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NATHALIA CAROLINA FERNANDES FAGUNDES

ESTRESSE OXIDATIVO E ALTERAÇÕES MORFOLÓGICAS EM GLÂNDULAS SALIVARES DE RATOS APÓS EXPOSIÇÃO INTENSA E EPISÓDICA (BINGE) AO ETANOL

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Dissertação apresentada ao Programa de Pós-Graduação em Odontologia (Mestrado) da Universidade Federal do Pará como pré-requisito para obtenção do grau de Mestre, sob a orientação do Prof. Dr. Rafael Rodrigues Lima.

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Aprovada em: _____/_____/_____

Banca Examinadora

_____ - Orientador
Prof. Dr. Rafael Rodrigues Lima
INSTITUTO DE CIÊNCIAS BIOLÓGICAS – UFPA

_____
Prof. Dra. Roberta Souza D'Almeida Couto
INSTITUTO DE CIÊNCIAS DA SAÚDE – UFPA

_____
Dra. Senda Charone
FACULDADE DE ODONTOLOGIA DE BAURU - USP

_____
Prof. Dra. Renata Duarte de Souza Rodrigues
INSTITUTO FEDERAL DE SÃO PAULO -IFSP

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RESUMO

O consumo de etanol em um padrão episódico e intenso tem se mostrado comum entre adolescentes e aumentado em mulheres, com um consumo de 3 vezes por semana. Glândulas salivares são fracamente investigadas acerca destes efeitos, embora alterações morfológicas já foram associadas ao consumo crônico de etanol durante a adolescência. Este estudo investigou os efeitos morfológicos e bioquímicos do consumo de etanol em padrão *binge* em glândulas parótida e submandibular de ratas da adolescência a fase adulta. Ratas wistas (n=26) receberam etanol em uma dose de 3g/kg/day (20% w/v) por três dias consecutivos por semana, entre 35-60 dias de vida. Os animais foram divididos em quatro grupos: G1, tratados com etanol durante 1 semana; G2, tratados durante 4 semanas com etanol; C1, tratados com água destilada de modo semelhante ao G1; C2, tratados com água destilada semelhantes a G2. Na análise morfológica do tecido glandular, realizou-se avaliação morfométrica e imunohistoquímica. Para os ensaios morfométricos, a percentagem média equivalente ao parênquima e estroma região glandular foi avaliada com software Image J. Imuno-histoquímica para actina de músculo liso (α-SMA), citoqueratina 18 (CK-18) e vimentina foram realizados e medidos com programa Image J, sobre padrão de imunoreatividade. Avaliou-se ainda as alterações bioquímicas por dois parâmetros de estresse oxidativo: concentração dos nitritos e níveis de malondialdeído (MDA), um biomarcador de peroxidação lipídica. A diferença entre os grupos etanol e controle foram avaliadas pelo teste t de Student e Mann-Whitney ($p \leq 0,05$), de acordo com cada caso. Após uma semana de exposição ao etanol, a glândula parótida mostrou menor expressão para CK-18 e α-SMA no grupo etanol, além de menores níveis de MDA para o mesmo período. Depois de quatro semanas de exposição, menor expressão de CK-18 e níveis mais elevados de MDA foram observadas na glândula parótida exposta ao etanol, em comparação ao respectivo grupo controle. A glândula submandibular mostrou menor expressão de α-SMA depois de 1 e 4 semanas de exposição ao etanol, bem como níveis de MDA mais elevados após 1 semana de consumo de etanol. O consumo de etanol em padrão binge durante a adolescência promoveu mudanças teciduais e bioquímicas. Nós demonstramos pela primeira vez que a estrutura da glândula salivar pode sofrer alterações bioquímicas e do citoesqueleto com apenas três dias de exposição ao etanol durante a adolescência.

Palavras-chaves: Beber em binge; Glândulas salivares; Adolescência; Estresse Oxidativo; Imunohistoquímica.

ABSTRACT

Ethanol consumption in episodic and intense pattern has been common among teenagers and increased in females, with a consumption pattern of 3 times a week. Salivary glands are poorly investigated about these effects, in which chronic consumption during adolescence has been reported as responsible for morphological changes. This study investigate morphological and biochemistry effects of binge ethanol consumption in parotid and submandibular salivary glands of rats from adolescence to adulthood. Wistar female rats (n=26) received ethanol at 3g/kg/day (20% w/v) for 3 consecutive days/week among 35-60 days of age. Animals were divided in four groups: G1, treated with ethanol for 1 week; G2, treated for 4 weeks with ethanol; C1, treated with distilled water similarly to G1; C2, treated with distilled water as G2. In morphological analysis of glandular tissue, morphometric and immunohistochemistry evaluation were made. To morphometric assays, the mean percentage equivalent to the glandular parenchyma and stroma region was evaluated with Image J software. Immunohistochemistry for smooth muscle actin (α-SMA), cytokeratin 18 (CK-18) and vimentin were conducted and measured with Image J software, regarding pattern of immunoreactivity. Biochemical changes were analyzed by two oxidative stress parameters: concentration of nitrites and levels of malondialdehyde (MDA), a biomarker of lipid peroxidation. The difference between groups in each analyisis were evaluated by Mann-Whitney test ($p \leq 0.05$). Parotid gland showed, at one week of ethanol exposure, lower CK-18 and α-SMA expression, as well as to MDA levels. After four weeks of exposure, a lower CK-18 and higher MDA levels were observed in parotid gland exposed to ethanol, in comparison to control group. Submandibular gland shows lower α-SMA expression after 1 and 4 weeks of ethanol exposure as well as higher MDA levels after 1 week of ethanol consumption. Ethanol binge consumption during adolescence promotes tissue and biochemical changes with only one binge in acinar and mioepithelial cell parotid glands. Submandibular glands seemed to be more resistant to the intoxication model proposed. We demonstrated for the first time that the salivary gland structure may suffer biochemical and cytoskeletal changes with only three days of exposure to ethanol during adolescence.

Key-words: Binge drinking; Salivary glands; Adolescence; Oxidative stress; Immunohistochemistry;

SUMÁRIO

INTRODUÇÃO

Segundo relatório da Organização Mundial da Saúde (OMS), indivíduos acima de 15 anos de idade consomem, em média, 6,2 litros de álcool puro por ano, que traduz-se em 13,5 g de álcool puro por dia, onde a América e a Europa figuram como regiões de consumo acima da média ("Global status report on alcohol and health 2014," 2014).

Neste contexto, verifica-se aumento do consumo eventual e intenso de etanol no sexo feminino no Brasil, especialmente em mulheres mais jovens, figurando como grupo de maior incremento na elevação desses índices (INPAD, 2013). O uso desta substância é comum entre adolescentes, cujo fácil acesso e a falta de consequências legais, a configuram como uma das drogas mais utilizadas neste grupo etário (SKALA e WALTER, 2013).

O consumo alcoólico eventual e intenso, em altas doses de EtOH em por um curto período de tempo seguido por um período de abstinência, conhecido como padrão binge, apresenta alta prevalência e aumento médio em sua frequência durante toda a adolescência, com pico na idade adulta jovem e subsequentemente queda com o avançar da idade. (HAHM et al., 2012; KUNTSCHE; GMEL, 2013; LÓPEZ-CANEDA et al., 2013).

O efeito do consumo em binge de altas doses de EtOH em glândulas salivares ainda não foi elucidado, embora o consume crônico de eanol já tenha sido associado a diminuição do fluxo salivar (NÖR et al., 2013; SCOTT; WOODS; BAXTER, 1988), atrofia de células acinares (FAUSTINO; STIPP, 2003; MAIER; BORN; MALL, 1988), aumento dos ductos maiores (FERRARIS et al., 1999) em glândulas salivares maiores.

No Brasil, o abuso de álcool representa um problema da saúde pública sendo que este consumo de maneira pesada inicia muitas vezes na adolescência e seus efeitos em tecidos orais, mais especificamente, nas glândulas salivares, necessitam ser investigados. Assim, este estudo teve como objetivo avaliar o efeito ocasionado pela intoxicação episódica e intensa de EtOH, em padrão binge, em glândulas salivares parótida e submandibular de ratas, da fase de adolescência a adulto jovem.

REVISÃO DE LITERATURA

1. ASPECTOS RELACIONADOS AO ABUSO DE ÁLCOOL E AO CONSUMO EM *BINGE*

O conceito do padrão de ingestão alcoólica tipo binge foi definido pelo Instituto Nacional dos EUA sobre Abuso de Álcool e Alcoolismo como a concentração de 0,08 g de EtOH no sangue após seu consumo, equivalente ao consumo de cinco ou mais doses de bebida em um período de duas horas para homens e de quatro doses ou mais de bebida no mesmo período para mulheres (HINGSON, 2004).

O conceito de dose de bebiba apresenta por definição 10g de etanol, mas pode variar de 6g a 20g, de acordo com o país consultado. Além disso, em estudos que utilizam o padrão em *binge* para suas análises, não se observa um mesmo conceito para a ingestão de álcool, cujos termos variam de: *drink*, *standard drink* ou ainda *Unit of alcohol* (SKALA; WALTER, 2013).

O abuso do álcool, mais especificamente seu consumo em binge, mostra-se nocivo e encontra-se associado ao desenvolvimento de doenças crônicas a longo prazo, como diabetes mellitus tipo 2 e hipertensão (FAN et al., 2008; SPROW; THIELE, 2012).

No Brasil, o consumo de etanol configura-se como um problema de saúde pública, com alto consumo entre jovens, especialmente mulheres (INPAD, 2013), o que denota a necessidade de investigação acerca dos efeitos do abuso deste tipo de substância.

O consumo de etanol em *binge* já foi associado a alterações de ordem psicossocial e problemas de ordem comportamental entre adolescentes (HAHM et al.,

2012; SKALA; WALTER, 2013). Este padrão de consumo de etanol é considerado um precursor do alcoolismo crônico, sendo associado ao maior risco de desenvolvimento de dependência ao etanol ao longo da vida, bem como ao desenvolvimento de danos ao Sistema Nervoso Central (SNC) (BAE et al., 2014; SPROW; THIELE, 2012).

O efeito tóxico do etanol é geralmente atribuído à ação de acetaldeído, que parece representar a origem de danos aos tecidos orais, sendo o prmeiro metabólito gerado durante a decomposição do álcool no organismo, e muitas vezes relacionado a um papel modulador dos efeitos desta substância no organismo. Entretanto, a formação de espécies reativas de oxigênio (ROS) e ésteres etílicos de ácidos graxos (FAEEs) também possam estar associados a ação direta de etanol (QUERTEMONT; DIDONE, 2006; WASZKIEWICZ et al., 2011).

O acetaldeído apresenta potencial mutagênico e carcinogênico, mostrando-se relacionado a deficiências funcionais de proteínas e danos ao DNA, o apontando tal metabólito como um dos responsáveis pelos efeitos nocivos que o etanol pode trazer ao organismo (QUERTEMONT; DIDONE, 2006; SETSHEDI; WANDS; MONTE, 2010)

Os efeitos da intoxicação por etanol já foram associados a um consumo crônico desta substância, caracterizado por desencadear danos aos órgãos e tecidos do corpo, como por exemplo, ao trato gástrico superior, musculatura esquelética e glândulas salivares, o que pode ocasionar modificações morfológicas e funcionais (BAGYÁNSZKI et al., 2010; BOHL et al., 2008).

Em contrapartida, o consumo moderado de álcool já foi relacionado a propriedades citoprotetoras, bem como a diminuição de riscos a doenças coronarianas e neuronais, devido a mecanismos anti-inflamatórios estimulados no coração, cérebro

e sistema vascular, que tendem a promover os percursos de sobrevivência celular (COLLINS et al., 2009).

A exposição de órgãos e tecidos do organismo ao álcool pode acarretar injúrias oxidativas ao organismo, que ocorre devido a um quadro de estresse oxidativo, onde ocorre produção de Radicais Livres em níveis superiores à capacidade de eliminação do sistema antioxidante. Este aumento da formação de radicais livres pode ocasionar danos, mutações e morte celular nos locais afetados (MCDONOUGH, 2003).

2. GLÂNDULAS SALIVARES E INTOXICAÇÃO POR ETANOL

A saliva exerce papel importante na manutenção da saúde bucal, como proteção, reparo tecidual, manutenção da integridade dentária, além de possuir ação antimicrobiana e contribuir na digestão e citoproteção gástrica (HOLMBERG; HOFFMAN, 2014; PROCTOR; CARPENTER, 2014).

As glândulas salivares compreendem um conjunto de ductos ramificados que culminam em uma porção secretora terminal, os ácinos, que podem apresentar origem mucosa ou serosa. Ao redor de sua porção secretora encontram-se células mioepiteliais, responsáveis por fornecer suporte a porção terminal durante a secreção salivar (HOLMBERG; HOFFMAN, 2014; NANCI, 2008).

O tecido conjuntivo apresenta-se sob a forma de cápsula, com presença de fibras colágenas e elásticas, além de células, como os fibroblastos, mastócitos, plasmócitos, macrófagos, células dendríticas e células adiposas. Este tecido sustenta e carrega o suprimento nervoso, linfático e vascular para os respectivos componentes parenquimais (NANCI, 2008; PATEL; REBUSTINI; HOFFMAN, 2006).

Anatomicamente, as glândulas salivares podem se apresentar em glândulas salivares maiores: parótida, submandibular e sublingual; e glândulas salivares menores. No que se refere a sua constituição, a glândula parótida caracteriza-se por

um padrão acinar seroso, enquanto as glândulas submandibular e sublingual apresentam ácinos mucosos cobertos com semiluas serosas, embora também apresentem ácinos mucosos e serosos em sua composição (NANCI, 2008).

Os efeitos de substâncias químicas nas glândulas salivares têm sido observados por diversos estudos. Neste contexto, frente ao consumo mundial, o álcool representa uma droga bastante estudada quimicamente e os efeitos dessa substância, sejam tóxicos ou protetores vem sendo descritos ao longo dos anos (BRIONES; WOODS, 2013; NÖR et al., 2013).

Neste âmbito, alterações nas glândulas salivares devido ao abuso de etanol foram primeiramente descritos em 1971, representando também uma das primeiras estruturas na descrição da influência do consumo crônico de etanol. Neste estudo, foi destacado o aumento da glândula parótida frente a presença da condição de alcoolismo (MANDEL; BAURMASH, 1971).

Segundo Maier et al (1986), ao avaliar o efeito do consumo crônico de etanol em glândulas parótida e submandibular de ratos, observou um acúmulo de tecido adiposo em células acinares de glândula parótida, diminuição de seu peso úmido e no teor de proteína deste tecido. As glândulas submandibulares não apresentaram tais alterações. Observou-se ainda alterações no fluxo e composição salivar (MAIER et al., 1986).

Neste sentido, ao analisar glândulas parótida e submandibular humanas após abuso crônico de álcool, Scott et al (1988) observou diminuição de células acinares, presença de sialose e aumento de tecido adiposo em glândula parótida, bem como aumento no tecido adiposo e fibrovascular de glândulas submandibulares. A partir desta análise descritiva, foi sugerido que o aumento de tecido adiposo estaria

diretamente associado ao aumento de volume dos respectivos tecidos glandulares (SCOTT; BURNS; FLOWER, 1988).

Tais resultados diferem da análise realizada por Bohl et al (2008), que compararam glândulas parótidas humanas que apresentavam sialose alcoólica com material controle. Em sua análise, não foram observadas diferenças nas dimensões acinares e ductais. A proporção de tecido adiposo em parótidas com sialose também não se mostrou alterada, cuja proporção mostrou-se mais acentuada no grupo controle (BOHL et al., 2008).

Além disso, o aumento de tecido adiposo em glândulas salivares é uma característica também associada ao processo de envelhecimento deste tecido (DAYAN et al., 2000; KIKUCHI et al., 2007), o que pode estar associado aos relatos descritivos apontados em estudos anteriores. Entretanto, devido à presença de fibrose e infiltrados inflamatórios em glândulas afetadas pelo álcool, já foi sugerido que esta substância poderia estar diretamente associada ao processo de envelhecimento (SEGERBERG-KONTTINEN, 1989).

Alterações no processo de regeneração de glândulas salivares também parecem estar associadas ao uso crônico de etanol. Nör et al (2013) apontaram modificações na expressão de laminina e na produção de glicoproteínas em glândulas submandibulares, em ratos submetidos ao uso crônico de etanol, o que ocasionou avançada morfogênese e atraso na citodiferenciação durante o processo de regeneração deste tecido glandular (NÖR et al., 2013).

No que se referem às alterações morfológicas neste tecido frente ao consumo de etanol, verifica-se que, clinicamente, esta substância promove atrofia do tecido glandular (CARDA et al., 2004). Um acúmulo de grânulos basófilos e aumento na

infiltração periductal de linfócitos, já foi observado em análise descritiva de glândulas parótidas de indivíduos em cirrose alcoólica (CARDA et al., 2004).

Indivíduos alcoólicos também apresentam modificações do fluxo salivar. Embora alguns relatos iniciais tenham apontado um aumento de fluxo salivar quanto ao uso tópico (MARTIN; PANGBORN, 1971) e crônico (ABELSON; MANDEL; KARMIOL, 1976) de etanol, estudos posteriores demonstraram redução do fluxo salivar (MAIER et al., 1986; NÖR et al., 2013; PRESTIFILIPPO et al., 2009; SCOTT; BURNS; FLOWER, 1988).

Enberg et al (2001) observou que o uso agudo de álcool também promove redução de fluxo salivar em seres humanos. Esta característica pode estar relacionada a diminuição de proteínas totais, da enzima amilase, bem como, da queda do número de eletrólitos presentes da saliva verificadas nos indivíduos avaliados.

Alterações na composição salivar já foram relacionadas ao uso agudo de etanol por Proctor et al. (1993), que avaliou a taxa de síntese de proteínas em glândulas salivares maiores de ratos e observou redução da síntese de proteínas neste tecido (PROCTOR; SHORI; PREEDY, 1993).

Assim, uso crônico de etanol resulta em alterações estruturais na morfologia macro e microscópicas das glândulas salivares, resultando em alterações de fluxo e composição salivar, caracterizado por um menor número de células acinares (FERRARIS et al., 1999; MAIER et al., 1986; SCOTT; BURNS; FLOWER, 1988; SCOTT; WOODS; BAXTER, 1988). Um comprometimento estrutural das glândulas salivares por abuso de álcool pode repercutir diretamente no desenvolvimento das funções deste tecido glandular, o que pode estar diretamente relacionando ao desenvolvimento de injúrias e a maior suscetibilidade a danos nos tecidos orais.

Imerso nesta lógica, Campos et al (2005) avaliaram o efeito do consumo crônico de etanol sobre o estresse oxidativo nas glândulas parótida e submandibular de ratos, verificaram aumento de peroxidação de lipídios e atividade de enzimas antioxidantes, além de aumento de volume da glândula parótida. O aumento de atividade das enzimas analisadas figura como mecanismo compensatório ao aumento de radicais livres, o qual é estimulado pelo consumo crônico de álcool (CAMPOS et al., 2005).

Embora o efeito do uso crônico do álcool sobre as glândulas salivares maiores seja conhecido por promover alterações morfológicas e funcionais neste tecido, o efeito do uso eventual e intenso de álcool, padrão binge, em glândulas salivares ainda não foi elucidado.

Sabe-se que este padrão de consumo alcoólico pode desencadear alterações como maior suscetibilidade ao acúmulo de tecido adiposo no fígado (GRASSELLI et al., 2014; MINATO et al., 2014), anomalias na atividade neural (LÓPEZ-CANEDA et al., 2013), além de mostrar potencial neurotóxico (CIPPITELLI et al., 2014).

O conhecimento do efeito deste efeito em glândulas salivares, especialmente em mulheres adolescentes e adultos jovens, grupo que apresenta alto consumo de álcool em padrão binge (“II levantamento nacional de álcool e drogas,” 2013) é necessário, visto que alterações na homeostase deste tecido podem resultar em alterações associadas aos demais tecidos orais, como: alterações dentárias e periodontais, além de mecanismos moleculares que possam estar associados a modificações no padrão de resposta celular.

CORPO DO ARTIGO

Title: Binge Drinking of Ethanol in Female Rats: How Does it Affects Salivary Glands?

Nathalia Carolina Fernandes Fagundes[a]

Luanna Melo Pereira Fernandes[b]

Ricardo Sousa de Oliveira Paraense[c]

Paulo Mecenas Alves de Farias Junior[a]

Francisco Bruno Teixeira[a]

Sergio Melo Alves Junior[d]

João de Jesus Viana Pinheiro[d]

Cristiane do Socorro Ferraz Maia[b]

Rafael Rodrigues Lima[a]

[a]Laboratory of Functional and Structural Biology, Institute of Biological Sciences, Federal University of Pará, Street Augusto Corrêa n. 1, Guamá, Belém-Pará, 66075-900, Brazil;

[b]Laboratory of Inflammation and Behaviour Pharmacology, Pharmacy Faculty, Institute of Health Science, Federal University of Pará, Street Augusto Corrêa n. 1, Guamá, Belém-Pará, 66075-900, Brazil;

[c]Laboratory of Molecular Pharmacology, Institute of Biological Sciences, Federal University of Pará, Federal University of Pará, Street Augusto Corrêa n. 1, Guamá, Belém-Pará, 66075-900, Brazil;

[d]Department of Oral Pathology, School of Dentistry, Federal University of Pará, Street Augusto Corrêa n. 1, Guamá, Belém-Pará, 66075-900, Brazil.

CORRESPONDING AUTHOR

Rafael Rodrigues Lima

Laboratory of Functional and Structural Biology, Institute of Biological Sciences,

Federal University of Pará. Street Augusto Corrêa, n. 1, Guamá, Belém-Pará,

66075-900, Brazil.

E-mail: rafalima@ufpa.br, rafaelrodrigueslima@hotmail.com

ABSTRACT

This study investigates morphological and biochemistry effects of binge ethanol consumption in parotid (PG) and submandibular (SG) salivary glands of rats from adolescence to adulthood. Female Wistar rats (n=26) received ethanol at 3g/kg/day (20% w/v) for 3 consecutive days/week among 35-62 days of age. Animals were treated in two periods: 1-week (G1) and 4-week (G2), with a control (treated with distilled water) and an ethanol group to each period. In morphological analysis, morphometric and immunohistochemistry evaluation for smooth muscle actin (α-SMA), cytokeratin 18 (CK-18) and vimentin (VIM) were made. Biochemical changes were analyzed by concentration of nitrites and levels of malondialdehyde (MDA). The difference between groups in each analysis was evaluated by Mann-Whitney or Student's t-test ($p \leq 0.05$). PG showed, at one week of ethanol exposure, lower CK-18 and α-SMA expression, as well as to MDA levels. After four weeks, a lower CK-18 and higher MDA levels were observed in PG exposed to ethanol, in comparison to control group. SG showed lower α-SMA expression after 1 and 4 weeks of ethanol exposure as well as higher MDA levels after 1 week. Ethanol binge consumption during adolescence promotes tissue and biochemical changes with only one-week binge in acinar and mioepithelial PG cells.

Key-words: Ethanol – Salivary Glands – Binge drinking.

1. Introduction

According to World Health Organization (WHO), individuals over 15 years old consume on average 6.2 liters of pure alcohol per year, which translates into 13.5 g of pure alcohol a day. America and Europe are regions shown as consuming above average. In 2012, 5.9% of global mortality was associated with alcohol use [1].

In this context, there are an increased potential and intense consumption of ethanol (EtOH) in women, especially in countries like the United States [2], Brazil [3] and western Europe [4,5]. This type of alcohol intake is often seen in younger women. Starting, in many cases, during adolescence, the easy access and lack of legal consequences may categorize this substance as one of the most commonly used drugs among this age group [6].

Heavy alcohol consumption, with high doses of EtOH for a short period of time followed by a period of abstinence, in a binge style, has a high prevalence and increases in frequency throughout adolescence, peaking in young adults and subsequently declining with advancing age [7–9].

EtOH consumption has been associated with triggering damage to organs and body tissues, such as the upper gastric tract, skeletal muscles and salivary glands, which can cause morphological and functional changes [10,11].

Salivary glands and their resulting secretion have a huge importance in the maintenance of oral and general homeostasis [12]. The structure of these glands comprise a series of ramified ducts that culminate in a secretory terminal portion, the acini cells, which compose the glandular parenchyma [12,13]. However, the effects of usual consumption of EtOH intermittent in a binge drink pattern on salivary glands have not been elucidated yet.

The investigation of changes in salivary glands after alcohol intake may involve analysis of immunohistochemical markers in parenchyma and stroma, as cytokeratin, vimentin and alpha smooth muscle actin, intended to evaluate morphological alterations [13]. Biochemical analysis, as oxidative stress, also shows changes that can occur in pathological situations, as in the case of EtOH

chronic consumption, showing involvement of oxidative stress in parotid gland sialadenosis [14].

This study aimed to evaluate the effects caused by episodic and intense intoxication of EtOH in a 3-day/week binge pattern on the parotid and submandibular salivary glands of female rats, during the adolescence to young adulthood phase.

2. Material and Methods

2.1. Animals and experimental groups

Female Wistar rats, 35 days of age, kept in standard conditions of temperature, in a climate-controlled room on a 12-h reverse light/dark cycle of 12 hours (lights on 7:00 AM), with food and water *ad libitum*. This study was approved by the Ethics Committee on Experimental Animals of the Federal University of Pará (CEPAE-UFPA: 196-14), and followed the guidelines suggested by the NIH *Guide for the Care and Use of Laboratory Animals*, with all animals kept in collective cages (maximum of five animals per cage).

A sample size calculation was performed assuming a normal distribution of the variables tested. A power of 80% and a bilateral alpha level of 5% were assumed with standard deviation of 1.29 (ethanol group) and 0.20 (control group). Standard deviation was determined through a previous study [13]. Thus, a sample size of eight animals in ethanol group and five animals in control group (n=26) was established for this study.

All animals received EtOH at a dose of 3 g/kg/day (20% w/v), mimetizing a pattern of binge consumption previously described [22]; or distilled water, administered through gavage with orogastric cannula, for three consecutive days/week in the animals, 35 to 62 days of age, which corresponds to late adolescence and early adulthood in this animal model [15,16]. The weighing of the animals was performed weekly for dose adjustment.

The total sample was divided into two groups, according to the period of solution administration: G1, with 1 week of 3 days of exposure to ethanol/distilled water; and G2, with 4 week of 3 days of exposure to ethanol/distilled water. Each group was composed of a control group, where the animals received distilled water, and an ethanol group, where animals received

EtOH. Twelve hours after treatment, animals were divided and submitted to collection of fresh glands or perfusion (n=5-8 animals per group; Figure 1, A).

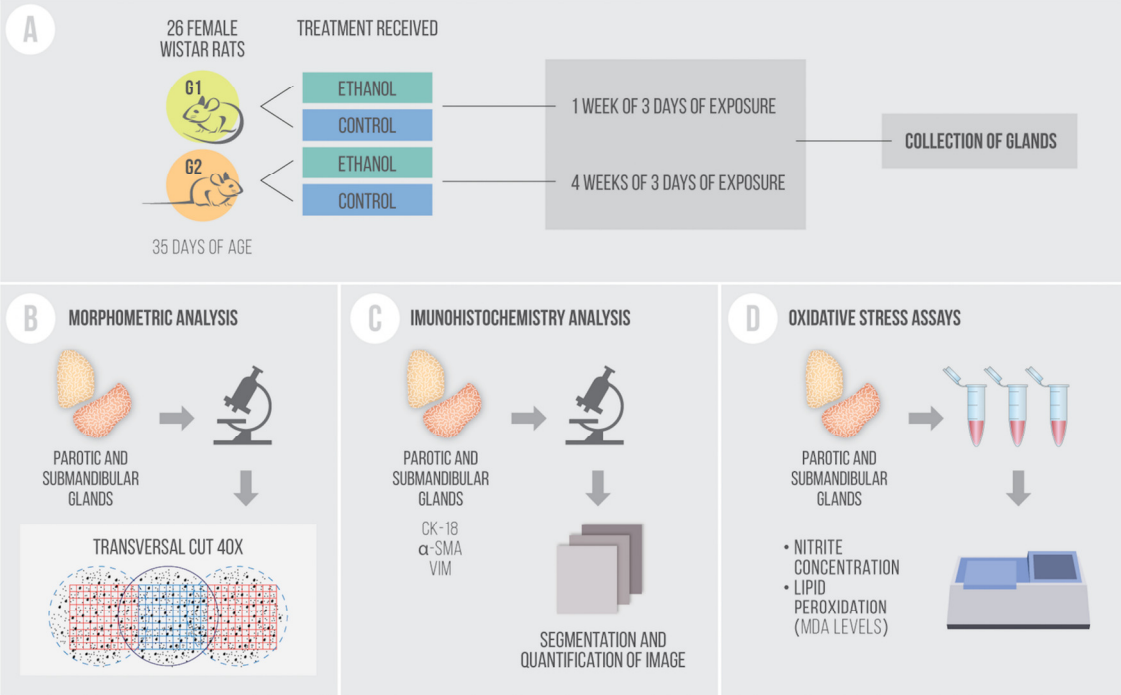

Figure 1: Sample description and experimental steps. Animals and sample description (A); morphometric analysis steps (B); immunohistochemistry analysis steps (C); oxidative stress assays (D).

2.2. Euthanasia of animals and collection of the glands

The animals were anesthetized with a combination of ketamine hydrochloride (90 mg/kg) and xylazine (10 mg/kg). After the absence of corneal and paw withdrawal reflex, surgery was performed to collect the parotid and submandibular salivary glands. The gland of the right side was removed and used for oxidative stress analysis. The gland of the left side was removed after perfusion and used to immunohistochemistry and morphometric analysis.

All glands were weighed after removal by analytical balance (FA 2104 N, Eletronic Balance Bioprecisa, Shangai, China), and the relative glandular weight calculated (gland weight x 100/final body weight).

2.3. Immunohistochemistry and morphometric analysis

After perfusion of the animals, one submandibular and one parotid gland from the left side of each animal were post-fixed in 6% formaldehyde until

processing [17,18]. The glands were dehydrated in increasing ethanol battery, cleared in xylene, and embedded in Paraplast resin, available for further 3 μm sections.

With regard to morphometric assays, the mean percentage equivalent to the glandular parenchyma and stroma region was evaluated. The area of the samples was evaluated by the planimetry method for counting points, using Image J software version 1.33-1.34 (NIMH, NIH, Bethesda, MD, USA, http://rsbweb.nih.gov/ij/). All sections were stained in hematoxylin & eosin, with five aleatory images of each gland, captured in 40x magnification on a Axio Scope microscope (Carl Zeiss, Germany) equipped with a CCD colour camera AxiocCam HRC (Carl Zeiss, Germany). The coincident points with glandular parenchymal and stromal elements were counted, and the percentage of each region was calculated by dividing the number of incidents on the same points and the total number of points on the grid [13] (Figure 1, B).

Immunohistochemical studies were performed on paraffin-embedded tissues using the streptavidin (Reveal Spring, Pleasanton, CA, USA) and 3,3-diaminobenzidine (DAB; Sigma, USA) methods. Briefly, 3-μm sections were deparaffinized and rehydrated in battery with decreasing concentration of alcohol. After antigen retrieval chamber Pascal (Dako Corporation Carpinteria CA, USA) and blocking of endogenous peroxidase activity, the sections were incubated in primary antibody anti-α smooth muscle actin (α-SMA) (1:50, Dako), anti-cytokeratin 18 (CK-18) (1:100 Bioss), and anti-Vimentin (VIM) (1:100, Bioss). Subsequently, the sections were incubated for 30 min with biotin-free horseradish peroxidase (HRP) enzyme-labeled polymer (REVEAL, Spring, Pleasanton, CA, USA). Diaminobenzidine (Sigma Chemical Corp., St Louis, USA) was used as the chromagen and sections were counterstained with Mayer's hematoxylin (Sigma Chemical Corp., St Louis, USA).

Evaluation of immunostaining was performed by evaluating the extent of the area (μm) and fraction (%) of a marked section. Brightfield images of five randomly selected areas for each sample were acquired with Axio Scope microscope (Carl Zeiss, Germany) equipped with a CCD colour camera AxiocCam HRC (Carl Zeiss, Germany), with a magnification of 40X. Areas stained by DAB were separated and segmented using "colour deconvolution plug-in" (Gabriel

Landini, http://www.dentistry.bham.ac.uk/landinig/software/software.html) from ImageJ software version 1.33-1.34 (NIMH, NIH, Bethesda, MD, USA, http://rsbweb.nih.gov/ij/). After image segmentation, the area and fraction of total staining were measured (Figure 1, C).

2.4. Oxidative stress assays

After collection and weighing of glands, the extracted tissue was rinsed in saline and subjected to freezing in liquid nitrogen and subsequently stored at -80 ° C. For analysis, samples were thawed and resuspended in 20mM Tris-HCl buffer, pH 7.4, at 4 ° C for sonic disintegration (approximate concentration of 1 g/ml) (Figure 1, D).

The concentration of nitrite is determined based on reaction with Griess reagent (0.1% Naphthyl-ethylene-diamine and 1% Sulfanilamide in 5% phosphoric acid; 1:1). An aliquot of crude homogenate was centrifuged at 21,000 g for 20 min at 4uC, and supernatant was used to analyze nitrite levels as described elsewhere [19]. Briefly, Fifty microliters of the supernatant or standard nitrite solution are added to 50mL of Griess reagent and incubated for 20 minutes at room temperature. The absorbance was measured at 550ηm and compared to that of standard solutions of sodium nitrite.

The level of lipid peroxidation is determined by the method proposed by Esterbauer and Cheeseman (1990), based on measurement of malonaldehyde (MDA) and 4-hidroxialcenos (4-HA) levels [20]. Briefly, an aliquot of crude homogenate was centrifuged at 2,500 g for 30 min at 4°C, and supernatant was processed as described by the Bioxytech LPO-568 kit (Cayman Chemical). This kit takes advantage of a chromogenic reagent that reacts with MDA and 4-HDA at 45°C, yielding a stable chromophore with maximal absorbance at the 586 ηm wave length.

Quantities of total protein content in the supernatants (used for determination of lipid peroxidation and nitrite levels) were assayed as described previously [21]. Thus, after corrected for protein concentration, results of lipid peroxidation and nitrite levels were expressed as picomole per milligram of protein.

2.5. Statistical analysis

The mean values obtained from the Control and EtOH groups, among parotid and submandibular gland, in each period evaluated, for each morphologic and biochemical assays. The normality of the data was verified by the Shapiro-Wilk test. The Student t test was applied for normal data and the Mann-Whitney test for abnormal data. All analyses were tabulated in GraphPad Prism software version 1.5 (San Diego, CA, USA). Statistical significance values of $p<0.05$ were considered acceptable.

3. Results

3.1. Body weight and relative glandular weight

In both periods of treatment (35 to 41 days of age and 35 to 62 days of age), the ethanol intake did not alter the body weight of animals (Figure 2). In the analysis of relative glandular weight and the treatment received, no difference was detected in submandibular or parotid gland for both periods of analysis (Figure 2).

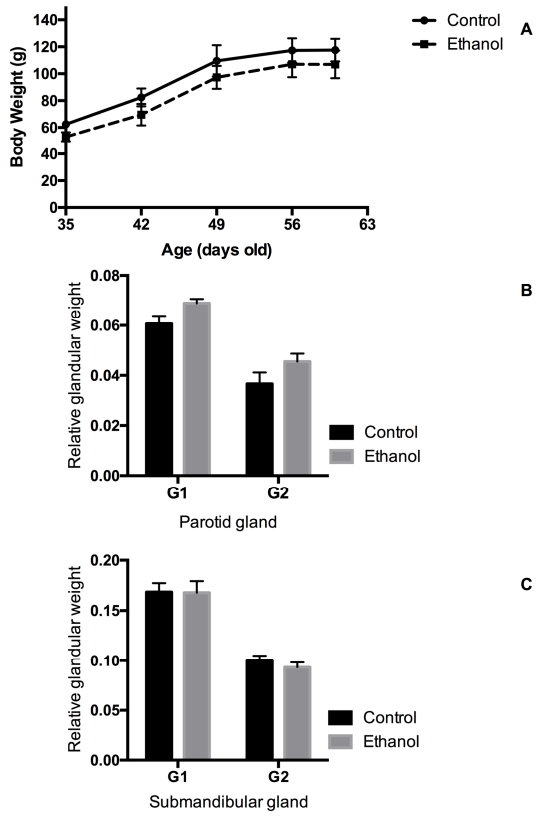

Figure 2: Effects of binge drinking (EtOH administration) during adolescence in the body weight gain of rats. (A) and relative gland weight of parotid (B) and

submandibular (C) glands. The results were expressed as mean ± SEM after t-Student test.

3.2. Morphologic changes

In the morphometric assay, as the results show in Figure 3, no changes were observed in the total epithelial area to parotid and submandibular glands after exposure to ethanol in doses of 3g/kg/day (Figure 3).

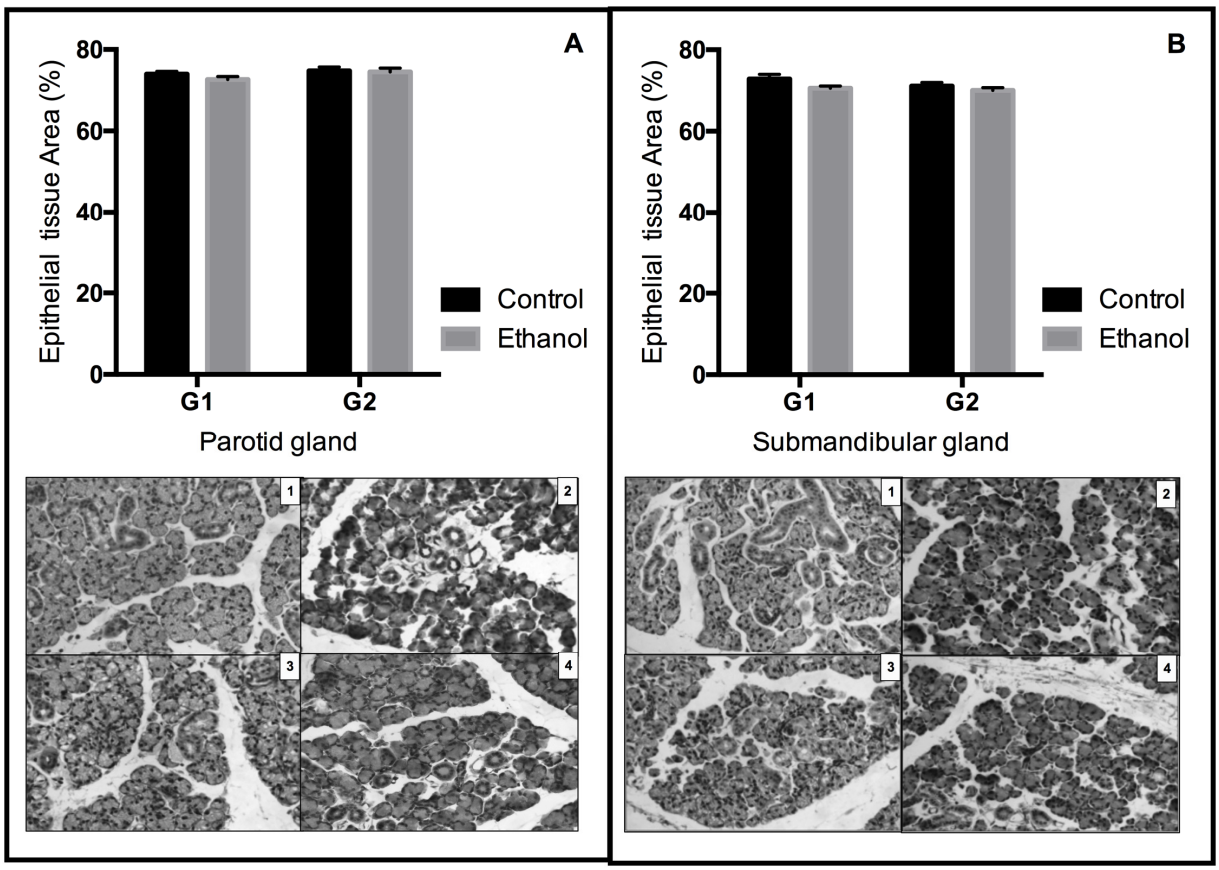

Figure 3: Morphometric analysis: effects of binge drinking (EtOH administration) during adolescence and young adulthood. Parotid (A) and submandibular glands (B), after one-week (control: 1, ethanol: 2) and four weeks of ethanol exposure (control: 3, ethanol: 4), according to treatment group. These results are expressed with photomicrographs and mean ± SEM (Mann-Whitney test). Inset scale= 20μm.

In parotid glands, ethanol consumption affected expression of CK-18 at one week and four weeks of exposure (Figure 4, A,). When we analyzed the α-SMA, the difference among groups was only detected at one-week of exposure to ethanol (Figure 4, C). With regard to Vimentin expression, no difference was detected in any of the periods of exposure (Figure 4, E).

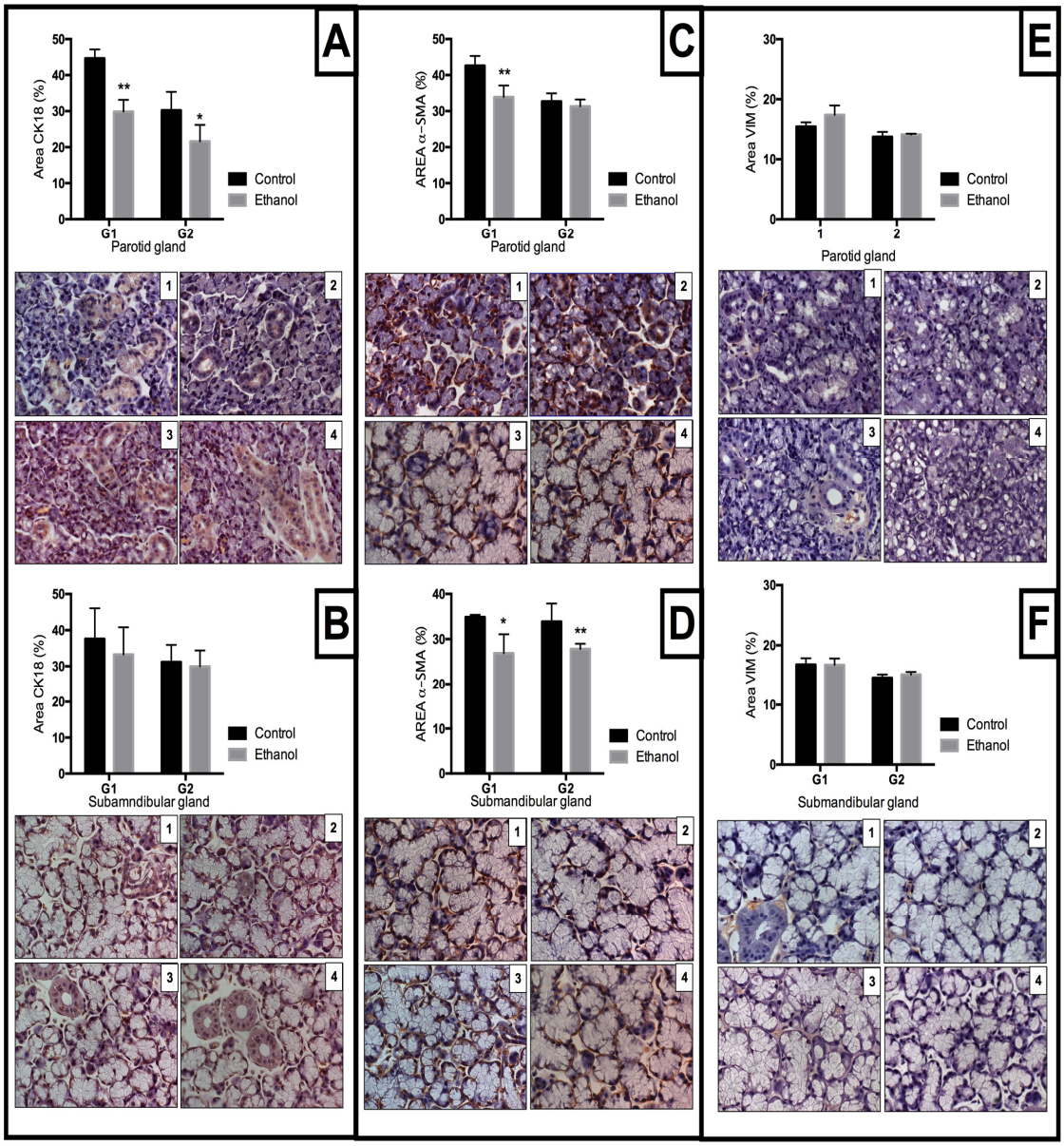

Figure 4: Effects of binge drinking (EtOH administration) during adolescence on the parotid and submandibular gland immune expressions. Anti-α muscle actin (α-SMA), anti-cytokeratin 18 (CK18) and vimentin (VIM) positive cells of female Wistar rats after 1-week and 4-week exposure to ethanol in control (1 and 3, at 1-week and 4-week, in each group) and ethanol (2 and 4, at 1-week and 4-week, in each group) groups. The results are expressed as mean ± SEM. *p≤0.05 compared to control group (Mann-Whitney test), **p≤0.01 compared to control group (Mann-Whitney test).

On the other hand, submandibular gland showed no difference between the control and ethanol groups in both periods of analysis regarding the expression of CK-18 (Figure 4, B). The same was observed for Vimentin expression (Figure 4, F). As for expression of α-SMA, a lower expression was observed in ethanol group after one and four weeks of exposure (Figure 4, D).

3.3. Oxidative stress

No difference in Nitrites concentration among the control and ethanol groups was detected in parotid glands and submandibular glands (Figure 5A, C) for both periods of evaluation.

The levels of MDA showed an increase in expression in parotid glands at one and four weeks of exposure to ethanol (Figure 5B). Submandibular glands showed a higher level of MDA in ethanol group only at one week of exposure to ethanol (Figure 5D).

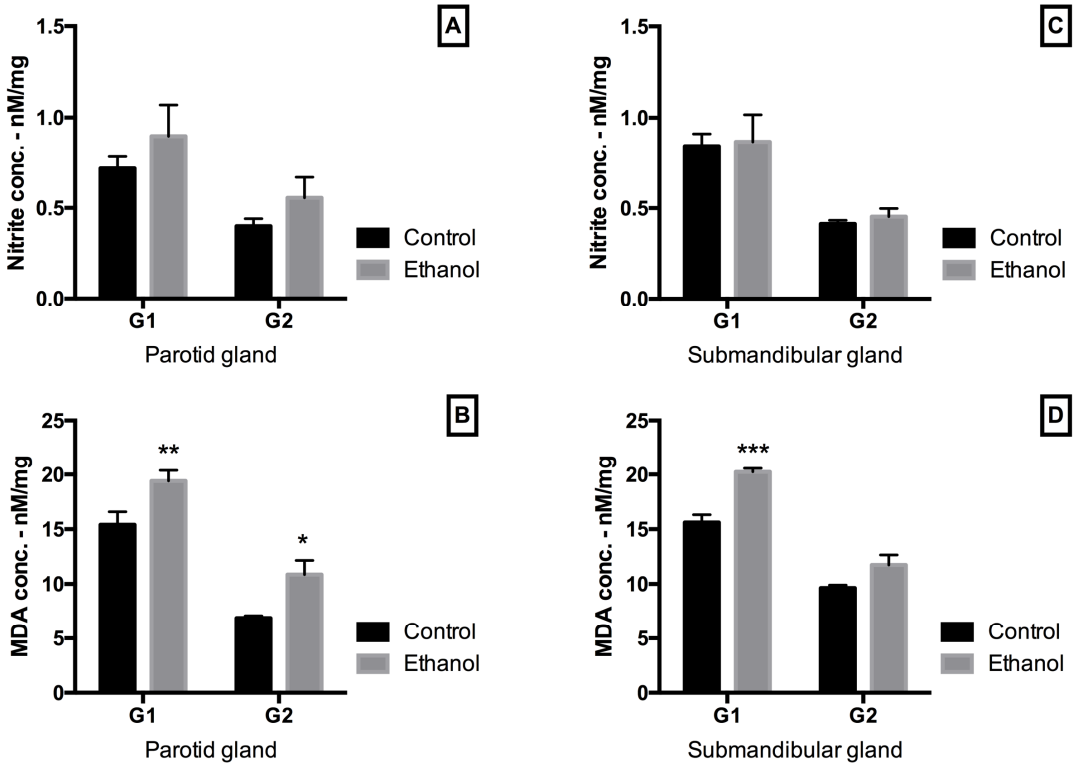

Figure 5: Effects of binge drinking (EtOH administration) during adolescence and young adulthood on the parotid and submandibular glands. The results are expressed as means ± SEM of the: Nitrite concentration (pM) per milligram of protein after 1-week (G1) and 4-week of ethanol exposure (G2) on parotid (A) and submandibular glands (C); Malondialdehyde (MDA) concentration (in pM) per milligrams of protein after 1-week (G1) and 4-week of ethanol exposure (G2) on parotid (B) and submandibular glands (D). *$p \leq 0.05$ compared to control group (Student's t test), **$p \leq 0.01$ compared to control group (Mann-Whitney test), ***$p \leq 0.001$ compared to control group (Mann-Whitney test).

4. Discussion

In this study, the effects of an episodic binge drinking model of consumption of ethanol in salivary glands were investigated in female rats considering two different periods, one and four weeks of exposure. Our results showed, for the first time, that a single episode of ethanol binge drinking can affect the parenchyma of the parotid gland, reduce mioepithelial cells and increase the levels of MDA in the parotid and submandibular glands. Furthermore, a 4-week exposure in the same conditions reveals a reduction in cytokeratin expression and MDA levels of the parotid gland and a reduction of the mioepithelial cells in the submandibular gland.

Our group have studied the effects of alcohol on the salivary glands of female rats from adolescence until adulthood. Our first findings demonstrated that a chronic heavy ethanol paradigm (6.5 g/kg/day) induces morphologic changes in both glands, however in a different way. In parotid glands an increase in total gland weight was observed as well as atrophy of glandular parenchyma. On the other hand, heavy ethanol intake promoted an increase in the submandibular gland stroma area. In addition, our work demonstrated through an immunohistochemistry assay that alcohol exposure increased duct-like cells related to caspase-3 overexpression in submandibular glands. These results highlight the difference between parotid glands and submandibular glands in the face of chemical noxious stimuli [13].

After that, we decided to investigate whether habitual and recreational consumption of alcohol among adolescents promotes the same range of damage in the salivary glands. Therefore, we employed the current protocol in doses that mimic a binge-drinking pattern (3 g/kg/day for three days a week) [22].

The evaluation of two different periods of exposures to EtOH shows the effects of an acute (1 week) and chronic (4 week) binge-drinking model in salivary glands [22]. In this study, a 1-week and 4 week binge result in similar responses at morphologic and biochemical evaluations, although a pattern breakage was observed in α-SMA expression in parotid gland and in MDA levels of submandibular glands. In both cases, only an acute exposure result in changes of these glands, suggesting an adaptation to damage in a period of chronic binging.

In this study, the ethanol group showed conformity of response with the chronic heavy drinking model in the expression of cytokeratin and α-SMA [13], like the MDA levels [14] reported in previous studies. Binge consumption of ethanol, especially in adolescence and early adulthood, has been associated with alcoholism with the hypothesis that binge drinking may indicate a qualitatively similar imbalance, but quantitatively lower when compared with a heavy chronic drinking model [23].

In contrast, neither period of ethanol exposure was able to cause changes in the EtOH group when compared to the control group or modify parotid or submandibular glands in terms of size or parenchyma volume, although it has been shown that heavy chronic consumption of ethanol can increase the size and cause atrophy of the parenchyma area of the parotid gland [13,14]. This lack of alterations may be associated with the dosage of consumption of ethanol observed in a heavy model, but further studies are necessary to clarify this process.

In line with a heavy chronic model of exposure to EtOH, morphologic changes after binge drinking are more related to expression patterns of CK-18 and α-SMA. CK-18 is commonly detected in the cytoskeleton of serous acinar and ductal cells in salivary glands. This protein helps to maintain cellular integrity [24,25].

Parotid glands showed a reduction in CK-18 expression in 1- and 4-week episodic binges, while submandibular glands did not present any significant modification. A previous study exhibits a reduction of cytokeratin expression in parotid glands and an increase in submandibular glands after chronic ethanol exposure [13]. These data are related to what was observed in our investigation, but showed that one episodic binge can change parotid gland parenchyma, but is not capable of interfering in submandibular expression, suggesting the possibility of induction of a cytoprotection process in the parenchyma of these glands.

The changes observed in α-SMA, with a lower expression in parotid glands after one episodic ethanol binge or after 1 and 4 weeks of binge drinking in submandibular glands, are directly related to mioepithelial cells. These structures are associated with the propagation of neural stimuli, tumor

suppression, and contraction and transportation of metabolites [26]. A reduction of the mioepithelial cell population has been associated with ductal atrophy [27].

Intended to analyze stromal fibroblasts, Vimentin expression was also verified in this study and no difference among groups was detected in either the periods or glands evaluated. Vimentin symbolizes a mesenchymal marker of cell migration and invasion identifying activated fibroblasts or the myofibroblasts in salivary glands [28]. Such evidence shows conformity with morphometric findings and suggests that morphological changes after episodic binges are associated with changes in the parenchyma of the evaluated glands.

Alcohol consumption is also connected with a metabolic imbalance of free radical production, leading to oxidative stress. In this study, a higher level of MDA, a lipid peroxidation marker, was demonstrated in the parotid glands of the ethanol group after 1 and 4 weeks of ethanol intake in a binge model. Submandibular glands exhibited a higher level of lipid peroxidation in the ethanol group only after a 1-week binge treatment. No changes between groups were detected in nitric oxide analysis for both glands in 1-week or 4-week binge ethanol exposure. Previously, it was shown that increased levels of lipid peroxidation, detected by the production of toxic compounds such as MDA, were related with membrane damage and can be deleterious for membrane permeability [14,29]. These toxic compounds can also initiate the production of abnormal substances, such as DNA and RNA [30].

A difference in response between parotid and submandibular glands was also shown by Fernandes (2015). This study reported a difference in the caspase-2 expression pattern of response after heavy drinking consumption, in which only submandibular glands showed a greater expression of caspase-2, indicating greater levels of apoptosis in this tissue.

In the present research, differences between parotid and submandibular responses were related. As we know, these glands have metabolic and structural differences. Parotid glands present purely serous acini and submandibular glands show mucous acini carrying a terminal cap of serous cells [31,32]. It was reported that parotid glands present an essentially aerobic metabolism, while the metabolism of submandibular glands is primarily anaerobic [32]. This

contrast between glands may be connected to the different regulatory mechanisms of enzymatic release reported by Busch and colleagues [33].

5. Conclusions

For the first time, in this study, 1 week of episodic binge drinking in female rats has been connected with damage to the submandibular and parotid glands showing a reduction in mioepithelial cells, cytokeratin expression in parenchyma and higher levels of lipid peroxidation. On the other hand, different patterns of response were observed after 1-week and 4-week exposure to ethanol. Further studies are necessary to clarify the damage with longer periods of exposure to ethanol.

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ANEXO 1 – PARECER DO COMITÊ DE ÉTICA EM PESQUISA COM ANIMAIS DE EXPERIMENTAÇÃO

cepae comitê de ética em pesquisa com animais de experimentação

PARECER 196-14

Projeto: AVALIAÇÃO NEUROCOMPORTAMENTAL, IMUNOISTOQUÍMICA E OXIDATIVA DE RATAS INTOXICADAS COM ETANOL DA ADOLESCÊNCIA À FASE ADULTA, EM PADRÃO *BINGE*.
Coordenador: Professora. Dra. Cristiane do Socorro Ferraz Maia

Área Temática: Farmácia

Vigência: 04/2014 a 04/2016

Nº no CEPAE-UFPA: 196-14

O projeto acima identificado foi avaliado pelo Comitê de Ética Em Pesquisa Com Animais de Experimentação da Universidade Federal do Pará (CEPAE). O tema eleito para a investigação e de alto teor científico justificando a utilização do modelo animal proposto. Os procedimentos experimentais utilizados seguem as normas locais e internacionais para tratamento e manipulação de animais de experimentação. Portanto, o CEPAE, através de seu presidente, no uso das atribuições delegadas pela portaria Nº 3988/2011 do Reitor da Universidade Federal do Pará, resolve **APROVAR** a utilização de animais de experimentação (N=80, ratos Wistar) nas atividades do projeto em questão, no período de vigência estabelecido.

As atividades experimentais fora do período de vigência devem receber nova autorização deste comitê.

Belém, 02 março de 2014

Prof. Dr. Walace Gomes Leal
Presidente do CEPAE-UFPA

ANEXO 2- NORMAS PARA PUBLICAÇÃO

Oxidative Medicine and Cellular Longevity: Author Guidelines

Submission

Manuscripts should be submitted by one of the authors of the manuscript through the online Manuscript Tracking System. Regardless of the source of the word-processing tool, only electronic PDF (.pdf) or Word (.doc, .docx, .rtf) files can be submitted through the MTS. There is no page limit. Only online submissions are accepted to facilitate rapid publication and minimize administrative costs. Submissions by anyone other than one of the authors will not be accepted. The submitting author takes responsibility for the paper during submission and peer review. If for some technical reason submission through the MTS is not possible, the author can contact omcl@hindawi.com for support.

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Units of Measurement

Units of measurement should be presented simply and concisely using System International (SI) units.

Title and Authorship Information

The following information should be included

- Paper title
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Abstract

The manuscript should contain an abstract. The abstract should be self-contained and citation-free and should not exceed 200 words.

Introduction

This section should be succinct, with no subheadings.

Materials and Methods

This part should contain sufficient detail so that all procedures can be repeated. It can be divided into subsections if several methods are described.

Results and Discussion

This section may each be divided by subheadings or may be combined.

Conclusions

This should clearly explain the main conclusions of the work highlighting its importance and relevance.

Acknowledgments

All acknowledgments (if any) should be included at the very end of the paper before the references and may include supporting grants, presentations, and so forth.

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Authors are responsible for ensuring that the information in each reference is complete and accurate. All references must be numbered consecutively and citations of references in text should be identified using numbers in square brackets (e.g., “as discussed by Smith [9]”; “as discussed elsewhere [9, 10]”). All references should be cited within the text; otherwise, these references will be automatically removed.

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