Leonardo de Oliveira Mendes

"Análise de receptores hormonais e marcadores inflamatórios no lobo ventral da próstata de ratos consumidores voluntários de etanol (UChB): influência da terapia hormonal com testosterona"

"Hormonal receptors and inflammatory markers analysis in the ventral prostate of ethanol-preferring rats (UChB): influence of hormonal therapy with testosterone"

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Orientador: Dr. Francisco Eduardo Martinez
Coorientador: Dr. Wellerson Rodrigo Scarano

"Hormonal receptors and inflammatory markers analysis in the ventral prostate of ethanol-preferring rats (UChB): influence of hormonal therapy with testosterone"

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Biologia Celular e Estrutural do Instituto de Biologia da Universidade Estadual de Campinas para obtenção do Título de Doutor em Biologia Celular e Estrutural, na área na área de Biologia Tecidual.

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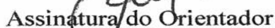

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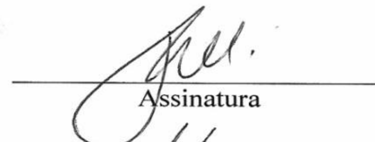

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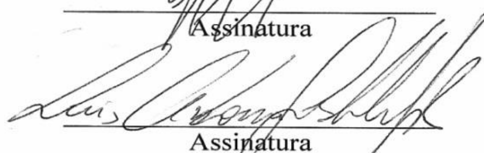

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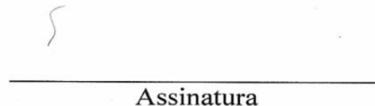

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Resumo

O etanol provoca danos reprodutivos, diretamente nos tecidos ou indiretamente via eixo hipotalâmico-hiposário-gonadal. A próstata, hormônio-dependente, é susceptível aos efeitos do etanol. Evidências atribuem papel anti-inflamatório à testosterona, com crescente interesse na ação da terapia hormonal sobre o etanol. Assim, o presente trabalho teve como objetivo avaliar os efeitos da terapia hormonal com testosterona sobre o consumo de etanol: 1) nas dosagens hormonais plasmátcas e tecidual, na histopatologia, proliferação celular e na localização e expressão de receptores hormonais (artigo I); 2) nas concentrações plasmáticas e expressão de citocinas pró e anti-inflamatórias (artigo II) no lobo ventral da próstata de ratos UChB. Ratos com 90 dias de idade foram divididos em dois grupos experimentais (n = 20/grupo): **C**, consumo de água *ad libitum,*e **EtOH**, consumo de etanol a 10% (v/v), > 2g de etanol/kg de peso corpóreo/dia, e água *ad libitum*. Aos 150 dias de idade, 10 ratos de cada grupo receberam injeções subcutâneas de cipionato de testosterona (5mg/kg de peso corpóreo) diluídos em olho de milho durante quatro semanas em dias alternados, **T** e **EtOH+T**, enquanto os restantes (10/grupo) receberam somente óleo de milho como veículo. Aos 180 dias de idade, os ratos foram eutanasiados por decapitação para coleta do sangue e as próstatas ventrais foram dissecadas, pesadas e processadas. Secções da próstata ventral foram coradas com hematoxilina e eosina, impregnação pela prata e azul de toluidina. Radioimunoensaio para avaliação das concentrações plasmáticas de testosterona, estradiol, diidrotestosterona (DHT) e testosterona intraprostática, imuno-histoquímica para Ki-67, AR, ERα, ERβ, DACH-1,TGF-β1, pSmad 2, e-caderina e α-

actina, western blot para AR, ERα, ERβ, DACH-1, PAR4, IL-6 IL-10, TNFα e TGF-β1 e elisa para determinação das concentrações plasmáticas de IL-6, IL-10, TNFα e TGF-β1foram realizados. A terapia com testosterona aumentou o peso da próstata ventral. Houve diminuição do compartimento epitelial e aumento do luminal no EtOH com a terapia hormonal revertendo esses efeitos. O compartimento estromal do EtOH caracterizou-se pela presença de feixes de fibras reticulares delgados e esparsos, com ruptura da camada de células musculares lisas.Focos inflamatórios, metaplasia, atipia reativa inflamatória, perda de adesão entre as células epiteliais e aumento de mastócitos intactos e desgranulados foram frequentes no EtOH e ausentes no EtOH+T. Tanto testosterona como etanol não alteraram a taxa de proliferação celular, porém maior expressão de PAR4 foi observada no EtOH. Houve aumento da testosterona plasmática, intraprostática e DHT no T e EtOH+T e diminuição do estradiol plasmático no EtOH+T. Os T e EtOH+T também apresentaram níveis plasmáticos superiores de TNFα e TGF-β1, sem diferença em relação à IL-6 e IL-10. A expressão de AR, ERβ, DACH-1 e IL-6 na próstata foi semelhante entre os grupos experimentais, diferentemente do que foi observado para ERα, TNFα e TGF-β1, que diminuíram após terapia hormonal.Nossos resultados indicam que o etanol foi capaz de induzir a emergência de focos inflamatórios, além de suprimir a imunorreatividade para e-caderina e α-actina. A testosterona reverteu esses efeitos, reduzindo a expressão do ERα, TGF-β1, TNFα e NFR2, tornando-se um possível alvo a ser avaliado para as disordens causadas pelo etanol.

Abstract

Ethanol induces reproductive damages, directly in the tissues or indirectly by hormonal imbalance. Prostate, a hormone-dependent gland, is susceptible to effects caused by ethanol. Emerging evidences assign to testosterone an anti-inflammatory role, with growing interest in the action of hormone therapy on the effects of ethanol. Therefore, the current research aimed to assess the effects of hormone therapy with testosterone on the ethanol consumption: 1) in tissue and plasma hormone assays, histopathology and immunolocalization and expression of hormone receptors (manuscript I); plasma levels and expression of pro and anti-inflammatory cytokines (manuscript II) in the ventral prostate of UChB rats (ethanol-preferring rat). UChB rats aged 90 days were divided into two experimental groups (n=20): **C**: drinking water only and **EtOH**: drinking 10% (v/v) ethanol at > 2 g/kg body weight/day + water. At 150 days of age, 10 rats from each group received subcutaneous injections of testosterone cypionate (5mg/kg body weight) diluted in corn oil every other day during 4 weeks, constituting **T** and **EtOH+T**, while the remaining animals (10/group) received corn oil as vehicle. All animals were euthanized at 180 days old by decapitation. Blood was collected to obtain plasma hormone and cytokines concentrations and ventral prostate was dissected, weighted and processed. Prostate sections were stained with hematoxylin and eosin, Gomori's reticulin and toluidine blue. The following techniques were performed: radioimmunoassay to plasma concentrations of testosterone, dihydrotestosterone (DHT), estradiol and intraprostatic testosterone, immunohistochemistry to Ki-67, AR, ERα, ERβ, DACH-1, TGF-β1, pSmad 2, e-cadherin e α-actinin, western blot

to AR, ERα, ERβ, DACH-1, PAR4, IL-6 IL-10, TNFα and TGF-β1 and elisa to plasma concentrations of IL-6, IL-10, TNF-α and TGF-β1. Testosterone therapy increased the ventral prostate weight. There were reducing of epithelial compartment and increasing of luminal compartment in the EtOH and hormonal therapy was able to reverse this pattern. Stroma compartment of EtOH showed thin and sparse reticular fiber bundles with rupture of smooth muscle cell layer. Inflammatory foci metaplasia, inflammatory reactive atypia, loss of cell-cell adhesion and increasing of degranulated mast cells were frequent in EtOH and absent in EtOH+T. Testosterone and ethanol did not alter the proliferation rate, but high expression of PAR4 was shown in EtOH. There was increasing of plasma and intraprostatic testosterone and plasma DHT in T and EtOH+T and decreasing of estradiol in EtOH+T. The T and EtOH+T exhibited increasing of plasma TNFα and TGF-β1, without differences regarding to IL-6 and IL-10. AR, ERβ, DACH-1 and IL-6 expressions were similar among the experimental groups, differently from ERα, TNFα and TGF-β1, which decreased after hormonal therapy. Our results show that ethanol was able to induce the emergence of inflammatory foci, besides suppress the immunorreactivity to e-cadherin and α-actinin. Testosterone was able to reverse these effects, downregulating ERα, TGF-β1, TNFα and NFR2, showing to be promising in the treatment of alcohol-related disorders.

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"O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis."

José de Alencar

"Todas as vitórias ocultam uma abdicação"

Simone de Beauvoir

Á minha FAMILIA, meus pais e meu irmão, pelo amor e incentivo dedicados a mim.

"O amor é sofredor, é benigno; o amor não é invejoso; o amor não se vangloria, não se ensoberbece, não se porta inconvenientemente, não busca os seus próprios interesses, não se irrita, não suspeita mal; não se regozija com a injustiça, mas se regozija com a verdade; tudo sofre, tudo crê, tudo espera, tudo suporta."

Coríntios 1, 13:4-7

Àquela que me ajudou a chegar aqui. A minha vó para todo o sempre, Orlanda.

"Foi o tempo que perdeste com a tua rosa que a fez tão importante"

Antoine de Saint-Exupéry

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Introdução

O consumo de etanol, principalmente aquele relacionado à ingestão crônica, é importante fator de risco para o desencadeamento de diversos problemas de saúde (Rehm, 2011). Pesquisa da Organização Mundial de Saúde demonstrou que 17% da população mundial são alcoólicas, sendo que no Brasil o alcoolismo é um dos mais graves problemas de saúde, decorrente de complicações no campo somático-psíquico e pela repercussão no meio social e econômico (Halsted, 2004; WHO, 2006). Acredita-se que o uso abusivo do etanol é fator de risco para mais de 60 doenças e injúrias, resultando em aproximadamente 2,5 milhões de mortes/ano no mundo (Edwards *et al*., 2011).

O etanol e os seus metabólitos provocam distúrbios generalizados em vários sistemas orgânicos e no metabolismo da glicose, lipídeos e proteínas (Martinez *et al*., 2000; Gomes *et al*., 2002). Por induzir a formação de espécies reativas de oxigênio (ROS), o etanol é capaz de desestabilizar proteínas e lipídeos celulares (lipoperoxidação), favorecendo o processo de apoptose e necrose, prejudicando a homeostasia tecidual. Tanto o etanol quanto o seu metabólito, o acetaldeído, tem efeitos sobre vários fatores de crescimento celular, inibindo a síntese de ácido retinóico e a proliferação celular (Riley *et al*., 2001).

Modelos animais têm contribuído para explicar alguns aspectos biológicos: bioquímicos, fisiológicos e morfológicos envolvendo o consumo de bebidas alcoólicas por humanos (Cândido *et al*., 2007). Li *et al*. (1987) menciona a existência de três pares de linhagens de ratos de alto e baixo consumo de etanol. Os UChA e UChB (UCh =

Universidade do Chile) os mais antigos, os de Helsink iniciados por Eriksson (1968) e os de Lumeng & Li em Indianápolis. As linhagens AA (*Alko Alcohol*) e ANA (Alko Nonalcohol) de Helsink foram obtidas por cruzamentos alternados de *inbreeding* e *outbreeding*. As linhagens P (*Alcohol Prefering* – 5-8g/kg/dia) e NP (*Non-Alcohol Prefering* – menos de 0,5/kg/dia) de Lumeng & Li tem sido obtidas por cruzamentos *outbreeding.* Além de ratos, existem variedades de camundongos, tanto de alto consumo, a C57BL/6, como de baixo consumo de etanol, a BALB e PBA. Dos três pares descritos, os únicos que se mantém em *inbreeding* são os UChA e UChB e, por conseguinte, são as únicas variedades consideradas puras para os efeitos de estudos genéticos, bioquímicos, fisiológicos, nutricionais e farmacológicos do efeito do etanol, bem como apetite e tolerância, que são importantes fatores do alcoolismo humano.

A próstata é a glândula sexual acessória masculina freqüentemente afetada pela hiperplasia benigna e pelo câncer (Guess, 2001). Os lobos ventral e dorsolateral da próstata são os mais susceptíveis e os primeiros a responder às alterações (Roy-Burman *et al*., 2004) morfológicas e inflamatórias em roedores (Morón *et al*., 2000; Cagnon *et al*., 2001; Scarano *et al*., 2009). A testosterona e a dihidrotestosterona possuem papel essencial no desenvolvimento, proliferação, diferenciação, manutenção e fisiologia da próstata (García-Flórez *et al*., 2005; Fávaro & Cagnon, 2007; Rittmaster, 2008). As conseqüências da toxicidade do etanol são complexas, sendo que persistem dúvidas sobre a morfofisiologia do estroma e sua interação com as células epiteliais em resposta ao alcoolismo crônico (Cândido *et al.*, 2007). O etanol pode agir diretamente nos testículos, alterando a síntese de testosterona (Bannister & Lowosky, 1987; Anderson *et al*., 1989; Saxena *et al*., 1990; Tadic *et al*., 2000) e indiretamente, interferindo no eixo hipotálamo – hipófise – testículo,

reduzindo a produção de andrógenos pelas células de Leydig (Salomen & Huhtaniemi, 1990; Tadic *et al*., 2000). Como conseqüência da diminuição dos andrógenos, ocorre alterações na próstata, como diminuição do tamanho e massa, atrofia epitelial, apoptose, diminuição da secreção e na expressão dos receptores de andrógenos (AR) (Salomen & Huhtaniemi, 1990; Kiess & Gallaher, 1998; Tadic *et al*., 2000).

É conhecido que estrógenos também estão envolvidos no crescimento normal e anormal da próstata em várias espécies (Adams *et al*., 2002). Testosterona circulante é convertida em estrógeno em vários tecidos pela enzima aromatase (Garcia-Flórez *et al*., 2005). Os receptores de estrógeno α (ERα) e β (ERβ) são expressos na próstata de ratos adultos, mas com diferença na localização. ERα é predominantemente expresso no estroma e quase ausente no epitélio, enquanto ERβ é altamente expresso no epitélio (Sugiyama *et al*., 2010). A aromatase foi detectada na próstata humana e de roedores, sugerindo a existência de uma possível fonte de estradiol, podendo influenciar tanto células prostáticas epiteliais como estromais via seus respectivos receptores (Garcia-Flórez *et al*., 2005). Os estrógenos podem agir diretamente através de seus receptores ou indiretamente via prolactina na próstata (Harkonen & Makela, 2004). Portanto, o controle hormonal prostático envolve eventos complexos que dependem, principalmente, do equilíbrio existente entre os hormônios esteróides (Figura 1).

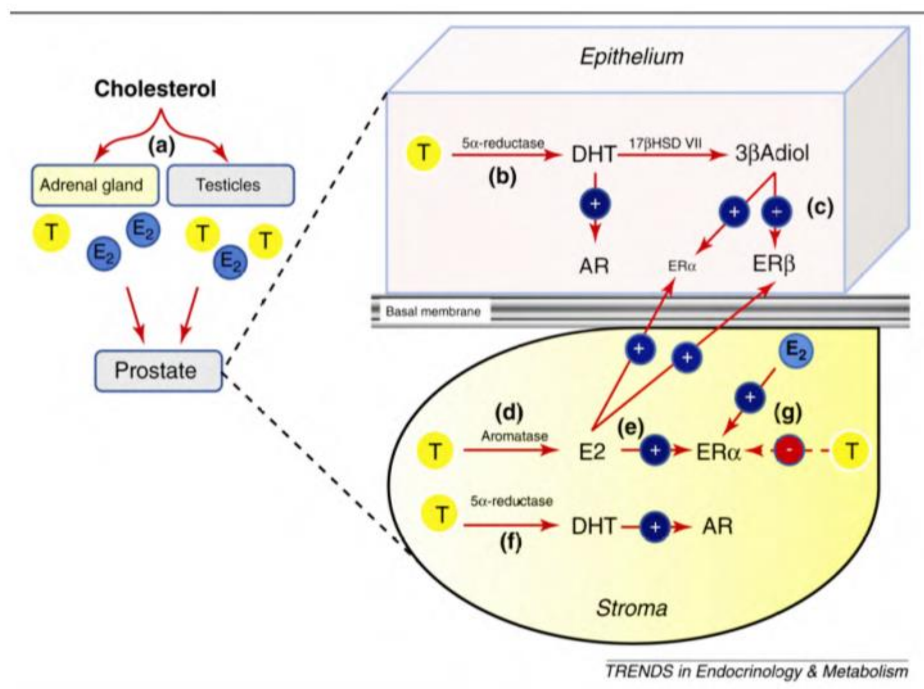

Figura 1. Ação dos hormônios esteróides no epitélio e estroma prostáticos. Abreviações: AR – receptor de andrógeno; ERα – receptor de estrógeno α; ERβ – receptor de estrógeno β; 3βAdiol - 5α-androstene-3β,17β-diol; DHT – dihidrotestosterona; E2 – estradiol; 17βHSDVIII - 17β-hidroxiesteróide desidrogenase tipo 7; 5α-reductase - 5α-redutase; T – testosterona. Linhas sólidas representam aumento da atividade ou ativação; linhas pontilhadas mostram atividade reduzida ou inibição (Sugiyama *et al*., 2010).

Segundo Yatkin *et al*. (2009), o desequilíbrio entre as concentrações de andrógenos e estrógenos, alterando a relação andrógenos/estrógenos, pode ser o fator responsável pelo desencadeamento do processo inflamatório. Exemplos de descompensação hormonal são verificados em casos de envelhecimento e obesidade (Prins & Korach, 2008; Yatkin *et al*., 2009). A concentração de testosterona diminui, porém a concentração de estrógenos permanece inalterada, devido ao aumento do peso corpóreo associado ao aumento das células adiposas que expressam aromatase e convertem, perifericamente, andrógenos em estrógenos (Prins & Korach, 2008). O aumento da estimulação estrogênica na próstata está

associado à reativação do seu crescimento, inflamação (Ellem & Risbridger, 2009), a transformações neoplásicas (Harkonen & Makela, 2004; Prins & Korach, 2008) e, possivelmente, câncer (Bosland, 2006). Ver figura 2.

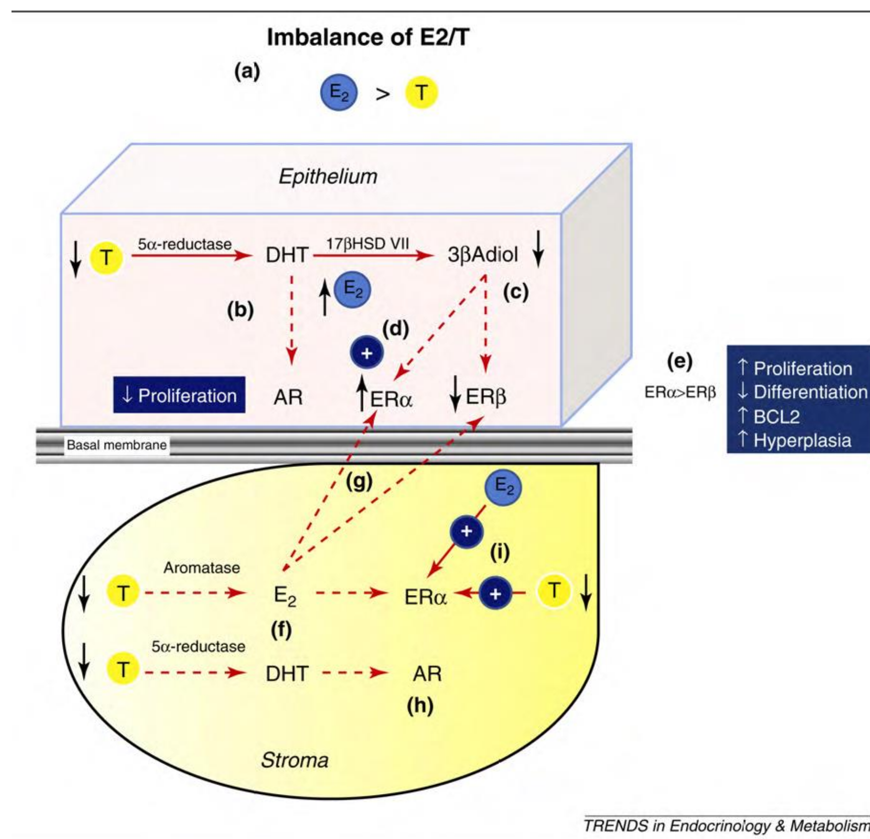

Figura 2. (a) Um desequilíbrio na relação E2:T favorece a ação do E2, que possui um papel fundamental no desenvolvimento do câncer de próstata. (b) Redução na conversão de T em DHT no epitélio diminui a ativação de AR e diminui a proliferação celular, estabelecendo um ambiente protetor. (c) Diminuição de DHT, entretanto, também reduz a síntese de 3βAdiol e a ativação de ERβ, embora o (d) aumento de E2 ative o ERα. (e) O predomínio da ativação do ERα sobre o ERβ aumenta a proliferação celular, hiperplasia e a expressão do fator apoptótico BCL-2, além de diminuir a diferenciação celular. (f) No estroma, a produção local de E2 e DHT é reduzida devido à diminuição dos níveis de T, levando a uma redução (g) da ativação parácrina dos ERs no epitélio e (h) uma redução da ativação local de AR. (i) Por outro lado, a diminuição dos níveis de T junto com o aumento de E2 permite maior ativação de ERα, aumentando a predisposição ao ambiente carcinogênico na próstata. Linhas sólidas representam aumento da atividade ou ativação; linhas pontilhadas mostram atividade reduzida ou inibição (Sugiyama *et al*., 2010). Para abreviações, ver figura 1.

O processo inflamatório é reação integrada, defendendo o corpo contra distúrbios que interfiram na homeostase, particularmente infecções e injúrias. A resposta é inicialmente caracterizada pela liberação local de citocinas que amplificam e regulam a cascata inflamatória (Cesari *et al*., 2004). Citocinas compreendem a família de moléculas de comunicação intracelular que possuem papel importante na imunidade, inflamação, reparo e homeostase tecidual geral (Wilson & Balkwill, 2002). Vários estudos correlacionam a presença de citocinas, quimiocinas e fatores de crescimento com o aumento do risco de desenvolvimento de câncer de próstata (Haverkamp *et al*., 2008), estabelecendo a hipótese de que a inflamação pode ser fundamental no processo (Krishnan & Feldman, 2010).

Dentre os principais marcadores inflamatórios, estão a interleucina seis (IL-6) e o fator de necrose tumoral alfa (TNFα), citocinas pró-inflamatórias e interleucina dez (IL-10), anti-inflamatória (Cesari *et al*., 2004). TNFα é o primeiro fator na promoção e no desenvolvimento da via inflamatória (Cesari *et al*., 2004), induzindo complexa cascata de outras citocinas inflamatórias e fatores de crescimento que recrutam e ativam uma gama de células no sítio de infecção e dano tecidual (Wilson & Balkwill, 2002). Sugimoto *et al.* (2011) e Mosli *et al.* (2012) comprovaram tal fato ao induzirem experimentalmente prostatite em ratos Wistar, verificando aumento da concentração de TNFα na próstata. No que diz respeito à carcingênese prostática, o papel do TNFα torna-se mais complexo. Evidências recentes atribuem a essa citocina função ambígua, podendo atuar tanto estimulando a angiogênese quanto inibindo a neovascularização e induzindo apoptose (Tse *et al*., 2012).

Dentro do quadro de promotores da inflamação, encontra-se também a IL-6, multifuncional, produzida por vários tecidos e tipos celulares, como células T e B, macrófagos e fibroblastos (Eliav *et al*., 2009). Em adição ao papel imunológico, a IL-6 está envolvida na regulação de várias funções celulares, entre elas a proliferação, apoptose, angiogênese e diferenciação (Azevedo *et al*., 2011) e na promoção de alguns tumores, como melanomas, carcinomas renais, ovarianos e de mama (Bouraoui *et al*., 2008). É considerada um dos fatores promotores da hiperplasia benigna e do câncer de próstata (Starsichová *et al*., 2010), podendo atuar de forma autócrina sobre as células malignas ou parácrina sobre as células normais do microambiente tumoral (Azevedo *et al*., 2011). Concentrações circulantes elevadas de IL-6 e TNFα estão associadas ao alcoolismo crônico (Szabo, 1999) e ao avanço da doença em pacientes com câncer de próstata (Haverkemp *et al*., 2008). Ao contrário, a IL-10 tem importante efeito regulatório nas respostas imunológicas e inflamatórias, pois é capaz de inibir a produção de outras citocinas pró-inflamatórias (Cesari *et al*., 2004), dentre elas IL-6 e TNFα, além de induzir a proliferação de mastócitos (Hamidullah *et al*., 2012). IL-10 também é conhecida por exercer papel na regressão tumoral, através do aumento da atividade das células Nk, verificado em modelo experimental de câncer de mama (Kundu *et al*., 1996).

Dentro do quadro de citocinas, o fator de crescimento transformante beta 1 (TGF-β1) exerce importante contribuição no desenvolvimento e na homeostase tecidual prostática, além do envolvimento na regulação do crescimento celular, angiogênese, modulação da matriz extracelular (Wikström *et al*., 2001) e proteção contra doenças imunes (Starsichová *et al*., 2010). Na diminuição dos andrógenos, como na castração, ocorre

aumento do TGF-β1 na próstata, com reversão após reposição hormonal com testosterona (Ikeda *et al*., 2000). Na carcinogênese prostática, o TGF-β1 possui papel paradoxal, pois pode inibir a proliferação celular e induzir apoptose, como pode também estimular a angiogênese e estimular a metástase, através da indução do processo de transição epitélio-mesenquimal (EMT) (Wong *et al*., 2009).

A sinalização do TGF-β1 ocorre da membrana celular para o núcleo através do complexo de proteínas *smad* (Starsichová *et al*., 2010).

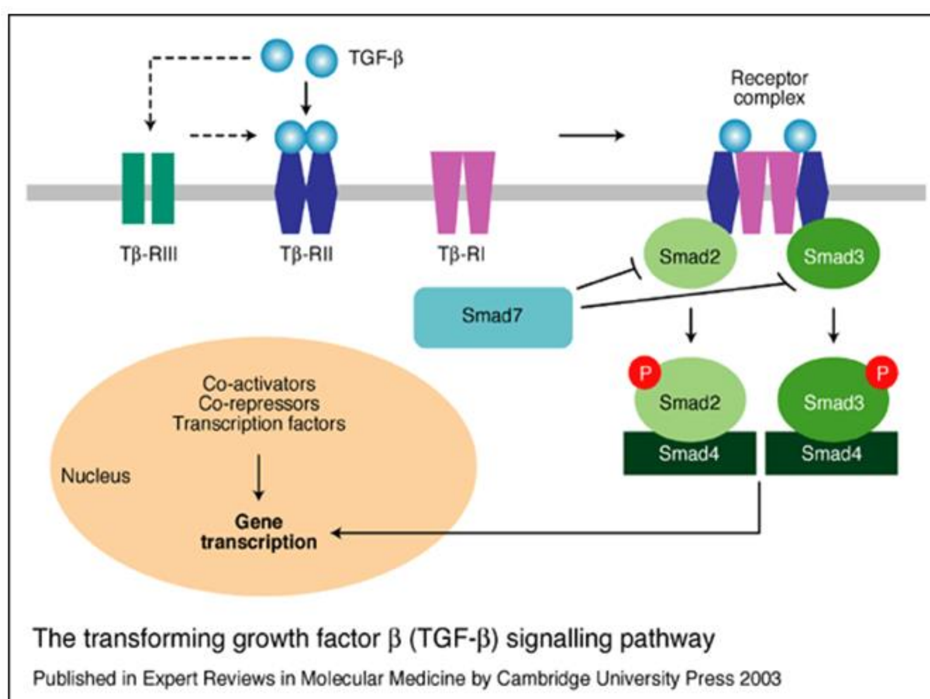

Figura 3. Via de sinalização do TGF-β. TGF-β liga-se ao receptor Tβ-RII; essa ligação pode ser aprimorada na presença de Tβ-RIII. Após ligação ao TGF-β, Tβ-RII recruta e fosforila Tβ-RI, levando à ativação de smad 2 e smad 3 por fosforilação. Esse processo é inibido pela smad 7. Smad 2 e 3 ativadas formam heterodímeros com smad 4 e transloca-se para o núcleo, regulando a expressão gênica.

Aumento do processo apoptótico verificado em células epiteliais tumorais prostáticas de animais castrados foi atribuído à indução da via de sinalização do TGF-β1,

mais especificamente ao aumento da expressão de smad 2 e 3 (Brodin *et al.,* 1999). Grande parte das dúvidas existentes a respeito do papel paradoxal do TGF-β1 se deve ao fato dessa citocina atuar também na EMT, processo no qual as células epiteliais polarizadas adotam características mesenquimais, o que propiciaria a atividade migratória dessas células (Smith & Odero-Marah, 2012). Característica importante durante a EMT é a perda da adesão célula – célula. Essa adesão é feita por proteínas, como a e-caderina, que são suceptíveis a diversos fatores de crescimento (Putzke *et al*., 2011). Em adenocarcinomas prostáticos, o padrão de expressão de e-caderina é irregular, com clara perda de marcação em algumas áreas do tumor (Blando *et al*., 2011).

Recentes pesquisas no campo dos processos inflamatórios e tumorigênicos têm depreendido grande atenção para o estudo da Dach1, proteína predominantemente nuclear que contém dois domínios: DachBox-N e DachBox-C, altamente conservados de drosófilas à humanos (Popov *et al*., 2009). O interesse pelo estudo dessa proteína surgiu a partir das descobertas de que sua expressão está alterada no câncer de mama, próstata, ovário e endométrio (Popov *et al*., 2009; Wu *et al*., 2009). Foi verificado que em linhagem de células tumorais mamárias dach 1 inibe a via de sinalização do TGF-β1 através da ligação à proteína smad 4 (Wu et al., 2003). No caso do câncer prostático sua expressão está reduzida, correlacionando-se com progressão e invasão tumoral (Wu *et al*., 2009).

Em adição aos efeitos indiretos causados pelo etanol, através da diminuição da testosterona plasmática e consequente desbalanço hormonal (Saxena *et al*., 1990; Tadic *et al*., 2000) alguns autores sugerem uma ação direta baseada na hipótese de que a próstata é capaz de metabolizar etanol em acetaldeído, levando a geração de radicais livres e aumento do stress oxidativo no órgão (Castro *et al*., 2002; Gomés *et al*., 2007). Em resposta ao stress

oxidativo há liberação de diversos fatores, dentre eles o NFR2, responsável por induzir a expressão de genes antioxidantes e relacionados à proteção celular (Zhang et al., 2005). Cheng et al. (2006) relataram função adicional para o NFR2, suprimindo genes inflamatórios, principalmente interleucinas, sugerindo essa via como um novo alvo terapêutico para o tratamento de doenças inflamatórias.

Justificativas

É descrito na literatura científica especializada que, com o avanço da idade, há diminuição da testosterona, causando desequilíbrio na taxa de andrógenos/estrógenos. Esse desequilíbrio hormonal pode ser responsável pela geração de um processo inflamatório, principalmente em órgãos hormônio-dependentes, como a próstata. Assim, como no envelhecimento, o etanol também inibe a produção de testosterona, porém não se sabe se essa redução é capaz de gerar uma descompensação hormonal e, consequentemente, inflamação. Portanto, trabalhos que avaliem os efeitos do etanol e da testosterona sobre receptores hormonais e marcadores inflamatórios na próstata são de extrema importância.

Objetivos

O presente trabalho teve como objetivos:

1.Analisar os efeitos do consumo crônico de etanol sobre a histopatologia, a localização e expressão de receptores hormonais e marcadores inflamatórios no microambiente prostático;

2. Avaliar os efeitos da terapia hormonal com testosterona frente ao consumo crônico de etanol, focando em parâmetros hormonais e inflamatórios.

Laboratórios envolvidos

Para desenvolvimento deste trabalho, diversos métodos foram empregados, entre eles a análise histológica, imuno-histoquímica, hormonal e western blot, envolvendo a colaboração de cinco laboratórios:

. **Laboratório de Biologia da Reprodução** – Departamento de Anatomia, IBB/UNESP, Botucatu, SP – Brasil: experimentação animal, análise histológica, imuno-histoquímica e western blot;

. **Laboratório de Desreguladores Endócrinos e Carcinogênese (LabDECA)**, Departamento de Morfologia, IBB/UNESP, Botucatu, SP – Brasil: análise histológica, imuno-histoquímica e western blot;

. **Centre for Nuclear Receptors and Cell Signaling (CNRCS/UH)**, University of Houston, Houston, TX – EUA: análise imuno-histoquímica e western blot;

. **Laboratório de Imunologia**, Departamento de Microbiologia e Imunologia, IBB/UNESP, Botucatu, SP –Brasil: elisa;

. **Laboratório de Neuroendocrinologia da Reprodução**, Departamento de Morfologia, Estomatologia e Fisiologia, FORP/USP, Ribeirão Preto, SP – Brasil: radioimunoensaio.

Os resultados estão organizados na forma de dois artigos científicos, apresentados na sequência.

Artigo I

Hormonal therapy with testosterone reverses harmful effects caused by ethanol consumption in the prostate microenvironment

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Hormonal therapy with testosterone reverses harmful effects caused by ethanol consumption in the prostate microenvironment

Running head: Testosterone reverses ethanol's effects

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Abstract

Chronic ethanol leads to reproductive damages, since it can act directly in the testis and sexual accessory tissue or indirectly, causing a hormonal imbalance. Prostate is a hormone-dependent gland and, consequently, susceptible to ethanol's effects. The potential of testosterone therapy in the ethanol-related disorders was investigated in the prostate microenvironment. UChB rats aged 90 days were divided into two experimental groups (n=20): C: drinking water only and EtOH: drinking 10% (v/v) ethanol at > 2 g/kg body weight/day + water. At 150 days old, ten rats from each group received subcutaneous injections of testosterone cypionate (5mg/kg body weight) diluted in corn oil every other day during 4 weeks, constituting T and EtOH+T, while the remaining animals (10/group) received corn oil as vehicle. All animals were euthanized at 180 days old, by decapitation. Blood was collected to obtain hormone concentrations and ventral prostate was dissected and processed for light microscope and molecular analyses. Body weight gain and caloric intake were reduced in the EtOH. Ventral prostate weight, plasma testosterone and DHT and intraprostatic testosterone concentrations were increased after testosterone treatment. Plasma estradiol level was reduced in the EtOH+T. Inflammatory foci, metaplasia and epithelial atrophy were constantly found in the prostate of EtOH and were not observed after hormonal therapy. No differences were found in the expression of AR, ERβ and DACH-1. Additionally, testosterone treatment down-regulated ERα and increased the e-cadherin and α-actinin immunorreactivies. Testosterone was able to reverse some damages caused by ethanol consumption in the prostate microenvironment.

Keywords: Ethanol, Testosterone, Prostate, Hormonal Receptors, UChB Rats.

Introduction

In addition to inciting social and economic problems, ethanol and its metabolites provoke disorders in several organic systems (Gomes *et al*., 2002). Animal models have helped to explain some of the biologic aspects of alcohol ingestion by humans. Among these animal models are the UChA and UChB strains of rats, which are the oldest and only rat strains that remain in inbreeding and therefore represent a unique model for understanding the basis of alcoholism-linked characteristics, such as those found in alcohol-related human diseases (Li *et al*., 1987).

The prostate is an accessory gland that is often affected by benign prostatic hyperplasia and cancer (Guess, 2001). In rodents, the ventral and dorsal lobes are the first to respond to morphologic and inflammatory alterations (Roy-Burman *et al*., 2004). Ethanol can act directly in the testis, modifying testosterone production (Saxena *et al*., 1990; Tadic *et al*., 2000), and indirectly in the hypothalamic – pituitary – testis axis, reducing LHRH and testosterone production by Leydig cells (Salomen and Huhtaniemi, 1990; Tadic *et al*., 2000). As a consequence of androgen decrease, changes occur in the prostate, such as involution, epithelial atrophy, apoptosis and reduction of androgen receptor (AR) expression (Salomen & Huhtaniemi, 1990; Tadic *et al*., 2000).

Testosterone and dihydrotestosterone (DHT) have essential roles in the development, proliferation, differentiation, maintenance and physiology of the prostate (Fávaro and Cagnon, 2007; Rittmaster, 2008). It is known that estrogens are also involved in the normal and abnormal growth of the prostate in different species (Adams *et al*., 2002).

Circulating testosterone is converted to estrogen in many tissues by aromatase enzyme (Carreau *et al*., 2003), and its cellular actions are mediated by two receptors, estrogen receptor α (ERα) and β (ERβ). In the prostate, ERα is expressed mainly in the stroma and is almost absent in the epithelium, whereas ERβ is highly expressed in the epithelium (reviewed in Sugiyama *et al*., 2010). Both receptors act at a particular times during development with specific and mostly opposite roles in the prostate (Morani *et al*., 2008). Therefore, hormonal prostate control involves intricate events that depend of a balance between steroid hormones.

According to Yatkin *et al*. (2009), an imbalance between androgen and estrogen concentrations (i.e., changes in the androgen/estrogen ratio) may be the essential factor responsible for triggering the inflammatory process in the diseased prostate. Increasing estrogenic stimulation in the prostate is associated with reactivation of prostate growth, inflammation (Ellem and Risbridger, 2009), neoplastic transformations (Harkonen and Makela, 2004) and, perhaps, cancer (Bosland, 2006).

Therefore, the aim of this study was to evaluate whether ethanol is able to induce hormonal imbalance by focusing on the prostate microenvironment and to verify the impact of testosterone therapy against the effects of ethanol in the ventral prostate of UChB adult rats.

Methods

Animals and experimental design

Forty adult UChB male rats were bred and maintained at the Department of Anatomy, Bioscience Institute of Botucatu, UNESP – Univ Estadual Paulista. When the UChB rats reached 60 days old, they were submitted to selection process for ethanol preference. Thus, during 20 days of selection, the rats were given a choice between two bottles containing either water *ad libitum* or 1:10 (v/v) ethanol. After this period, rats exhibiting ethanol consumption at 2-6 g/kg body weight/day were selected for this experiment according to Mardones and Segovia-Riquelme (1983). The selected rats, at 80 days old, were divided into two groups (n = 20): the EtOH group, receiving ethanol 1:10 (v/v) and water *ad libitum*, and the control group (C), receiving only water. At 150 days old, 10 rats of each group received subcutaneous injections of testosterone cypionate (Deposteron®, 5 mg/kg body weight) diluted in corn oil, every other day over 4 weeks at a consistent time each day (8:00 – 8:30 am) (Sáttolo *et al*., 2004; Scarano *et al*., 2006). Rats receiving injections constituted the T and EtOH+T groups, while the other twenty (10 rats/group) males received just corn oil.

All rats were housed in polypropylene cages with laboratory-grade pine shavings as bedding, maintained under a controlled temperature (23ºC ± 1ºC) and day/night cycle (12 h/12 h), and provided filtered tap water and rat chow *ad libitum*. Experimental protocols followed the ethical principles in animal research of the Brazilian College of Animal Experimentation (208 – CEEA).

Food intake and organs weights

Feeding content was prepared in lots of 5 days, always at the same time of day (15h) using a marked test tube and analytical balance (Ohaus Traveler; Ohaus Corporation, México, D.F, MÉXICO, MX). The profile food ingestion (caloric value of standard chow = 2,930 kcal ⁄ kg) were assigned according to the standards of necessary care. The animals were weighed at the beginning and end of the experiment to perform the calculation of the body weight gain. At the end of treatment, the ventral prostate and seminal vesicle were dissected and weighed. The determination of the body and organs weight was carried out using an analytical balance (OwaLabor, Oschatz, Germany).

Sex hormones assay

Plasma testosterone, dihydrotestosterone (DHT) and estradiol

At 180 days old, the rats were euthanized in CO2 chamber followed by decapitation. Blood samples were collected from the trunks of decapitated rats into heparinized tubes at the time of the death (between 9:00 and 11:30 am). Afterwards, plasma was obtained by centrifugation at 1,200 x g for 15 min at 4ºC and stored at -20ºC until it was assayed. Testosterone and DHT levels were determined by a double-antibody radioimmunoassay using Coat-A-Count® (Diagnostics Products Corporation, Los Angeles, USA). All samples were dosed in the same assay to avoid inter-assay errors. The intra-assay variation was 1.75%, and the results were in ng/mL. Estradiol levels were assayed by chemiluminescence

(Elecsys kit - Roche®, Basileia, Swiss; Estradiol E2 II, test sensitivity: 5 pg/mL, linearity: 4.300 pg/mL). The assays were performed at the Neuroendocrinology Laboratory, Dental School of Ribeirão Preto, University of São Paulo – USP.

Intraprostatic testosterone

After euthanasia, the ventral prostate was rapidly removed, and tissue samples of 150 mg were immediately frozen in liquid nitrogen and stored at -80ºC. The tissue fragments were homogenized (9500 rpm) with PBS during 2 min in “tube A,” and diethyl ether was added. The tissues were then placed on the vortex for 2 min. The homogenate was incubated at room temperature and the liquid phase transferred into “tube B”. The precipitate from tube A was resuspended with diethyl ether, homogenized, and incubated in dry ice, and the liquid phase transferred to tube B. Tube B was left in the fume hood overnight to evaporate all of the volatile components. The next day, PBS was added, the specimen was aliquoted and the testosterone levels were determined by double-antibody radioimmunoassay using Coat-A-Count® (Diagnostics Products Corporation, Los Angeles, CA, USA). The assays were performed at the Neuroendocrinology Laboratory, Dental School of Ribeirão Preto, University of São Paulo – USP.

Light Microscopy

Histological analysis

At 180 days of age, rats were euthanized by decapitation, and their ventral prostate and seminal vesicles were removed and weighed. The fragments of the intermediate segment of the ventral prostate were rapidly fixed by immersion in metacarn (7 methanol: 2 chloroform: 1 acetic acid) for 2 hours and kept in 70% ethanol. Next, the tissues were dehydrated in graded ethanol and were embedded in paraplast (Oxford Labware, St. Louis, MO, USA). The blocks were sectioned at 4 µm thicknesses and the slides were stained with hematoxylin-eosin (HE) for general studies and with Gomori's reticulin to assess reticular fibers. The slides were analyzed and images captured using Axiophot II (Zeiss-Jenaval, Jena, Germany) digital photomicroscope.

Morphometric-stereological analysis

Using an imaging analysis system (Image Pro Plus version 4.5 for Windows software), slides stained by HE were evaluated. Random HE images of 50 histological fields per experimental group were captured and analyzed by the stereological method, such that the histological samples of five rats were evaluated equally (10/rat). Stereological analyses were performed using Weibel's multipurpose test with 120 points and 60 test lines (Weibel, 1963) to compare the relative proportions among the prostate compartments (epithelium, stroma and lumen) in the experimental groups.

Immunohistochemistry (IHC)

The following primary antibodies were used for IHC: Anti-androgen receptor (AR, Sc-816, Santa Cruz Biotechnology, Dallas, TX, USA, 1:100), anti-estrogen receptor alpha (ERα, Ab37438, Abcam, Cambridge, MA, USA 1:25), anti-estrogen receptor beta (ERβ, Homemade, Karolinska Institute, 1:50), Ki-67 (Ab16667, Abcam, 1:200) and DACH-1 (10914-AP, Proteintech, Chicago, IL, USA, 1:50). After the removal of paraffin and rehydration of the sections, antigens were retrieved at high temperature (100ºC) for 50 min. Endogenous peroxidase was quenched with 3% H_2O_2 diluted in 50% methanol for 30 min, and nonspecific proteins were blocked by the incubation of the slides in bovine serum albumin (BSA) diluted to 3% in PBS plus 0.1% NP-40. The primary antibody was diluted in 1% BSA in PBS plus 0.1% NP-40, and the slides were incubated overnight at 4ºC. For the immunoperoxidase assay, the slides were rinsed in PBS, incubated with biotinylated secondary antibodies followed by VECTASTIN ABC kit (Vector Laboratories Ltd.), and visualized with diaminobenzidine. The sections were counterstained with Harris hematoxylin. Negative controls were obtained by omitting the primary antibody-incubation step.

Proliferation index determination

The number of Ki67 positive epithelial cells were counted in 50 random fields at 40x magnification from five different prostate histological samples (10 fields/each fragment) in each experimental group and expressed as a percentage of the total cells

counted. All image acquisition and quantitative measurements were performed by investigators blinded to both the animal identity and experimental condition.

Western blotting analyses and protein quantification

Frozen ventral prostate fragments of 5 different adult rats from each experimental group were mechanically homogenized with RIPA lysis buffer (Millipore, CA, USA), 10X (0.5 M Tris-HCl, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA, pH 7.4) and a protease inhibitor cocktail (Sigma Chemical Co.) using a homogenizer (IKA® T10 basic Ultra, Staufen, Germany). Aliquots containing 1:10 (v/v) of Triton X-100 were added to homogenates and samples were placed on dry ice under agitation for 2 h to improving extraction. These suspensions were centrifuged at 21.912 x g for 20 min at 4ºC and were measured by the Bradford micro-method for colorimetric determination. Total proteins were dissolved in 2x sample buffer as previously described by Laemmli and were used for SDS-PAGE (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (100 µg) of each sample were loaded per well onto preformed gradient gels, 4-15% (Bio-Rad Laboratories, Hercules, CA, USA), with a Tris-glycine running buffer system for electrophoresis (100 V fixed during 1 h 30 min). After electrophoresis, the total proteins were electro-transferred (80 mA fixed for 1 h 30 min) onto 0.2 µm nitrocellulose membranes in a Tris-glycine-methanol buffer. Kaleidoscope Prestained Standards (Bio-Rad) were used as molecular weight markers. Subsequently, the membranes were blocked with a TBS-T solution containing 5% BSA or 5% non-fat dry milk at room temperature

(RT) for 1 h and were then incubated at 4ºC overnight. AR (1:300), ERα (1:300), ERβ (1:1000), DACH1 (1:500) and PAR4 (SC-1666, Santa Cruz Biotechnology, Dallas, TX, 1:200) were carried out at 1% BSA or 1% non-fat dry milk. This was followed by washing for 6 x 5 min with TBS-T solution and then incubating for 2 h at RT with rabbit or mouse HRP-conjugated secondary antibodies (diluted between 1:5000 and 1:20000 in 1% BSA or 1% non-fat dry milk; Abcam, Cambridge, MA, USA). After sequential washing with TBS-T, band locations were detected using a chemiluminescence substrate kit (Pierce ECL Western Blotting Substrate – GE Healthcare®, Pittsburgh, PA, USA), according to the manufacturer's instructions. The substrates were removed from the membranes, and ECL signals were captured by CCD camera (G:BOX Chemi, Syngen®, Sacramento, CA, USA). The integrated optical density (IOD) of the bands of target proteins was measured using the Image J software downloaded from the NIH website (http://rsb.info.nih.gov.ij/) to compare proteins levels. Beta-actin was used as an endogenous control.

Statistical analysis

All statistical analyses were carried out using GraphPad Prism software (version 5.0). Depending on the data, either a parametric ANOVA or a non-parametric Kruskall-Wallis test was used to examine the significance of any difference between groups. The results were presented as the means ± SD or means ± SEM. Differences were considered statistically significant when the p-value was < 0.05.

Results

Biometric data

There was a decrease in the body weight gain in the animals that consumed ethanol, EtOH group (Table I). Lower energetic intake was observed in the EtOH group (Table I). There was no change in the absolute and relative of ventral prostate weight in the alcoholic animals (Table I). In contrast, the experimental groups submitted to testosterone treatment showed increasing absolute and relative of ventral prostate weight (Table I).

Hormone assays

The groups submitted to hormonal therapy, T and EtOH+T displayed higher plasma testosterone levels than the other groups (Fig. 1A). The same pattern was observed for intraprostatic testosterone and DHT (Fig. 1B, C), which were significantly different than the EtOH group. Ethanol did not have an effect on estradiol levels (Fig 1D), except that the EtOH+T group exhibited lower level compared to the T group. Moreover, testosterone therapy induced lower E2/T ratio in the T and EtOH+T groups (Fig. 1E).

Histopathological and stereological analysis

The ventral prostate structure in the C group was characterized by acini with simple cubic epithelium and polarized nuclei in the basal region of the cells (Fig. 2B and J). The reticular fibers were shown to be in contact with the smooth muscle cells that surround the glandular acini and were adjacent to the basal region of the epithelial cells (Fig. 2Q). Atrophic acini were abundant in the EtOH group (Fig. 2R), having decreased epithelial compartments (33%) and increased luminal compartments (30%) compared to C group (Table II). In some prostatic regions of the EtOH group, epithelial metaplasia was noted in different cells types in the same prostatic acinus (Fig. 2K and L). Furthermore, reactive inflammatory atypia was observed in association with inflammatory foci, with invaginations of the epithelium occupied with inflammatory cells, changing the normal epithelium pattern (Fig. 2C and D). The layer of reticular fibers was more slender around the atrophic acini and did not spread throughout the stroma (Fig. 2R). The EtOH+T group did not present inflammatory foci, and the testosterone treatment was able to reverse atrophy of the epithelial compartment caused by ethanol (Fig. 2G, H, O and P) increasing the epithelial compartment in 25% compared to EtOH (Table II). Moreover, in the animals submitted to hormonal therapy, the reticular fibers were arranged in thick bundles scattered throughout the stroma (Fig. 2S and T).

Immunohistochemistry, Proliferation index and Western Blot

In the atrophic acini of the EtOH group, a loss of cell-cell adhesion was observed, represented by a decrease of e-cadherin immunoreactivity (Fig. 5H). This alteration was followed by a rupture of the smooth muscle cell layer, which was confirmed by a weak immunoreactivity for α-actinin around the acini (Fig. 5M and N). The testosterone treatment reversed the effects of ethanol in both parameters, increasing the cell-cell adhesion (Fig. 5J) and restoring the integrity of smooth muscle cell layer (Fig. 5Q and R).

Neither ethanol nor testosterone caused changes in the proliferation index (Fig. 3A). However, there was a reduction in the PAR4 expression of the EtOH group compared to C group (up to 50% decreasing), indicating a possible resistance to apoptosis (Fig. 3B).

In regard to hormonal receptors, there were few alterations in the expression of AR (Fig. 4A – F), ERβ (Fig. 4G – M) as well as DACH-1 (Fig. 5A – F). Unlike, several differences were observed in the immunostaining pattern of these proteins. Regarding AR, atrophic acini of EtOH-treated group exhibited weak immunorreactivity compared to the others groups (Fig. 4B, thick arrow). In the EtOH group, there was notably reduced ERβ immunostaining in the epithelium, with many negative cells (4I, arrowhead). In addition, there was a shift toward more ERβ positive cells in the stroma than in the epithelium (Fig. 4H, star; I), whereas the other groups showed more ERβ positive staining in the epithelial cell populations, including basal and luminal cells (Fig. 4G, J and K).

In contrast to ERβ, ERα was decreased (up to 52% decreasing) in the EtOH+T group (Fig. 4Q, R and S). The presence of inflammatory foci in the EtOH group contributes

to higher expression of ERα, as indicated by the many immune cells in the stroma that were positive for ERα (Fig. 4O, star).

Discussion

It has been reported that alcoholism is detrimental to the nutritional pattern of humans and laboratory animals, leading to processes of malnutrition (Lieber *et al*., 1984), which are supported by the lower energetic ingestion verified in the EtOH group. However, the calories necessary to maintain the adult rat body functions are 11 kcal/100g body weight (National Research of Council, 1985), which is less than the amount consumed by the EtOH group in this study. Furthermore, Campana *et al*. (1975) show that protein malnutrition in rodents is accompanied by behavioral disturbances, diarrhea, edema, irregular distribution and loss of hair, none of which were observed in our rats. In conclusion, UChB are viable models to study alcoholism, and the observed changes were due the effects of ethanol. Moreover, testosterone is linked to activation of growth factors, such GH and IGF-I (Lewis *et al*., 2002); ethanol may unbalance this axis (Ronis *et al*., 2007), nullifying the testosterone effects and therefore preventing the ETOH+T group from gaining body weight.

Androgens are essential to the growth and function of normal prostate while maintaining homeostasis (Debes and Tindall, 2002). They can have a direct and indirect action, bind to receptors and activate growth factors, respectively (Griffths *et al*, 1997). The increased prostate weight in the EtOH+T group may be due the higher plasma levels of

testosterone, stimulating the production of growth factors because the AR expression was not changed. In addition, the decreases in the epithelial compartment in the EtOH group, confirming the observed prostatic atrophy, is due the lower volume of cytoplasm and, consequently, reduced synthetic activity, which was reversed after testosterone treatment (Sáttalo *et al*., 2004).

The presence of inflammatory foci in the prostatic stroma of UChB rats has been described by Cândido *et al*. (2007). Wilson and Balkwill (2002) established an interesting relation between inflammatory infiltrated, maintenance and tumor progression with inflammatory cells, particularly leukocytes, which release angiogenic and growth factors (Lin and Pollard, 2004). The inflammatory processes induced by ethanol did not appear to drive the prostate to develop neoplastic lesions. In addition to the morphologic aspects observed, the expression of DACH1, a tumor suppressing protein, was similar between the experimental groups. Supporting this observation, recent findings suggest that DACH1 is highly expressed in normal prostate epithelium and reduced in prostate cancer (Wu *et al*., 2009).

One of the keys to cell proliferation is increasing the circulating androgens and thus raising intracellular AR (Cordeiro *et al*., 2008). The unchanged AR expression may be the cause for the similar cellular proliferation indices, even with the testosterone treatment. An alternative method for observing apoptosis is the measurement of PAR4, a pro-apoptotic protein that shows strong activity in response to several stimuli, including UV irradiation, cytokines, hormone ablation and cytotoxic agents in a variety of cellular systems (García-Cao *et al*., 2005). The expression of this protein was lower in the EtOH, evidencing the

resistance of prostatic cells to apoptosis, as PAR4 demonstrates strong apoptotic activity and reduced expression in certain types of cancer (Gurumurthy and Rangnekar, 2004).

It is well-established that in normal prostate, both ERα and ERβ coexist: ERα is found mainly in the stroma, while ERβ is mostly, but not exclusively, found in the epithelium (Weihua *et al*., 2002; Kawashima and Nakatani, 2012). In pathological conditions, such as benign prostatic hyperplasia and cancer, ERα stimulates proliferation, inflammation, development and invasion (Thomas and Gustafsson, 2011; Kawashima and Nakatani, 2012). The variation in serum estradiol levels in the EtOH+T group led to modified ERα expression but did not affect ERβ.

Recent findings suggest that when testosterone is present, the regulating growth signaling pathway is active via ERβ (Rochel-Maia *et al*., 2013). In the prostate, DHT is converted to 3βdiol, an endogenous ligand of ERβ and an important part of the feedback mechanism by which androgens promote growth via AR, whereas 3βdiol suppresses growth in prostate via ERβ (Weihua *et al*., 2001; Muthusamy *et al*., 2011). Our results suggest that in the UChB rat strain, the exogenous testosterone treatment did not activate the AR signaling pathway and therefore did not cause changes in ERβ expression.

The major caveat to using androgenic therapy to treat prostatic pathologies is the fact that testosterone may raise the risk of prostate cancer by augmenting cell proliferation (Landau *et al*., 2012). In addition to the proliferative pathway that was not activated in the EtOH+T group, some markers of tumor progression, such as the loss of cell-cell adhesion and rupture of smooth muscle cell layer (Gonçalvez *et al*., 2013) that were noted in EtOH

group, were reversed after hormonal treatment, showing that E-cadherin and α-actinin expression can be regulated by testosterone.

In sum, the current results suggest that ethanol was able to induce the emergence of inflammatory foci and epithelial atrophy in the ventral prostate of UChB rats, as well as suppress E-cadherin and α-actinin immunoreactivity. These observations allow us to hypothesize that with long term ethanol ingestion, more severe injuries may arise in the prostate microenvironment. Testosterone hormone therapy, via its anti-inflammatory role, was able to reverse the effects caused by ethanol without increasing cell proliferation and reducing ERα expression and, therefore, should be further investigated for the treatment of disorders caused by alcoholism.

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Table I. Body weight gain (g), feed energetic intake (kcal/100g body weight/day) total (g) and relative (g/100g body weight) ventral prostate and seminal vesicle weights of UChB rats submitted or not to testosterone treatment.

Parameters	C	EtOH	T	EtOH+T
Body weight gain	70.0 ± 6.40	33.5 ± 6.66[a]	56.5 ± 3.25	34.3 ± 8.38
Feed energetic intake	18.70 ± 0,10	16.75 ± 0.51[a]	17.48 ± 0.29	17,20 ± 0,33
Ventral prostate weight	0.57 ± 0.01	0.51 ± 0.03	1.21 ± 0.06[a]	1.31 ± 0.05[b]
Relative prostate weight	0.14 ± 0.00	0.12 ± 0.00	0.30 ± 0.01[a]	0.35 ± 0.01[b]

Values represent mean ± SD (n = 10). Mean followed by lowercase shows statistical differences among the groups. Kruskall Wallis with Dunn test.[a]p< 0,001 vs. C; [b]p < 0,001 vs. EtOH.

Table II. Stereological analysis (%) of UChB ventral prostate submitted or not to testosterone treatment.

Groups	Epithelium	Lumen	Stroma
C	19.64 [13.24 – 23.60]	44.94 [34.22 – 56.99]	32.73 [23.21 – 44.04]
EtOH	13.09 [10.11 – 15.92][a]	58.63 [44.64 – 68.30][a]	25 [17.26 – 41.07]
T	26.48 [19.04 – 26.19][ab]	40.77 [30.35 – 57.58][b]	31.54 [22.61 – 39.58]
EtOH+T	16.36 [12.64 – 23.66][bc]	54.46 [39.88 – 69.75][c]	25.29 [16.22 – 35.56]

Values represent median [Q1 – Q3](n = 5). Median followed by lowercase shows statistical differences among the groups.Kruskall Wallis with Dunn test.[a]p< 0,05 vs. C; [b]p < 0,05 vs. EtOH; [c]p < 0,05 vs. T.

Figure Legends

Figure 1. Hormonal assays of UChB rats submitted or not to testosterone therapy. Plasma testosterone (T), estradiol (E2), dihidrotestosterona (DHT) and intraprostatic testosterone concentrations and E2/T ratio. $^{a}p < 0.05$ *vs* C; $^{b}p < 0.05$ vs EtOH; $^{c}p < 0.05$ *vs* T. All results are expressed as mean ± SEM. Kruskall- Wallis test complemented with Dunn.

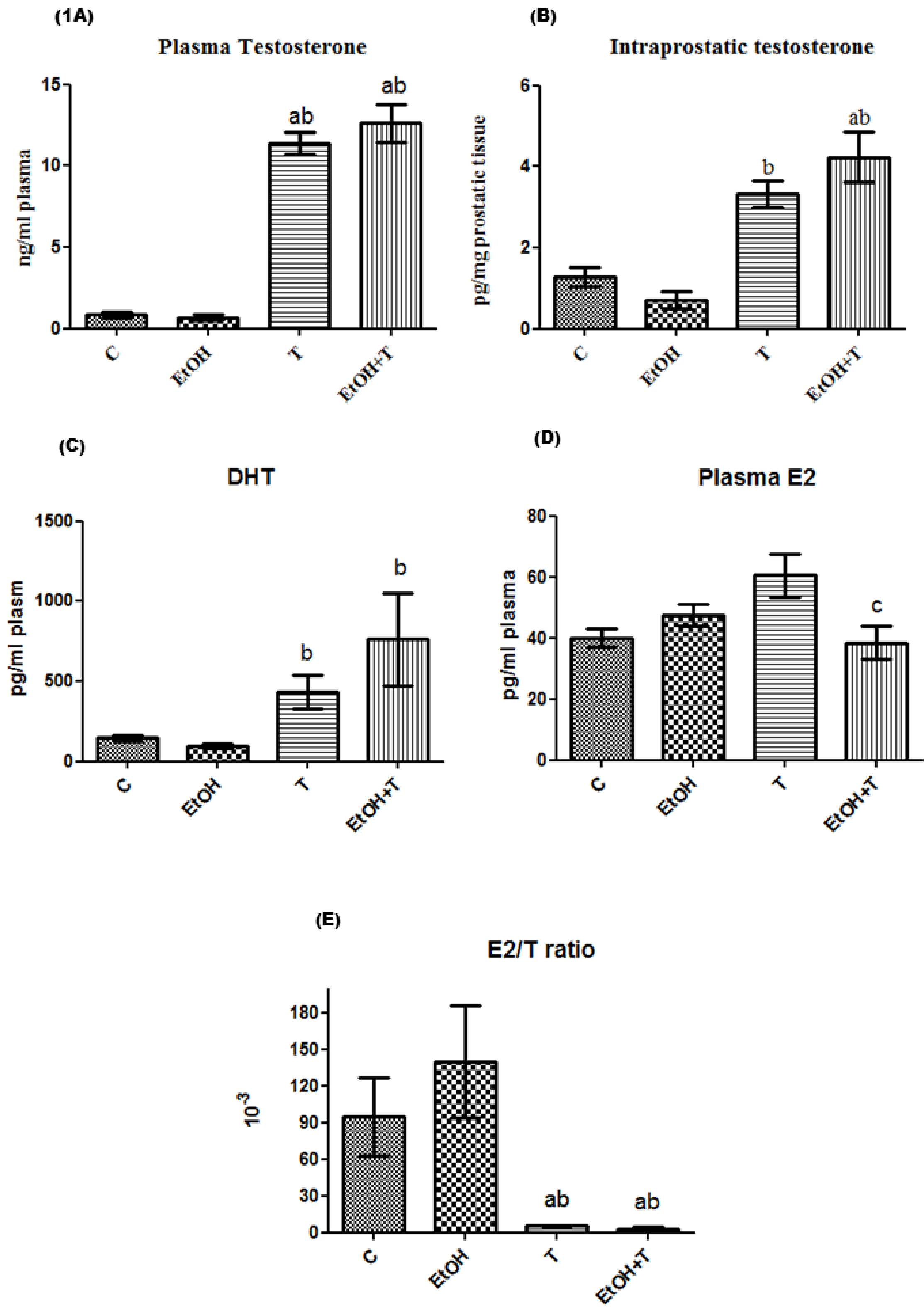
Figure 2. Histological sections of ventral prostate of UChB rats submitted or not to testosterone therapy (A - T). General aspects of distal segment of the gland. C shows simple cuboidal epithelium, stroma with few inflammatory cells (B and J) and reticular fiber bundles close to the epithelium (Q). Inflammatory foci associated with inflammatory reactive atypia were noted in the EtOH (C and D) beyond acini with metaplasia (K and L) and fine bundles of reticular fibers (R). High epithelium was found in the T (F and N), being the EtOH+T (G and O) exhibited similar pattern to C with reticular fiber bundles spread out to the stroma (T). Abbreviations: ep, epithelium; inf, inflammatory focus; ira, inflammatory reactive atipy; lu, lumen; met, metaplasia; st, stroma.

Figure 3. Proliferative index (A) and western blotting analysis of PAR4 (B) in the ventral prostate of UChB rats submitted or not to testosterone therapy. Densitometry values for PAR4 levels (C) were studied following normalization the house-keeping gene (β-actin). All results are expressed as mean ± SEM. Kruskall- Wallis test complemented with Dunn.

Figure 4. Immunolocalization (A - D, G – K and N – Q) and western blotting analysis of AR (E), ERβ (L) and ERα (R) in the ventral prostate of UChB rats submitted or not to testosterone therapy. Densitometry values for AR levels (F), ERβ (M) and ERα (S) were studied following normalization the house-keeping gene (β-actin). Besides the weak immunostaning of AR in the atrophic acini of EtOH (B, thick arrow), there are no great difference in the expression among the groups (E). ERβ immunopositive cells were found in the epithelium and stroma of the ventral prostate of experimental groups (G – K, thin arrow). EtOH showed weak immunostaining in the epithelium with negative cells (I, arrowhead), but a lot of positive immune cells were seen in the stroma (H, star; I, thin arrow), which reflect in similar expression among the groups (K and L). Few positive ERα stromal cells were observed in the experimental groups (M - P), except some inflammatory foci in the EtOH that showed ERα positive cells (N, star). All results are expressed as mean ± SEM. Kruskall- Wallis test complemented with Dunn.

Figure 5. DACH1 (A – D), e-cadherin (G – J) and α-actinin (K – R) immunolabelled and western blotting analysis of DACH1 (E) in the ventral prostate of UChB rats submitted or not to testosterone therapy. Densitometry values for DACH-1 levels (F) were studied following normalization the house-keeping gene (β-actin). Almost all the epithelial cells were positives to DACH1 (A - D, thick arrow), with few negatives cells (arrowhead) and no differences in the protein levels (E and F). Weak e-cadherin immunostaining was verified in the EtOH (H), showing a recovery after hormonal therapy (J). Rupture of smooth muscle cell layer (N, thin arrows) was observed in the EtOH, different that was

seen in the other groups (L, P, R, asterisk), with strong immunostaining around the acini. All results are expressed as mean ± SEM. Kruskall- Wallis test complemented with Dunn.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ng/ml plasma
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EtOH+T
(B)
Intraprostatic testosterone
pg/mg prostatic tissue
6
4
2
0
b
ab
C
EtOH
T
EtOH+T
(C)
DHT
pg/ml plasm
1500
1000
500
0
b
b
C
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Plasma E2
pg/ml plasma
80
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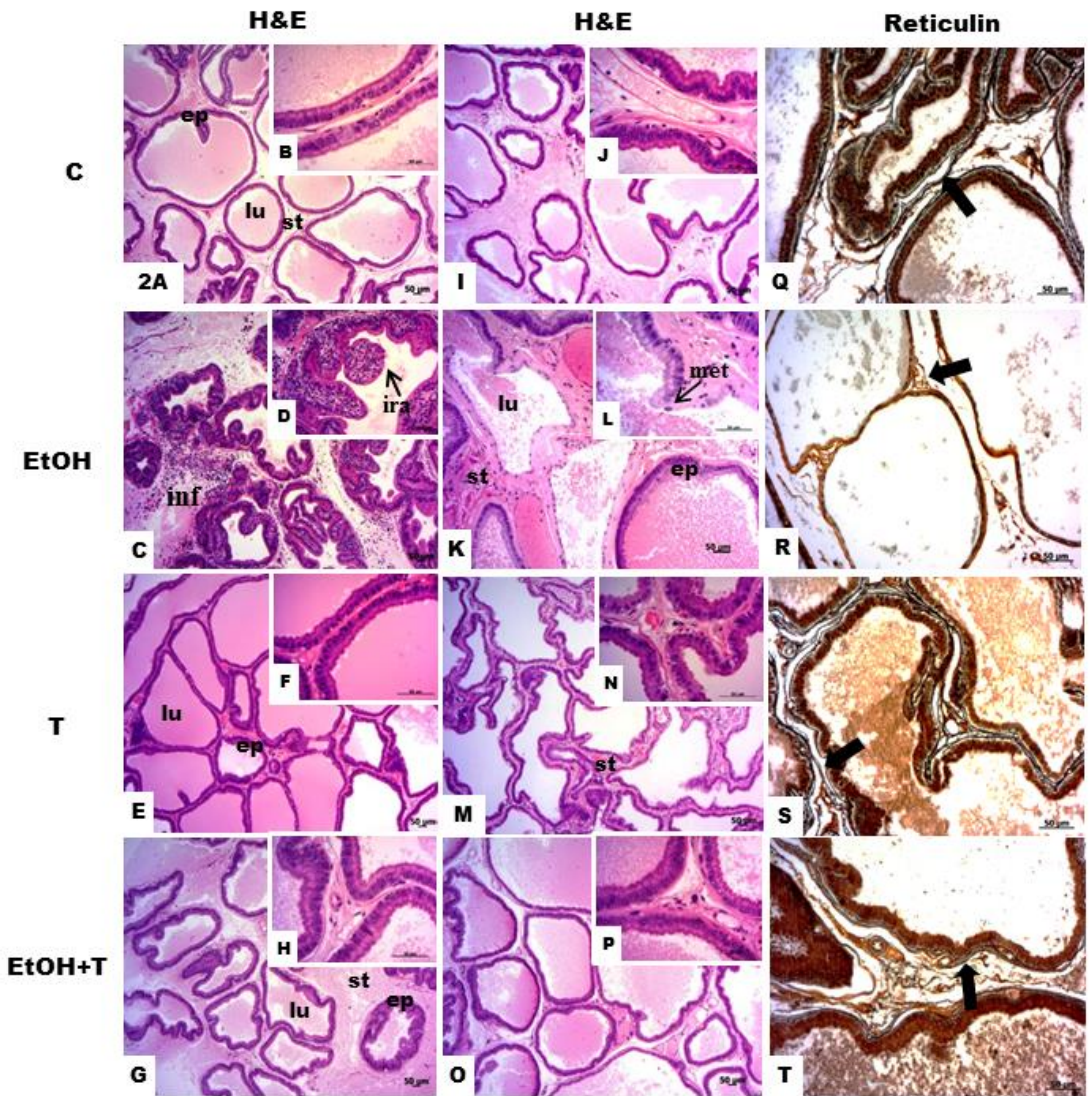
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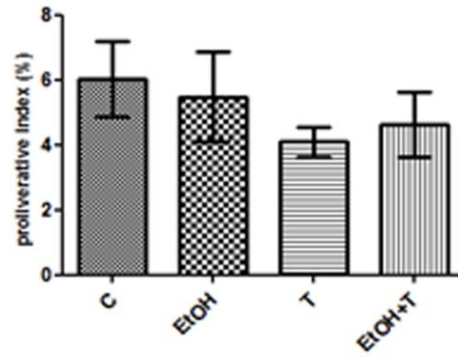

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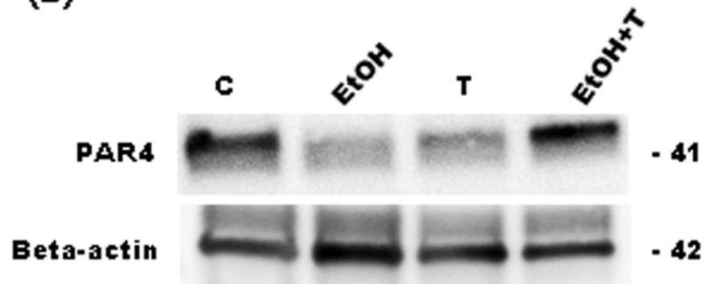

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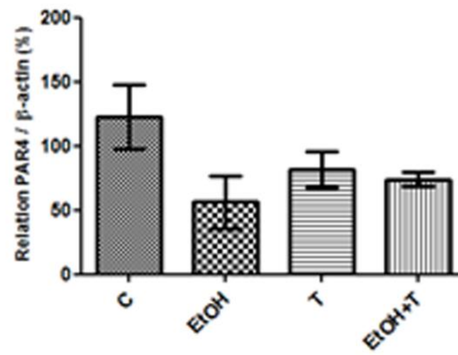


AR ERβ ERα

C

4A G N

EtOH

B H I O

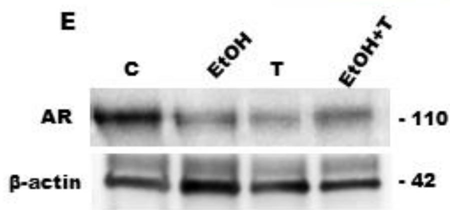
T

C J P

EtOH+T

D K Q

E



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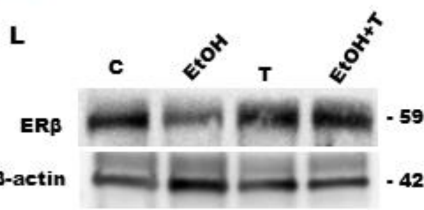

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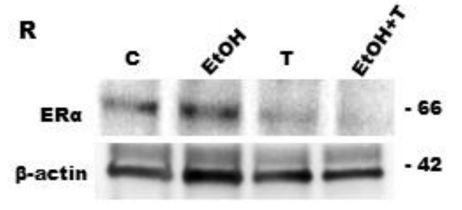

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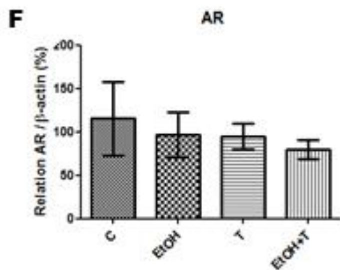

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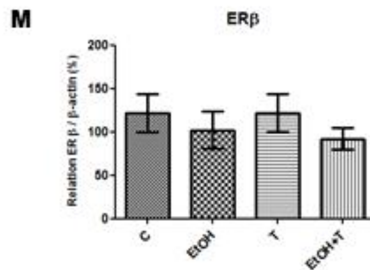

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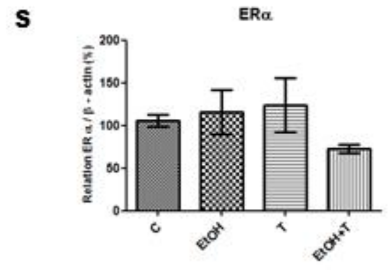


DACH-1 e-cadherin α-actinin

C

EtOH

T

EtOH+T

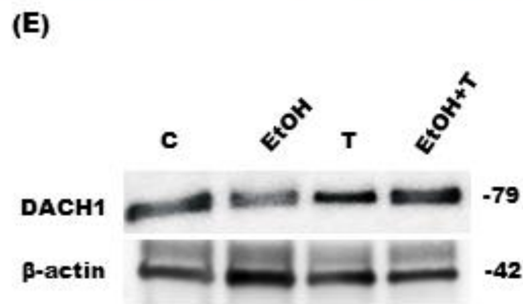
5A G K L

B H M N

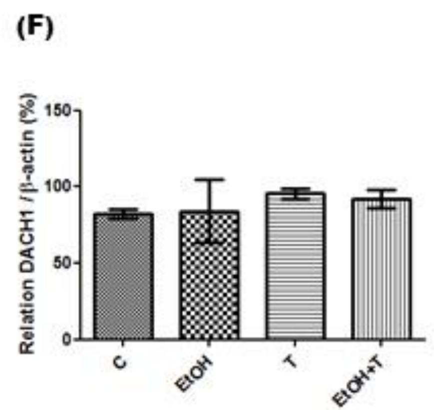
C I O P

D J Q R

(E)



(F)



Artigo II

Testosterone therapy differently regulates the anti - and pro-inflammatory cytokines in the plasma and prostate of rats submitted to chronic ethanol consumption (UChB)

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Testosterone therapy differently regulates the anti - and pro-inflammatory cytokines in the plasma and prostate of rats submitted to chronic ethanol consumption (UChB)

Running title: Testosterone and ethanol modulate cytokines

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Summary

Chronic ethanol consumption damages the prostate and testosterone is known by anti-inflammatory role. So, we investigated the effects of ethanol and testosterone therapy on the plasma levels and ventral prostate expression of cytokines. UChB rats aged 90 days were divided into two experimental groups (n=20): C: drinking water only and EtOH: drinking 10% (v/v) ethanol at > 2 g/kg body weight/day + water. At 150 days old, ten rats from each group received subcutaneous injections of testosterone cypionate (5mg/kg body weight) diluted in corn oil every other day during 4 weeks, constituting T and EtOH+T groups, while the remaining animals (10/group) received corn oil. Animals were euthanized at 180 days old.Blood was collected to obtain plasma cytokine levels and ventral prostate was dissected and processed for light microscopy, immunohistochemistry and western blot. Inflammatory foci were found in the EtOH and absent in the EtOH+T. Plasma levels of IL-6 and IL-10 were not changed. Regarding to ventral prostate, IL-6 did not alter, while high increasing of IL-10 was verified in the T. Plasma levels of TNFα and TFG-β1 increased in the EtOH+T. Both cytokines were reduced in the prostate of EtOH+T. Nfr2 was increased in the EtOH, besides the high number of degranulated mast cell. Reduced number of intact and degranulated mast cells was seen in the EtOH+T. Nevertheless, the T showed increasing number of intact and degranulated mast cells. So, ethanol and testosterone differentially modulates the cytokines in the plasma and prostate.

Keywords: ethanol, testosterone, cytokines, prostate, UChB rats

Introduction

The acute and chronic ethanol consumption is associated with disorders in several organic systems, such as digestive, nervous (Wang et al. 2012; Kane et al. 2014) and reproductive (Chuffa et al. 2011; Fontaneli et al. 2013). The effects of ethanol have been better understood with the use of ethanol-preferring animals (e.g UChB) that derived from original Wistar rats and have been selectively bred at the University of Chile for almost 70 generations (Mardones and Segóvia-Riquelme, 1983). This rat represent a special model for studying the relationship among the genetic, biochemical, physiologic, nutritional and pharmacological factors arising from alcoholism, such as those found in alcohol-related human diseases(Quintanilla et al. 2007).

Because ethanol alters hormone-dependent tissues, the prostate is one of the main reproductive organs that indirectly suffer from its effects (Sáttolo et al. 2004; Martinez et al. 2006; Cândido et al. 2007). However, the mechanism(s) by which ethanol cause prostate changes remain unknown. Nevertheless, the ethanol consumption has been linked to the two most important health problems in aging male: 1) benign prostatic hyperplasia and 2) prostate cancer (Platz et al. 1999 and Gass, 2002).

There is a recent and growing body of evidences suggesting that chronic inflammation may promotes the initiation, promotion, and progression of prostate cancer by inducing genetic and epigenetic alterations, and controlling the tumor microenvironment (Fibbi et al. 2010; Kwon et al. 2013; Nguyen et al. 2013). The inflammatory process is an integrated response, defending the body against failures that interfere with homeostasis, particularly related to infections and injuries (Cesari et al. 2004). The cytokines profile is

assumed to be of types Th1 (pro-inflammatory) or Th2 (anti-inflammatory) according to their role in the immune system and impact tissue in an intrinsic way regulating cell death, cell proliferation, cell migration and healing mechanisms (Achur et al. 2010).

Ethanol intake is described to changing cytokines levels in several tissues, including plasma, lung, liver and brain (Crews et al. 2006). Emanuele et al. (2005) have reported that part of the deleterious effects of ethanol upon the reproduction may be triggered by the increase of pro-inflammatory cytokines, such as IL-6 and TNFα, which were higher in the hypothalamus, pituitary and ovary of rats submitted to chronic ethanol consumption.

The benefic role of androgenic stimulation has been extensively demonstrated in animals with a variety of induced inflammatory diseases (Dalal et al. 1997; Kimura et al. 1998, Ben-Nathan et al. 1999). Although the exact mechanism(s) by which testosterone plays in inflammatory responses are uncertain, Malkin et al. (2004) proposed that testosterone stimulates the production of anti-inflammatory cytokines, and conversely, inhibits the synthesis of pro-inflammatory cytokines. The understanding of inflammatory effects of ethanol and the anti-inflammatory role of testosterone could bring new insights into therapeutic advances for treating disorders caused by chronic ethanol intake.

To better clarify this issue, the aim was to investigate the effects of ethanol on the plasma levels and ventral prostate expression of cytokines and the role of hormonal therapy with testosterone in the alcohol-related disorders.

Material and Methods

Animals and experimental design

Forty adult UChB male rats were bred and maintained at the Department of Anatomy, Bioscience Institute of Botucatu, UNESP. When the UChB rats reached 60 days old, they were submitted to selection process for ethanol preference. The rats were given a choice between two bottles containing either water *ad libitum* or 1:10 (v/v) ethanol during 20 days of selection.After this period, rats displaying ethanol consumption ranging from 2 to 6 g EtOH/kg body weight/day were selected to the study according to Mardones and Segovia-Riquelme (1983). At 80 days old, UChB rats were divided into two groups (20 rats/group): 1) EtOH group, receiving ethanol 1:10 (v/v) and water *ad libitum*, and 2) control group (C), receiving only water. At 150 days old, 10 rats of each group started to receive subcutaneous injections of testosterone cypionate (Deposteron®, at doses of 5 mg/kg body weight) every other day diluted in corn oil over 4 weeks at a consistent time each day (8:00 – 8:30 am). Rats receiving injections constituted the T and EtOH+T groups, while the other twenty (10 rats/group) males received just corn oil.

All rats were housed in polypropylene cages with laboratory-grade pine shavings as bedding, and maintained under a controlled temperature (23ºC ± 1ºC) and day/night cycle (12 h/12 h). They were provided filtered tap water and rat chow *ad libitum*. Experimental protocols followed the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation (Permit number: 208 – CEEA).

Histochemistry and mast cell score

At 180 days old, the rats were euthanized in CO_2 chamber followed by decapitation, and the fragments of the intermediate segment of the ventral prostate (n=5 samples/group) were rapidly removed and fixed by immersion in metacarn (7 methanol: 2 chloroform: 1 acetic acid) for 2 h and kept in 70% ethanol. After fixation, tissues were dehydrated in graduated ethanol series, cleared in xylene, and embeddedin Historesin embedding kit (Leica, Nussloch,Germany). The blocks were sectioned at 4 µm thicknesses on the LEICA RM 2165microtome.

To identify mast cells, tissue sections were stained with 1% toluidine blue. Because the granules within mast cells contain heparin and sulfated glycosaminoglycan, they were stained metachromatically using toluidine blue to evaluate and quantify the intact and degranulated mast cells. These dynamics of mast cells were achieved by the absence or presence of metachromatic granules outside the mast cells in the surrounding connective tissue, as described by Moron et al. (2000) and Keith et al. (2001). The number of intact and degranulated mast cells was measured in 5 animals of each experimental group, and the values were given in cells/mm^2. Random images of 50 histological fields per experimental group (10 images/rat) were captured by digital photomicroscope Axiophot II – Zeiss using 40x objective, with each field covering an area of 0.085 mm^2.

Immunohistochemistry

Fragments of the intermediate segment of ventral prostate (n=5 samples/group), fixed in metacarn, and embedded in paraplast (Oxford Labware, St. Louis, MO, USA) were used for immunostaining TGF-β1 (Ab64715, Abcam, Cambridge, MA, USA, 1:150) and

pSmad 2 (Ab3840, Milipore, Billerica, MA, USA, 1:200). After the removal of paraffin and rehydration of the sections, antigens were retrieved at high temperature (100ºC) for 50 min. Endogenous peroxidase was quenched with 3% H_2O_2 diluted in 50% methanol for 30 min, and non-specific binding proteins were blocked by the incubation of the slides in bovine serum albumin (BSA) diluted in 3% PBS plus 0.1% NP-40. The primary antibody was diluted in 1% buffered BSA plus 0.1% NP-40, and the slides were incubated overnight at 4ºC. For immunoperoxidase assay, the slides were rinsed in PBS, and finally incubated with biotinylated secondary antibodies and VECTASTIN ABC kit (Vector Laboratories Ltd.). The immunoreactions were visualized with diaminobenzidine as chromogen. All sections were counterstained with Harris hematoxylin. Negative controls were obtained by omitting the primary antibody-incubation step.

Plasma cytokines

Blood samples were collected from the trunks of decapitated rats (n = 10/group) into heparinized tubes at the time of the death (between 9:00 and 11:30 am). Afterwards, plasma was obtained by centrifugation at 1.200 x g for 15 min at 4ºC, and stored at -20ºC until they were assayed. Plasma levels of IL-6, IL-10, TNFα and TGF-β1 were measured by specific enzyme-linked immunoassay (ELISA) using Quantike®kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Plasma cytokines levels were expressed as absolute concentrations (pg/ml). Specifically for TGF-β1 measurement the samples were activated before the assay. Sample activation basically comprised biochemical steps (acidification followed by neutralization of the pH) in order to activate latent TGF-β1 to immunoreactive TGF-β1 detectable by the Quantikine TGF-β1

immunoassay, as recommended by the manufacturer. The minimum detection limits were 21 pg/ml (IL-6), 10 pg/ml (IL-10), 5 pg/ml (TNF-α), 4.6 pg/ml (TGF-β1).

Western blotting analyses and protein quantification

The ventral prostate of the experimental animals (n= 5 samples/group) was rapidly removed and immediately frozen in liquid nitrogen and stored at -80ºC. The samples were mechanically homogenized with RIPA lysis buffer (Millipore, CA, USA), 10X (0.5 M Tris-HCl, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA, pH 7.4) and protease inhibitor cocktail (Sigma Chemical Co.), using a homogenizer (IKA® T10 basic Ultra, Staufen, Germany). Aliquots containing 1:10 (v/v) of Triton X-100 were added to homogenates and samples were placed on dry ice under agitation for 2 h to improving extraction. These suspensions were centrifuged at 21.912 x g for 20 min at 4ºC and were measured by the Bradford micro-method for colorimetric determination. Total proteins were dissolved in 2x sample buffer as previously described by Laemmli and were used for SDS-PAGE (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (100 μg) of each sample were loaded per well onto preformed gradient gels, 4-15% (Bio-Rad Laboratories, Hercules, CA, USA), with a Tris-glycine running buffer system for electrophoresis (100 V fixed during 1 h 30 min). After electrophoresis, the total proteins were electro-transferred (80 mA fixed for 1 h 30 min) onto 0.2 μm nitrocellulose membranes in a Tris-glycine-methanol buffer. Kaleidoscope Prestained Standards (Bio-Rad) were used as molecular weight markers. Subsequently, the membranes were blocked with a TBS-T solution containing 5% BSA or 5% non-fat dry milk at room temperature (RT) for 1 h, and were then incubated at 4ºC overnight. IL-6 (Ab1692, Abcam, 1:500), IL-

10 (Ab34843, Abcam, 1:1000), TNFα (Ab1793, Abcam, 1:500), TGF-β1 (Ab9758, Abcam, 1:500) and NFR2 (sc-722, Santa Cruz Biotechnology, Dallas, TX, USA, 1:1000) were carried out at 1% BSA or 1% non-fat dry milk. This was followed by washing for 6 x 5 min with TBS-T solution and then incubating for 2h at RT with rabbit or mouse HRP-conjugated secondary antibodies (diluted between 1:5000 and 1:20000 in 1% BSA or 1% non-fat dry milk; Abcam, Cambridge, MA, USA). After sequential washing with TBS-T, band locations were detected using a chemiluminescence substrate kit (Pierce ECL Western Blotting Substrate – GE Healthcare®, Pittsburgh, PA, USA), according to the manufacturer's instructions. The substrates were removed from the membranes, and ECL signals were captured by CCD camera (G:BOX Chemi, Syngen®, Sacramento, CA, USA). The integrated optical density (IOD) of the bands of target proteins was measured using the Image J software downloaded from the NIH website (http://rsb.info.nih.gov.ij/) to compare proteins levels. Beta-actin was used as an endogenous control.

Statistical analysis

All statistical analyses were carried out using GraphPad Prism software (version 5.0) and non-parametric Kruskall-Wallis test was used to examine the significance of any difference between groups. The results were presented as the means ± SEM. Differences were considered statistically significant when the p-value was < 0.05.

Results

Hematoxylin and eosin stained demonstrated the presence of inflammatory foci in the stroma of EtOH (Fig. 1C). The inflammatory cells are shown to be in close contact with the prostate epithelium (Fig. 1D), changing the morphologic pattern compared to C (Fig. 1A, B). The striking feature of the stroma after testosterone therapy was the absence of inflammatory foci, even in the EtOH+T (Fig. 1G, H).

The ethanol associated or not to testosterone was unable to alter the plasma concentrations of IL-6 (Fig. 2C) and IL-10 (Fig. 3C). Regarding to the ventral prostate, IL-6 expression did not alter among the experimental groups (Fig. 2A, B), differently what was observed to IL-10, where the T presented higher expression compared to C (up to 93% increased; Fig. 3A, B). The hormonal therapy showed to be effective for the EtOH+T by increasing in 35% the IL-10 expression compared to EtOH (Fig. 3A, B).

TNFα seems to be differentially regulated in the plasma and in the prostatic tissue. EtOH+T exhibited higher plasma levels of TNFα than EtOH (Fig. 4C). Surprisingly, analyzing the expression of this factor in the prostate, EtOH+T presented lower TNFα levels in the prostate compared to the groups C (up to 55% increased) and EtOH (up to 30% increased), respectively (Fig. 4A, B).

The hormonal therapy with testosterone was also responsible to increase the plasma levels of TGF-β1 in the EtOH+T, similar to that observed with TNF-α level (Fig. 5C). In contrast, a slight reduction in TGF-β1 expression (up to 22% decreased) was verified in the prostate of EtOH+T compared to EtOH. (Fig. 5A, B). Furthermore, this growth factor was shown to be present in the prostatic stroma, regardless of either ethanol or testosterone

therapy. (Fig. 5D – G). Based on its signaling pathway, the immunohistochemistry to pSmad 2 revealed that the TGF-β1 was able to send signal transduction into the cell independent of experimental condition, being found these molecules in the nucleus of both epithelial and stromal cells. (Fig. 5H – K).

NFR2 expression was changed according the experimental groups. EtOH showed a higher expression (up to 32% increased) compared to C and the testosterone therapy produced a reduction of NFR2 expression (up to 64% decreased) compared to EtOH (Fig 6A, B).

The EtOH presented a large number of intact and degranulated mast cells compared to C (Fig 7A, B, F). Testosterone therapy was able to reverse this event by decreasing the number of degranulated mast cells when compared to EtOH (Fig. 7D – F). Interestingly, testosterone therapy promoted an increase in the number of intact and degranulated mast cells in T when compared to C.

Discussion

The effects of alcoholism vary with the consumption and the amount of ethanol in the blood, influencing the cytokines cascade in a complex way (Crews et al. 2006). In addition to being involved in the function and development of the innate and adaptive immune response, cytokines may also have a broad impact to the tissue, regulating cell proliferation, apoptosis and healing mechanisms (Achur et al. 2010).

Recent efforts have been assigned in an attempt to discover possible biomarkers of alcoholism. The inflammatory cytokines, such as IL-6 and TNFα, are considered potential

targets since recent findings have shown high levels of these cytokines in patients with chronic or acute hepatic disease induced by ethanol. Importantly, this increase is tightly related to the metabolic injuries in the liver due to ethanol intake (Achur et al. 2010; Rocha et al. 2012). More recently, Gonzáles-Reimers et al. (2012) found no difference in the plasma concentrations of IL-6 in stable alcoholic patients. Similarly, the absence of hepatic lesions observed in ethanol-preferring animals may be linked to the circulating levels of this cytokine. The presence of IL-6 is not an abnormal occurrence, but its uncontrolled production is associated to chronic inflammation in different subtypes of cancer, including prostate cancer (Hodge et al. 2005). Despite the common inflammatory focus observed in the prostate of alcoholic rats, neoplastic lesions were not found. Regarding TNFα, we could not link the increasing of plasma levels of this cytokine in the EtOH+T to onset of inflammatory process since in this group were not observed inflammatory foci besides to show lower expression of TNFα in the prostate.

Vignozzi et al. (2012) have proposed interesting association between testosterone levels and inflammation, showing an increased inflammatory process in elderly men with low levels of this hormone. They also suggested that DHT, a testosterone metabolite, plays a central role in the immune regulation of prostate stromal cells, after triggering the inflammatory response cascade. Therapy with testosterone reduced TNFα and TGF-β1 expressions in the ventral prostate of animals submitted to chronic alcoholism, in addition to an increased expression of IL-10, an anti-inflammatory cytokine. As reported by Cesari et al. (2004) IL-10 has the ability to inhibit the production of other inflammatory cytokines, such as IL-6 and TNFα. A similar mechanism was observed after hormonal therapy in men

with androgen deficiency, which was verified a decrease in TNFα and IL-1β levels, in contrast to an increase in IL-10 (Malkin et al. 2004).

In rodent prostate, TGF-β1 is synthesized in the stroma and acts in the epithelial cells in a paracrine fashion (Nemeth et al., 1997), corroborating with our results. Regarding to the effects of TGF-β1, these can function as tumor suppressor in normal or pre-malignant cells, and promoter of invasion/metastasis in advanced carcinomas, depending on cell type or physiological conditions (Massagué, 2012). The presence of pSmad2 protein in the nucleus of stromal and epithelial cells shows that the TGF-β1 signaling pathway is active in the prostate of ethanol-preferring animals, since pSmad2 mediator is necessary to take the TGF-β1 signal from the cytoplasm to the nucleus (Pasche 2001; Danielpour 2005). The slight increase in the expression of TGF-β1 found in EtOH group is probably associated to its suppressor function since that ethanol did not act as tumor promoter in the prostate microenvironment.

TGF-β1 is also responsible for both initiation and development of inflammatory responses by recruiting and activating several types of immune cells, including mast cells (Halova et al. 2012). The high number of degranulated mast cells in the EtOH confirmed this process already described by Mendes et al. (2011). The increase of intact and degranulated mast cells observed in T could be attributed to a high expression of IL-10 in the prostate, since this cytokine is proposed to stimulate the proliferation of mast cells (Hamidullah *et al*., 2012).

In addition to the indirect effects caused by ethanol (e.g. decreasing plasma testosterone and triggering hormonal imbalance, Saxena et al. 1990; Tadic et al. 2000), some authors suggest a direct action based on the hypothesis that prostate is able to

metabolizes ethanol to acetaldehyde which leads to the generation of free radicals, and a subsequent oxidative stress in the organ (Castro et al. 2002; Gomés et al. 2007). The increased expression of NFR2 in the EtOH group seems to agree with the authors, since this transcription factor is a powerful redox sensor activated in response to oxidative stress (Furfaro et al. 2012). Moreover, Chen and colleagues (2006) showed that NFR2 pathway is involved in immune and inflammatory process, suppressing redox-sensitive inflammatory responses that have key role in a variety of chronic inflammatory disease.

In summary, our results suggest that ethanol differentially modulates the cytokines profile in the plasma and prostate microenvironment, being the prostate a local source of cytokines. The prostate, a hormone-responsive organ, was susceptible to the anti-inflammatory effects of testosterone therapy and, therefore, becoming the testosterone a potential target to be assessed for the inflammatory disorders caused by ethanol

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Figure legends

Figure 1.H&E sections of ventral prostate of UChB rats submitted or not to testosterone therapy (A - H). C shows simple cuboidal epithelium and stroma with few inflammatory cells (A and B). Inflammatory foci associated to the epithelium were observed in the EtOH (C and D). High epithelium was found in the T (E and F), and the EtOH+T exhibited similar pattern to C with absence of inflammation (G and H). Abbreviations: ep, epithelium; inf, inflammatory focus; lu, lumen; st, stroma. Stain: hematoxylin and eosin.

Figure 2. Western blotting analysis of IL-6 (A) in the ventral prostate and plasma IL-6 (C) levels in the UChB rats submitted or not to testosterone therapy. Densitometry values for IL-6 levels (B) were studied following normalization the house-keeping gene (β-actin). All results are expressed as mean ± SEM. Kruskall- Wallis test complemented with Dunn.

Figure 3. Western blotting analysis of IL-10 (A) in the ventral prostate and plasma IL-10 (C) levels in the UChB rats submitted or not to testosterone therapy. Densitometry values for IL-10 levels (B) were studied following normalization the house-keeping gene (β-actin). All results are expressed as mean ± SEM. Kruskall- Wallis test complemented with Dunn.

Figure 4. Western blotting analysis of TNFα (A) in the ventral prostate and plasma TNFα (C) levels in the UChB rats submitted or not to testosterone therapy. Densitometry values for TNFα levels (B) were studied following normalization the house-keeping gene (β-

actin). * $p < 0.05$ *vs* EtOH. All results are expressed as mean ± SEM. Kruskall- Wallis test complemented with Dunn.

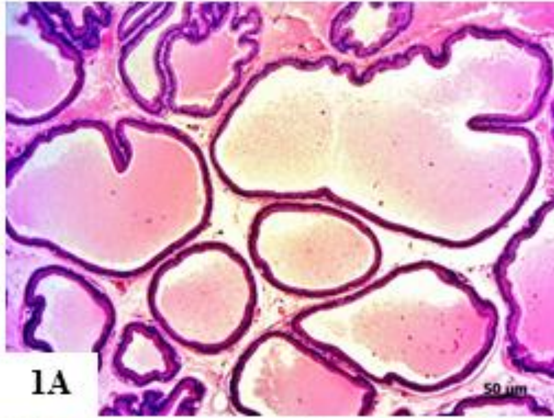
Figure 5. Western blotting analysis of TGF-β1 (A) and immunolabelled of TGF-β1 (D – G) and pSmad 2 (H – K) in the ventral prostate and plasma TGF-β1 levels (C) (H – K) in the UChB rats submitted or not to testosterone therapy. Densitometry values for TGF-β1 levels (B) were studied following normalization the house-keeping gene (β-actin). Stroma labeling of TGF-β1 (D – G, thick arrow) around the acini in all experimental groups. pSmad2 immunopositve cells were found in the epithelium and stroma (H – K, thin arrow) with few negative cells in the epithelium (I – K, arrowhead). * $p < 0.05$ *vs* EtOH. Kruskall-Wallis test complemented with Dunn. Bars: 50 µm.

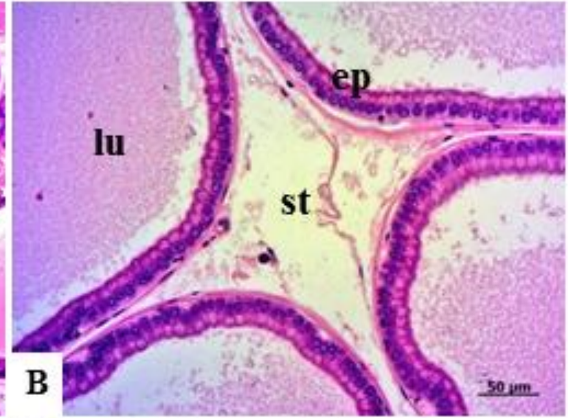
Figure 6. Western blotting analysis of NFR2 (A) in the ventral prostate of UChB rats submitted or not to testosterone therapy. Densitometry values for NFR2 levels (B) were studied following normalization the house-keeping gene (β-actin). All results are expressed as mean ± SEM. Kruskall- Wallis test complemented with Dunn.

Figure 7. Number of intact and degranulated mast cells in the ventral prostate of UChB rats submitted or not to testosterone therapy. Ethanol increased the number of intact (thick arrow) and degranulated (thin arrow) mast cells (B, E and F). The hormonal therapy reduced the number of intact and degranulated mast cells in the animals submitted to ethanol consumption (D – F). High number the intact and degranulated mast cells were also found in the T (C, E and F). All results are expressed as mean ± SEM. Kruskall- Wallis test

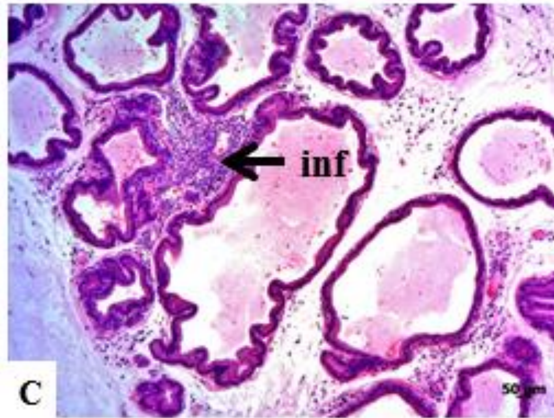
complemented with Dunn. Stain: toluidine blue. Stain: Toluidine Blue. * $p < 0.05$ *vs* C; ** $p < 0.05$ *vs* T; *** $p < 0.05$ *vs* EtOH.

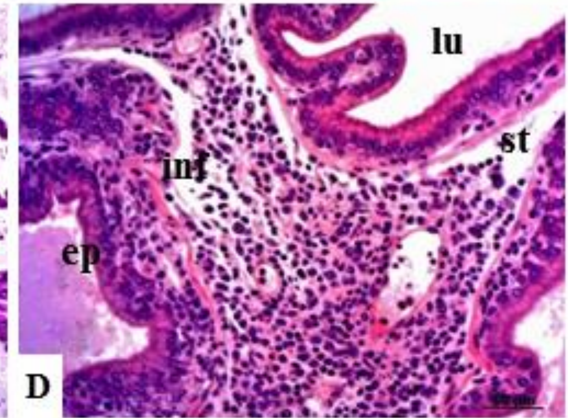
C



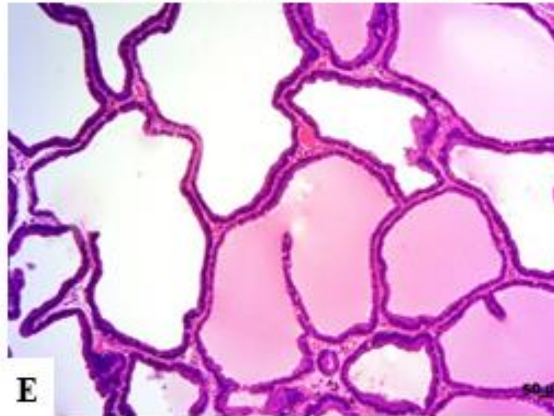


EtOH

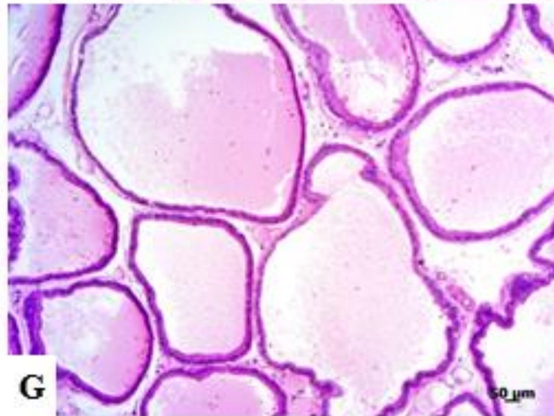


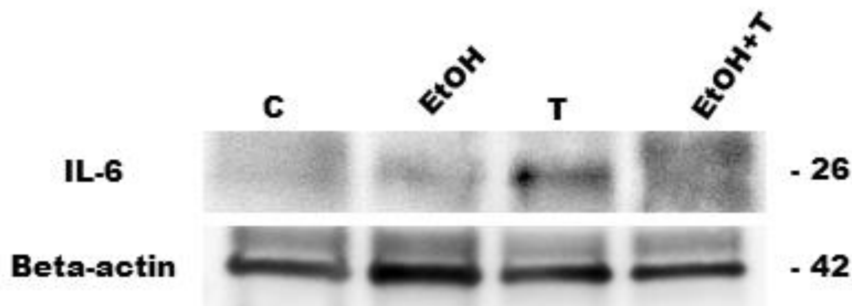


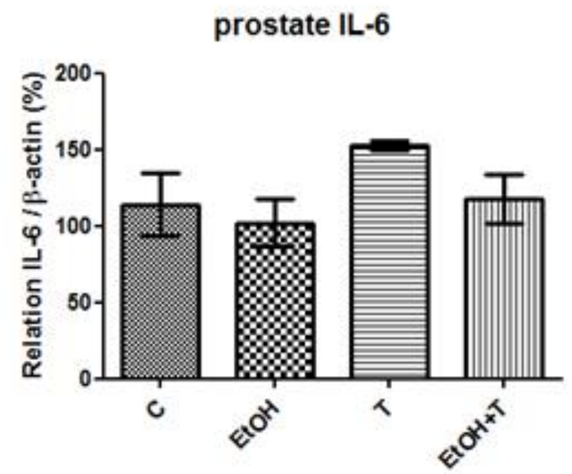
T

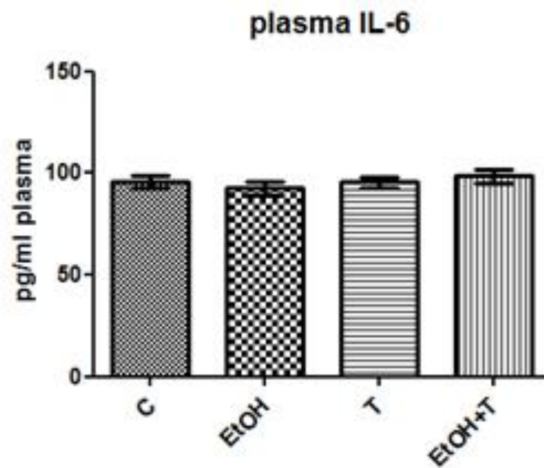


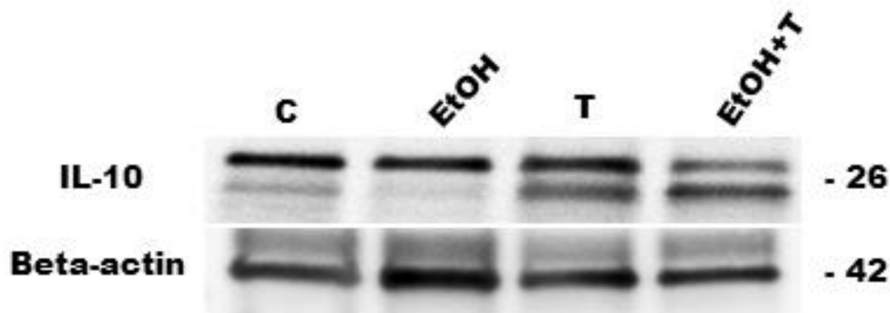
EtOH+T

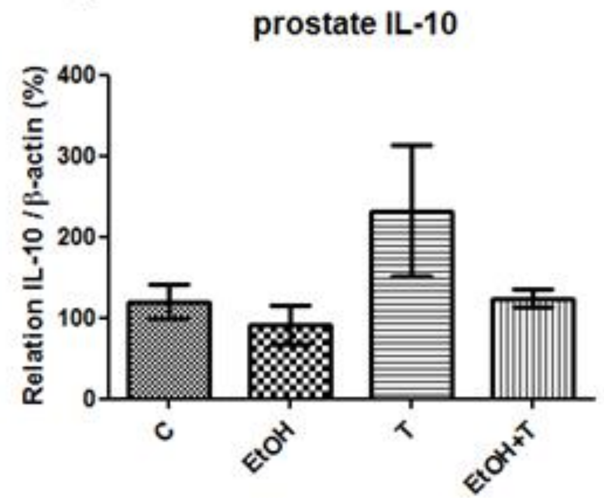


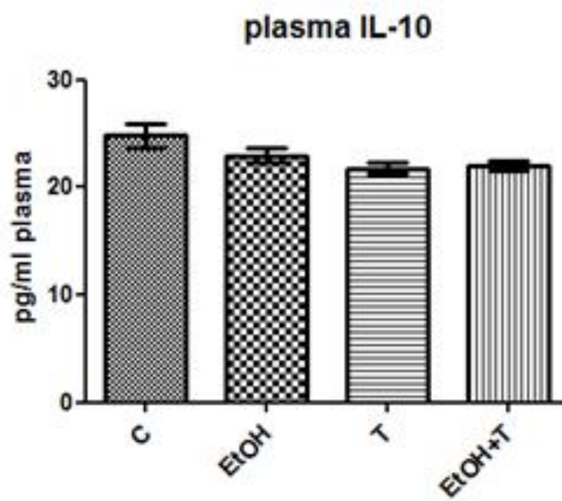

2A
C
EtOH
T
EtOH+T
IL-6
- 26
Beta-actin
- 42


B
prostate IL-6
Relation IL-6 / β-actin (%)
200
150
100
50
0
C
EtOH
T
EtOH+T

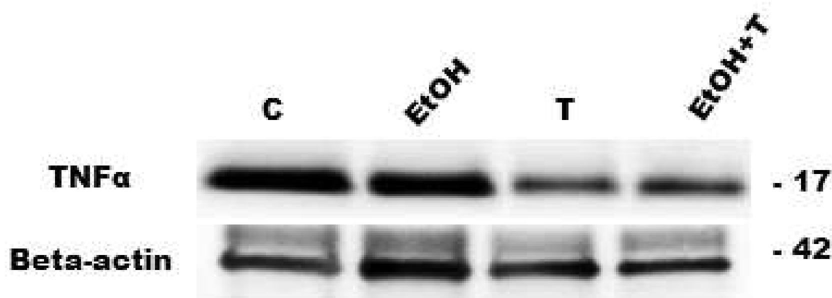

C
plasma IL-6
pg/ml plasma
150
100
50
0
C
EtOH
T
EtOH+T


3A
C
EtOH
T
EtOH+T
IL-10
- 26
Beta-actin
- 42

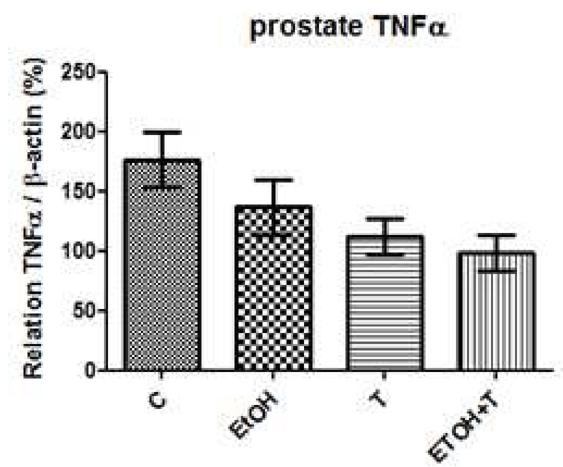

B
prostate IL-10
Relation IL-10 / β-actin (%)
400
300
200
100
0
C
EtOH
T
EtOH+T


C
plasma IL-10
pg/ml plasma
30
20
10
0
C
EtOH
T
EtOH+T

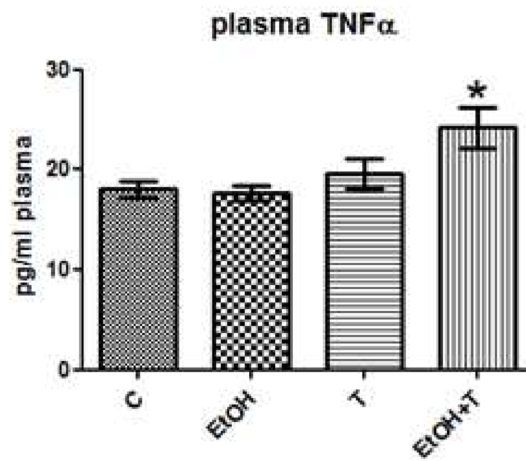
4A

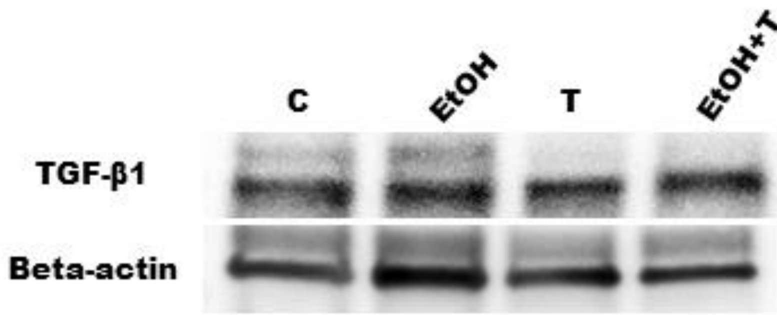


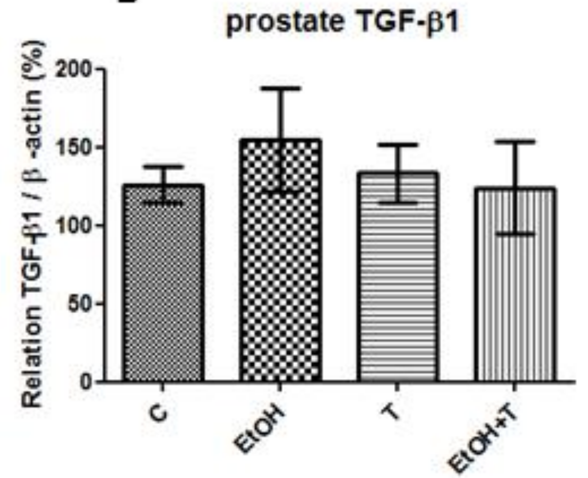
B

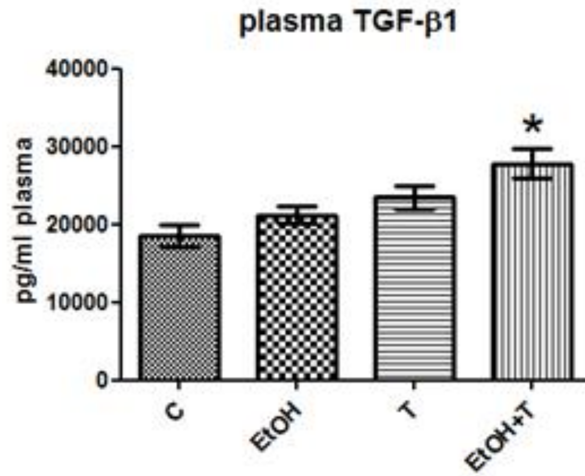


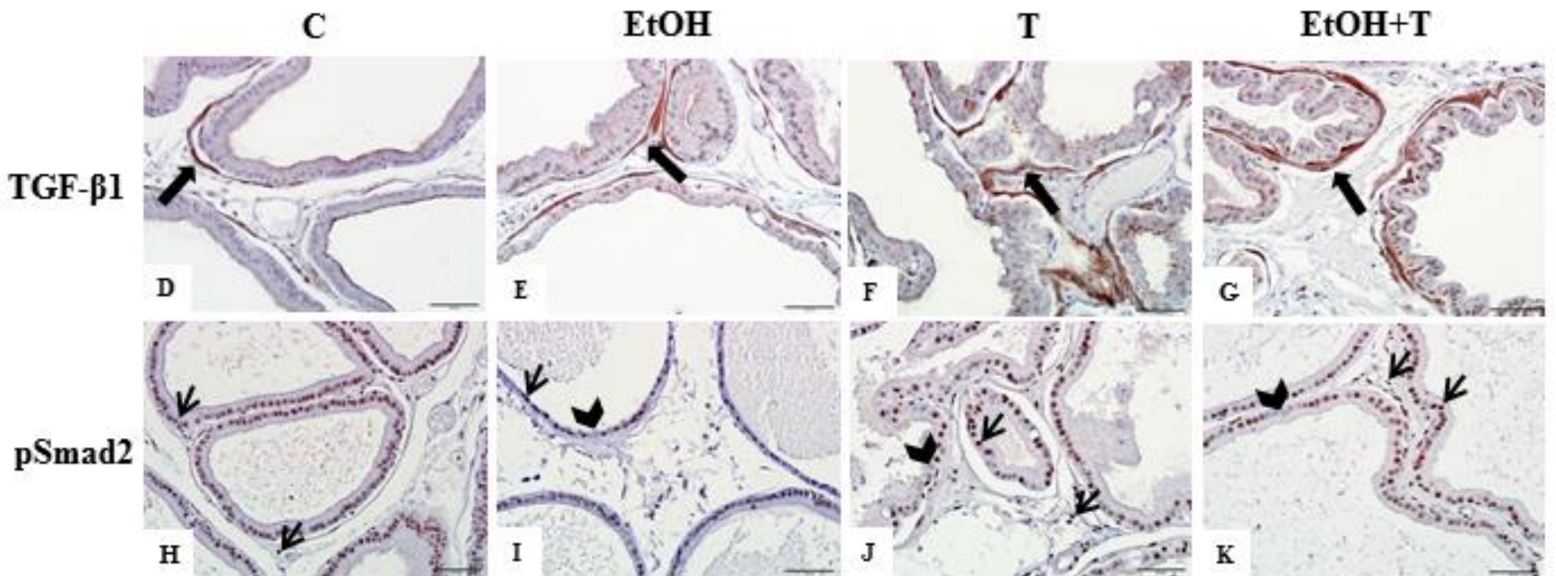
C

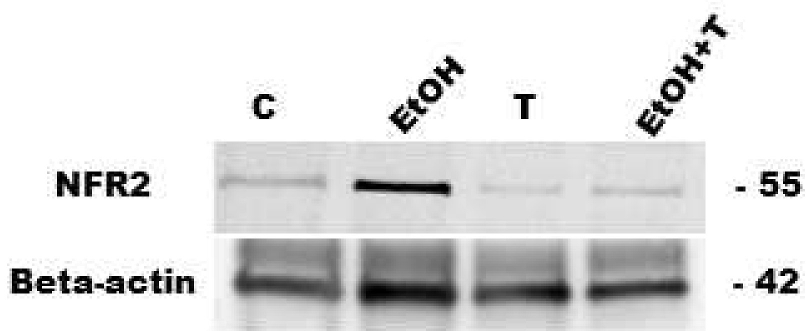


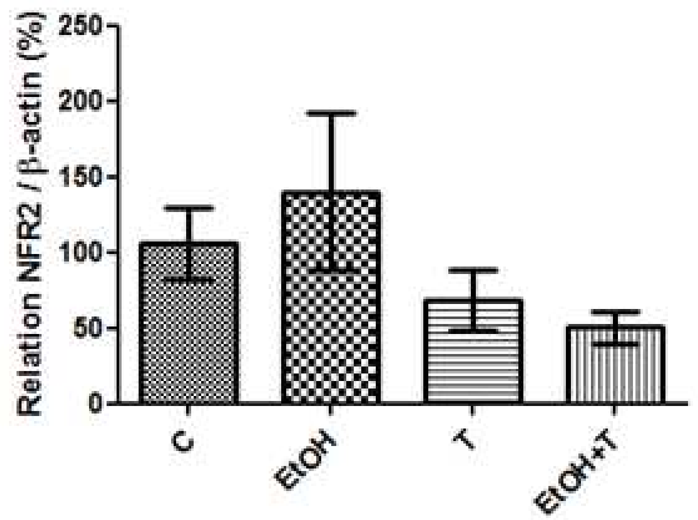

5A
C
EtOH
T
EtOH+T
TGF-β1
Beta-actin


B
prostate TGF-β1
Relation TGF-β1 / β -actin (%)
200
150
100
50
0
C
EtOH
T
EtOH+T


C
plasma TGF-β1
pg/ml plasma
40000
30000
20000
10000
0
*
C
EtOH
T
EtOH+T


C
EtOH
T
EtOH+T
TGF-β1
pSmad2
D
E
F
G
H
I
J
K


6A
C
EtOH
T
EtOH+T
NFR2
- 55
Beta-actin
- 42


B
Relation NFR2 / β-actin (%)
250
200
150
100
50
0
C
EtOH
T
EtOH+T

C

7A

50 µm

B

50 µm

EtOH

T

C

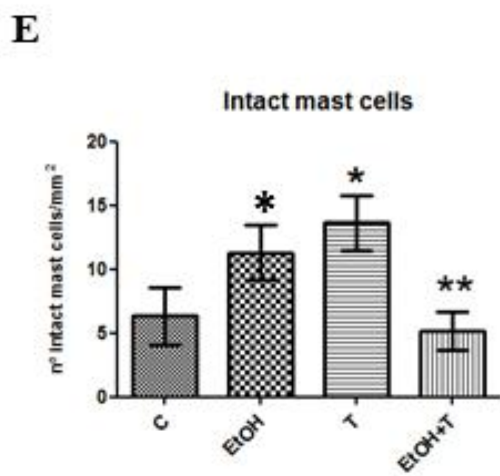
50 µm

D

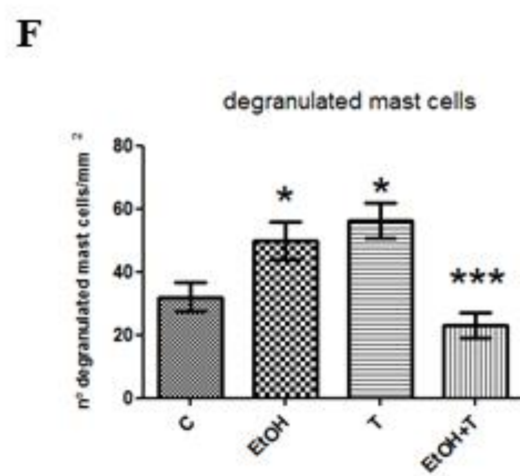
50 µm

EtOH+T

E



F



Conclusão Geral

O etanol induz o surgimento de focos inflamatórios no estroma prostático com alteração na camada de células musculares lisas e perda da adesão celular epitelial. A terapia hormonal com testosterona reverte as alterações, reduzindo a expresção de ERα. As citocinas pró e anti-inflamatórias são diferencialmente reguladas na próstata e plasma pelo etanol e pela testosterona. A glândula prostática, hormônio-responsiva, é susceptível à terapia hormonal, demonstrando o papel anti-inflamatório da testosterona.

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Anexos

UNIVERSITY of
HOUSTON
Center for Nuclear Receptors and Cell Signaling

December 17, 2012

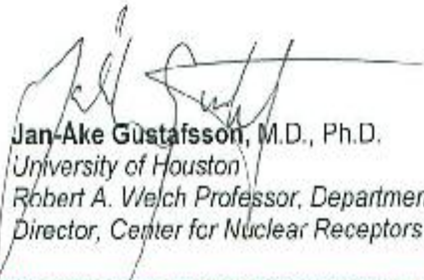
I attest that Mr. Leonardo de Oliveira Mendes was a visiting research scholar at the Center of Nuclear Receptor and Cell Signaling (CNRCS) during the period of June 01, 2012 – October 31, 2012.

In our laboratory, he became very adept in the use of several techniques, such as, immunohistochemistry and western blotting procedures which he has utilized in assessing expression of the Estrogen Receptors Alpha (ERα) and Beta (ERβ), Transforming Growth Factor Beta signaling molecules (TGF-β, pSMAD-2, E-cadherin) and Dachshund Homolog 1 (DACH-1) in the prostates of rats. These studies are part of his PhD project at the University of Campinas (UNICAMP) in Brazil.

In addition, he participated in several scientific meetings regarding different areas of interest in the field of nuclear receptors and prostate cancer research, as well as in weekly laboratory meetings, where he presented his results on three occasions.

He has successfully accomplished much of his project purpose in the study of these proteins and his results will certainly be recognized in the form of a co-authorship future publication.

Best regards,



Jan-Ake Gustafsson, M.D., Ph.D.
University of Houston
Robert A. Welch Professor, Department of Biology and Biochemistry
Director, Center for Nuclear Receptors and Cell Signaling

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DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Tese de Doutorado** intitulada "Análise de receptores hormonais e marcadores inflamatórios no lobo ventral da próstata de ratos consumidores voluntários de etanol (UChB): infuência da terapia hormonal com testosterona":

( ) não se enquadra no § 4º do Artigo 1º da Informação CCPG 002/13, referente a bioética e biossegurança.

Tem autorização da(s) seguinte(s) Comissão(ões):

( ) CIBio - Comissão Interna de Biossegurança , projeto nº _______________, Instituição: ____.

(x) CEUA - Comissão de Ética no Uso de Animais , projeto nº 208, Instituição: instituto de Biociências de Botucatu – IBB/UNESP.

( ) CEP - Comissão de Ética em Pesquisa, protocolo nº _______________, Instituição: ____.

** Caso a Comissão seja externa ao IB/UNICAMP, anexar o comprovante de autorização dada ao trabalho. Se a autorização não tiver sido dada diretamente ao trabalho de tese ou dissertação, deverá ser anexado também um comprovante do vínculo do trabalho do aluno com o que constar no documento de autorização apresentado.*

Leonardo de Oliveira Mendes

Aluno: Leonardo de Oliveira Mendes

Orientador: Prof. Dr. Francisco Eduardo Martinez

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

Carimbo e assinatura

Profa. Dra. ANA MARIA APARECIDA GUARALDO
Presidente da CEUA/UNICAMP

Para uso da Comissão ou Comitê pertinente:
( ) Deferido ( ) Indeferido

Carimbo e assinatura

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

CERTIFICADO

Certificamos que o Protocolo nº **208-CEEA,** sobre "Caracterização do processo inflamatório e influência da terapia de reposição hormonal no lobo ventral da próstata de ratos consumidores voluntários de etanol a 10% (UChB)", sob a responsabilidade de **Francisco Eduardo Martinez,** 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 NA EXPERIMENTAÇÃO ANIMAL*** (CEEA), nesta data.

Botucatu, 19 de abril de 2010.

Profª Drª PATRÍCIA FERNANDA F. PINHEIRO
Presidente - CEEA

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