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LORAZEPAM INDUZ APOPTOSE DE CÉLULAS ACINARES EM GLÂNDULAS PARÓTIDAS DE RATOS

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Tese apresentada ao Programa de Pós-Graduação em Odontologia da Pontifícia Universidade Católica do Paraná, como parte dos requisitos para obtenção do título de Doutor em Odontologia, Área de Concentração em Biociências.

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Sumário

Artigo em português

Lorazepam induz apoptose de células acinares em glândulas parótidas de ratos

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Resumo

Introdução: Dentre as diversas situações médicas existentes, insônia, depressão e ansiedade estão cada vez mais presentes no cotidiano da população mundial, cujo tratamento inclui terapia psicológica e prescrição de benzodiazepínicos. Estudos anteriores demonstraram que esses fármacos são capazes de reduzir a proliferação e o número de células acinares, assim como o fluxo salivar estimulado em glândulas parótidas de ratos. Desta forma, hipotetizou-se um possível efeito apoptótico, pelo uso crônico de um benzodiazepínico, sobre essas células acinares. O objetivo deste estudo foi avaliar a ação apoptótica do benzodiazepínico Lorazepam, por meio da quantificação de núcleos acinares apoptóticos, número de núcleos de células acinares e a imunoexpressão antígeno nuclear de proliferação celular acinar de glândulas parótidas de ratos.

Métodos: Quarenta ratos Wistar machos foram divididos em quatro grupos. Grupos controle receberam soro fisiológico por trinta dias G_{S30} e sessenta dias G_{S60}. Dentre os grupos experimentais, L_{30} recebeu Lorazepam por trinta dias e $G_{L30+S30}$ recebeu Lorazepam por trinta dias e, a partir do trigésimo primeiro dia, soro fisiológico. Para contagem dos núcleos celulares apoptóticos foi aplicada a técnica de TUNEL. Os dados, de número de núcleos celulares acinares e imunoexpressão antígeno nuclear de proliferação celular acinar, foram extraídos de estudos prévios. Os testes estatísticos utilizados foram ANOVA e Tukey HSD.

Resultados: Aos trinta dias, não houve diferença estatisticamente significante entre os grupos G_{L30} e G_{S30}, em relação ao número de núcleos celulares apoptóticos e proliferação celular. Houve redução estatística significativa quanto ao número de núcleos de células acinares, no grupo G_{L30} em relação ao $G_{S30.}$ Nos grupos tratados por sessenta dias, a porcentagem de núcleos celulares apoptóticos do grupo $G_{L30+S30,}$ mostrou-se significantemente maior, em relação ao controle $G_{S60.}$ O oposto foi verificado para o número de núcleos de células acinares. Não houve diferença estatística significante para proliferação celular entre os grupos $G_{L30+S30}$ e G_{S60}.

Conclusão: O uso do Lorazepam ocasionou diminuição do número de núcleos de células acinares e maior porcentagem de núcleos celulares apoptóticos em glândulas parótidas de ratos.

Palavras-chave: apoptose, receptores de $GABA_A$, células acinares, glândulas salivares, Lorazepam.

Abstract

Introduction: Insomnia, depression and anxiety are medical situations increasingly present in the daily lives of the world population, whose treatment includes psychological therapy and the prescription of benzodiazepines. Previous studies have shown that benzodiazepines are capable of reducing the proliferation and number of acinar cells, as well as the salivary flow stimulated in parotid glands of rats. Thus, a possible apoptotic effect was hypothesized with the chronic use of a benzodiazepinic - Lorazepam - on acinar cells of the parotid glands of rats. The objective of this study was to evaluate the apoptotic action of Lorazepam on acinar cells of the parotid glands of rats by means of the quantification of apoptotic acinar nuclei, number of acinar cells and nuclear antiantigen antibody of cell proliferation.

Methods: Forty male Wistar rats were divided into four groups. Control groups received saline for thirty days and sixty days G_{S30} $G_{S60.}$ Among the experimental groups, L_{30} received Lorazepam for thirty days and $G_{L30 + S30}$ received Lorazepam for thirty days, and from the thirty-first day, saline. For the counting of apoptotic cell nuclei, the TUNEL technique was applied. The statistical tests used were ANOVA and Tukey HSD.

Results: On the thirtieth day, there was no statistically significant difference between the G_{L30} and G_{S30} groups, in relation to the number of apoptotic cell nuclei and cell proliferation. There was a statistically significant difference in the number of acinar cell nuclei in the G_{L30} group compared to $G_{S30.}$ In the groups treated for sixty days, the percentage of apoptotic cell nuclei from the $G_{L30+S30}$ group was significantly higher in relation to the G_{S60} control. A statistically significant reduction in the value of acinar cell nuclei was also observed between these groups. There was no statistically significant difference between the values of cell proliferation in the $G_{L30+S30}$ and G_{S60} groups.

Conclusion: The use of Lorazepam caused significant decrease in the number of acinar cell nuclei and larger percentage of cellular apoptotic nuclei in rat parotid glands.

Keywords: apoptosis, $GABA_A$ receptors, acinar cells, salivary glands, Lorazepam.

Introdução

Insônia, depressão e ansiedade são situações médicas cada vez mais presentes no cotidiano da população mundial. A depressão, classificada como doença de caráter afetivo, manifesta-se, principalmente, por meio de queixas somáticas, baixa autoestima, sensação de inutilidade, tendência autodepreciativa e alteração de apetite (Kessler et al., 2005).

Estima-se, que aproximadamente 18,4% da população brasileira, já tenha manifestado pelo menos um quadro de depressão durante a vida. Este dado caracteriza o Brasil como o país em desenvolvimento com maior número de pessoas sofrendo dessa doença, enquanto as médias para os países desenvolvidos estão em torno de 14,6% e de 11,1% para os países em desenvolvimento (WHO, 2012).

A depressão é comumente acompanhada por distúrbios de ansiedade como fobias, transtorno do pânico, transtorno de ansiedade generalizada (TAG), transtorno obsessivo compulsivo (TOC) e estresse pós-traumático agudo. Estas também são situações

psiquiátricas crônicas, graves e recorrentes, com prevalência estimada em torno de 29% (Kessler et al., 2005) que resultam em prejuízo funcional, associadas a significativos custos sociais, uma vez que o tratamento das mesmas inclui terapia psicológica e prescrição de medicamentos depressores do Sistema Nervoso Central (SNC), denominados psicotrópicos. A indicação destes fármacos, dentre eles os benzodiazepínicos (BZDs), é amplamente realizada desde os anos de 1960 (Llorente et al., 2000), até a atualidade (WHO, 2012).

A prevalência de uso dos BZDs é de 15% em algumas regiões do continente europeu, Estados Unidos, Canadá e demais países de alta renda (Manthey et al., 2011). BZD é amplamente consumido por 9 a 25% da população idosa a partir dos 65 anos de idade, principalmente entre o gênero feminino (15 a 21,7% no Brasil; 9,9% nos Estados Unidos; 24% no Canadá e 25% na Suécia) (Thomson e Smith, 1995; Gleason et al., 1998; Jorm et al., 2000; Lima et al., 1999; Blazer et al., 2000; Fourrier et al., 2001; Tu et al., 2001; Alvarenga et al., 2008; Johnell e Fastbom, 2009; Leggett et al., 2015).

Os BZDs são divididos em ansiolíticos, responsáveis pela diminuição dos estágios de ansiedade e depressão, e hipnóticos usados no tratamento de insônia, indução de hipnose e sedação. Promovem, também, relaxamento muscular e podem ser utilizados como anticonvulsivantes (De Almeida et al., 2008). Na Odontologia, esses fármacos são indicados para diminuir a ansiedade de pacientes não colaboradores, em relação ao tratamento dentário (Zaclikevis et al., 2009). Entre os BZDs licenciados, o Lorazepam, é um dos fármacos mais indicados tanto para o tratamento de insônia como para casos de ansiedade (Amato et al., 2010). Entretanto, o mesmo apresenta diversos efeitos colaterais como diminuição da atividade psicomotora, perda de memória, dependência física em tratamentos em longo prazo, aumento do risco de quedas em idosos (Longo e Johnson, 2000; Uzun et al., 2010), hipossalivação e xerostomia (Guggenheimer e Moore, 2003).

A ação do Lorazepam sobre glândulas parótidas de ratos também foi analisada por Rinaldi et al., (2015) e Mattioli et al., (2016). Os autores observaram que o uso crônico desse BZD contribuiu significantemente para a diminuição do número e da proliferação de células acinares, redução do fluxo salivar e aumento do volume celular. Diante dessa redução do número de células acinares verificadas nos estudos supracitados, oriundas da interação farmacológica do Lorazepam com glândulas salivares, hipotetizou-se um possível efeito apoptótico, pelo uso crônico desse fármaco, sobre essas células de glândulas parótidas de ratos.

A apoptose desempenha papel fundamental na eliminação de células indesejadas, danificadas ou infectadas, em organismos multicelulares e também em diversos processos biológicos, incluindo desenvolvimento, diferenciação e proliferação celulares (So et al., 2016). Para detecção dessas células, a técnica de Terminal Deoxynucleotidil Tranferase-mediated Deoxyuridine Triphosphate Biotin Nick End-labeling (TUNEL) é uma das mais utilizadas (Tilly, 1996), sendo padrão ouro (Burattini et al., 2009).

Na falta de estudos prévios, que comprovem a apoptose induzida pelo Lorazepam em células acinares, o objetivo deste estudo foi avaliar a ação apoptótica desse BZD, sobre células acinares de glândulas parótidas de ratos, por meio da quantificação de núcleos celulares apoptóticos (NCA), número de células acinares (N) e imunoexpressão antígeno nuclear de proliferação celular (PCNA).

Material e Método

Para realização deste estudo, o projeto foi avaliado e aprovado pelo Comitê de Ética no Uso de Animais da Pontifícia Universidade Católica do Paraná (PUCPR) (CEUA/PUCPR nº 01084/2016 – 2ª versão). Para tanto, foram utilizados 40 ratos machos do tipo *Rattus norvegicus albinus Rodentia mammalia* da linhagem *Wistar* (oriundos do Biotério Central /PUCPR), com massa corporal de *ca.* 250g. Os animais foram mantidos em gaiolas plásticas e alimentados com ração peletizada Nuvilab® CR-1 (Nuvital Nutrientes S.A.) e água *ad libitum*, com ciclos claro-escuro de 12 h e temperatura ambiente (25±1 °C).

Tratamento experimental

O tratamento dos animais foi realizado e descrito por Mattioli et al., (2016), sendo os mesmos alocados aleatoriamente em quatro grupos de dez espécimes, conforme o quadro a seguir. (Quadro 1).

Quadro 1 – Divisão dos Grupos Controle e Experimental de acordo com fármaco utilizado, dose diária, via de administração e período de tratamento

Grupos	Fármaco	Dose Diária	Via de administração	Período de tratamento
G_{S30} (Controle)	Solução injetável de soro fisiológico 0,9%	0,1 mL	Intraperitoneal (1 vez ao dia/mesmo horário)	30 dias
G_{S60} (Controle)	Solução injetável de soro fisiológico 0,9%	0,1 mL	Intraperitoneal (1 vez ao dia/mesmo horário)	60 dias
G_{L30} (Experimental)	Solução injetável de Lorazepam 1%*	0,5 mg/Kg	Intramuscular (1 vez ao dia/mesmo horário)	30 dias
$G_{L30 + S30}$ (Experimental)	Solução injetável de Lorazepam 1%*	0,5 mg/Kg	Intramuscular (1 vez ao dia/mesmo horário)	30 dias
	Solução injetável de soro fisiológico 0,9%	0,1mL	Intraperitoneal (1 vez ao dia/mesmo horário)	31 – 60 dias

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Morte dos animais e excisões das glândulas parótidas

Os animais dos grupos controle e experimental foram mortos por overdose de tiopental sódico 120 mg/kg (Thionembutal®, Abbott Laboratórios do Brasil Ltda., São Paulo, Brasil), via intraperitoneal e intracardíaca, após os períodos de trinta e sessenta dias.

As glândulas parótidas direita e esquerda foram removidas com uso de lâmina de bisturi n° 12 e acondicionadas em coletores universais, previamente identificados, com formalina tamponada 10% por 48 horas.

Processamento e técnica de TUNEL

As glândulas parótidas fixadas em formalina foram emblocadas em parafina (bloco doador). A partir deste bloco doador, foi removido um cilindro de 3 mm de diâmetro com broca trefina (Neodent®, Curitiba, Brasil) acoplada em um motor de suspensão de 130 watts de Bethil (Prometal Ind. Met. LTDA®, Marília, Brasil). Dez desses cilindros foram organizados em linhas e colunas, e emblocados em parafina (bloco receptor), constituindo o *tissues microarray* (TMA) (Sawaki et al., 1995; Kosuge et al., 2009). Na sequência foram realizados cortes microscópicos com 4 µm de espessura e, em seguida, as lâminas obtidas foram submetidas à técnica de TUNEL.

A técnica de TUNEL foi realizada para detecção de apoptose, em célula acinar, com base na marcação dos fragmentos de DNA, resultantes da quebra do DNA genômico. *In situ* Cell Death Detection Kit, POD - Roche® (Roche Diagnostics GmbH, 11684817910, Mannheim, Alemanha) foi usado de acordo com as instruções do fabricante.

Depois da desparafinização das secções, a atividade da peroxidase endógena foi bloqueada em solução de peróxido de hidrogênio e metanol 5%, durante 15 minutos em temperatura ambiente. As secções foram, então, tratadas com 0,1 tampão citrato, pH 6.0 em cuba de banho-maria, durante 25 minutos a 99º C para recuperação antigenômica.

Após lavagem em TBS-tris (pH 7,0), as lâminas foram cobertas com 50 µL da mistura reacional da enzima solution com a label solution e, em seguida, incubadas durante 1 hora a 37° C numa atmosfera umidificada, no escuro. Depois de serem lavadas em PBS, as lâminas foram cobertas com 50 µl de converter-POD, e incubadas durante 30 minutos a 37º C em câmara úmida.

Em sequência à lavagem em PBS, as lâminas foram incubadas em solução de diaminobenzidina (DAB, K3468 DAKO DAB + substrato cromogênio sistema líquido, Carpinteria, CA, EUA) durante 15 segundos à temperatura ambiente. As mesmas foram contrastadas com hematoxilina de Harris. Em controles negativos, a solução de enzima foi omitida.

Dez imagens de cada cilindro (totalizando 400 imagens) foram capturadas, por uma microcâmera Dinolite® (AM 423× AmMo Eletronics Corporation, New Taipei City, Taiwan) acoplada em um microscópio Olympus® BX50 (Olympus Corporation, Ishikawa, Japão), em magnificação de 400×, conectado a um notebook Dell Inspiron 15459 (Round Rock, Texas, EUA). As imagens foram analisadas, por um único observador, no programa de morfometria Image Proplus™ 4.5 (Media Cybernetics, Silver Spring, MD), no qual foi

aplicada uma grade virtual às imagens, para contagem dos núcleos positivos para apoptose, corados de marrom escuro e os núcleos não apoptóticos, de coloração roxa.

O examinador realizou a contagem de uma imagem, de cada cilindro, e repetiu a contagem da mesma imagem depois de vinte e um dias, para verificar a reprodutibilidade e ausência de erro sistemático. Toda a amostra foi contada somente após esta verificação. Observou-se que o erro de Dahlberg foi de 5,10%, indicando que o examinador reproduziu de forma confiável a contagem das células em apoptose. O teste t de Student revelou que não houve erro sistemático na contagem (p=0,16) (Midtgard et al., 1974; Houston, 1983).

A partir do número total de núcleos (somatória de núcleos apoptóticos + não apoptóticos), foram calculadas as porcentagens de núcleos celulares apoptóticos (NCA). Os valores finais foram determinados a partir das médias das porcentagens dos dez campos.
Também foram extraídos os dados de N e PCNA, dos trabalhos de Rinaldi et al., 2015 e Mattioli et al., 2016, respectivamente.

Análise estatística

Foi empregado o pacote estatístico SPSS® 23.0 (SPSS Inc., Chicago, IL), com nível de significância de 5% ($p<0,05$).

Inicialmente, testou-se a normalidade da variável porcentagem segundo grupo, utilizando-se o teste de Shapiro-Wilk. Uma vez que os dados apresentaram distribuição normal, a comparação entre os grupos foi feita com ANOVA, seguido do teste de Tukey HSD, já que o teste de homogeneidade de variâncias de Levene acusou variâncias homogêneas.

Resultados

Aos trinta dias, não houve diferença estatisticamente significante entre os grupos G_{L30} e G_{S30}, em relação ao NCA e PCNA (**Tabela 1**), (**Figura 1 A e B**). Houve diferença estatística significativa quanto ao N, no grupo G_{L30} em relação ao G_{S30} (**Tabela 1**).

Tabela 1 – Valores Médios e Desvio Padrão (DP) das variáveis estudadas segundo os grupos tratados por 30 dias com soro fisiológico (G_{S30}) e Lorazepam (G_{L30})

Grupos Váriáveis	G_{S30} Média ±DP	G_{L30} Média ±DP
NCA (%)	37,94 ± 5,94[a]	33,14 ± 8,02[a]
PCNA	55,80 ± 14,38[a]	43,90 ± 22,46[a]
N (x 10^6)	44,73± 7,29[a]	27,26 ±7,48[b]

Teste Anova p<0,0000; Observed Power: 1,0000
Letras diferentes significam diferenças estatisticamente significantes
NCA- Núcleos celulares apoptóticos
PCNA- Imunoexpressão antígeno nuclear de proliferação celular
N- Número de núcleos de células acinares

Nos grupos tratados por sessenta dias, a porcentagem de NCA do grupo $G_{L30+S30,}$ mostrou-se significantemente maior em relação ao controle G_{S60} (**Tabela2**), (**Figura 1 C e D)**. O oposto foi observado para o valor de N (**Tabela2**). Não houve diferença estatística significante em relação ao PCNA entre os grupos $G_{L30+S30}$ e G_{S60} (**Tabela2**).

Tabela 2 - Valores Médios e Desvio Padrão (DP) das variáveis estudadas segundo os grupos tratados por 60 dias com soro fisiológico (G_{S60}) e com Lorazepam e soro fisiológico ($G_{L30+S30}$)

Grupos Variáveis	G_{S60} Média ±DP	$G_{L30+S30}$ Média ±DP
NCA (%)	31,85 ± 9,18[a]	54,05 ± 8,00[b]
PCNA	50,00 ± 17,53[a]	41,50 ± 18,06[a]
N (x 10^6)	40,55 ± 4,64[a]	30,62 ± 8,13[b]

Teste Anova p<0,0000; Observed Power: 1,0000
Letras diferentes significam diferenças estatisticamente significantes
NCA- Núcleos celulares apoptóticos
PCNA- Imunoexpressão antígeno nuclear de proliferação celular (PCNA)
N- Número de núcleos de células acinares

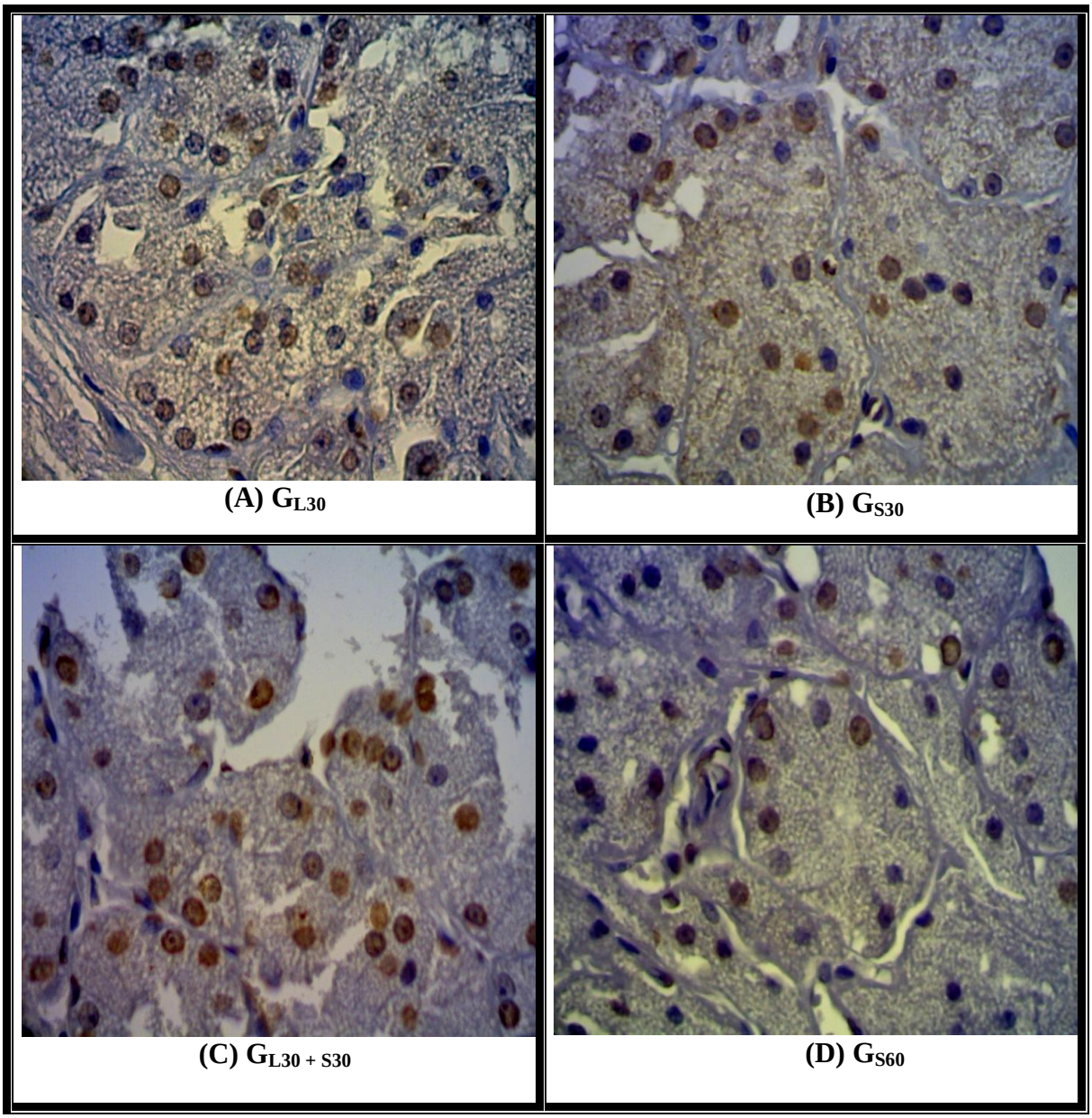

Figura 1 – Fotomicrografia de glândula parótida de rato submetida a **(A):** trinta dias com Lorazepam $G_{L30;}$ **(B):** trinta dias com soro fisiológico $G_{S30;}$ **(C):** trinta dias com Lorazepam e a partir do trigésimo primeiro dia com soro fisiológico ($G_{l30 +} G_{S30}$); **(D):** sessenta dias com soro fisiológico $G_{S60.}$ Os núcleos apoptóticos foram corados de marrom em magnificação de 400×.

Discussão

Este é o primeiro estudo que avalia apoptose em glândulas salivares de ratos tratados com Lorazepam, onde foi verificado que o uso deste BZD ocasionou em comparação ao controle: a) Aos trinta dias, diferença estatística significativa quanto ao número de núcleos de células acinares (N), no grupo G_{L30} em relação ao G_{S30}, assim como nenhuma diferença quanto ao número de núcleos celulares apoptóticos (NCA) e proliferação celular (PCNA); b) Aos sessenta dias, a porcentagem de células apoptóticas do grupo $G_{L30+S30,}$ mostrou-se significantemente maior, em relação ao controle $G_{S60.}$ O oposto foi observado para a variável N.

Os resultados para N, aos trinta e sessenta dias, e para NCA aos sessenta dias, observados nesta pesquisa, estão dentro de um mesmo raciocínio, demonstrando o efeito deletério do Lorazepam sobre glândulas parótidas de ratos. Neste sentido, conseguiu-se levantar a possível hipótese de que esse fármaco além de sialorredutor é ainda indutor de apoptose, fato que corrobora com todos os achados até então observados por esse grupo de pesquisa (Zaclikevis et al., 2009; Rinaldi et al., 2015; Mattioli et al., 2016). E esse efeito apoptótico foi confirmado pelo presente estudo.

Os estudos de Rinaldi et al., (2015) e Mattioli et al., (2016), avaliando respectivamente N e PCNA, utilizando as mesmas amostras do presente estudo, revelaram que o menor número de células acinares verificado no grupo G_{L30}, pode ter sido resultante de um menor PCNA, que não se mostrou estatisticamente significante em relação ao G_{S30}, mas que pode ter levado ao menor número de células acinares devido ao equilibrio de proliferação/apoptose, dentro do próprio grupo. Neste período de tempo (trinta dias), o Lorazepam ainda não aumentou a apoptose de células acinares, comparado ao controle, provavelmente por um efeito farmacocinético, uma vez que ele é um fármaco bastante lipossolúvel, e que apresenta uma meia-vida plasmática duradoura (De Almeida et al., 2012).

O efeito tardio do Lorazepam, verificado trinta dias após o seu uso, para o grupo $G_{L30+S30}$**,** revelou menor número de células acinares, resultante de um maior índice de apoptose celular, em relação ao controle, o que confirma a hipótese considerada no estudo de Rinaldi et al., (2015). Deve-se lembrar de que, nos grupos tratados por sessenta dias, houve suspensão do BZD no trigésimo dia e administração de soro fisiológico, a partir do trigésimo primeiro dia. Lorazepam é um fármaco que não apresenta metabólitos ativos, sendo metabolizado por meio de conjugação direta com um radical glicosídeo, em um processo mais rápido que a oxidação, tendo meia vida de 12 a18 horas (De Almeida et al., 2012). Considerando que este BZD apresenta meia vida maior que outros, como o Midazolam, por exemplo, sua ação atuaria por mais tempo, fato que poderia explicar o efeito tardio desse fármaco.

O estudo realizado por Zaclikevis et al., (2009) sobre a ação dos BZDs, em glândulas parótidas de ratos, revelou redução do fluxo salivar e aumento do volume celular. Diante disso, postulou-se que esse fato poderia ser resultante da alteração do número de células acinares. Então, Rinaldi et al., (2015) confirmaram que o uso desse fármaco contribuiu para a diminuição do número de células acinares, hipotetizando uma possível ação apoptótica do Lorazepam, sobre essas células, além de alteração na proliferação celular. Esses dados corroboram com os achados da presente pesquisa, uma vez que o Lorazepam, quando administrado cronicamente, provocou efeito apoptótico sobre as células das glândulas parótidas de ratos. Acrescentando a esses resultados, Mattioli et al., (2016) ainda verificaram redução da proliferação de células acinares, pelo uso crônico desse fármaco.

Similarmente à presente pesquisa, Pavlovic et al., (2012) também avaliaram o poder apoptótico do BZD sobre timócitos de ratos, uma vez que os mesmos foram incubados por um período de vinte e quatro horas com esse fármaco. Após esse período, os autores verificaram que a exposição ao BZD, resultou em um aumento da morte celular por apoptose. Não há estudos prévios que avaliam a ação apoptótica do Lorazepam sobre células acinares. Posteriores estudos devem ser realizados nesse sentido.

Fafalios et al., (2009) demonstraram que o Lorazepam apresenta ações antiproliferativa e pró apoptótica, uma vez que inibiu o crescimento e a viabilidade de células cancerosas de próstata *in vitro* por dezoito horas e *in vivo*, quando apresentou efeito antitumoral, em ratos que foram enxertados com células cancerígenas de próstata, para desenvolvimento do tumor, e o mesmo regrediu em tamanho e volume, quando tratado com o BZD. Apesar de se ter conhecimento de todas as alterações genéticas e moleculares que acontecem nas células cancerígenas, e na falta de outros estudos para comparar a apoptose induzida pelo Lorazepam, esse estudo vem de encontro à presente pesquisa, uma vez que a mesma demonstrou ter, o Lorazepam, pró apoptótico aos sessenta dias sobre células acinares de glândulas parótidas de ratos, assim como ocorreu nas células acinares cancerígenas da próstata.

Com ação inibitória sobre SNC, agindo principalmente no hipotálamo e tronco cerebral, os BZDs apresentam ação sobre um receptor específico, o chamado receptor dos benzodiazepínicos, do ácido gama-aminobutírico ($GABA_R$), na sua subunidade A ($GABA_A$). O ácido gama-aminobutírico (GABA) é um neurotransmissor inibitório do SNC, amplamente distribuído em todo o cérebro, que age abrindo os canais de cloro, gerando hiperpolarização dos neurônios e inibição da geração do potencial de ação. Os BZDs se ligam ao $GABA_A$, facilitando a ação do neurotransmissor GABA (Howard et al., 2014). Além de presente no SNC, $GABA_A$ também é encontrado nas glândulas salivares (Chen et al., 2012), em menor quantidade que no SNC (Sawaki et al., 1995). Isto justifica a alteração e diminuição da concentração do fluxo salivar, em usuários de BZD, pela inibição desse receptor (Kazunori et al., 2011), levando-os, como já descrito anteriormente, a quadros de hipossalivação e/ou xerostomia (Guggenheimer e Moore, 2003; Lambrecht et al., 2013; Raghavan et al., 2014).

Foi demonstrado, em células cancerígenas, que um ligante do receptor periférico de BZD causa um desacoplamento mitocondrial e perda do citocromo C, via indução da transição da permeabilidade mitocondrial, fato que apresenta importante papel no processo de apoptose celular (Li, Wang, Zang, 2007). Entretanto, nenhum estudo prévio avaliou se a apoptose induzida pelo Lorazepam, poderia se dar via este receptor. Estudos adicionais devem ser conduzidos nesse sentido, a fim de certificar o papel do GABA na apoptose, e concluir a referida hipótese.

Dentro dos limites do presente estudo, pode-se concluir que o uso do Lorazepam ocasionou diminuição significativa do número de núcleos de células acinares e maior porcentagem de núcleos celulares apoptóticos em glândulas parótidas de ratos.

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Artigo em inglês

Lorazepam induces acinar cells apoptsis of rat parotid glands

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Abstract

Introduction: Insomnia, depression and anxiety are medical situations increasingly present in the daily lives of the world population, whose treatment includes psychological therapy and the prescription of benzodiazepines. Previous studies have shown that benzodiazepines are capable of reducing the proliferation and number of acinar cells, as well as the salivary flow stimulated in parotid glands of rats. Thus, a possible apoptotic effect was hypothesized with the chronic use of a benzodiazepinic - Lorazepam - on acinar cells of the parotid glands of rats. The objective of this study was to evaluate the apoptotic action of Lorazepam on acinar cells of the parotid glands of rats by means of the quantification of apoptotic acinar nuclei, number of acinar cells and nuclear antiantigen antibody of cell proliferation.

Methods: Forty male Wistar rats were divided into four groups. Control groups received saline for thirty days and sixty days G_{S30} $G_{S60.}$ Among the experimental groups, L_{30} received Lorazepam for thirty days and $G_{L30+S30}$ received Lorazepam for thirty days, and from the thirty-first day, saline. For the counting of apoptotic cell nuclei, the TUNEL technique was applied. The data of acinar cell nuclei and cell proliferation were extracted from previous studies. The statistical tests used were ANOVA and Tukey HSD.

Results: On the thirtieth day, there was no statistically significant difference between the G_{L30} and G_{S30} groups, in relation to the number of apoptotic cell nuclei and cell proliferation. There was a statistically significant difference in the number of apoptotic cell nuclei in the G_{L30} group compared to $G_{S30.}$ In the groups treated for sixty days, the percentage of apoptotic cell nuclei from the $G_{L30+S30}$ group was significantly higher in relation to the G_{S60} control. A statistically significant reduction in the value of acinar cell nuclei was also observed between these groups. There was no statistically significant difference between the values of cell proliferation in the $G_{L30+S30}$ and G_{S60} groups.

Conclusion: The use of Lorazepam caused significant decrease in the number of acinar cell nuclei and larger percentage of cellular apoptotic nuclei in rat parotid glands.

Keywords: apoptosis, GABA $_A$ receptors, acinar cells, salivary glands, Lorazepam.

Introduction

Insomnia, depression and anxiety are medical situations increasingly present in the daily lives of the world population. Depression, classified as an affective disorder, is mainly manifested by somatic complaints, low self-esteem, feeling of worthlessness, self-deprecating tendency and altered appetite (Kessler et al., 2005).

It is estimated that approximately 18.4% of the Brazilian population has already shown at least one picture of depression during their lifetime. This data characterizes Brazil as the developing country with the largest number of people suffering from this disease, while the averages for developed countries are around 14.6% and 11.1% for developing countries (WHO, 2012).

Depression is commonly accompanied by anxiety disorders such as phobias, panic disorder, generalized anxiety disorder (GAD), obsessive compulsive disorder (OCD), and acute posttraumatic stress. These are also chronic psychiatric conditions, severe and recurrent, with an estimated prevalence of around 29% (Kessler et al., 2005) that result in functional impairment, associated with significant social costs, since the treatment thereof include psychological therapy and Central nervous system (CNS) depressant drugs, called psychotropic drugs. These drugs, such as benzodiazepines (BZDs), have been widely reported since the 1960s (Llorente et al., 2000), to date (WHO, 2012).

The prevalence of BZD use is 15% in some regions of the European continent, the United States, Canada and other high-income countries (Manthey et al., 2011). BZD is widely consumed by 9 to 25% of the elderly population aged 65 and over, mainly among women (15 to 21.7% in Brazil, 9.9% in the United States, 24% in Canada and 25% In Sweden) (Thomson and Smith, 1995; Gleason et al., 1998; Jorm et al., 2000; Lima et al., 2001; Alvarenga et al., 2008; Johnell and Fastbom, 2009; Leggett et al., 2015).

BZDs are divided into anxiolytics, which are responsible for decreasing the stages of anxiety and depression, and hypnotics used in the treatment of insomnia, induction of hypnosis and sedation. They also promote muscle relaxation and can be used as anticonvulsants (De Almeida et al., 2008). As with all central-acting drugs, this drug has side effects, decreased psychomotor activity, memory loss, physical dependence on long-term treatments, increased risk of falls in the elderly (Longo and Johnson, 2000; Uzun et al. (Ghaz et al., 2010) and hyposalivation and / or xerostomia, one of the main adverse effects observed in the literature (Guggenheimer and Moore, 2003; Lambrecht et al., 2013 and Raghavan et al., 2014) and as one of the highest rates of complaints by patients in dental offices (Smith and Burtner, 1994). In dentistry, these drugs are indicated to reduce the anxiety of non-collaborating patients, in relation to dental treatment (Zaclikevis et al., 2009). Among licensed BZDs, Lorazepam is one of the most indicated drugs for both insomnia treatment and anxiety (Amato et al., 2010).

The action of Lorazepam on parotid glands of rats was also analyzed by Rinaldi et al., (2015) and Mattioli et al., (2016). The authors observed that the chronic use of this BZD contributed significantly to the decrease in number and proliferation of acinar cells, reduction of salivary flow and increase in cell volume. In view of this reduction in the number of acinar cells observed in the aforementioned studies, caused by the

pharmacological interaction of Lorazepam with salivary glands, a possible apoptotic effect was hypothesized by the chronic use of this drug on these cells of the parotid glands of rats.

Apoptosis plays a fundamental role in the elimination of unwanted, damaged or infected cells in multicellular organisms and also in several biological processes, including cellular development, differentiation and proliferation (Su et al., 2016). For the detection of these cells, the Terminal Deoxynucleotidyl Tranferase-mediated Deoxyuridine Triphosphate Biotin Nick End-labeling (TUNEL) technique is one of the most used (Tilly, 1996), being gold standard (Burattini et al., 2009).

In the absence of previous studies demonstrating Lorazepam-induced apoptosis in acinar cells, the aim of this study was to evaluate the apoptotic action of BZD on acinar cells of the parotid glands of rats by quantification of apoptotic cell nuclei (NCA), number of acinar cells (N) and nuclear proliferation cell proliferation (PCNA) immunoexpression.

Material and Method

To carry out this study, the project was evaluated and approved by the Committee of Ethics in the Use of Animals of the Pontifical Catholic University of Paraná (PUCPR) (CEUA / PUCPR nº 01084/2016 - 2nd version). For this, 40 male *Rattus norvegicus albinus Rodentia mammalia rats* of the Wistar lineage (from the Central Vivarium of the Pontifical Catholic University of Paraná / PUCPR) were used, with body mass of ca. 250g. The animals were kept in plastic cages and fed with Nuvilab® CR-1 (Nuvital Nutrientes S.A.) pellets and water ad libitum, with light-dark cycles of 12 h and at temperature room (25 ± 1 ° C).

Experimental treatment

The treatment of the animals was performed and described by Mattioli et al., (2016), and they were randomly assigned to four groups of ten specimens, according to the table below (Table 1).

Table 1 - Division of control and experimental groups according to drug used, daily dose, administration and treatment period

Groups	Drug	Daily dose	Administration	Treatment period
G_{S30} (Control)	injectable 0.9% saline solution	0,1 mL	intraperitoneally, (once a day, at the same time)	30 days
G_{S60} (Control)	injectable 0.9% saline solution	0,1 mL	intraperitoneally, (once a day, at the same time)	60 days
G_{L30} (Experimental)	injectable solution of Lorazepam 1%*	0,5mg/Kg	Intramuscularly (once a day, at the same time)	30 days
$G_{L30 + S30}$ (Experimental)	injectable solution of Lorazepam 1%*	0,5 mg/Kg	Intramuscularly (once a day, at the same time)	30 days
	injectable 0.9% saline solution	0,1mL	intraperitoneally, (once a day, at the same time)	31 – 60 days

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Death of animals and excisions of the parotid glands

The animals of the control and experimental groups were killed by overdose of thiopental sodium 120 mg / kg (Thionembutal ®, Abbott Laboratórios do Brasil Ltda., São Paulo, Brazil), intraperitoneally and intracardiacly after the periods of thirty and sixty days.
The right and left parotid glands were removed using a No. 12 scalpel blade and placed in previously identified universal collectors with 10% buffered formalin for 48 hours.

TUNEL processing and technique

The formalin-fixed parotid glands were embedded in paraffin (donor block). From this donor block, a 3 mm diameter cylinder with trephine drill (Neodent®, Curitiba, Brazil) was coupled to a 130 watt suspension motor from Bethil (Prometal Ind. Met. LTDA®, Marília,Brazil). Ten of these cylinders were organized into rows and columns, and embedded in paraffin (receptor block), constituting the tissues microarray (TMA) (Sawaki et al., 1995; Kosuge et al., 2009). In the sequence, microscopic sections with 4 µm thickness were made and then the slides obtained were submitted to the TUNEL technique.

The TUNEL technique was used to detect apoptosis in acinar cell, based on the marking of the DNA fragments resulting from the breakage of genomic DNA *in situ* Cell Death Detection Kit, POD -. Roche® (Roche Diagnostics GmbH, 11684817910, Mannheim, Germany) was used according to the manufacturer's instructions.

After dewaxing the sections, endogenous peroxidase activity was blocked in 5% hydrogen peroxide solution and methanol for 15 minutes at room temperature. Sections were then treated with 0.1 citrate buffer, pH 6.0 in a water bath, for 25 minutes at 99 ° C for antigenic recovery.

After washing in TBS-tris (pH 7.0), the slides were covered with 50 μL of the solution enzyme reaction mixture with the label solution and then incubated for 1 hour at 37 ° C in a humidified atmosphere in the dark. After being washed in PBS, the slides were covered with 50 μl POD-converting, and incubated for 30 minutes at 37 ° C in a humid chamber.

Following washing in PBS, the slides were incubated in diaminobenzidine solution (DAB, K3468 DAKO DAB + chromogen substrate liquid system, Carpinteria, CA, USA) for 15 seconds at room temperature. They were contrasted with Harris haematoxylin. In negative controls, the enzyme solution was omitted.

Ten images for each cylinder (totaling 400 images) were captured by a micro Dino-Lite ® (AM 423 × AmMo Electronics Corporation, New Taipei City, Taiwan) coupled into a microscope Olympus ® BX50 (Olympus Corporation, Ishikawa, Japan) at a magnification of 400 ×, connected To a Dell Inspiron 15459 notebook (Round Rock, Texas, USA). The images were analyzed by a single observer in morphometry Image ProPlus ™ 4.5 software (Media Cybernetics, Silver Spring, MD), in which a virtual grid was applied to images for the counting of nuclei positive for apoptosis, of dark brown coloration and non-apoptotic, of purple coloration.

The examiner counted one image of each cylinder and repeated counting the same image after twenty-one days to check for reproducibility and absence of systematic error. The entire sample was counted only after this verification. It was observed that the Dahlberg error was 5.10%, indicating that the examiner reliably reproduced the cell count in apoptosis. Student's t-test showed that there was no systematic error in the count ($p = 0.16$) (Midtgard et al, 1974; Houston, 1983).

From the total number of nuclei (sum of apoptotic + non-apoptotic nuclei), the percentages of apoptotic cell nuclei (NCA) were calculated. Final values were determined from the means of the percentages of the ten fields.

The data of number of N and PCNA were also extracted from the works of Rinaldi et al., 2015 and Mattioli et al., 2016, respectively.

Statistical analysis

It used the SPSS ® 23.0 (SPSS Inc., Chicago, IL) with a significance level of 5% ($p <0.05$).

Initially, we tested the normality of the variable percentage second group, using the Shapiro-Wilk test. Since the data presented normal distribution, the comparison between the groups was done with ANOVA, followed by the Tukey HSD test, since Levene´s test for homogeneity of variances accused homogeneous variances.

Results

On the thirtieth day, there was no statistically significant difference between the G and $G_{L30\ S30}$ groups in relation to the NCA and PCNA **(Table 1) (Figure 1A and B).** There was no statistically significant difference in N in G_{L30} group than in the G_{S30} **(Table 1).**

Table 1 - Average values and standard deviation (SD) of the variables according to the groups treated for 30 days with saline (G_{S30}) and Lorazepam (G_{L30})

Groups Variables	G_{S30} Mean ± SD	G_{L30} Mean ± SD
NCA%	37.94 ± 5.94^a	33.14 ± 8.02^a
PCNA	55.80 ± 14.38^a	43.90 ± 22.46^a
N (x 10^6)	44.73 ± 7.29^a	27.26 ± 7.48^b

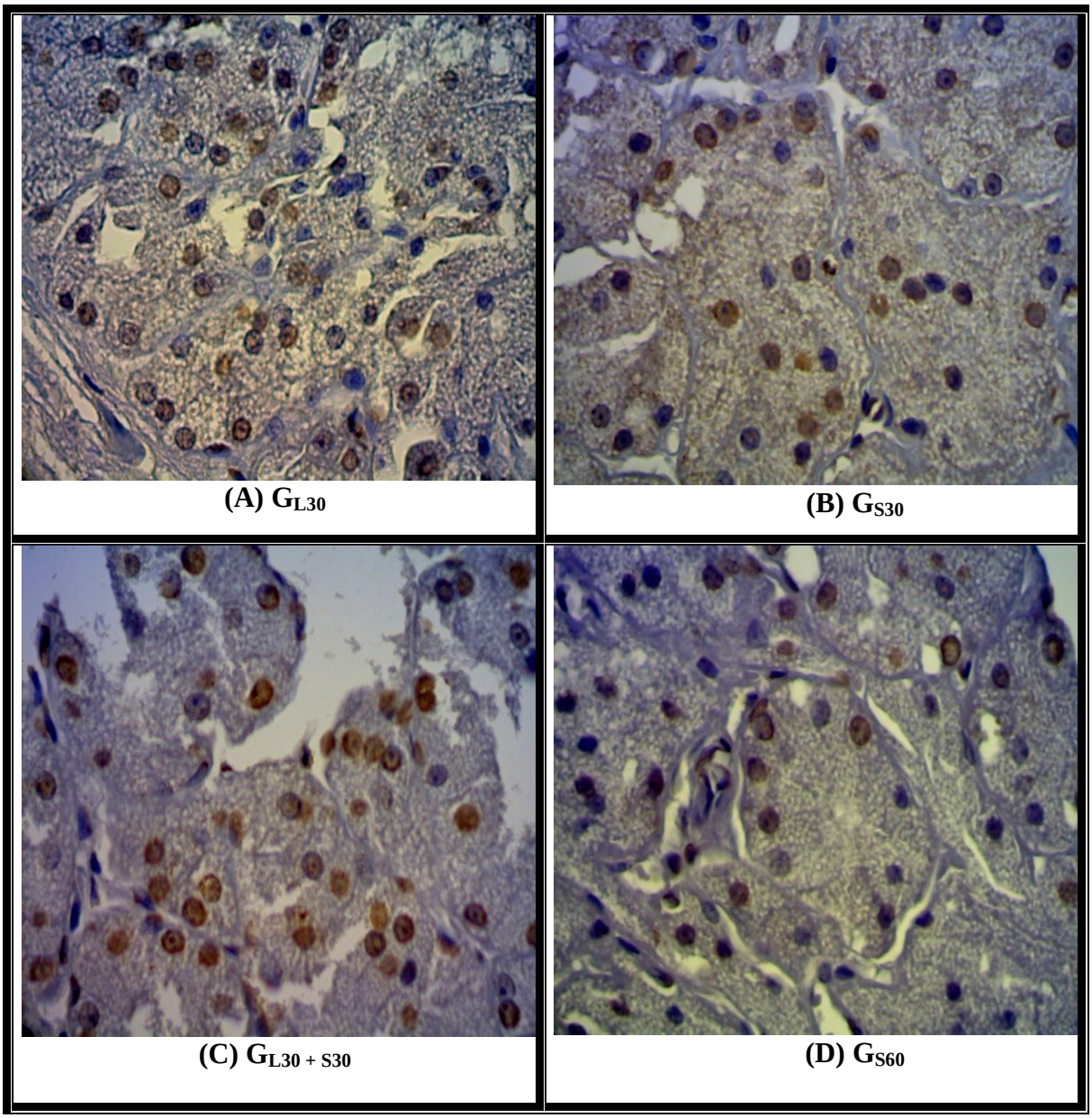
Anova test p <0.0000; Observed Power: 1,0000
Different letters mean statistically significant differences
NCA- Apoptotic cell nuclei
PCNA- Immunoexpression nuclear antigen of cell proliferation
N Number of acinar cell nuclei

In the groups treated for sixty days, the percentage of NCA in the $G_{L30+\ S30}$ group was significantly higher when compared to the control G_{S60} **(Table 2) (Figure 1 C and D).** The opposite was observed for the value of N **(Table 2).** Statistically similar values of PCNA were found between the $G_{L30\ +\ S30\ S60}$ groups and G **(Table** 2).

Table 2 - Average values and standard deviation (SD) of the variables according to **the groups treated for 60 days with saline (G_{S60}) and Lorazepam and saline ($G_{L30\ +\ S30}$)**

Groups Variables	G_{S60} Mean ± SD	$G_{L30\ +\ S30}$ Mean ± SD
NCA%	31.85 ± 9.18^a	54.05 ± 8.00^b
PCNA	50.00 ± 17.53^a	41.50 ± 18.06^a
N (x 10^6)	40.55 ± 4.64^a	30.62 ± 8.13^b

Anova test p <0.0000; Observed Power: 1,0000
Different letters mean statistically significant differences
NCA- Apoptotic cell nuclei
PCNA- Immunoexpression Nuclear Antigen of Cellular Proliferation (PCNA)
N-Number of acinar cell nuclei



(A) G_{L30} **(B) G_{S30}**

(C) $G_{L30 + S30}$ **(D) G_{S60}**

Figure 1- Parotid gland photomicrograph of rat subjected to **(A):** thirty days with Lorazepam $G_{L30;}$ **(B):** Thirty days with saline $G_{S30,}$ **(C):** Thirty days with Lorazepam and from the thirty-first day with saline $G_{l30+ S30,}$ **(D):.** Sixty days with saline G_{S60}. Apoptotic cells nuclei were stained brown at 400 × magnification.

Discussion

This is the first study evaluating apoptosis in the salivary glands of rats treated with Lorazepam, where it was verified that the use of this BZD caused in comparison to the control: a) at thirty days, a statistically significant difference in the number of acinar cell nuclei (N) in the G_{L30} group than the $G_{S30,}$ so as no statistically significant difference in the number of apoptotic cell nuclei (NCA) and cell proliferation (PCNA); b) sixty days, the percentage of apoptotic cells $G_{L30 + S30}$ group, proved to be significantly higher than in the control $G_{S60.}$ A statistically significant reduction in the amount of N was also observed between these groups.

The results for N, at thirty and sixty days, and for NCA at sixty days, observed in this research, are within the same rationale, demonstrating the deleterious effect of Lorazepam on parotid glands of rats. In this sense, it was viable to raise the possible hypothesis that these drugs besides sialorreductive are still inducer of apoptosis, a fact that corroborates all the findings previously observed by this research group (Zaclikevis et al., 2009; Rinaldi et al., 2015; Mattioli et al., 2016). Thus, the present study revealed that the use of Lorazepam caused when compared to the control: a) at thirty days, a statistically significant difference in the number of acinar cell nuclei (N), the G_{L30} group than in the $G_{S30,}$ so as no difference in the number of apoptotic cell nuclei (NCA) and cell proliferation (PCNA); b) sixty days, the percentage of apoptotic cells $G_{L30 + S30}$ group, proved to be significantly higher in the control $G_{S60.}$ A statistically significant reduction in the amount of N was also observed between these groups. Statistically similar values of PCNA were found between the experimental and control groups.

The studies of Rinaldi et al., (2015) and Mattioli et al., (2016) evaluating respectively N and PCNA, using the same samples from this study showed that the least number of acinar cells found in the G_{L30} group may have been the result of a lower PCNA, which was not statistically significant compared to the $G_{S30,}$ but that may have led to fewer acinar cells due to the balance of proliferation / apoptosis, within the group. At this time (thirty days), Lorazepam has not yet increased apoptosis of acinar cells, compared to the control group, probably because of a pharmacokinetic effect, since it is a very liposoluble drug and has a long-lasting plasma half-life (De Almeida et al ., 2012).

Late effect of Lorazepam, checked thirty days following its use for the $G_{L30 + S30}$ group showed fewer acinar cells, resulting in increased apoptosis rate compared to the treatment for thirty days $G_{L30,}$ which confirms the hypothesis considered in the study by Rinaldi et al., (2015). It should be remembered that in the groups treated for sixty days, BZD was suspended on the thirtieth day and saline was administered from the thirty-first day. Lorazepam is a drug that does not present active metabolites, being metabolized by means of direct conjugation with a glycoside radical, in a process faster than oxidation, having a half-life of 12 to 18 hours (De Almeida et al., 2012). Considering that this BZD has a half-life greater than others, such as Midazolam, for example, its action would act for a longer time, a fact that could explain the late effect of this drug.

The study by Zaclikevis et al., (2009) on the action of BZDs on parotid glands of rats revealed a reduction of salivary flow and Cell volume. In view of this, it was postulated that this fact could be due to the alteration of the number of acinar cells. Thus, Rinaldi et al., (2015) confirmed that the use of this drug contributed to a decrease in the number of acinar cells, hypothesizing a possible apoptotic action of Lorazepam on these cells, in addition to alteration in cell proliferation. These data corroborate the findings of the present study, since Lorazepam, when administered chronically, caused an apoptotic effect on the cells of the parotid glands of rats. Adding to these results, Mattioli et al., (2016) still verified a reduction of acinar cell proliferation, due to the chronic use of this drug.

Similarly to the present study, Pavlovic et al., (2012) also evaluated the apoptotic power of BZD on rat thymocytes, since they were incubated for a twenty-four hour period with a BZD. After this period, the authors found that exposure to the drug resulted in an increase in cell death by apoptosis. There are no previous studies evaluating the apoptotic action of Lorazepam on acinar cells. Further studies should be carried out in this regard.

Fafalios et al., (2009) demonstrated that Lorazepam has antiproliferative and pro - apoptotic actions since it inhibited the growth and viability of cancerous cells in the prostate *in vitro* for eighteen hours and *in vivo*, when introduced antitumor effect in mice that were grafted with prostate cancer cells for tumor development, and it regressed in size and volume when treated with BZD. In spite of being aware of all the genetic and molecular changes that occur in cancer cells, and in the absence of other studies to compare Lorazepam-induced apoptosis, this study is in line with the present research, since it has demonstrated that Lorazepam has pro-apoptotic effect at sixty days on acinar cells of parotid glands of rats, as occurred in acinar cancer cells of the prostate.

With inhibitory action on CNS acting mainly in the hypothalamus and brainstem, benzodiazepines exhibit action on a specific receptor, called the benzodiazepine receptor, gamma-aminobutyric acid ($GABA_R$) on subunit A ($GABA_A$). Gamma-aminobutyric acid (GABA) is a CNS inhibitory neurotransmitter, widely distributed throughout the brain, which acts by opening the chlorine channels, generating hyperpolarization of neurons and inhibiting the generation of action potential. The benzodiazepines bind to $GABA_A$ facilitating the action of GABA (Howard et al., 2014). In addition to this the CNS $GABA_A$ is also found in the salivary gland (Chen et al., 2012), a lesser amount in the CNS (Sawaki et al., 1995). This fact justifies the alteration and decrease of the salivary flow concentration in BZD users by the inhibition of this receptor (Kazunori et al., 2011), leading them, as previously described, to hyposalivation and / or xerostomia (Guggenheimer et al., Moore, 2003; Lambrecht et al., 2013; Raghavan et al., 2014).

It has been demonstrated in cancer cells that a BZD peripheral receptor ligand causes mitochondrial decoupling and cytochrome C loss through induction of the mitochondrial permeability transition, a fact that plays an important role in the cellular apoptosis process (Li, Wang, Zang, 2007). However, no previous study evaluated whether Lorazepam induced apoptosis could occur via this receptor. Additional studies should be conducted to confirm the role of GABA in apoptosis, and to conclude this hypothesis.

Within the limits of the present study, it can be concluded that the use of Lorazepam led to a significant decrease in the number of acinar cell nuclei and a higher percentage of apoptotic cell nuclei in rat parotid glands.

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Anexos

Anexo A – Paracer Comitê de Ética

Pontifícia Universidade Católica do Paraná
Pró-Reitoria de Pesquisa e Pós-Graduação
Comitê de Ética em Pesquisa no Uso de Animais

Curitiba, 13 de outubro de 2016.

PARECER DE PROTOCOLO DE PESQUISA

REGISTRO DO PROJETO: **01084/2016 – 2ª versão**

TÍTULO DO PROJETO: Análise histomorfométrica e Imunohistoquimica de glândulas parótidas de ratos submetidos ao tratamento crônico com Benzodiazepideos.

PESQUISADOR RESPONSÁVEL

Ana Maria Trindade Grégio

EQUIPE DE PESQUISA

Marina Rinaldi, Patrícia Vida Cassi Bettega

INSTITUIÇÃO

Pontifícia Universidade Católica do Paraná

ESCOLA / CURSO

Escola de Ciências da Vida / Odontologia

VIGÊNCIA DO PROJETO	Dados já coletados	**QUANTIDADE DE ANIMAIS**	Dados já coletados
ESPECIE/LINHAGEM	*Rattus norvegicus* (Ratos)	**Nº SISBIO** (Somente animais de vida livre)	Não se aplica
SEXO	Dados já coletados	**ATIVIDADES** (Somente animais de vida livre)	Não se aplica
IDADE / PESO	Dados já coletados	**ESPECIÉ – GRUPO TAXONÔMICOS** (de vida livre)	Não se aplica
ORIGEM DO ANIMAL	Pesquisas anteriores	**LOCAL (IS)** (Somente animais de vida livre)	Não se aplica

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Atenciosamente,

Profa. Dra. Marta Luciane Fischer
Coordenadora
Comissão de Ética no Uso de Animais

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PUCPR

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Anexo B – Produção Científica

Artigos Científicos

Piva, R. M., Johann, A. C., Costa, C. K., Miguel, O. G., Rosa, E. R., de Azevedo-Alanis, L. R., Trevilatto, P. C., Ignacio, S. A., Bettega, P. V.C., Gregio, A. M. (2013). Bixin action in the healing process of rats mouth wounds. *Curr Pharm Biotechnol*.**14**:9.785-91.

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Bettega, P.V.C., Johann, A. C. B. R., Alanis, L. R. A., Bazei, I. F., Miguel, O. G., Kocler, C. C., Lima, A. A. S., Machado, M. A. N., Machado, R. P., Rosa, E. A. R., Yusuf, S., Atiah, H. A., Abuhammad, S., Grégio, A. M. T. (2016). Experimental Confirmation of the Utility of Nasturtium officinale Used Empirically as Mouth Lesion Repairing Promotor. *Clin Exp Pharmacol* **6**: 1.

Capitulo de livro

Grégio, A. M. T., Veiga, F. F., Rinaldi, M., Bettega, P.V.C. (2015). ¨Antifungals for Candidosis Treatment¨, in Oral Candidosis Physiopathology,Decision Making, and Therapeutics, Ed.Springer (Verlag, Berlin Heidelberg), pgs29-37.

Editoração

Grégio, A. M. T., Bettega, P.V.C., Rinaldi, M., Johann A. C. B. R. (2016). Phytotherapy Approaches for Oral Ulcers Treatment. New York: Nova Science Publishers.

ANEXO C – NORMAS DA REVISTA FRONTIERS IN PHARMACOLOGY

Author Guidelines

1.Summary Table
Please view the table below for a summary on currently accepted article types and general manuscript style guidelines. Article types may vary depending on journal. Original Research 350 words

2. Manuscript Guidelines
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The title is written in title case, centered, and in 16 point bold Times New Roman font at the top of page.

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As a primary goal, the abstract should render the general significance and conceptual advance of the work clearly accessible to a broad readership. In the abstract, minimize the use of abbreviations and do not cite references. The text of the abstract section should be in 12 point normal Times New Roman. See Summary Table for abstract requirement and length according to article type.

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Funding

Details of all funding sources should be provided, including grant numbers if applicable. Please ensure to add all necessary funding information, as after publication this is no longer possible.

Acknowledgments

This is a short text to acknowledge the contributions of specific colleagues, institutions, or agencies that aided the efforts of the authors.

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Article or chapter in a book: Sorenson, P. W., and Caprio, J. C. (1998). "Chemoreception," in *The Physiology of Fishes*, ed. D. H. Evans (Boca Raton, FL: CRC Press), 375-405.

Book: Cowan, W. M., Jessell, T. M., and Zipursky, S. L. (1997). *Molecular and Cellular Approaches to Neural Development.* New York: Oxford University Press.

Abstract: Hendricks, J., Applebaum, R., and Kunkel, S. (2010). A world apart? Bridging the gap between theory and applied social gerontology. *Gerontologist* 50, 284-293. Abstract retrieved from Abstracts in Social Gerontology database. (Accession No. 50360869)

Patent: Marshall, S. P. (2000). *Method and apparatus for eye tracking and monitoring pupil dilation to evaluate cognitive activity.* U.S. Patent No 6,090,051. Washington, DC: U.S. Patent and Trademark Office.

Data: Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837

Theses and Dissertations: Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master's thesis]. [Chicago (IL)]: University of Chicago

For examples of citing other documents and general questions regarding reference style, please refer to the Chicago Manual of Style.
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All experiments reporting results on animal research must be performed in accordance with relevant institutional and national guidelines and regulations. In the manuscript, authors must identify the full name of the ethics committee that approved the work. For most article types, this statement should appear in the Materials and Methods section.

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