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Efeito apoptótico do Midazolam em glândula salivar de ratos e reversão do efeito pela Pilocarpina

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Efeito apoptótico do Midazolam em glândula salivar de ratos e reversão desse efeito pela Pilocarpina

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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DEDICATÓRIA

À Deus, aos meus pais, e alguns amigos em especial.

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EPÍGRAFE

"Se o conhecimento pode criar problemas,

não é através da ignorância que podemos

solucioná-lo.”

- Isaac Asimov -

Resumo

Introdução: O Midazolam provoca em glândulas parótidas de ratos redução da proliferação de células acinares, aumento do número de células mioepiteliais, hipertofia acinar e redução do fluxo salivar. O presente estudo teve por objetivo avaliar a apoptose em células acinares de glândulas parótidas de ratos tratados cronicamente com Midazolam associados ou não à Pilocarpina. Metodologia: Sessenta ratos machos Wistar foram distribuídos aleatoriamente em 6 grupos: controle receberam solução salina durante 30 dias (S_{30}) e 60 dias (S_{60}) e os demais grupos receberam Pilocarpina por 60 dias (P_{60}); Midazolam por 30 dias (M_{30}); Midazolam por 30 dias e mais 30 dias de salina (M_{30}+S_{30}) e por fim Midazolam por 30 dias e mais 30 dias de Midazolam e Pilocarpina (M_{30}+MP_{30}).Os Cortes histológicos foram submetidos à técnica Terminal Deoxynucleotidyl Transferase (TUNEL). Foram quantificados o número de células positivas e negativas, calculando-se o Índice Apoptótico (IA). Os dados foram analisados por ANOVA a dois critérios e teste de Tukey, com nível de significância de 5%. Resultados: Maior IA foi verificado nos grupos M_{30} (52,79 ± 9,01), M_{30}+S_{30} (62,43 ± 8,52), quando comparados aos grupos S_{30} (37,94 ± 5,94), S_{60} (31,85 ± 9,18) respectivamente ($p<0,05$). Não houve diferenças entre os grupos M_{30}+MP_{30} (30,98 ± 6,19) e S_{60} (31,85 ± 9,18) para a variável IA. Conclusão: A administração crônica do Midazolam demonstrou aumento no número de células apoptóticas em glândulas parótidas de ratos. Porém a Pilocarpina inibiu esse efeito, reestabelecendo os parâmetros de normalidade e inibindo a apoptose.

Palavras-chave: Benzodiazepínico, Apoptose, Pilocarpina, Midazolam, Glândula salivar, TUNEL

Título: Efeito apoptótico do Midazolam em glândula salivar de ratos e reversão desse efeito pela Pilocarpina

Título curto: Efeito apoptótico do Midazolam em glândula de ratos

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Introdução

Recentes dados da Organização Mundial da Saúde (OMS) de 2016 revelam que nos últimos 23 anos houve um aumento expressivo (quase 50%) nos casos de distúrbios mentais. Mais de 615 milhões de pessoas em todo o mundo sofrem de algum tipo de depressão ou ansiedade, representando 10% da população mundial[1]. A OMS também estima que, em casos de conflitos e emergências humanitárias, há necessidade de se ampliar o tratamento, já que uma a cada cinco pessoas são afetadas por ansiedade e depressão. O tratamento medicamentoso contudo exerce impacto na economia mundial, totalizando cerca de 1 trilhão de dólares a cada ano.[2]

No Brasil, os dados publicados na Pesquisa Nacional de Saúde de 2013 demonstram que cerca de 11,2 milhões de brasileiros (7,6% da população total) apresentam depressão e, em decorrência disso, ela já se tornou uma das cinco doenças crônicas não transmissíveis mais prevalentes no país juntamente com diabetes, hipertensão, problemas crônicos na coluna e colesterol alto. [3]

Os Benzodiazepínicos (BZD) são os medicamentos ansiolíticos mais prescritos para ansiedade, depressão e estresse, sendo o Midazolam o mais utilizado para tratamento de depressão [4]. Este fármaco é rapidamente absorvido após a sua administração, atingindo 90% de sua biodisponibilidade em 30 minutos e possui uma meia vida plasmática de 1 a 3 horas. [4] Seu uso contínuo deve ser avaliado com cautela, pois seus efeitos adversos interferem tanto na condição geral do paciente quanto em sua saúde bucal. [5] Os BZDs apresentam como efeitos colaterais gerais: diminuição da tonicidade muscular, sedação, amnésia, comprometimento cognitivo e ataxia. Entre os efeitos adversos bucais mais comuns, destaca-se a xerostomia. [5,6]

A xerostomia é conhecida como a sensação subjetiva de boca seca, mas não necessariamente está associada a uma diminuição real do fluxo salivar, ou mesmo a uma hipofunção da glândula salivar.[] A hipossalivação é a diminuição da quantidade de fluxo salivar estimulado por meio da mensuração da quantidade de saliva. Ambas as situações trazem prejuízo à vida dos pacientes como dificuldade na adaptação de próteses, maior risco à candidose, cáries radiculares, mucosites, disfagia entre outras.[7,8]

A apoptose é um tipo de morte celular, induzida por um programa de suicídio rigorosamente regulado. [] Esse processo pode ser fisiológico como na destruição programada de células durante a embriogênese, perda celular em populações celulares proliferativas, morte de células que já tenham cumprido seu papel. A apoptose também pode ser deflagrada por processos patológicos como lesão de DNA, por atrofia após obstrução [11], lesão celular em infecções[12], e uso de fármacos[13].

Estudos têm verificado a apoptose de diferentes tipos celulares induzidos por Midazolam [6,14,15,16]. Ohno et al.(2012) verificou a alta toxicidade celular desse BZD em células tumorais bucais.[14] Mishraet al. (2013) observou que o Midazolam é capaz de induzir a apoptose em células tumorais humanas. [6] So et al. (2014, 2016) avaliou células tumorais de Leydig e observou que este BZD desencadeia a cascata de caspase e seus mecanismos específicos além de promover apoptose em células tumorais. [15,16] Contudo ainda não existem estudos na literatura que avaliem especificamente a apoptose em células acinares.

O presente grupo de pesquisa anteriormente verificou que o Midazolam acarreta nas glândulas parótidas de ratos: um aumento do volume celular,

redução do fluxo salivar, da proliferação de células acinares; [9] do número de núcleos de células acinares; [10]. Contudo, o uso da Pilocarpina reestabeleceu fluxo salivar [9], normalizou o número de células acinares [10] e aumentou a proliferação de células acinares [9]. A partir destes estudos, sugeriu-se então a esta diminuição do número de células acinares poderia ser, pelo menos em parte, decorrente da apoptose destas células.

Frente aos resultados encontrados nos estudos prévios o presente estudo tem como objetivo avaliar a apoptose de células acinares em glândulas parótidas de ratos tratados cronicamente com Midazolam (Dormonid ®) e o papel da Pilocarpina no restabelecimento dos parâmetros de normalidade.

A hipótese nula seria que não haveria diferença no Índice Apoptótico (IA) de células acinares de glândulas salivares de ratos tratados com Midazolam, solução salina e Pilocarpina.

Metodologia

Este estudo foi aprovado pelo Comitê de Ética em Pesquisa no uso de animais da Pontifícia Universidade Católica do Paraná (CEUA-PUCPR nº 01084/2016 – 2° versão). Foram utilizados ratos machos do tipo *Rattus norvegicus albinus*, *Rodentia, mammalia* da linhagem *Wistar* (oriundos do Biotério Central da Pontifícia Universidade Católica do Paraná), com peso aproximado de 250g, que foram mantidos em gaiolas plásticas com água e comida *ad libitum,* com ração Peletizada Nuvilab CR-1 (Nuvital Nutrientes S.A), respeitando o fotoperíodo de doze horas e aclimatados ao ambiente laboratorial (25±1°C).

Os animais foram alocados aleatoriamente em seis grupos. Dois grupos serviram como controle (S_{30}, S_{60}) e receberam somente 0,1 mL de solução injetável de soro fisiológico 0,9%, via intraperitoneal, uma vez ao dia, no mesmo horário, por trinta e sessenta dias, respectivamente.

Dentre os demais grupos, destaca-se P_{60} que recebeu 0,05 mL, de forma tópica, de gel em orabase, preparado com 1% de hidrocloridrato de Pilocarpina (Gerbras Química e Farmacêutica Ltda., São Paulo, Brasil), uma vez ao dia, no mesmo horário, por um período de sessenta dias.

O grupo M_{30} recebeu 0,5mg/Kg, via intramuscular, de solução injetável de Midazolam 1% (Cosmética Farmácia de Manipulação Ltda, Curitiba, Paraná, Brasil), uma vez ao dia, sempre no mesmo horário, por um período de trinta dias.

O grupo M_{30}+S_{30} foi submetido a 0,5mg/Kg, via intramuscular, de solução injetável de Midazolam 1% (Cosmética Farmácia de Manipulação Ltda, Curitiba, Paraná, Brasil), uma vez ao dia, sempre no mesmo horário, por trinta dias. A partir do trigésimo primeiro dia, este mesmo grupo recebeu, por mais trinta dias

somente 0,1 mL de solução injetável de soro fisiológico 0,9%, via intraperitoneal, uma vez ao dia, sempre no mesmo horário.

O grupo M_{30}+P_{30} recebeu 0,5mg/Kg, via intramuscular, de solução injetável de Midazolam 1% (Cosmética Farmácia de Manipulação Ltda, Curitiba, Paraná, Brasil), uma vez ao dia, sempre no mesmo horário, por trinta dias. A partir do trigésimo primeiro dia, este mesmo grupo recebeu, por mais trinta dias, somente 0,05mL de gel em orabase, preparado com 1% de hidrocloridrato de Pilocarpina (Gerbras Química e Farmacêutica Ltda., São Paulo, Brasil), topicamente, uma vez ao dia, sempre no mesmo horário.

No Quadro 1 encontra-se a distribuição dos grupos, números de animais, fámacos, período de tratamento, dose e vias de administração.

O tempo de tratamento crônico foi determinado de acordo com a vida média de um rato, em que os 30 dias representam aproximadamente 3 anos. O grupo que recebeu o fármaco por 30 dias e suspendeu a medicação por mais 30 dias, visou simular a situação de um paciente que deixa de tomar o fármaco após o mesmo período em que houve a administração[18].

Quadro 1- Distruibuição dos grupos, números de animais, fámacos, período de tratamento, dose e vias de administração.

Grupos	Quantidade de animais	Fármacos C = Concentração	Período de tratamento	Dose	Via de administração
S_{30}	10	Soro Fisiológico (C=0,9%)	1-30 dias	0,1 mL	Intraperitoneal
S_{60}	10	Soro Fisiológico (C=0,9%)	1-60 dias	0,1 mL	Intraperitoneal
P_{60}	10	Pilocarpina (C=0,1%)	1-60 dias	0,05 mL	Tópica
M_{30}	10	Midazolam (C=1%)	1-30 dias	0,5mg/Kg	Intramuscular
M_{30}+S_{30}	10	Midazolam + Soro Fisiológico	1-30 dias 31-60 dias	0,5mg/Kg 0,1mL	Intramuscular Intraperitoneal
M_{30}+MP_{30}	10	Midazolam + Midazolam+Pilocarpina	1-30 dias 31-60 dias	0,5mg/Kg 0,1mL	Intramuscular Intramuscular + Tópica

Nota:1% de Hidrocloridrato de Pilocarpina (Gerbras Química e Farmacêutica Ltda, São Paulo, São Paulo, Brasil); Midazolam 1% (Cosmética Farmácia de Manipulação Ltda, Curitiba, Paraná Brasil); Soro Fisiológico (LBS – Laborasa Indústria Famacêutica Limitada, São Paulo, São Paulo, Brasil).

Excisão das glândulas parótidas

Após o término de cada tratamento, os animais foram anestesiados e eutanasiados por overdose da administração intraperitoneal de Tiopental Sódico 120mg/kg (Thionembutal®, Abbott Laboratórios do Brasil Ltda., São Paulo, Brasil).

As glândulas parótidas direita e esquerda foram removidas com uso de lâmina de bisturi número 12 e colocadas em um recipiente previamente identificado, contendo formol a 10% por 48h e seccionadas no sentido longitudinal.

Processamento e protocolo TUNEL

As glândulas parótidas fixadas em formalina foram emblocadas em parafina (bloco doador). Com o auxílio de uma broca trefina (Neodent®, Curitiba, Brasil) de 3 mm de diâmetro acoplada a um motor de suspensão de 130 watts de Bethil (Prometal Ind. Met. LTDA®, Marília, Brasil), foram realizados cortes cilíndricos de tamanhos iguais. Dez desses cilindros foram dispostos em linhas e colunas, e imersos em parafina (bloco receptor), constituindo o *tissues microarray* (TMA). [19,20] Foram realizados cortes microscópicos com 4 µm de espessura e, em seguida, as lâminas obtidas foram submetidas à técnica de TUNEL (Terminal deoxynucleotidyl transferase) utilizando-se o Kit In situ Cell Death Detection, POD (Roche Diagnostics GmbH, 11684817910, Mannheim, Alemanha) de acordo com as instruções do fabricante. A técnica TUNEL (Terminal deoxynucleotidyl transferase) têm sido o padrão-ouro para avaliação de apoptose celular.[17]

O ensaio de TUNEL foi utilizado para identificar células apoptóticas por meio da marcação de fragmentos de DNA resultantes da quebra do DNA genômico.

Após a desparafinização das secções, a atividade da peroxidase endógena foi bloqueada com 5% de H2O2 em metanol durante 15 minutos à temperatura ambiente. Os cortes foram então tratados com 0,1ml de tampão citrato (pH 6,0) em banho-maria durante 25 minutos a 99º C para reativação de epitopos.

Após lavagem em TBS-tris (pH 7,0), as lâminas foram cobertas com 50 uL da mistura reacional da solução enzima com a solução de marcação (label solution). Em seguida, foram incubadas durante uma hora a uma temperatura de 37°C numa atmosfera humidificada no escuro. Após lavagem em PBS, as lâminas foram cobertas com 50 ul de converter-POD e incubadas durante 30 minutos a 37 ° C em câmara úmida.

Em seguida, a incubação ocorreu em uma solução de diaminobenzidina (DAB, K3468 DAKO DAB + substrato cromogênio sistema líquido, Carpinteria, CA) durante 15 segundos à temperatura ambiente e as lâminas contrastadas com hematoxilina de Harris. Nos controles negativos, a solução de enzima foi omitida.

Análise da marcação de Apoptose

Dez imagens de cada cilindro (correspondendo a um rato cada) de TMA 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, TX). As 600 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 acinares positivos para apoptose, corados de marrom escuro e os núcleos não apoptóticos, de coloração roxa.

A partir do número total de núcleos contados (somatória de núcleos apoptóticos + não apoptóticos), foi calculado o índice apoptótico (IA). Os valores finais foram determinados a partir das médias das porcentagens das dez imagens.

O examinador realizou a contagem de uma imagem de cada corte e repetiu a contagem da mesma imagem depois de vinte e um dias para verificar a reprodutibilidade. Toda a amostra foi contada somente após a verificação da reprodutibilidade e da ausência de erro sistemático. O erro de Dahlberg foi de 5,10%, indicando que o examinador reproduziu de forma confiável a contagem das células. O teste t de Student revelou que não houve erro sistemático na contagem (p=0,16).

Análise de antígeno nuclear de proliferação celular e número de células acinares (PCNA e N)

O presente estudo adicionou os dados obtidos em estudos prévios por meio da imuno-histoquímica para PCNA (antígeno nuclear de proliferação celular)[9] e análise histomorfométrica do número de células acinares (N)[10]na mesma amostra, para fins de comparação.

Análise estatística

A análise estatística foi realizada utilizando-se o programa SPSS 21.0 (SPSS Inc, Chicago, IL). O teste de Shapiro- Willk observou que as variáveis apresentaram distribuição normal. O teste de homogeneidade de variâncias de Levene verificou que a amostragem apresentou variância homogênea. Visando comparar se os valores médios das variáveis (utilização ou não de medicamento) nos grupos apresentaram diferenças estatisticamentes significantes, realizou-se ANOVA a dois critérios com modelo fatorial completo e, em seguida, o teste Tukey HSD.O nível de significância adotado em todos os testes foi de 5% ($p<0,05$).

Resultados

Houve aumento no índice apoptótico (IA) nos grupos que receberam somente o Midazolam (M_{30}, M_{30}+S_{30}) quando comparados aos grupos controle (S_{30}, S_{60}) respectivamente (Tabelas 1 e 2; Figura 1).

Houve uma diminuição significante no grupo M_{30} e M_{30}+ S_{30} quando comparado ao S_{30} e S_{60} respectivamente quando analisados o valor de N. Um aumento no PCNA e N foi verificado na associação no grupo M_{30}+MP_{30} quando comparado a M_{30}+ S_{30}.

Tabela 1 – Valores Médios ± Desvio Padrão das variáveis estudadas nos grupos tratados por 30 dias.

Grupos	S_{30}	M_{30}
IA (%)	37,94 ± 5,94 [a]	52,79 ± 9,01 [b]
PCNA (%)	55,8 ± 14,38 [a]	39,6 ± 30,00 [a]
N (x 10^{6})	44,73± 7,29 [a]	27,72±5,5 [b]

Nota: *Letras diferentes equivalem a diferenças estatisticamente significantes em linha ($p<0,05$); IA= Índice Apoptótico; PCNA= Antígeno nuclear de proliferação celular; N =Número de células acinares.

Tabela 2 - Valores Médios ± Desvio Padrão das variáveis estudadas nos grupos tratados por 60 dias.

Grupos	P_{60}	S_{60}	$M_{30}+S_{30}$	$M_{30}+MP_{30}$
IA (%)	30,78 ± 4,52 [a]	31,85 ± 9,18 [a]	62,43 ± 8,52 [b]	30,98 ± 6,19 [a]
PCNA (%)	46,8 ± 13,50 [a,b]	50 ± 17,53 [a,b]	39 ± 14,11 [a]	67 ± 19,10 [b]
N (x 10^6)	40,05 ± 8,09 [a]	40,55 ± 4,64 [a]	31,10 ± 3,30 [b]	37,96 ± 5,44 [a]

Nota: *Letras diferentes equivalem a diferenças estatisticamente significantes em linha ($p<0,05$); IA= Índice Apoptótico; PCNA= Antígeno nuclear de proliferação celular; N =Número de células acinares.

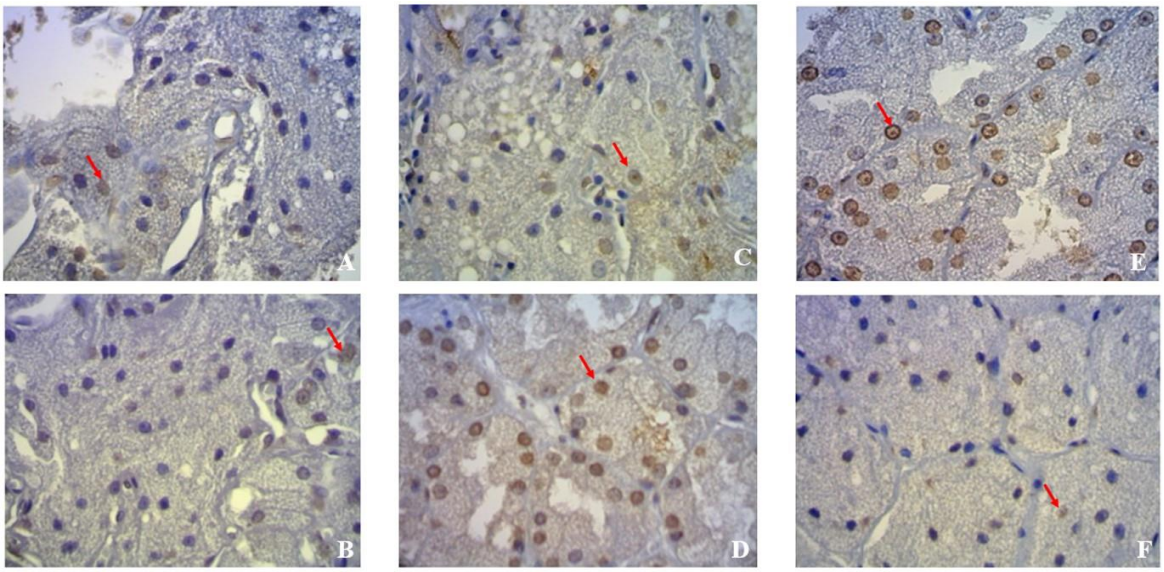

Figura 1 – Fotomicrografia das células acinares de glândulas salivares parótidas de ratos tratados com: (A)Soro fisiológico por 30 dias, (B) Soro fisiológico por 60 dias, (C) Pilocarpina por 60 dias, (D) Midazolam por 30 dias, (E) Midazolam por 30 dias + soro fisiológico por 30 dias, (F) Midazolam por 30 dias + Pilocarpina por 30 dias (Magnificação de 400×). Todas as lâminas foram processadas por TUNEL. As setas em vermelho indicam as células apoptóticas com núcleo corado em marrom.

Discussão

O presente estudo verificou, por meio da técnica de TUNEL para apoptose, um aumento do IA nos grupos M_{30}, M_{30}+S_{30} quando comparados aos grupos controle em as glândulas parótidas de ratos.

Analisando-se isoladamente o tratamento crônico com Midazolam por 30 dias e Midazolam por 30 dias mais 30 dias de solução salina em comparação aos grupos controle tratados com solução salina, foi observado um aumento expressivo de apoptose em glândulas parótidas de ratos. Esse aumento da apoptose associado à manutenção da taxa de proliferação[9], provavelmente responsável pelo menor número de células acinares.[10] Foi demonstrado em um estudo prévio que o uso tópico do Midazolam em mucosa nasal de ratos acarreta uma maior apoptose quando comparado ao grupo controle, concordando com os resultados do presente estudo em glândula parótida de ratos.[21] Já foi também reportado que esse fármaco também induziu apoptose em célula tumorais de Leydig, desencadeando a cascata de caspase e seus mecanismos específicos. [15,16]

Quando verificamos que o grupo tratado cronicamente com Midazolam associado à Pilocarpina apresenta índices de apoptose, proliferação celular e um número de células acinares semelhantes aos do grupo com solução salina, sugere-se que a Pilocarpina foi eficaz no reestabelecimento do número de células acinares. Estudos já revelaram que a Pilocarpina tem uma proteção eficaz contra a poptose neuronal induzida por glutamato através do receptor muscarínico M1. [22]

Os resultados obtidos no grupo tratado somente com Pilocarpina mostraram não haver diferenças entre valores de células apoptóticas quando comparado

grupos tratados somente com solução salina, mostrando que o uso isolado da Pilocarpina não causa apoptose em células acinares. Segundo Montoya et al.[23], apesar de inúmeras outras opções medicamentosas, a Pilocarpina continua sendo uma medicação segura e eficaz para o tratamento da hipossalivação. [23]

Dentro dos limites do presente estudo, os resultados encontrados explicam ao menos em parte, os efeitos adversos do Midazolam. Deve ser levado em consideração o fato de que a pesquisa foi realizada em animais de laboratório, porém em um grupo bem controlado quanto à alimentação, ciclo claro-escuro, ambientação e administração medicamentosa.

O Midazolam tem provocado reações em diferentes tecidos e células tumorais aumentando ou diminuindo a apoptose dependendo do tecido. [14,15,16,17,21] Em virtude disso, sugere-se que novos estudos sejam realizados para investigar a apoptose em células acinares induzidas pelo Midazolam, pois há uma carência na literatura científica atual com relação a esse assunto. Estudos adicionais podem levar ao melhor entendimento do mecanismo que desencadeia essa reação apoptótica e a adoção de alternativas que possam reverter esse mecanismo.

Conclusão

Dentro das limitações do presente estudo, pode-se concluir que a administração crônica do Midazolam demonstrou aumento no número de células apoptóticas em glândulas parótidas de ratos. Observou-se também uma ação ácino protetora da Pilocarpina reestabelecendo o número de células acinares.

Conflito de interesses

Nenhum conflito.

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Anexo A

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
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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

O colegiado do CEUA certifica que este protocolo que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto homem), para fins de pesquisa cientifica, encontra-se de acordo com os preceitos da Lei nº 11.794/2018 e Decreto nº 6.899/2009, e com as normas editadas pelo CONCEA e foi **APROVADO** pela CEUA - PUCPR em reunião de ***13.10.2016.*** Se houver mudança do protocolo o pesquisador deve enviar um relatório á CEUA descrevendo de forma clara e sucinta, a parte do protocolo a ser modificado e as suas justificativas. Se a pesquisa, ou parte dela for realizada em outras instituições, cabe ao pesquisador não iniciar antes de receber a autorização formal para a sua realização.

O documento que autoriza o início da pesquisa deve ser carimbado e assinado pelo responsável da instituição e deve ser mantido em poder do pesquisador responsável, podendo ser requerido por esta CEUA em qualquer tempo. Lembramos ao pesquisador que é obrigatório encaminhar o relatório final da pesquisa a esta CEUA.

Atenciosamente,

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

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Anexo B

Artigo em Inglês

Introduction

Recent data from the World Health Organization (WHO) in 2016 show that in the last 23 years there has been an expressive increase (almost 50%) in cases of mental disorders. Over 615 million people worldwide suffer from some form of depression or anxiety, accounting for 10% of the world's population [1]. The WHO also estimates that in cases of conflict and humanitarian emergencies, there is a need to expand treatment, as one in five people are affected by anxiety and depression. Drug treatment, however, has an impact on the world economy, totaling about 1 trillion dollars each year. [2]

In Brazil, data published in the National Health Survey of 2013 show that about 11.2 million Brazilians (7.6% of the total population) present with depression and, as a result, it has already become one of the five chronic diseases Most prevalent in the country along with diabetes, hypertension, chronic spinal problems, and high cholesterol. [3]

Benzodiazepines (BZDs) are the most commonly prescribed anxiolytic medications for anxiety, depression, and stress, with Midazolam being the most widely used treatment for depression [4]. This drug is rapidly absorbed after administration, reaching 90% of its bioavailability in 30 minutes and has a plasma half-life of 1 to 3 hours. [4] Its continuous use should be evaluated with caution, since its adverse effects interfere both in the general condition of the patient and in his oral health. [5] BZDs present as general side effects: decreased muscle

tone, sedation, amnesia, cognitive impairment and ataxia. Among the most common oral adverse effects, xerostomia stands out. [5,6]

Xerostomia is known as the subjective sensation of dry mouth, but it is not necessarily associated with a real decrease in salivary flow or even hypofunction of the salivary gland. Hyposalivation is the decrease in the amount of salivary flow stimulated through Measurement of the amount of saliva. Both situations lead to impairment in patients' lives, such as difficulty in prosthesis adaptation, increased risk of candidiasis, root caries, mucositis, dysphagia, etc. [7,8]

Apoptosis is a type of cell death, induced by a strictly regulated suicide program. This process can be physiological as in the programmed destruction of cells during embryogenesis, cell loss in proliferative cell populations, death of cells that have already fulfilled their role. Apoptosis can also be triggered by pathological processes such as DNA damage, atrophy after obstruction [11], cell injury in infections [12], and drug use [13].

Studies have verified the apoptosis of different cell types induced by Midazolam [6,14,15,16]. Ohno et al. (2012) verified the high cellular toxicity of this BZD in oral tumor cells. [14] Mishraet al. (2013) noted that Midazolam is capable of inducing apoptosis in human tumor cells. [6] So et al. (2014, 2016) evaluated Leydig tumor cells and observed that this BZD triggers the caspase cascade and its specific mechanisms in addition to promoting apoptosis in tumor cells. [15,16] However, there are no studies in the literature that specifically evaluate apoptosis in acinar cells.

The present research group has verified that Midazolam causes rats to parotid glands: an increase in cell volume, reduction of salivary flow, proliferation of acinar cells; [9] the number of acinar cell nuclei; [10]. However, the use of

Pilocarpine re-established salivary flow [9], normalized the number of acinar cells [10] and increased proliferation of acinar cells [9]. From these studies, it was then suggested that this decrease in the number of acinar cells could be, at least in part, due to the apoptosis of these cells.

The aim of this study was to evaluate apoptosis of acinar cells in the parotid glands of rats chronically treated with Midazolam (Dormonid ®) and the role of Pilocarpine in the reestablishment of normality parameters.

The null hypothesis would be that there would be no difference in the Apoptotic Index (AI) of acinar cells of salivary glands of rats treated with Midazolam, saline and Pilocarpine.

Mehtodology

This study was approved by the Committee of Ethics in Research on the use of animals of the Pontifical Catholic University of Paraná (CEUA-PUCPR nº 01084/2016 - 2nd version). Male rats of the Rattus norvegicus albinus, Rodentia, Wistar lineage mammals (from the Central Biotério of the Pontifical Catholic University of Paraná), weighing approximately 250 g, were used in plastic cages with water and ad libitum food, with ration Pelletized Nuvilab CR-1 (Nuvital Nutrientes SA), respecting the photoperiod of twelve hours and acclimated to the laboratory environment (25 ± 1 ° C).

The animals were randomly allocated into six groups. Two groups served as controls (S30, S60) and received only 0.1 mL of 0.9% saline solution intraperitoneally once daily at the same time for thirty and sixty days, respectively.

Among the other groups, we highlight P60, which received 0.05 mL, topically, of gel in orabase, prepared with 1% of Pilocarpine hydrochloride

(Gerbras Química e Farmacêutica Ltda., São Paulo, Brazil), once Day, at the same time, for a period of sixty days.

The M30 group received 0.5mg / kg intramuscularly of intravenous solution of Midazolam 1% (Cosmética Farmácia de Manipulção Ltda, Curitiba, Paraná, Brazil), once daily, always at the same time, for a period of thirty days .

The M30 + S30 group was submitted to 0.5mg / kg, intramuscularly, of intravenous solution of Midazolam 1% (Cosmética Farmácia de Manipulação Ltda, Curitiba, Paraná, Brazil), once daily, always at the same time for thirty Days. From the thirty-first day, this same group received, for a further thirty days, only 0.1 mL of 0.9% saline solution intraperitoneally, once daily, always at the same time.

The M30 + P30 group received 0.5 mg / kg, intramuscularly of solution of Midazolam 1% (Cosmética Farmácia de Manipulação Ltda, Curitiba, Paraná, Brazil), once a day, always at the same time, for thirty days. From the thirty-first day, this same group received, for a further thirty days, only 0.05 ml of orabase gel, prepared with 1% of Pilocarpine hydrochloride (Gerbras Química e Farmacêutica Ltda., São Paulo, Brazil), topically, Once a day, always at the same time.

Table 1 shows the distribution of groups, numbers of animals, drugs, treatment period, dose and routes of administration.

Chronic treatment time was determined according to the average life of a mouse, in which the 30 days represent approximately 3 years. The group that received the drug for 30 days and suspended the medication for another 30 days, was aimed at simulating the situation of a patient who stops taking the drug after the same period of administration [18].

Table 1- Distribution of groups, numbers of animals, drugs, treatment period, dose and routes of administration.

Groups	Amount of Animals	Drugs C = Concentration	Treatment Period	Dose	Route of Administration
S_{30}	10	Saline (C=0,9%)	1-30 days	0,1 mL	Intraperitoneal
S_{60}	10	Saline (C=0,9%)	1-60 days	0,1 mL	Intraperitoneal
P_{60}	10	Pilocarpine (C=0,1%)	1-60 days	0,05 mL	Topical
M_{30}	10	Midazolam (C=1%)	1-30 days	0,5mg/Kg	Intramuscular
M_{30}+S_{30}	10	Midazolam + Saline	1-30 days 31-60 days	0,5mg/Kg 0,1mL	Intramuscular Intraperitoneal
M_{30}+MP_{30}	10	Midazolam + Midazolam+Pilocarpine	1-30 days 31-60 days	0,5mg/Kg 0,1mL	Intramuscular Intramuscular + Topical

Note: 1% of Pilocarpine hydrochloride (Gerbras Química e Farmacêutica Ltda, São Paulo, São Paulo, Brazil); Midazolam 1% (Cosmetics Pharmacy Manipulation Ltda, Curitiba, Paraná Brazil); Physiological Serum (LBS - Laborasa Indústria Famacêutica Limitada, São Paulo, São Paulo, Brazil).

Excision of parotid glands

After completion of each treatment, the animals were anesthetized and euthanized by overdose of the intraperitoneal administration of Thiopental Sodium 120mg / kg (Thionembutal®, Abbott Laboratories of Brazil Ltda., São Paulo, Brazil).

The right and left parotid glands were removed using a No. 12 scalpel blade and placed in a previously identified container containing 10% formaldehyde for 48 h and longitudinally sectioned.

Processing and protocol TUNEL

The formalin-fixed parotid glands were embedded in paraffin (donor block). A 3-mm-diameter trephine drill bit (Neodent®, Curitiba, Brazil) was coupled to a 130-watt suspension motor from Bethil (Prometal Ind. Met. LTDA®, Marília, Brazil). Sizes. Ten of these cylinders were arranged in rows and columns, and immersed in paraffin (receptor block), constituting the tissues microarray (TMA). [19,20] Microscopic sections with 4 µm thickness were obtained and then the slides obtained were submitted to the TUNEL (Terminal deoxynucleotidyl transferase) technique using the Cell Death Detection In POD Kit (Roche Diagnostics GmbH, 11684817910, Mannheim, Germany) according to the manufacturer's instructions. The TUNEL technique (Terminal deoxynucleotidyl transferase) has been the gold standard for evaluation of cellular apoptosis. [17]

The TUNEL assay was used to identify apoptotic cells by labeling DNA fragments resulting from the breakdown of genomic DNA.

After dewaxing the sections, endogenous peroxidase activity was blocked with 5% H 2 O 2 in methanol for 15 minutes at room temperature. The sections were then treated with 0.1ml of citrate buffer (pH 6.0) in a water bath for 25 minutes at 99 ° C for reactivation of epitopes.

After washing in TBS-tris (pH 7.0), the slides were covered with 50æl of the reaction solution of the enzyme solution with the label solution. Thereafter, they were incubated for one hour at a temperature of 37øC in a humidified atmosphere in the dark. After washing in PBS, the slides were covered with 50 µl of POD-converting and incubated for 30 minutes at 37 ° C in a humid chamber.

Then the incubation was carried out in a solution of diaminobenzidine (DAB, K3468 DAKO DAB + substrate chromogen liquid system, Carpinteria, CA) for 15 seconds at room temperature and the slides stained with Harris hematoxylin. In the negative controls, the enzyme solution was omitted.

Apoptosis labeling

Ten images from each cylinder (corresponding to one mouse each) of TMA were captured by a Dinolite® microcamera (AM 423 × AmMo Eletronics Corporation, New Taipei City, Taiwan) coupled on an Olympus® BX50 microscope (Olympus Corporation, Ishikawa, Japan) , In magnification of 400 ×, connected to a Dell Inspiron 15459 notebook (Round Rock, TX). The 600 images were analyzed by a single observer in the Image Proplus ™ 4.5 morphometry

program (Media Cybernetics, Silver Spring, MD), in which a virtual grid was applied to the images for counting positive actin nuclei for apoptosis, stained dark brown and The non-apoptotic nuclei, purple in color.

From the total number of nuclei counted (sum of apoptotic + non-apoptotic nuclei), the apoptotic index (AI) was calculated. The final values were determined from the averages of the ten images.

The examiner counted one image from each cut and repeated counting the same image after twenty-one days to check for reproducibility. The entire sample was counted only after verification of reproducibility and absence of systematic error. Dahlberg's error was 5.10%, indicating that the examiner reliably reproduced the cell count. Student's t test revealed no systematic error in the score ($p = 0.16$).

Analysis of nuclear proliferation cell antigen and number of acinar cells (PCNA and N)

The present study added the data obtained in previous studies using immunohistochemistry for PCNA (nuclear proliferation antigen) [9] and histomorphometric analysis of the number of acinar (N) cells [10] in the same sample for comparison purposes .

Statistical analysis

Statistical analysis was performed using SPSS 21.0 software (SPSS Inc, Chicago, IL). The Shapiro-Willk test found that the variables had a normal distribution. The Levene variance homogeneity test verified that the sample had homogeneous variance. In order to compare whether the mean values of the

variables (use or not of medication) in the groups presented statistically significant differences, ANOVA was performed on two criteria with a complete factorial model and then the Tukey HSD test. The level of significance adopted in all The tests were 5% ($p < 0.05$).

Results

There was an increase in the apoptotic index (AI) in the groups that received only Midazolam (M30, M30 + S30) when compared to the control groups (S30, S60) respectively (Tables 1 and 2, Figure 1).

There was a significant decrease in the M30 and M30 + S30 groups when compared to the S30 and S60 respectively when analyzed for the value of N. An increase in PCNA and N was verified in the association in the M30 + MP30 group when compared to M30 + S30.

Table 1 - Mean values ± Standard deviation of the variables studied in the groups treated for 30 days.

Groups	S_{30}	M_{30}
IA (%)	37,94 ± 5,94 [a]	52,79 ± 9,01 [b]
PCNA (%)	55,8 ± 14,38 [a]	39,6 ± 30,00 [a]
N (x 10^6)	44,73± 7,29 [a]	27,72±5,5 [b]

Note: * Different letters are equivalent to statistically significant differences in line ($p < 0.05$); IA = Apoptotic Index; PCNA = Cell proliferation nuclear antigen; N = Number of acinar cells.

Table 2 - Mean values ± Standard deviation of the variables studied in the groups treated for 60 days.

Groups	P_{60}	S_{60}	$M_{30}+S_{30}$	$M_{30}+MP_{30}$
IA (%)	30,78 ± 4,52 [a]	31,85 ± 9,18 [a]	62,43 ± 8,52 [b]	30,98 ± 6,19 [a]
PCNA (%)	46,8 ± 13,50 [a,b]	50 ± 17,53 [a,b]	39 ± 14,11 [a]	67 ± 19,10 [b]
N (x 10^6)	40,05 ± 8,09 [a]	40,55 ± 4,64 [a]	31,10 ± 3,30 [b]	37,96 ± 5,44 [a]

Note: * Different letters are equivalent to statistically significant differences in line ($p < 0.05$); IA = Apoptotic Index; PCNA = Cell proliferation nuclear antigen; N = Number of acinar cells.

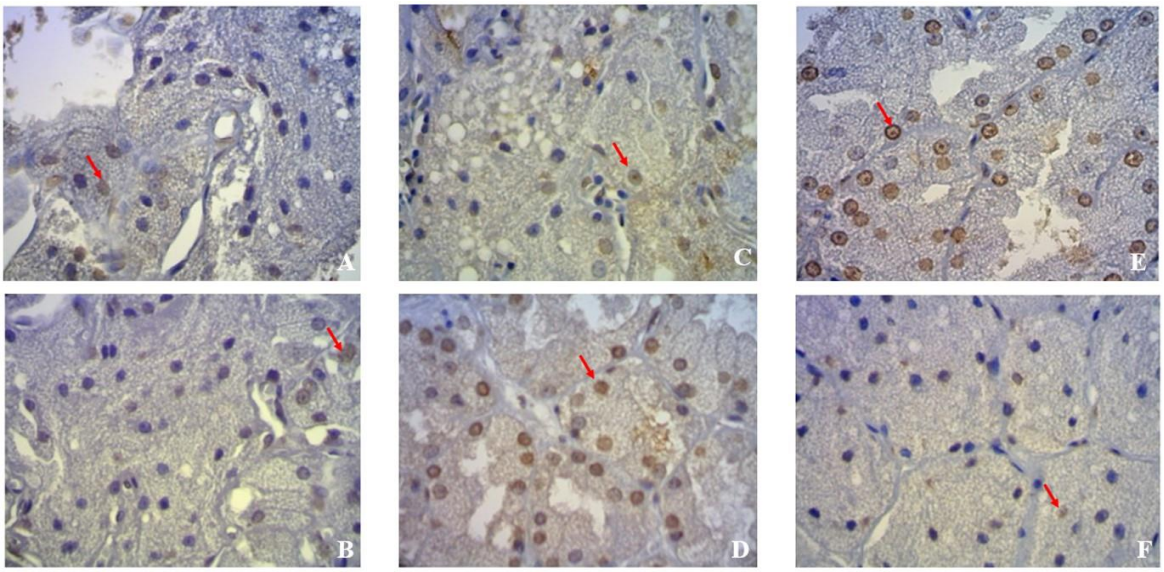

Figure 1 - Photomicrograph of the acinar cells of the parotid salivary glands of rats treated with: (A) Physiological saline for 30 days, (B) Physiological saline for 60 days, (C) Pilocarpine for 60 days, (D) Midazolam for 30 days, (E) Midazolam for 30 days + saline for 30 days, (F) Midazolam for 30 days + Pilocarpine for 30 days (Magnification 400 ×). All slides were processed by TUNEL. The red arrows indicate apoptotic cells with a brown colored nucleus.

Discussion

The present study verified, through the TUNEL technique for apoptosis, an increase in AI in the M30, M30 + S30 groups when compared to the control groups in the parotid glands of rats.

By analyzing the chronic treatment with Midazolam for 30 days and Midazolam for 30 days plus 30 days of saline in comparison to the saline-treated control groups, a significant increase of apoptosis was observed in the parotid glands of rats. This increase in apoptosis associated with the maintenance of proliferation rate [9], probably responsible for the lower number of acinar cells. [10] It has been demonstrated in a previous study that the topical use of Midazolam in the nasal mucosa of rats entails a greater apoptosis when compared to the control group, agreeing with the results of the present study in rats parotid gland. It has also been reported that this drug also induced apoptosis in Leydig tumor cells, triggering the caspase cascade and its specific mechanisms. [15,16]

When we verified that the chronically treated group with Midazolam associated with Pilocarpina presents apoptosis, cell proliferation and a number of acinar cells similar to those in the saline group, it is suggested that Pilocarpine was effective in reestablishing the number of acinar cells. Studies have already shown that Pilocarpine has effective protection against glutamate-induced neuronal poptosis through the M1 muscarinic receptor. [22]

The results obtained in the group treated only with Pilocarpine showed no differences between apoptotic cell values when compared to groups treated with saline only, showing that the use of Pilocarpine alone does not cause apoptosis in acinar cells. According to Montoya et al. [23], despite numerous other drug

options, Pilocarpine remains a safe and effective medication for the treatment of hyposalivation. [23]

Within the limits of the present study, the results found explain at least in part the adverse effects of Midazolam. Consideration should be given to the fact that the research was performed on laboratory animals, but in a well-controlled group regarding feeding, light-dark cycle, setting and drug administration.

Midazolam has elicited reactions in different tissues and tumor cells by increasing or decreasing apoptosis depending on the tissue. [14,15,16,17,21] In light of this, it is suggested that further studies are conducted to investigate apoptosis in acinar cells induced by Midazolam, as there is a lack in current scientific literature regarding this subject. Further studies may lead to a better understanding of the mechanism that triggers this apoptotic reaction and the adoption of alternatives that may reverse this mechanism.

Conclusion

Within the limitations of the present study, it can be concluded that the chronic administration of Midazolam demonstrated an increase in the number of apoptotic cells in the parotid glands of rats. A protective actinic action of Pilocarpine was also observed, reestablishing the number of acinar cells.

Conflict of interests

No conflict.

Instructions to Authors: *British Journal of Clinical Pharmacology* (BJCP)

Papers will be considered for publication if they are relevant to any aspect of drug action in humans.

Submission of a manuscript to BJCP will be taken to indicate that

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- no part of the manuscript is currently under consideration for publication elsewhere;
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Investigations of human subjects will usually be expected to have been conducted in accordance with good clinical practice, which defines investigator responsibilities, including, among others, qualifications, resources, providing medical care to trial participants, communication with the research ethics committee, protocol, informed consent, data integrity and reporting (http://ichgcp.net/4-investigator). Where this is the case we would expect the principal investigator to be an author.

Tips for authors on how to navigate the peer review process can be found here.

PREPARING AND SUBMITTING A MANUSCRIPT

Once you have read these guidelines in full, please go here to submit your manuscript.

You will need to submit three documents:

a. manuscript;

b. covering letter that includes specific statements; and

c. a competing interests statement formatted as per our guidelines.

If your paper is accepted, you will subsequently be required to submit both a table of key links (targets and ligands) to the BPH/IUPHAR Guide to Pharmacology, and a copyright transfer agreement form signed by the corresponding author. You will be taken to the Wiley Author Licensing Service to complete this, and full guidance is given there. Please see more below.

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Regrettably, the Journal is unable to undertake major corrections to language, which is the responsibility of the author. Because there should be no barriers to getting your research published, however, manuscripts are often returned for English language and formatting issues. Thus, a pre-acceptance Editing Service (comprising English language editing, translation services, manuscript formatting and preparation) is available and can provide you, the author, with expert help to ensure your manuscript is ready for submission. Japanese authors can also find a list of local English improvement services at http://www.wiley.co.jp/journals/editcontribute.html . All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication.
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A separate title page should be included (see below). The submitting author need not be the same as the corresponding author.

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Authors should ensure that they have provided the following information, when appropriate:

1. A cover letter detailing originality of the manuscript and agreement of all authors and also identifying the **Principal Investigator** who undertook any human studies reported.
2. A title page including a title of no more than 150 characters, all author names and affiliations, and the corresponding author contact information. Also including a running head, keywords and word count.
3. A structured summary of no more than 250 words.
4. 'What is known about this subject' and 'What this study adds' statements (up to three bullet point sentences for each).
5. 95% confidence intervals (CI) on differences between major end points.
6. Some numerical data in the summary, including 95% CIs, when appropriate.
7. Details of precision, accuracy, sensitivity, and specificity for drug/metabolite assays.
8. A statement (in Methods) of ethics committee approval and subject consent including the name of the ethics committee and the approval number or identification code.
9. A statement declaring any competing interests, or declaring that there are no competing interests.
10. Acknowledgement of financial and other support.
11. Preprints of relevant unpublished papers.

Figures and tables must be submitted as separate files, and guidance on preparing artwork can be found here. The following formats are accepted: gif, jpg, bmp, tif, pic (figures) and as Word and Excel files (tables). PDF and Powerpoint files are **not** accepted.
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If your manuscript refers to unpublished data (see section below “References to data on file”), you should obtain (and provide the Editorial Office with a copy) written permission to mention a responsible individual (e.g. “Prof X...contact address a.x@kcl.ac.uk) or should identify a department in a company that can be contacted, with a contact address.

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Examples of appropriate conflicts of interest statements would be

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For papers where no conflicts of interest or funding are declared, a default statement is added to that paper.'

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When writing your paper, original research papers should generally be divided into the following sections: Title Page (including PI Statement), Structured Summary, Introduction, Methods, Results, Discussion, Acknowledgements, References, tables and legends to figures (Please remember that figures must be submitted as separate files). In addition authors should provide material for 2 sections, placing this after the Structured Summary:

Section 1: What is already known about this subject: In up to three short bullet point sentences (not more than 50 words in total) summarise the state of scientific knowledge on this subject before you did your study and say why this study needed to be done.

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These two statements should be succinct, accurate and specific.

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Papers should be concise and consideration should be given to using online publication of supplementary tables or other material (see http://authorservices.wiley.com/bauthor/suppmat.aspfor more information about our Supporting Information service).

Review articles

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From time to time the Journal will publish themed issues, including review articles and related original research papers. Authors who want to suggest a theme for a special issue should contact the Editor-in-Chief.

Systematic reviews

The Journal welcomes systematic reviews. The manuscript should provide a concise account of the methods

used, and concentrate on highlighting key aspects of interest and relevance to clinical pharmacologists, under the following headings: Structured Summary, Introduction, Methods, Results, Discussion, and Conclusion.

- **Introduction** This should mention the background (*e.g.* relevant clinical and pharmacological issues) and describe the scope and aim of the review. What was the reason for the review? The strengths and weaknesses of the existing literature should be briefly described, earlier reviews identified and the need for the present paper explained.
- **Methods** Study selection (search strategy, type of intervention/exposure, types of studies included, types of outcomes, types of participants); data extraction and synthesis (statistical techniques and use of a quality assessment tool, if any).
- **Results** The key characteristics of the included studies and the main outcome measures; discuss variation within and between studies.
- **Discussion** Compare the findings to existing knowledge; outline the limitations of the review.
- **Conclusion** Summarize the key findings and the implications for clinical pharmacology and/or practical drug therapy.

Letter to the Editor

Comments on previously published papers, items of topical interest, and brief original communications will be considered under this heading. The length, including references, should not exceed 800 words, plus one figure or table. The letter should NOT be divided into sections.

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The Journal generally does not publish case reports as full papers but will do so as letters to the Editor. As for other letters the length, including references, should not exceed 800 words, plus one figure or table. Such case reports (for example adverse drug reactions or interactions) should include some novel aspect of drug action in man (for example a new adverse reaction or one that gives insight into a mechanism or method of management). Such reports may include single cases or short case series. Notes and guidelines on the format for publishing such reports, including a structured summary, will be found at http://www.bmj.com/content/suppl/2003/06/19/326.7403.1346.DC1.

TERMINOLOGY

Stereoisomers

When a drug can exist as stereoisomers or diastereomers (for example geometrical isomers), the form of compound studied must be designated as follows in the methods section.

In the case of racemates the prefix *rac*- should precede the drug name (for example *rac*-propranolol).

When possible the absolute configuration of enantiomers should be indicated (for example (*S*)-warfarin).

Similarly, geometrical isomerism should be indicated by the prefixes *Z*/*E* or *cis*/*trans*. When appropriate, the interpretation of data obtained using mixtures of isomers should take account of stereochemical aspects.

Drug names

Prescribed drugs should be designated by an International Non-proprietary Name (recommended, rINN, or proposed, pINN). If such a name is not available, a drug should be designated by its British Approved Name (BAN; for example hyoscyamine) or its chemical name (for example glyceryl trinitrate).

When a mixture of drugs has a combination BAN (for example co-trimoxazole, co-fluampicil), that should be used.

For brevity, a company's code name may be used, but in that case the full chemical name or a figure showing the structure of the drug should be given in the introduction or a reference provided that gives this information.

Some mediators with well established common names (*e.g*. prostacyclin) are also prescribed as licensed preparations with an rINN (*e*. *g*. epoprostenol). In such cases the rINN should be used in the context of therapeutic use. Sometimes English and American usage varies, as with adrenaline / epinephrine and noradrenaline / norepinephrine. “Adrenaline / noradrenaline” relate clearly to terms such as “noradrenergic”, “adrenergic” and “adrenal gland” but we will accept the term preferred by authors.

Units

SI units (mass or molar units) should be used. If other units are used, a conversion factor should be included in the **Methods** section.

Symbols

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STRUCTURE

Title page

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- Conclusions

The summary should be a maximum of 250 words; abstracting services truncate summaries that are longer. It should be couched in terms that will be understood by most readers of the Journal.

Analytical methods

Authors should include details of the precision, accuracy, sensitivity, and specificity of an analytical method used to measure drugs, metabolites or biomarkers or refer to other publications in which the information is given.

- **Precision** is a measure of random error, usually expressed as the coefficient of variation.
- **Accuracy** is a measure of systematic error, also called bias; it can be expressed as the percentage difference between the result for a test sample and the reference value for that compound.

- **Sensitivity** or lower limit of quantification.
- **Specificity** is the extent to which the method does not detect compounds other than those intended.

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In the **Methods** section statistical methods should be described clearly, with references when appropriate. Editors and referees will be particularly concerned that any study described had sufficient statistical power for its purpose; when appropriate, the power of the study and its calculation should be described in the Methods section.

In the **Results** section 95% confidence intervals of differences should be cited where relevant for important endpoints. This is particularly important when equivalence is being claimed (*i.e.* for non-significant comparisons). The Editors may utilize specialist statistical referees for manuscript review where necessary.

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To enhance the presentation of their articles, authors are encouraged to use colours in complementary pairs (example): a primary pair that is close to the colour of the journal and existing tables (example); a secondary pair for use when there are multiple figures side by side, or purely for variety within a paper (example); and a third pair to particularly highlight a figure, possibly in the conclusion/results part of a paper (example). With certain figures when it is helpful to strongly differentiate between sets of results, the first colour of each pair should initially be used (example), with the corresponding extra colours introduced as necessary. As blocks, the suggested colours all work together and should be clear to those readers who are colour-blind (example). Although white backgrounds are generally advisable, if necessary a suggested background colour that works both with the overall look of the journal, and the proposed colour palette is illustrated (example).

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Examples:

1. Johnson TN, Rostami-Hodjegan A, Tucker GT. A comparison of methods to predict drug clearance in neonates, infants and children. Br J Clin Pharmacol 2004; 57: 677-8.

2. Metters J (editor). Independent Steering Committee Report of an Independent Review of Access to the Yellow Card Scheme. London: The Stationery Office, 2004.

3. Hoffman BB, Lefkowitz RJ. Beta-adrenergic receptor antagonists. In: The Pharmacological Basis of Therapeutics, Eighth Edition, eds Gilman AG, Rail TW, Nies AS, New York: Pergamon Press, 1990: 229-43.

Accepted articles and Early view

References to papers published as Accepted Articles should be cited thus: Ferro A. Paediatric prescribing: why children are not small adults. *Br J Clin Pharmacol* 2015. doi: 10.1111/bcp.12540.

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Acknowledgements

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Useful resources

Guidelines on Publication Ethics

For full guidance on all aspects of Publication Ethics, please see **here**.

Guidelines on Reporting

For guidance on how to enhance the quality and transparency of health research, please see **here**.

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Produção Científica

Artigos Científicos

Rinaldi M, Johann AC, Rocha F, Ignacio SA, Rosa EA, Alanis LR, Sari Y, da Silva S, de Lima AA, do Prado AM, Bettega PV, Gregio AM. Histomorphometric Analysis of Salivary gland in Wistar Rats Treated Chronically With Two Benzodiazepines Curr Pharm Biotechnol. 2015;16(6):573-8.

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.