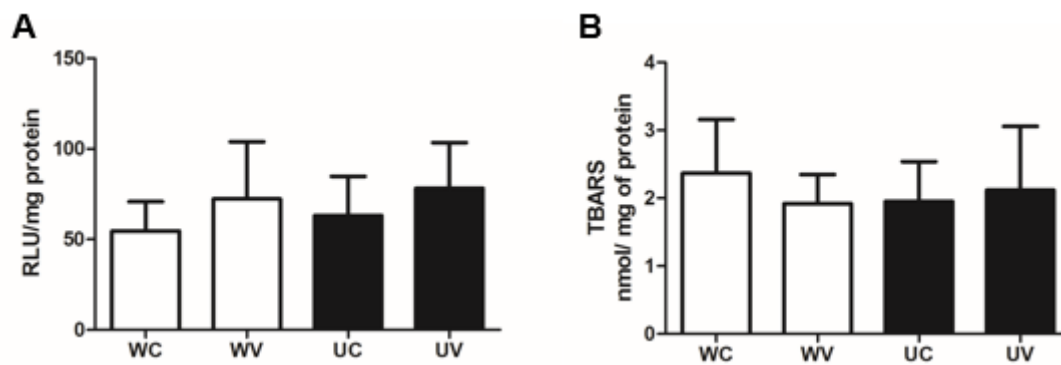


Figure 7. Graphic of averages and standard deviations of the effects of vitamin D₃ supplementation and ethanol in the analyses of oxidative stress in the EDL muscle of rats. A: O₂⁻ release as determined by chemiluminescence lucigenin-enhanced assay; B: TBARS concentration.

Table 4. Averages and standard deviations of blood dosages of the experimental groups. Data were analyzed by generalized linear model with Gamma distribution (vitamin D₃) and Student's t-test (calcium and phosphorus).

Blood dosages				
	WC	WV	UC	UV
25(OH)D₃ (ng/ml)	2.8 ± 0.5	4.2 ± 1.2*	23.6 ± 42.7	75.9 ± 80.7
Calcium (mg/dl)	10.3 ± 0.4	10.4 ± 0.3	10.1 ± 0.63	10.7 ± 0.3
Phosphorus (mg/dl)	8.7 ± 0.9	8.9 ± 1.0	9.2 ± 1.8	9.6 ± 0.5
PTH (pg/ml)	1	1	1	1

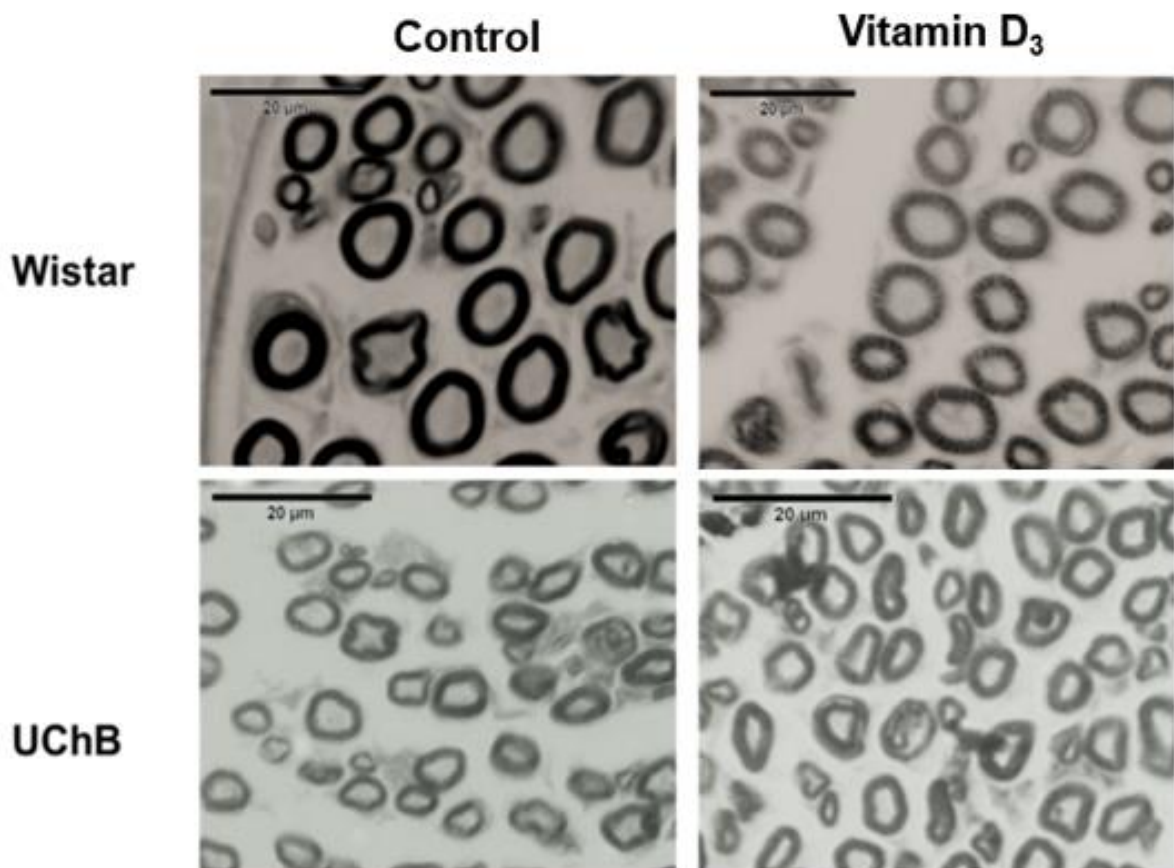


Figure 8- Photomicrography of cross-sectional slices of the deep fibular nerve of the experimental groups stained by osmium tetroxide.

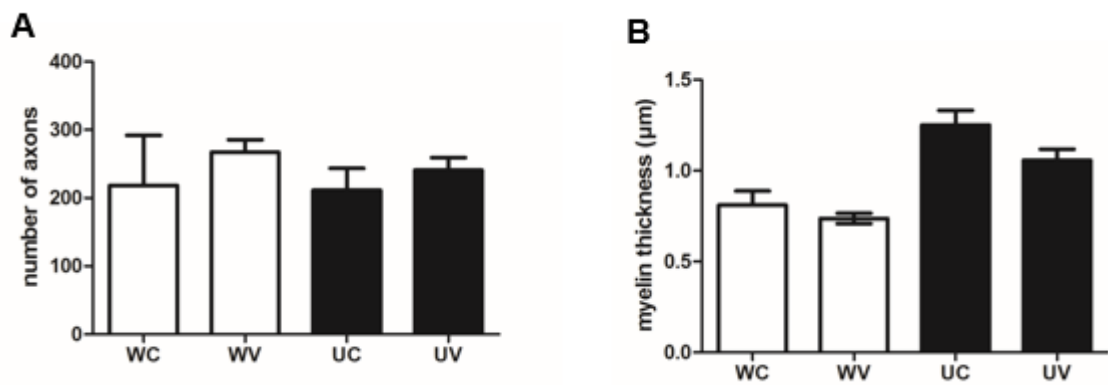


Figure 9. Graphic of the number of axons and myelin thickness (μm) of the experimental groups. Bars and vertical columns represent averages and standard deviations.

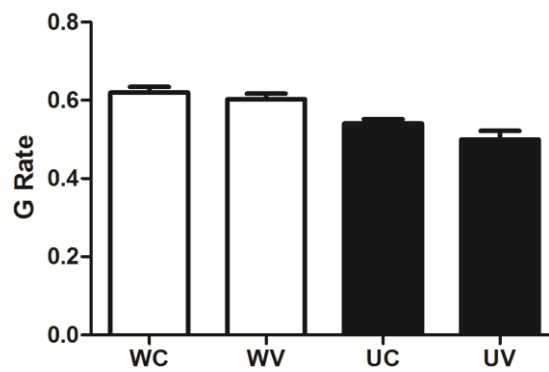


Figure 10. Graphic of the G rates of the experimental groups. Bars and vertical columns represent averages and standard deviations.

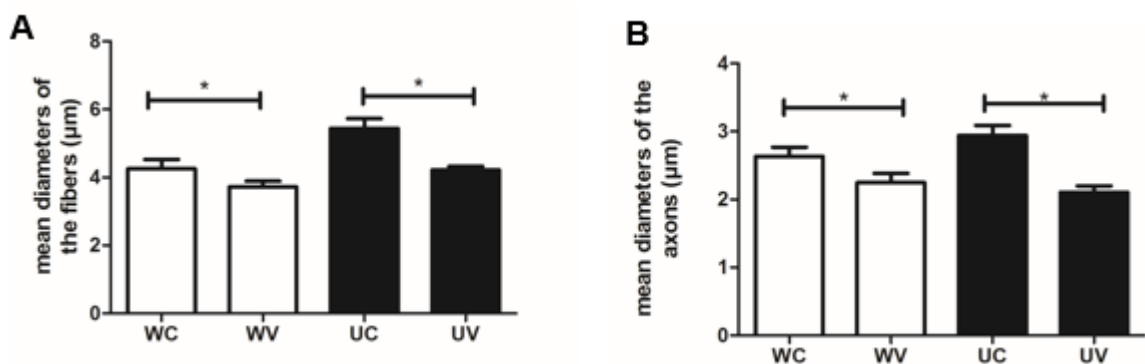


Figure 11. Graphic of the mean diameters of the fibers (μm) and of axons (μm) of the experimental groups. Bars and vertical columns represent averages and standard deviations of data where * corresponds to $p \leq 0.05$.

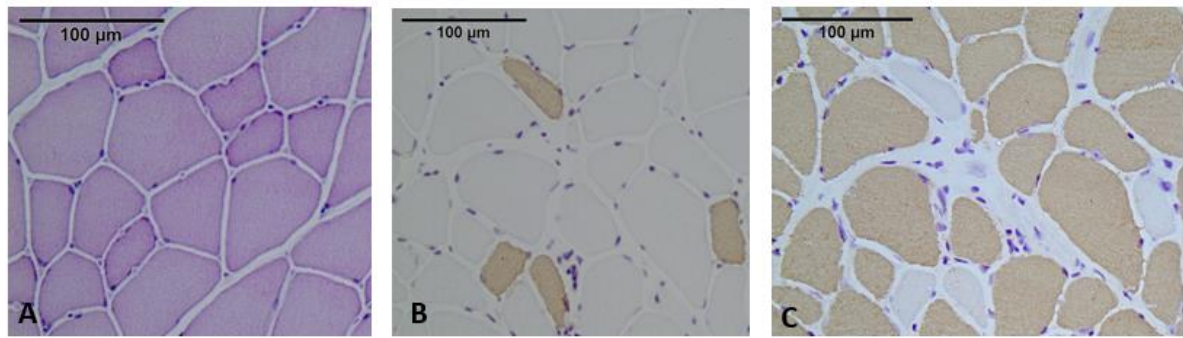


Figure 12. Photomicrography of cross-sectional slices of the muscle fibers of the UChB Control group. A: Hematoxylin and Eosin; B: Immunohistochemistry of the Slow fibers; C: Immunohistochemistry of the Fast fibers.

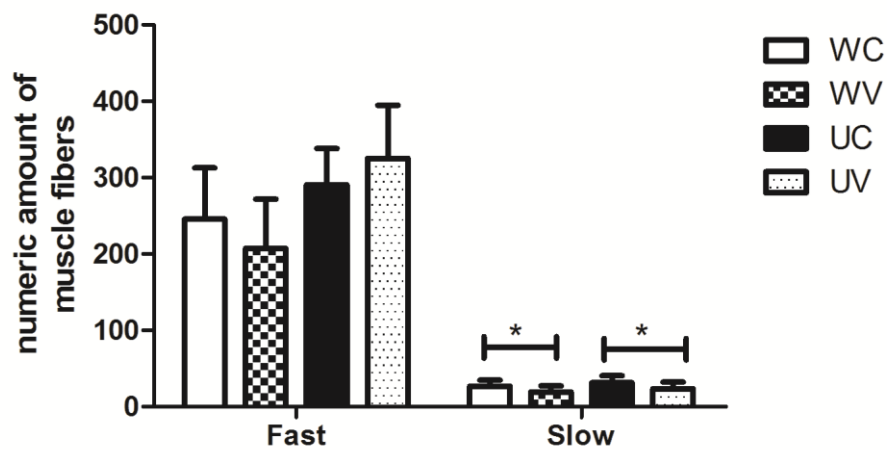


Figure 13. Graphic of the numeric amount of fast and slow muscle fibers in the EDL muscle. Bars and vertical columns represent averages and standard deviations of data where * corresponds to $p \leq 0.05$.

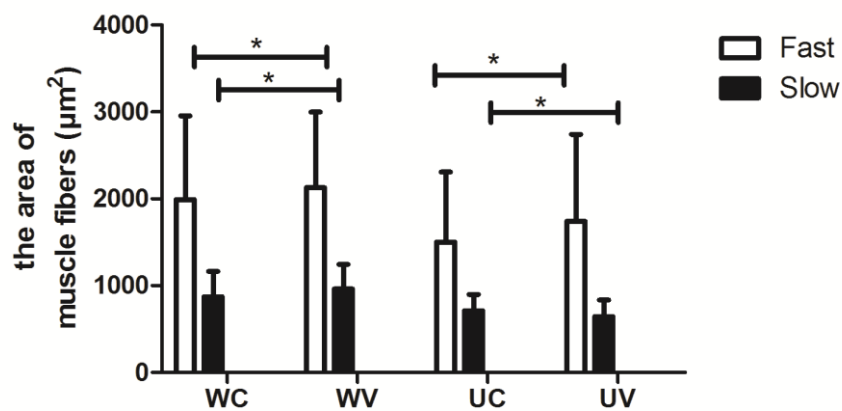


Figure 14. Graphic of the area of fast and slow muscle fibers (μm^2) in the EDL muscle. Bars and vertical columns represent averages and standard deviations of data where * corresponds to $p \leq 0.05$.

Anexos

Anexo 1



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu



Certificado

Certificamos que o Protocolo nº 531-CEUA, sobre “Efeito da Vitamina D em ratos UChB (bebedores voluntários de etanol): estudo comportamental e morfológico (Músculo EDL e nervo fibular profundo)”, sob a responsabilidade de **Selma Maria Michelin Matheus**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado “Ad referendum” da **COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA)**, nesta data.

Botucatu, 26 de agosto de 2013.

Prof. Dr. Wellerson Rodrigo Scarano
Presidente da CEUA

Anexo 2



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu



Declaração 01/2015-CEUA
ATRB/WRS

DECLARAÇÃO

Declaro para os devidos fins e a pedido da Profa. Dra. Selma Maria Michelin Matheus que o protocolo CEUA/IBB sob o número 531 intitulado *"Efeito da Vitamina D em ratos UChB (bebedores voluntários de etanol): estudo comportamental e morfológico (músculo EDL e nervo fibular profundo)"* sob sua coordenação foi avaliado e aprovado pela Comissão de Ética no Uso de Animais do Instituto de Biociências em 26/08/2013. Conforme documentação apresentada, a referida pesquisa coordenada pela Dra. Selma teve como colaboradora e co-executora a Srta. Carina Guidi Pinto, aluna de mestrado, cuja dissertação intitula-se: *"Associação da Suplementação de Vitamina D3 e do alcoolismo experimental em ratos: efeitos morfológicos e comportamentais"*. Dessa forma, para fins éticos e legais, como se trata do mesmo protocolo experimental, o certificado 531 é extensivo ao novo título atribuído ao protocolo.

Por ser verdade, firmo o presente.

Botucatu, 10 de Dezembro de 2015

Prof. Adj. Wellerson Rodrigo Scarano
Presidente da Comissão de Ética no Uso de Animais
IBB/UNESP



Anexo 3



ISSN: **0031-9384**

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