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KARIN MAIA MONTEIRO

REFINAMENTO DE MODELOS EXPERIMENTAIS PARA A DIMINUIÇÃO DO SOFRIMENTO ANIMAL E DA VARIABILIDADE DA RESPOSTA FARMACOLÓGICA

REFINEMENT OF EXPERIMENTAL MODELS AIMING AT REDUCING BOTH ANIMAL SUFFERING AND VARIABILITY OF THE PHARMACOLOGICAL RESPONSE

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ORIENTADOR: PROF. DR. JOÃO ERNESTO DE CARVALHO

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“ Quanto ao futuro, temia-o demasiado porque conhecia bem seus próprios limites. E porque, apesar de conhecê-los, não se resignara a abandonar aquela ambição enorme, indefinida, que, depois já inumana, dirigia-se para além das coisas da terra. Falhando na realização do que se lhe apresentava aos olhos, voltara-se para o que ninguém, adivinhava-o, poderia realizar. ”

Clarice Lispector

“ Ele olhou-a severo:
- Que você não saiba qual o maior homem da atualidade apesar de conhecer muitos deles, está bem. Mas que você não saiba o que você mesma sente, é que me desagrada.
Olhou-o aflita:
- Olhe, a coisa de que eu mais gosto no mundo...eu sinto aqui dentro, assim se abrindo... quase, quase posso dizer o que é, mas não posso...
- Tente explicar, disse ele de sobrancelhas franzidas.
- É como uma coisa que vai ser... É como...
- É como?... - Inclinou-se ele, exigindo sério.
- É como uma vontade de respirar muito, mas também o medo... não sei... não sei, quase dói. É tudo... é tudo.
- Tudo?... - Estranhou o professor.
Ela assentiu com a cabeça, emocionada, misteriosa, intensa: tudo... ”

Clarice Lispector

RESUMO

Aliviar a dor e o sofrimento dos animais de laboratório é ético, humanitário e promove boa ciência. A inclusão de agentes supressores da dor em modelos farmacológicos, entretanto, requer detalhada pesquisa envolvendo as diversas opções de analgésicos ou anestésicos a fim de minimizar o estresse do animal experimental sem interferir negativamente no resultado da pesquisa. Utilizando o conceito de refinamento lançado por Russell e Burch na década de 50, o presente projeto tem por objetivo principal propor modificações em modelos experimentais de atividade secretora gástrica, inflamação e câncer, através do tratamento da dor e/ou estresse inerentes à metodologia em questão. No modelo de ligadura de piloro em ratos foi possível observar os benefícios decorrentes da supressão da dor nos dados experimentais através do uso de anestesia geral durante todo o período de teste. Tal medida de refinamento permitiu a redução da variabilidade da resposta intragrupo, a redução do número de animais utilizados, maior facilidade na execução dos procedimentos trans e pós-cirúrgico e, finalmente, a eliminação do sofrimento animal de forma significativa. O teste de edema de pata em ratos, quando utilizado apenas para a avaliação de possível atividade anti-inflamatória – e não antinociceptiva – provoca dor intensa nos animais em decorrência da inoculação do agente flogístico. Neste modelo foi proposto, portanto, a redução do sofrimento animal através do uso de anestésicos gerais e locais. O bloqueio do nervo ciático visando a eliminação da dor provocada pela inflamação induzida por carragenina não comprometeu a evolução do edema de pata em ratos, embora análises específicas de citocinas inflamatórias ainda sejam necessárias. Outro modelo experimental que possui a dor como subproduto não avaliado é o tumor de Ehrlich, frequentemente utilizado para a investigação de propriedades anticâncer de novos compostos. Em sua forma sólida, tanto em pata como em dorso, foi sugerido o uso de analgésicos opióides e enriquecimento ambiental, respectivamente. Em tumor de Ehrlich de flanco, a inclusão de tubos de PVC não ofereceu prejuízo à qualidade dos dados experimentais gerados, corroborando a tendência atual de inclusão desta forma de refinamento. Por sua vez, em modelo de Ehrlich de pata, o uso diário de morfina foi responsável por um melhor estado clínico dos animais, além de atuar de forma sinérgica com o quimioterápico na redução da massa tumoral. Como conclusão, o estresse decorrente dos procedimentos experimentais representa importante viés na pesquisa, influenciando tanto o bem-estar dos animais experimentais como a qualidade dos dados gerados.

Palavras-chave: Anestesia. Analgesia. Dor. Sofrimento animal. Refinamento. Animais de laboratório. Farmacologia experimental.

ABSTRACT

Relieving pain and suffering from laboratory animals is ethical, humane and promotes good quality science. The inclusion of pain suppressors to pharmacological models, however, requires detailed research involving the various choices of analgesic drugs or anesthetics in order to minimize animal discomfort without impairing the parameters to be evaluated. Based on the concept of refinement introduced by Russell and Burch in the fifties, our project aims to propose modifications in three pharmacological experimental models: gastric secretion, inflammation and cancer. In the gastric secretion model in rats, it was possible to observe the benefits due to the use of general anesthesia during the entire experimental period. Such a refinement technique helped reduce the variability of intra-group response, the number of animals used, improved the execution of surgical procedures and, finally, decreased animal suffering significantly. The results obtained encouraged us to investigate the use of pain killers in other pharmacological models. The paw edema test in rats, for example, when used for assessing only the anti-inflammatory activity – not the antinociceptive one – causes intense pain due to the injection of the phlogistic agent. In this case, therefore, we propose the reduction of animal suffering through the use of anesthesia. Another experimental model in which pain is a non-evaluated byproduct involves the Ehrlich tumor, often used for the study of anticancer properties of a given substance. In both solid forms, either in the hind paw or the flank, we suggest the inclusion of opioid analgesic drugs or environmental enrichment, respectively. As a whole, this project is based on the investigation of the benefits of pain/stress relieving agents in the pharmacological models mentioned above. The refinement of the experimental protocols will be recommended in cases where benefits are not followed by any damage to relevant parameters.

Key words: Anesthesia. Analgesia. Pain. Animal suffering. Refinement. Laboratory animals. Pharmacology.

LISTA DE FIGURAS

REVISÃO DE LITERATURA

CAPÍTULO I

CAPÍTULO II

CAPÍTULO III

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

µL - *microliter* / microlitro

5-FU - *5-fluorouracil* / 5-fluorouracil

5-HT_3 - *5-hidroxitryptamine receptor* / receptor para 5-hidroxitriptamina

ACTH - *adrenocorticotropic hormone* / hormônio adrenocorticotrófico

Al^{3+} - *aluminium ion* / íon alumínio

AMP - *adenosine monophosphate* / adenosina monofosfato

ANOVA - *analysis of variance* / análise de variância

ANVISA - Agência Nacional de Vigilância Sanitária / *National Health Surveillance Agency*

BraCVAM - *Brazilian Center for Validation of Alternative Methods* / Centro Brasileiro de Validação de Métodos Alternativos

Ca^{2+} - *calcium ion* / íon cálcio

CCK_2 - *cholecystokinin type 2* / colecistoquinina tipo 2

CEMIB - Centro Multidisciplinar para Investigação Biológica / *Multidisciplinary Center for Biological Investigation on Laboratory Animal Science*

CEUA - Comissão de Ética no Uso de Animais / *Committee for Ethics in Animal Use*

CFA - *complete Freund's adjuvante* / adjuvante completo de Freund

CIOMS-ICLAS - *Committee of International Organizations of Medical Science – International Council for Laboratory Animal Science* / Comissão de Organização Internacional de Ciências Médicas Conselho Internacional para Ciência de Animais de Laboratório

CIUCA - Cadastro das Instituições de Uso Científico de Animais / *Database of Institutions that use Animals for Research and Education*

CNS - *central nervous system* / sistema nervoso central

COBEA - Colégio Brasileiro de Experimentação Animal / *Brazilian College of Animal Experimentation*

CONCEA - Conselho Nacional de Controle de Experimentação Animal / *National Council for the Control of Animal Experimentation*

CRF - *corticotropin-release factor* / fator liberador de corticotrofina

CSF - *cerebrospinal fluid* / líquido cefalorraquidiano

ES - *Ehrlich solid (tumor)* / Ehrlich sólido

FIOCRUZ - Fundação Oswaldo Cruz / *Oswaldo Cruz Foundation*

g - *gram(s)* / grama(s)

GM-CSF - *granulocyte-macrophage-colony-stimulating factor* / fator de estimulação de formação de colônia de macrófagos

h - *hour(s)* / hora(s)

$[H^+]$ - *hydrogenionic concentration* / concentração hidrogeniônica

H_2 - *histamine receptor* / receptor para histamina tipo 2

HCl - *hydrochloric acid* / ácido clorídrico

HCO_3 - *bicarbonate* / bicarbonato

HCT - *hematocrit* / hematócrito

H&E - *hematoxylin eosin* / hematoxilina eosina

HGB - *hemoglobin* / hemoglobina

H_2O_2 - *hydrogen peroxide* / peróxido de hidrogênio

HPA - *hypothalamic-pituitary-adrenal* / hipotálamo hipófise adrenal

IASP - *International Association for the Study of Pain* / Associação Internacional para Estudo da Dor

iBK - *immunoreactive bradykin* / bradicinina imunorreativa

IL - *interleukin* / interleucina

INCA - Instituto Nacional do Câncer / *National Institute of Cancer*

INCQS - Instituto Nacional de Controle de Qualidade em Saúde / *National Institute of Quality Control in Health*

i.p. - *intraperitoneal* / por via intraperitoneal

iSP - *immunoreactive P substance* / substância P imunorreativa

kg - *kilogram* / quilograma

LPS - *lipopolysaccharide* / lipopolissacarídeo

LTC_4 - *leukotriene C4* / leucotrieno C_4

M_3 - *muscarinic, cholinergic or acethylcholine receptor* / receptor muscarínico, colinérgico ou de acetilcolina

MCH - *mean corpuscular hemoglobin* / hemoglobina corpuscular média

MCHC - *mean corpuscular hemoglobin concentration* / concentração de hemoglobina corpuscular média

MCV - *mean corpuscular volume* / volume corpuscular médio

mEq - *milliequivalent* / miliequivalente

mg - *milligram* / miligrama

Mg^{2+} - *magnesium ion* / íon magnésio

min - *minutes* / minutos

mL - *milliliter* / mililitro

MPO - *myeloperoxidase* / mieloperoxidase

mRNA - *messenger RNA* / RNA mensageiro

NaCl - *sodium chloride* / cloreto de sódio

NaOH - *sodium hydroxide* / hidróxido de sódio

NMDA - *N-methyl-D-aspartate* / N-metil-D-aspartato

NSAID - *non-steroidal anti-inflammatory drug* / fármaco anti-inflamatório não esteroidal

p - *calculated probability* / probabilidade de significância

p.a. - para análise / *analytical grade*

PACAP - *pituitary-adenilate-cyclase-activating peptide* / peptídeo ativador de adenil ciclase hipofisária

PAR - *proteinase-activated receptor* / receptor ativado por proteinase

PBS - *phosphate buffered saline* / tampão salina fosfato

PGD - *prostaglandin* / prostaglandina

PGE_2 - *prostagladin* E_2 / prostaglandina tipo E_2

PGE_2R - *prostagladin* E_2 *receptor* / receptor para prostaglandina tipo E_2

pH - *hydrogenionic potential* / potencial hidrogeniônico

PLT - *platelet* / plaqueta

p.o. - *per os* / via oral

PVC - *polyvinyl chloride* / policloreto de vinila

RBC - *red blood cells* / eritrócitos

RENAMA - Rede Nacional de Métodos Alternativos / *National Network of Alternative Methods*

rpm - *rotations per minute* / rotações por minuto

SBCAL - Sociedade Brasileira de Ciência em Animais de Laboratório / *Brazilian Society of Laboratory Animal Science*

s.c. - *subcutaneously* / por via subcutânea

SEM - *standard error of the mean* / erro padrão da média

SNA - sistema nervoso autônomo / *autonomic nervous system*

SNC - sistema nervoso central / *central nervous system*

SNE - sistema nervoso entérico / *enteric nervous system*

SP - *P substance* / substância P

SRMD - *stress-related mucosal damage* / dano à mucosa relacionado ao estresse

TLR_4 - *toll-like receptor 4* / receptor “toll” do tipo 4

TNFα - *tumor necrosis factor alpha* / fator de necrose tumoral alfa

TPA - *12-O-tetradecanoylphorbol-13-acetate* / 12-O-tetradecanoilforbol-13-acetato

TRH - *thyrotropin-releasing hormone* / hormônio liberador de tireotrofina

$TRPV_1$ - *transient receptor potential vanilloid type 1* / receptor vanilóide $TRPV_1$

UNICAMP - Universidade Estadual de Campinas / *Campinas State University*

VIP - *vasoactive intestinal peptide* / peptídeo intestinal vasoativo

$VPAC_1$ - *vasoactive intestinal peptide receptor 1* / receptor para peptídeo intestinal vasoativo do tipo 1

WBC - *white blood cells* / leucócitos totais

SUMÁRIO

INTRODUÇÃO GERAL

O debate sobre o uso de animais na experimentação tem dividido opiniões há muitos séculos (Paixão, 2001). Sua relevância para o avanço da Medicina como a conhecemos hoje, contudo, é inegável: o aumento significativo na expectativa de vida de seres humanos e não humanos, a evolução constante de métodos de diagnóstico e a possibilidade de melhor qualidade de vida são alguns dos benefícios resultantes do esforço daqueles que foram pioneiros no estudo da anatomia e fisiologia (Marban & Gariepy, 1978).

Como todas as áreas do conhecimento humano, a ciência de animais de laboratório tem sofrido profundas modificações impulsionadas por estudos que alertam sobre a influência até mesmo de questões básicas de manejo sobre a fisiologia dos indivíduos. Se compararmos, por exemplo, as condições de manutenção dos biotérios da década de quarenta com as de hoje, a evolução é significativa (Hessler, 2000). No Brasil, a preocupação com o impacto dos procedimentos experimentais na qualidade dos resultados obtidos levou à criação, em 1983, pelo Prof. Dr. Fernando Sogorb Sanchis, do Colégio Brasileiro de Experimentação Animal (COBEA), que atualmente é denominado Sociedade Brasileira em Ciência de Animais de Laboratório (SBCAL). Nesta época já haviam sido estabelecidas normas para o uso de animais em pesquisa e ensino, com a Lei Federal n. 6.638, de 8 de maio de 1979.

A discussão ética envolvendo protocolos científicos e didáticos no Brasil ganhou impulso na década de 90, com a criação das primeiras Comissões de Ética no Uso de Animais (CEUAs). Em 2008, a Resolução n.879, instituída pelo Conselho Federal de Medicina Veterinária e Zootecnia, abordou a criação de (CEUAs) em instituições de ensino superior e pesquisa. Neste mesmo ano, foi sancionada a Lei 11.794, também conhecida como Lei Arouca, que estabelece regras para a criação e utilização de vertebrados para fins científicos e didáticos, prevendo penalidades administrativas em casos de maus-tratos e criando o Conselho Nacional de Controle de Experimentação Animal (CONCEA). Este, por sua vez, ligado ao Ministério da Ciência e Tecnologia, tem como competências "expedir e fazer cumprir normas relativas à utilização humanitária de animais com finalidade de ensino e pesquisa científica", "credenciar instituições brasileiras para criação ou utilização de animais em ensino e pesquisa científica", além de "monitorar e avaliar a introdução de técnicas alternativas que substituam o uso de animais em ensino e pesquisa". Através do Decreto n°. 6.899, de julho de 2009, foi criado o Cadastro das Instituições de Uso Científico de Animais (CIUCA), implementado pelo

Ministério da Ciência e Tecnologia e administrado pela Secretaria-Executiva do CONCEA (Fischer & Oliveira, 2012).

Apesar dos esforços das CEUAs e de pesquisadores preocupados com os aspectos éticos da pesquisa científica, ainda há muito o que ser feito no sentido de reduzir o sofrimento animal nos procedimentos experimentais e pedagógicos. Especificamente no campo da Farmacologia Experimental, observamos certa resistência em modificar protocolos que, em sua maioria, levam em conta apenas um aspecto farmacológico em estudo, ignorando a estreita relação entre os sistemas. Soma-se a isso a busca - positiva e natural do pesquisador - de eliminar o maior número possível de variáveis em seu experimento, rejeitando, muitas vezes, a inclusão de fármacos que possam ser utilizados como agentes supressores da dor ou sofrimento animal.

Assim como o ser humano deve ser considerado um universo complexo e não somente um aglomerado de órgãos e tecidos que funcionam simultaneamente de maneira isolada, os animais de laboratório podem nos fornecer respostas farmacológicas incompletas ou equivocadas, quando não consideramos todas as susceptibilidades dos organismos vivos. A psiconeuroimunoendocrinologia tem apontado para a necessidade de olharmos para os indivíduos, sejam eles animais humanos ou não humanos, como um todo, sujeitos a agentes modificadores de suas respostas fisiológicas efeitos tão sutis como o maior ou menor grau de luminosidade do ambiente ou o contato com um agente estressante (Padgett & Glaser, 2003).

Dentre os fatores estressantes mais frequentes na Farmacologia Experimental, a dor representa um importante viés na pesquisa sendo, na maioria das vezes, um subproduto não analisado. Definida como “um complexo conjunto de experiências sensoriais, emocionais e cognitivas causado por uma lesão tecidual real ou percebida e que se manifesta por determinadas reações autonômicas, fisiológicas e comportamentais” (Quintner et al., 2008), a dor é capaz de alterar as respostas farmacológicas além de elevar, de forma significativa, o desvio padrão de um experimento. Sendo assim, exceto em protocolos específicos para o estudo de substâncias-teste com possível atividade antinociceptiva, a presença do estímulo álgico é dispensável e prejudicial para a qualidade da pesquisa.

Seguindo esta lógica, foram selecionados para o presente projeto quatro modelos experimentais comumente utilizados para a triagem de novos compostos com atividades antissecretora gástrica, anti-inflamatória e anticâncer, propondo modificações nos protocolos originais, com o objetivo de reduzir o sofrimento animal sem prejuízo da resposta

farmacológica em casos em que não há necessidade de avaliação de possíveis efeitos antinociceptivos das substâncias-teste.

REVISÃO DE LITERATURA

1. Refinamento experimental

Propostos por Russell e Burch em 1959, o princípio dos 3 R's - em inglês *replacement* (substituição), *reduction* (redução) e *refinement* (refinamento) – trouxe para a ciência de animais de laboratório a discussão sobre termos como "humanitário" (*humanity*) e "não humanitário" (*inhumanity*). Dor, medo, ansiedade, sofrimento e estresse experimentados pelos animais passaram a ser considerados, em teoria, causadores de efeitos negativos, com grande impacto na pesquisa científica (Tannenbaum & Bennett, 2015). Tanto assim que foram incorporados à legislação para o uso de animais em pesquisa de vários países do mundo (Törnqvist et al., 2014).

Segundo seus idealizadores, **substituição** (*replacement*) consiste no uso de seres não sencientes em lugar de vertebrados. Dentre os organismos não sencientes estão incluídas as plantas, micro-organismos e endoparasitas que apresentam sistema nervoso primitivo. Originalmente, o termo definido por Russell e Burch referia-se apenas ao uso de seres não sencientes em substituição a seres sencientes, ou seja, substituição *relativa*. Substituição *absoluta* refere-se a situações em que não há necessidade de uso de quaisquer seres vivos (Russell et al., 1992). Nas últimas quatro décadas, o conceito de "testes alternativos" (Smith, 1978), obviamente desejável por permitir a substituição absoluta de animais, tem gerado divergências quanto à necessidade de validação de novos protocolos experimentais, em especial no que diz respeito à avaliação de risco de novas substâncias (Schiffelers et al., 2012; Presgrave et al., 2010; OECD, 2005; Balls, 1994). No Brasil, em 2011, foi criado o Centro Brasileiro de Validação de Métodos Alternativos (BraCVAM), vinculado ao Instituto Nacional de Controle de Qualidade em Saúde (INCQS), como resultado do Acordo de Cooperação Técnica entre a Fundação Oswaldo Cruz (FIOCRUZ) e a Agência Nacional de Vigilância Sanitária (ANVISA). No ano seguinte, ficou estabelecido que o processo de validação de métodos alternativos se daria no âmbito do BraCVAM, com a instituição da Rede Nacional de Métodos Alternativos (RENAMA) (Bones & Molento, 2012), o que torna promissora a contribuição do Brasil na aplicação cada vez mais ampla dos conceitos dos 3R's.

A **redução** (*reduction*), por sua vez, envolve o uso do menor número de animais *necessário à obtenção da informação desejada*. É importante frisar, entretanto, que simplesmente minimizar o número de animais de um dado experimento está longe do conceito de redução proposto por Russell e Burch. Neste aspecto, a escolha do melhor tratamento

estatístico torna-se imprescindível a fim de permitir o uso de um número de indivíduos *suficientes* para a significância dos dados obtidos e não o número *mínimo* de animais. Segundo eles, outro aspecto importante é que muitas vezes reduzir ao extremo o número de animais de um experimento pode infligir aos animais utilizados um grau maior de dor ou sofrimento, o que não seria considerado humanitário (Tannenbaum & Bennett, 2015; Parker & Browne, 2014).

Finalmente, o **refinamento** (*refinement*) consiste na diminuição na incidência ou severidade de procedimentos desumanos aplicados aos animais que ainda precisam ser usados na experimentação. Atualmente, muitos manuais para o uso de animais de laboratório definem refinamento como "eliminação ou redução da dor ou sofrimento animal". Todavia, vale ressaltar que incluídos nas definições de dor ou sofrimento estão "estados mentais" como medo, ansiedade, tédio, fome, sede, desconforto físico ou quaisquer outras sensações desagradáveis (Lidster et al., 2016; Tannenbaum & Bennett, 2015).

Mais uma vez, somos levados a considerar o que seria "agradável" para um animal de laboratório. Qual será, para um coelho, rato ou camundongo, a definição de "bem-estar"? Obviamente, como ainda não é possível obter respostas objetivas dos animais sobre o que lhes confere conforto, precisamos inferir tal conceito com base nas nossas próprias sensações.

Se a discussão sobre o que é "felicidade", "bem-estar", "prazer" para um animal de laboratório e até onde se deve ir ao aplicar formas de refinamento tem gerado debate entre profissionais conceituados (Carbone, 2015), há pontos básicos, indiscutíveis. Explorando a similaridade nas estruturas anatômicas a nível celular, não é problema, por exemplo, deduzir o sofrimento provocado por uma fratura exposta ou, ainda, da privação de líquidos ou isolamento por longos períodos de tempo.

Dentro dos aspectos elementares que compõem a rotina de manejo dos animais de laboratório mais comumente utilizados, há inúmeras possibilidades de refinamento, muitas delas já praticadas e difundidas, até mesmo nos países da América do Sul, onde a ciência de animais de laboratório é mais recente quando comparada àquela dos países europeus, por exemplo. De maneira superficial, contemplando tanto o biotério de produção animal como o de experimentação, é possível identificar alguns fatores que possuem influência direta ou indireta na fisiologia dos animais de laboratório e, portanto, podem – e devem – ser objeto de refinamento (Figura 1).

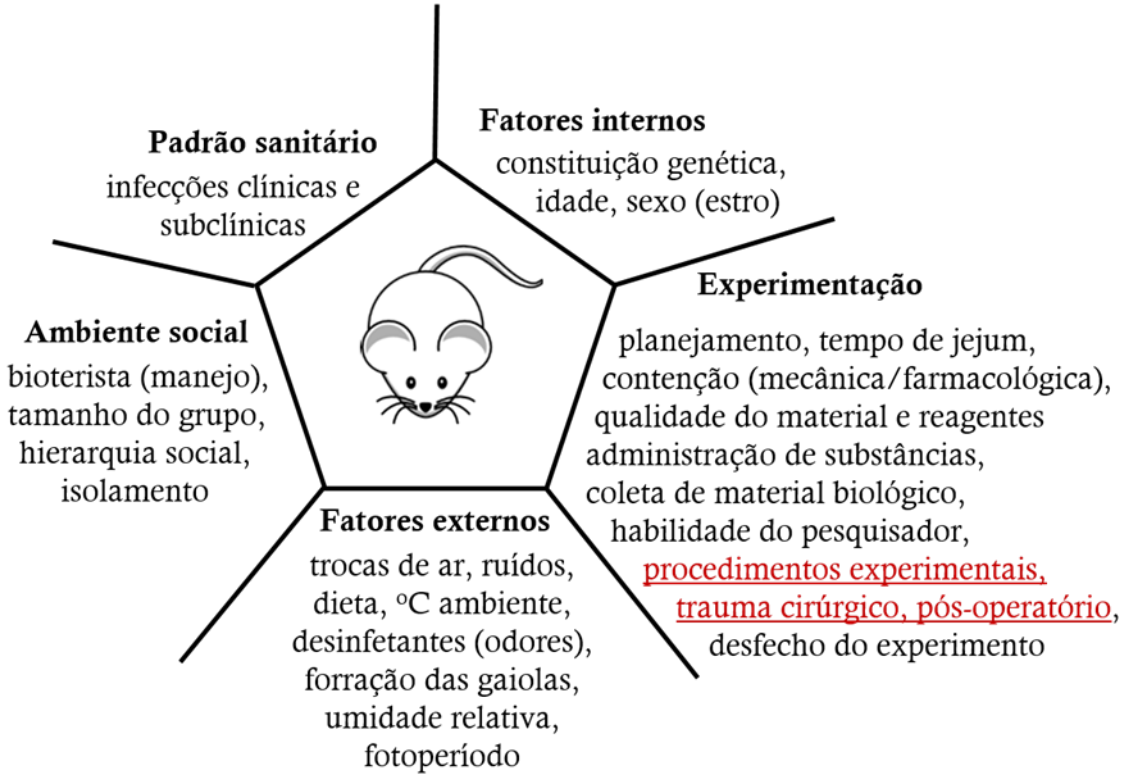


Figura 1. Fatores que influenciam direta ou indiretamente a resposta farmacológica/toxicológica do animal de laboratório (Monteiro, 2016).

Aplicar o conceito de refinamento no campo da Farmacologia Experimental, contudo, envolve modificar protocolos consagrados, inserir novas substâncias ou atualizar procedimentos, o que nem sempre é possível sem grande resistência (Stokes et al., 2009). Felizmente, o número de publicações relacionadas ao assunto tem crescido significativamente, abordando questões relacionadas não só ao enriquecimento ambiental, mas também a procedimentos experimentais, tais como a redução do tempo de jejum, uso de analgésicos, anestésicos e anti-inflamatórios, modificações de técnicas cirúrgicas visando reduzir o trauma cirúrgico, inclusão de cuidados pós-operatórios mais intensivos e recursos tecnológicos destinados à capacitação de pesquisadores e técnicos (Huang et al., 2016; Ciuffreda et al., 2014; Kappeler & Meaney, 2010). Além destes, são consideradas formas de refinamento experimental: ótimas condições de assepsia e antissepsia durante atos cirúrgicos e demais procedimentos que envolvam solução de continuidade da pele dos animais, aproveitamento do maior número de dados oriundos de um experimento (desde que, com isto, não haja maior sofrimento dos animais) (Carvalho et al., 2014), aplicar a “regra das três tentativas” quando estiver realizando um procedimento e, finalmente, antecipar o desfecho de um experimento,

sempre que possível. Com relação a este último item, o uso de Fichas de Avaliação Clínica Individual, adaptadas aos parâmetros avaliados em cada experimento específico, pode auxiliar na identificação de estados dolorosos nos animais (Wolfensohn & Lloyd, 2003).

2. Dor, estresse, distresse e eustresse

A subjetividade do termo "dor" está refletida em sua definição, segundo o comitê de taxonomia da *International Association for the Study of Pain (IASP)*: "experiência sensorial e emocional desagradável, que é associada ou descrita em termos de lesões teciduais" (Teixeira & Souza, 2001). O conceito de dor, portanto, está estreitamente ligado a aspectos psicológicos; nos seres humanos, o uso da palavra "dor" varia com "o aprendizado frente a experiências prévias" (Teixeira & Souza, 2001). Nos animais não humanos não é possível definir claramente os aspectos psicológicos e emocionais do estímulo doloroso. Entretanto, no presente trabalho, o termo "dor" será utilizado como equivalente à "nocicepção" que pode ser definida como a sequência de eventos neuronais que envolvem a codificação e o processamento do estímulo nocivo (Dubin & Patapoutian, 2010). Nocicepção relaciona-se com o estímulo proveniente de uma lesão, enquanto que dor é a "sensação percebida pelo indivíduo" (Figueiró et al., 2003).

Para fins didáticos, alguns autores apresentam três tipos de dor. A dor **nociceptiva**, resultante da lesão por calor, frio ou objeto contundente, possui um caráter "protetor" que demanda uma reação imediata, em virtude do reflexo de retirada que gera, da sensação desagradável e angústia que gera (Figura 2). A dor **inflamatória** é causada pela ativação do sistema imune como consequência de injúria tecidual ou infecção. Também possui um caráter protetor, uma vez que a hipersensibilidade presente nos tecidos inflamados desestimula o contato ou movimentação da região acometida (Figura 3). A dor **patológica**, por sua vez, é resultante do funcionamento anormal do sistema nervoso, podendo ser consequência de lesão do sistema nervoso (dor neuropática) ou ocorrer em condições onde não há quaisquer lesões ou inflamação (dor disfuncional) (Figura 4). A analogia feita por Woolf (2010) compara a dor a um alarme de incêndio: o tipo nociceptiva seria o acionamento correto e imediato pela presença de fogo; o tipo inflamatória seria a ativação pela elevação da temperatura e, finalmente, o tipo patológica, um falso alarme gerado pelo mau funcionamento do sistema.

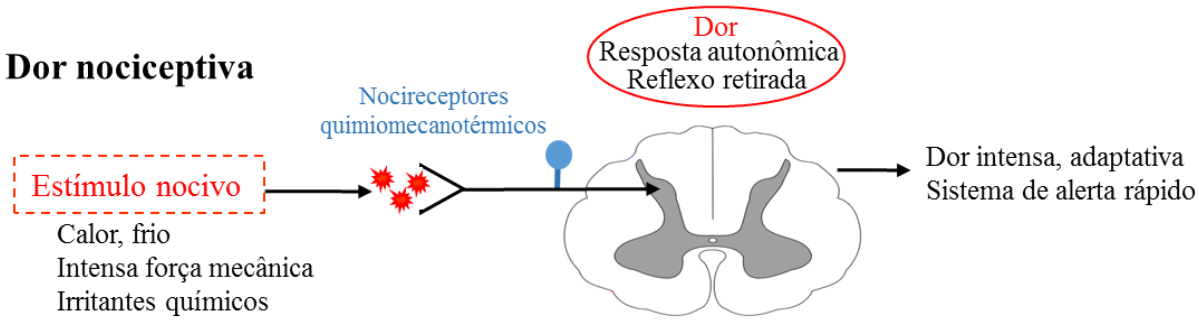


Figura 2. Dor nociceptiva: representa a sensação associada à detecção de estímulo nocivo potencialmente danoso (Woolf, 2010).

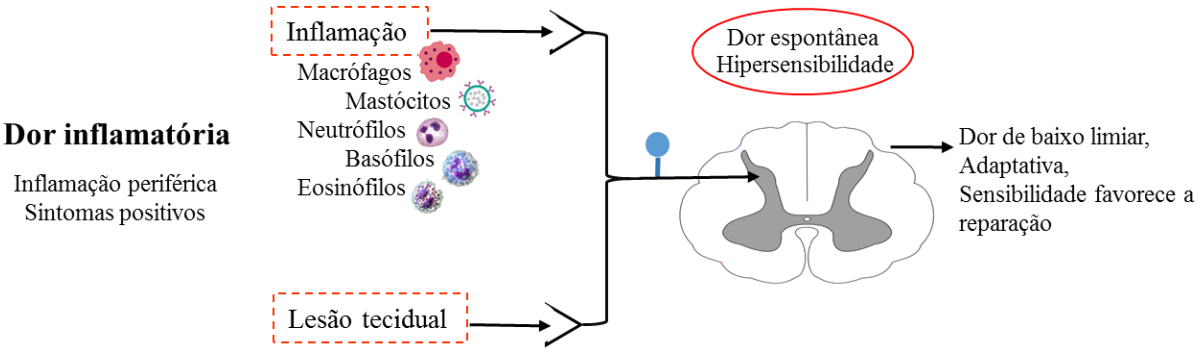


Figura 3. Dor inflamatória: associada à infiltração de células do sistema imune após lesão, facilitando o reparo tecidual por provocar hipersensibilidade até que haja completa neoformação da área lesionada (Woolf, 2010).

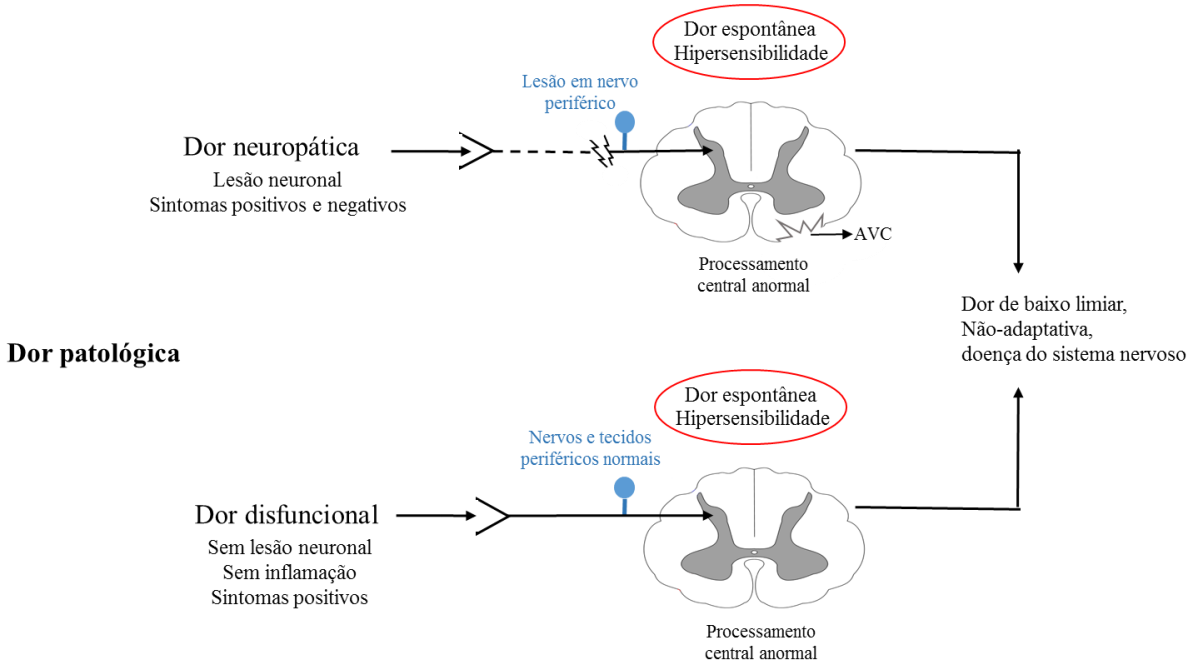


Figura 4. Dor patológica: causada por lesão a estruturas pertencentes ao sistema nervoso (neuropática) ou pelo seu mau funcionamento (disfuncional) (Woolf, 2010).

Uma das consequências do estímulo doloroso, independente do seu tipo, é a mobilização de regiões do sistema nervoso central (SNC), como o córtex cerebral onde ocorrem a percepção da sensação desagradável, a reação emocional e o comportamento motor. O hipotálamo, por sua vez, secreta moduladores de funções neurovegetativas (frequência cardíaca e respiratória), reações de agressividade, acesso ao sistema límbico (emoções) e, finalmente, reações neuroendócrinas que buscam mecanismos de adaptação ao **estresse**. A experiência dolorosa, fugaz ou constante, promove profundas modificações no indivíduo como um todo, corpo e emoções (Figueiró et al., 2003).

O termo **estresse**, apresentado à comunidade científica em 1936 por Hans Selye, identifica um conjunto de reações do organismo que resulta em alterações morfofuncionais como consequência da exposição a diversos agentes físicos e/ou biológicos (externos ou internos). Tais mudanças podem ter um caráter positivo, neutro ou negativo para o organismo em questão. Esta diferença deu origem aos conceitos de **eutresse** e **distresse** (Traian et al., 2013). **Eutresse** pode ser definido como conjunto de estímulos que desencadeiam respostas benéficas ao organismo em relação a aspectos do seu bem-estar físico e/ou reprodutivo a fim de manter a homeostase. O termo **estresse** (ou estresse neutro) refere-se a estímulos não lesivos

que despertam respostas sem influência negativa ou positiva em relação ao bem-estar ou reprodução do indivíduo. Finalmente, **distresse** representa estímulo que provoca respostas prejudiciais ao bem-estar, conforto e/ou reprodução, gerando, muitas vezes, patologias evidentes (Traian et al., 2013).

A busca da homeostase é objetivo final das respostas biológicas que envolvem o SNC, sistema nervoso autônomo (SNA), eixo hipotálamo-hipófise-adrenal (HPA), sistema imune e órgãos-alvo, como, por exemplo, pâncreas e rins, quando expostos ao estresse. Como consequência, respostas neuronais, bioquímicas, metabólicas, endócrinas, imunes e comportamentais fazem com que o cérebro, fígado, sistemas cardiovascular, gastrintestinal e imune sejam significativamente distintos no organismo sob estresse (Clark et al., 1997).

3. Dor, estresse e secreção gástrica

Em seres humanos, aspectos psicossociais das mais variadas origens possuem papel importante na etiologia dos distúrbios gástricos (Levenstein et al., 2015). O termo "dano à mucosa relacionado ao estresse" (*SRMD – stress-related mucosal damage*) tem sido utilizado para definir o quadro atribuído à lesão aguda e erosiva do trato gastrintestinal superior que, inicialmente assintomática e identificada incidentalmente durante procedimentos endoscópicos, pode levar à anemia por sangramento oculto e evoluir para a perda de sangue ostensiva (Plummer et al., 2014).

Em mamíferos, a comunicação entre cérebro e estômago, denominada de eixo-estômago-cérebro é bidirecional (Figura 5). A resposta neurobiológica acontece em três etapas: primeiro, o estresse é percebido e processado por centros nervosos cerebrais. Em seguida, o cérebro inicia uma resposta neuroendócrina através do eixo HPA e SNA. Finalmente, são disparados mecanismos de *feedback* a fim de restaurar a homeostase (Nardone & Compare, 2014).

No cérebro, através de circuitos nervosos subcorticais, o "estímulo estressante" induz neurônios efetores hipotalâmicos (localizados no núcleo para-ventricular) a liberarem o fator liberador de corticotrofina (*CRF – corticotropin release factor*). Presentes tanto no hipotálamo como no sistema nervoso entérico (SNE), os receptores para CRF, quando ativados, modulam respostas centrais e periféricas. No cérebro, produzem alterações comportamentais, neuroendócrinas, autonômicas, imunológicas e viscerais. Suas ações periféricas incluem atividade gastromioentérica; aumento da resposta inflamatória local e, consequentemente, da

permeabilidade vascular e redução da motilidade por inibição vagal gástrica, favorecendo a hipersensibilidade visceral, ou seja, o aumento da percepção à dor (Nardone & Compare, 2014). No que diz respeito à resposta inflamatória local, sabe-se que mastócitos possuem importante papel, uma vez que secretam mediadores pró-inflamatórios[1] além de possuir, em sua membrana, receptores para CRF, o que indica sua estreita relação com o estresse (Farhadi et al., 2007).

Dentre os fatores envolvidos na redução das defesas gástricas contra os danos causados pela secreção de ácido e pepsina estão a redução do aporte sanguíneo para a mucosa, diminuição na secreção de bicarbonato (HCO3), o aumento da secreção ácida aliado à sua maior difusão sobre a mucosa por redução da motilidade e, finalmente, menor taxa de proliferação celular resultando em comprometimento da renovação da mucosa gástrica.

Considerando que a fisiologia gástrica está sujeita a fatores biológicos, psicológicos e sociais, agentes capazes de alterar a percepção a dor como antidepressivos tricíclicos, antagonistas de receptor 5-hidroxitriptamina ($5\text{-}HT_3$) e análogos de somatostatina têm sido estudados como adjuvantes no tratamento de distúrbios gastrintestinais, porém ainda sem resultados conclusivos (Abdel-Sater et al., 2012; Takahashi et al., 2012).

Em seres humanos, há uma maior prevalência de patologias gástricas em pessoas divorciadas, separadas ou viúvas, o que corrobora a estreita relação entre a exposição ao estresse e o comprometimento das defesas naturais presentes no trato gastroduodenal contra a ação do ácido clorídrico e da pepsina (Nardone & Compare, 2014). Politraumatismos, queimaduras de grandes proporções, lesões do SNC, cirurgias de grande porte ou infecções severas são outros fatores que predispõe ao desenvolvimento de úlceras gástricas e duodenais por estresse (Dembiński et al., 2005).

[1] histamina, heparina, sulfato de condroitina, quimase, carboxipeptidase, triptase, fator de ativação plaquetária, prostaglanina (PGD2), leucotrieno (LTC4) e interleucinas, como IL-1β, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-13, IL-16, IL-18, IL-25, TNF-alfa, fator de estimulação de colônia de macrófago e granulócito (*granulocyte-macrophage colony-stimulating factor - GM-CSF*), entre outros.

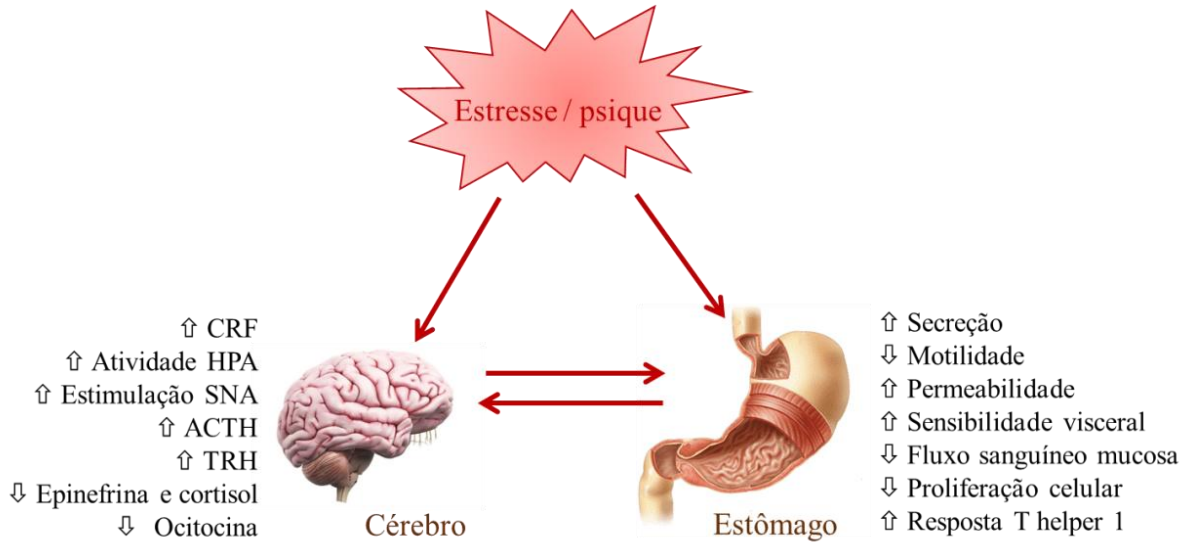


Figura 5. Efeitos do estresse e estado mental no eixo estômago-cérebro (Nardone & Compare, 2014). (Fator liberador de corticotropina: CRF; eixo hipotálamo-hipófise-adrenal: HPA; sistema nervoso autônomo: SNA; hormônio adrenocorticotrópico: ACTH; hormônio liberador de tireotrofina: TRH.)

O primeiro modelo experimental para estudo de atividade secretora gástrica foi descrito por Shay e colaboradores em 1945. Nesta época, o uso de anestésico era previsto apenas como forma de contenção (farmacológica) dos animais a fim de permitir a exposição do estômago e a ligadura do piloro. Quase 30 anos depois, os artigos científicos ainda eram publicados sem menção de quaisquer medidas analgésicas pós-operatórias no "modelo Shay" (Radwan & West, 1971). Finalmente, sete décadas após a primeira descrição do procedimento que permite a avaliação de substâncias gastroprotetoras, ainda é possível encontrar trabalhos publicados em revistas indexadas onde há menção à anestesia apenas durante o período transcirúrgico (Mard et al., 2016; Vijayakumar et al., 2016).

Partindo da premissa de que os ratos submetidos ao protocolo de ligadura de piloro experimentam intensa dor visceral durante o período pós-operatório, investigamos o efeito da redução do sofrimento animal, através da manutenção da anestesia geral durante todo o período de latência, sobre os parâmetros de interesse no referido modelo.

4. Dor, estresse e inflamação

A perda da homeostase tecidual como consequência de um estímulo lesivo tem como resultado imediato a dor periférica em decorrência da liberação local de diversos mediadores pró-inflamatórios e fatores de crescimento capazes de sensibilizar terminações nervosas sensitivas (Figura 6). Trata-se de uma complexa sequência de eventos que, uma vez desencadeada, promove alterações bioquímicas e estruturais no tecido acometido. Graças ao fenômeno pela primeira vez descrito pelos pesquisadores Melzack e Wall (1965) como 'plasticidade neural', sabemos que o sistema nervoso possui a capacidade de responder de formas distintas, de acordo com o estímulo. Hoje sabemos que a hipersensibilidade característica de estados inflamatórios é decorrente da ativação de receptores (como, por exemplo, vanilóides, ácido-sensíveis e purinérgicos), canais iônicos (sódio e cálcio) e transmissores (bradicinina, citocinas, prostaglandinas, fatores de crescimento e fatores neurogênicos), gerando a sensação dolorosa após estímulos que são, em situações normais, inócuos (Kidd & Urban, 2001).

A compreensão dos elementos envolvidos no processo inflamatório, independentemente de sua etiologia, advém tanto de observações clínicas como da exploração de modelos animais (Muley et al., 2016). Investigar o (s) possível (is) mecanismo (s) de ação anti-inflamatória de uma substância-teste através de modelos experimentais distintos é de extrema importância uma vez que diversos mediadores inflamatórios desempenham papel importante em outros processos patológicos como o câncer (Zhou et al., 2016; Vendramini-Costa & Carvalho, 2012).

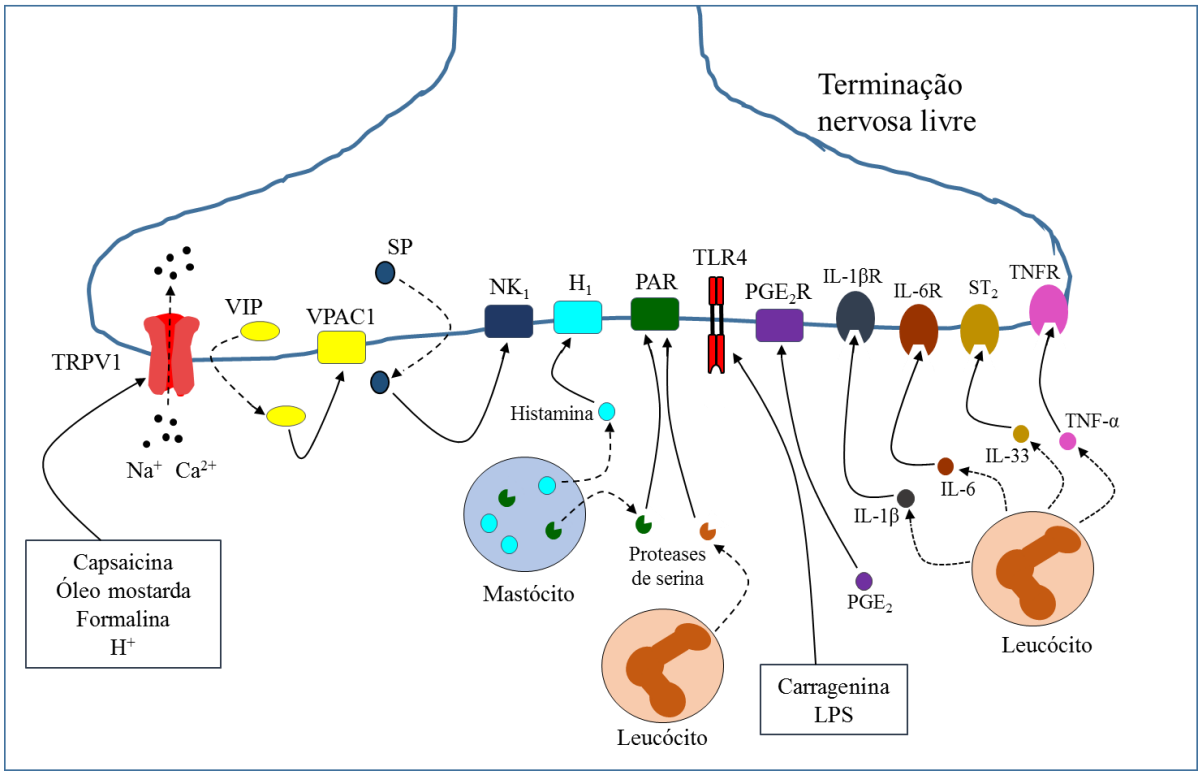


Figura 6. Representação esquemática da sensibilização de terminação nervosa livre por diversos mediadores inflamatórios e substâncias irritantes, tais como capsaicina, óleo mostarda, formalina e ácidos (H^+). O contato destes agentes irritantes aumenta a sensibilidade dos neurônios através da abertura de canais iônicos do tipo $TRPV_1$ (*transient receptor potential vanilloid type 1*). Como consequência, ocorre a liberação de neuropeptídeos como a substância P (SP) e o peptídeo intestinal vasoativo (VIP) que se ligam aos receptores neuroquinina-1 (NK_1) e $VPAC_1$ (*vasoactive intestinal peptide receptor 1*), respectivamente. Receptores H_1 neuronais são sensibilizados pela histamina liberada por mastócitos. Leucócitos e mastócitos liberam proteases de serina que, por sua vez, clivam diversos receptores PAR *(proteinase-activated receptors*) modulando nociceptores. Receptores do tipo TLR4 (*toll-like receptor 4*) podem ser ativados por substâncias exógenas como a carragenina e lipopolissacarídeos (LPS), enquanto que citocinas derivadas de leucócitos, como fator de necrose tumoral alfa (TNF-α), interleucina 1beta (IL-1β), interleucina 6 (IL-6) e interleucina 17 (IL-17) se ligam a seus respectivos receptores aumentando a transmissão da dor. Prostaglandinas E_2 (PGE_2) ativam principalmente receptores específicos (PGE_2R), elevando a sensibilidade de neurônios sensitivos a estímulos externos (adaptado de Muley et al., 2016).

Dentre os diversos modelos experimentais para estudo de atividade anti-inflamatória, o edema de pata induzido por carragenina é bastante utilizado na triagem de compostos, após resultados promissores em modelos *in vitro*. Este protocolo foi inicialmente proposto por Winter e colaboradores na década de 60 (Winter et al., 1963) e, desde então, sofreu poucas modificações metodológicas. A carragenina representa um grupo de polissacarídeos obtidos da alga *Chondrus crispus*, com características de gel e que, quando inoculada no tecido subcutâneo da pata do animal de laboratório, é capaz de gerar intensa resposta inflamatória: aumento gradual do volume (edema), vermelhidão (eritema), elevação da temperatura local (hipertermia) e dor/aumento da sensibilidade local (hiperalgesia). Graças à liberação de mediadores pró-inflamatórios, aproximadamente cinco horas após a inoculação do agente flogístico no coxim plantar de ratos, o aumento de volume atinge seu nível máximo, sendo seguido por uma redução gradual (Morris, 2003). A medição do volume da pata do animal a intervalos regulares permite o estudo dos diferentes mediadores envolvidos no processo inflamatório, em contraste com os primeiros modelos experimentais, pioneiros, nos quais a atividade farmacológica de uma substância-teste era avaliada através da sua capacidade de inibição de alguns dos sinais cardinais: edema, eritema, hipertermia e hiperalgesia causados pela introdução de pequeno rolo de algodão no tecido subcutâneo do dorso do roedor (Meier et al., 1950).

A Farmacologia Experimental dispõe de inúmeros modelos onde há intensa resposta inflamatória em roedores e que são utilizados para avaliar uma possível atividade sobre a nocicepção/dor inflamatória como, por exemplo, o teste de contorção abdominal induzido por ácido acético e o teste da formalina em pata de camundongo. Em alguns modelos, é possível estudar tanto a dor inflamatória como outros aspectos como edema, migração leucocitária e/ou dosagem de citocinas e marcadores específicos como, por exemplo, nos modelos de edema induzido por carragenina, granuloma induzido por rolo de algodão, edema de pata induzido por TPA (*12-O-tetradecanoylphorbol-13-acetate*) e pleurisia por azul de Evans (Lopes et al., 2013; Spindola et al., 2010; Cavaller-Machado et al., 2008).

Desde a demonstração científica da inibição da cascata flogística por drogas anti-inflamatórias não esteroidais (*non-steroidal anti-inflammatory drug - NSAID*) em 1963 por Winter e colaboradores, o modelo de edema de pata tem sido também utilizado para estudo de mecanismos de ação (Tavares et al., 2013). Vale ressaltar que existem situações nas quais o referido modelo experimental de edema de pata por carragenina também serve como ferramenta de estudo para possíveis propriedades antinociceptivas de uma dada substância-teste

(“hiperalgesia mecânica induzida por carragenina”) (Servat et al., 2012). Em tais casos, por razões óbvias, o uso de agentes supressores da dor como forma de refinamento não é possível, sendo necessárias outras formas de redução do sofrimento animal. Todavia, nos casos em que o efeito antinociceptivo da substância-teste não será avaliado, a dor inflamatória representa um subproduto que pode – e deve – ser removido do experimento, não só por motivos éticos como em prol da qualidade dos dados gerados (Kidd & Urban, 2001).

No presente projeto, a tentativa de reduzir o sofrimento animal e, se possível, a variabilidade intragrupo envolve duas abordagens distintas: anestesia geral e bloqueio nervoso local. Em ambos os casos, serão avaliados os efeitos da supressão da dor inflamatória nos demais parâmetros de interesse em modelo de edema de pata por carragenina em ratos.

5. Dor, estresse e câncer

Em seres humanos, processos dolorosos constantes, ainda que de média intensidade, são responsáveis por quadros de ansiedade e depressão (Barry et al., 2016), podendo, inclusive, promover alterações cerebrais estruturais como atrofia da substância cinzenta no córtex temporal inferior (Niddam et al., 2016; May, 2008), área relacionada ao estímulo visual, porém sob influência do processamento cognitivo e afetivo. Estudos demonstram que, mesmo em situações que não envolvem quaisquer estímulos nociceptivos, mas onde há situações de estresse, como na contenção mecânica ou isolamento, animais de laboratório apresentam maior desenvolvimento tumoral, bem como aumento da incidência de metástases (Thaker et al., 2006).

Dentre as diversas patologias que merecem atenção especial da comunidade científica, o câncer é, certamente, aquela capaz de provocar dores de altíssima intensidade que comprometem severamente a qualidade de vida de um indivíduo (Amiel et al., 2016; Lahoud et al., 2016; Falk et al., 2014). A neurofisiologia da dor oncológica envolve aspectos inflamatórios, neuropáticos, isquêmicos e compressivos (Raphael et al., 2010; Saunders & Booth, 2003). Os mecanismos patofisiológicos responsáveis pelos estímulos álgicos no câncer podem estar relacionados a invasão tecidual local pelo tumor, dor óssea metastática, osteoporose, degeneração articular, neuropatia induzida por quimioterapia, obstrução visceral, compressão de nervos, invasão de plexos, isquemia, artropatia e neuropatia paraneoplásicas, radionecrose e inflamação pós-cirúrgica ou estimulada pela presença tumoral (Raphael et al., 2010).

A estreita relação entre câncer e inflamação resulta, entre outros aspectos, da liberação local de fatores de crescimento, citocinas, interleucinas, quimiocinas, prostanóides e endotelinas que alteram o ambiente tumoral, acidificando o pH tecidual (Falk et al., 2014; Vendramini-Costa & Carvalho, 2012). Mais especificamente nos casos de metástases ósseas ou tumores ósseos primários, as alterações provocadas pelas células tumorais promovem lesões em terminações nervosas localizadas no periósteo, sendo responsáveis por dores de alta intensidade (Kang et al., 2016). Este estímulo periférico desencadeia uma hipersensibilidade dos neurônios sensitivos medulares e, muitas fibras sensibilizadas levam a informação dolorosa até partes do cérebro envolvidas na resposta emocional à dor (De Felice & Ossipov, 2016; Ossipov et al., 2010).

Outro desafio que o paciente oncológico enfrenta é a dor causada pelo tratamento. A neuropatia periférica causada pela quimioterapia em geral acomete entre 30-50% dos pacientes e evolui gradativamente com a repetição dos ciclos de administração dos fármacos, atingindo inicialmente os membros inferiores e avançando para as mãos (Izicky et al., 2016; Schloss et al., 2016). A permanência da chamada "dor basal" (*background pain*) representa um fator debilitante a mais no seu quadro geral, uma vez que o mantém, com muita frequência, num quadro de prostração e inapetência.

A dor oncológica, em todas as suas formas, representa, portanto, um elemento de suma importância no prognóstico do paciente. Tanto assim, que grande parte dos hospitais oncológicos mantém profissionais especializados no tratamento deste sintoma (Expósito Vizcaíno et al., 2016; Williams et al., 2016). Considerando o impacto inegável do estresse causado pela dor e demais sintomas indesejáveis que enfrenta o paciente oncológico, é natural surjam alguns questionamentos ao considerarmos certos modelos de câncer experimental: (a) A exemplo do que ocorre tanto na Medicina Humana como na Medicina Veterinária de animais de companhia, que medicação de apoio (*support care*) oferecemos aos nossos animais de laboratório portadores de câncer experimental para que possam responder adequadamente ao tratamento proposto? (b) Será que nossos animais de laboratório têm condições de se alimentar adequadamente, considerando os inúmeros efeitos adversos da quimioterapia? (c) Dada a individualidade da resposta ao estresse, provocado pela dor ou por quaisquer outros agentes, quão grande é a variabilidade da resposta farmacológica que obteremos em nosso modelo experimental?

Sendo assim, no presente projeto propomos a introdução de enriquecimento ambiental e administração regular de analgésico opióide nos modelos experimentais de Ehrlich

sólido de flanco e pata, respectivamente, a fim de reduzir os efeitos deletérios causados pela dor e/ou desconforto dos animais experimentais.

OBJETIVOS

1. OBJETIVO GERAL

O presente projeto de doutoramento tem como objetivo geral selecionar os agentes supressores da dor/estresse mais adequados aos modelos experimentais, visando a redução do sofrimento animal sem prejuízo da resposta farmacológica.

2. OBJETIVOS ESPECÍFICOS

Os objetivos específicos deste trabalho estão relacionados com os diferentes modelos experimentais escolhidos, a saber:

2.1. Investigar a possibilidade de refinamento através do uso de anestésico em modelo de ligadura de piloro em ratos, sem que haja comprometimento dos parâmetros de interesse no estudo da secreção ácida gástrica.

2.2. Investigar a possibilidade de refinamento através do uso de anestésico em modelo de edema de pata em ratos, eliminando a intensa dor causada pelo agente flogístico sem que haja prejuízo dos parâmetros de interesse no estudo da atividade anti-inflamatória.

2.3. Investigar a possibilidade de refinamento através do uso de enriquecimento ambiental ou analgesia em modelos de tumor sólido de Ehrlich em flanco e em pata em camundongos, reduzindo o estresse e/ou a dor produzidos pelo desenvolvimento tumoral, sem prejuízo dos parâmetros de interesse no estudo da atividade anti-inflamatória.

CAPÍTULO I

CHARACTERISATION OF A REFINEMENT OF THE "PYLOROUS LIGATION" MODEL OF RAT GASTRIC ULCERATION RESULTING IN "NO PAIN" AND A MORE SPECIFIC PHARMACOLOGICAL RESPONSE

Reproduced from Monteiro KM, Spindola HM, Possenti A, Tinti SV, Ruiz ALTG et al. Characterization of a refinement of the "pylorous ligation" model of rat gastric ulceration in "no pain" and a more specific pharmacological response. *J Pharm Tox Met* 2013; 67: 121-128, with permission from Elsevier.

ABSTRACT

Introduction: The pharmacological assessment of the factors for gastric protection of a test substance should involve experimental models that can determine the involvement of cytoprotective factors, as well as their influence on the secretion of hydrochloric acid. The original protocol of pylorus ligation in rats proposed by Shay et al. In 1945, still today, provides a latency time of 240 min without considering the effect of postoperative pain in the mechanisms of peptic ulcer. This paper proposes a modification of this experimental protocol by eliminating the pain throughout the postoperative period, as a refinement of the test with consequent improvement of the pharmacological response. **Methods:** Adult male HanUnib:WH (Wistar/Uni) rats underwent surgical ligation of the pylorus and were kept anesthetized throughout the experimental period (4h) in contrast to the other experimental groups that followed the original protocol proposed by Shay et al., 1945. **Results:** We were able to determine effective doses for a positive control, as well as of a variety of secretagogues in the new experimental protocol proposed. **Discussion:** The supression of post-surgical pain, through the use of anesthesia throughout the experimental period, brought several benefits for the study of gastric acid secretion, rendering in a more homogeneous pharmacologic response in non-inbred animals, thus being an effective experimental procedure.

Keywords: Anesthesia. Gastric secretion. Pylorus ligation. Rats. Refinement. Stress.

1. Introduction

The study of substances with possible gastric protective action involves, in a logical sequence, the determination of an effective dose of the prospective drug in an experimental model of ulcers induced by various agents such as ethanol, NSAIDs and stress. Once an effective dose is determined, it is required to identify the possible mechanisms involved in the anti-ulcerogenic action observed, considering peptic ulcer to be the result of an imbalance between aggressive factors (HCl, pepsin, *Helicobacter pylori* infection) and defense factors (blood supply, mucus, bicarbonate, prostaglandins, non-protein sulfhydryl substance and nitric oxide). In addition to identifying new treatment options for peptic ulcer, the use of appropriate experimental models that evaluate the action of drugs on gastric secretion allows an understanding of the mechanisms involved in this process. Such pharmacological models represent essential research tools, as the chemical mediators of gastric ulceration, such as histamine and acetylcholine, participate in various activities throughout the body (Carvalho, 2006; Mózsik, 2010).

In the history of the study of gastric disorders, acid secretion was designated in 1910, by researcher Karl Schwartz, as a key factor in the emergence of gastric ulcer, becoming known as the "no acid, no ulcer" dogma (Toneto, Oliveira & Lopes, 2011). Despite significant advances in the understanding of the etiology of gastric ulcer, a number of factors still remain unknown, such as gastric acid levels, that vary in different stages of gastric diseases and how, in many cases, the majority of patients suffering from this disorder have normal, or even below normal, secretion of hydrochloric acid and pepsin (Ghosh, Lewis, Axon & Everett, 2011). The discovery of *H. pylori* as a potential moderating etiologic agent has not answered all of the questions relating to the pathogenesis of this debilitating gastric disease (Uyanikoğlu et al., 2012). Thus, acid regulating drugs are still treatment noteworthy, being the most frequently used in the treatment of gastritis and ulcer.

Among the mechanisms that influence gastric acid secretion, stress has long been exploited in pharmacological tests with rats. We can find in the literature experimental models inducing stress by cold, restraint, or by immersion in water, where the result is an acute, gastric mucosal, lesion (Grandi et al., 2007; Padol, Wang & Hunt, 2012; Pavel, Saavedra, 2008). In humans, stress ulcer of the gastric mucosa can develop within hours following burns, polytraumas, CNS injury, shock, major surgery, or severe infections (Dembiński et al., 2005). "Pain" is usually described as a transient negative feeling in consequence of a noxious stimulus, such as a somatic, or visceral, wound (Spindola et al., 2011). Starting from the premise that

pain itself is a stressor, and that stress causes proven effects on gastric acid secretion, we proposed a modification on the experimental model of pylorus ligation in rats, in order to minimize animal suffering and improve pharmacological response, by eliminating post-operative pain through the use of prolonged anesthesia throughout the experimental period.

The aim of the study was to compare the effect of stress from post-surgical pain in the experimental protocol of gastric acid secretion evaluation in rats, proposing a new protocol with minimal animal suffering.

2. METHODS

2.1. Animals

Healthy adult (250-350g) male HanUnib:WH (Wistar) rats (*Rattus norvegicus*) (278 in total), 5 per cage, acquired from the Multidisciplinar Center for Biological Investigation on Laboratory Animal Science (CEMIB), University of Campinas (UNICAMP, São Paulo, Brazil), were used just once on each experiment. During the maintenance period (at least seven days prior to the commencement of the study), all animals were group housed in polycarbonate cages, under a climate-controlled environment (22ºC ± 3ºC and relative humidity 30-70%) and a 12-hour light/dark cycle.

The animals were submitted to an unlimited supply of conventional standard laboratory diet (Nuvilab®, from Nuvital Nutrients, Curitiba, Brazil) and tap water. Animal's welfare guidelines were adopted for all studies (Guide for the Care and Use of Laboratory Animals, 2000; International Guiding Principles for Biomedical Research Involving Animals, 1985). The protocols employed are in agreement with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (# 2630-1) and the work was approved by the university's animal experimentation ethics committee.

2.2. Materials

Cimetidine (SIGMA Chem. Co. – USA), histamine (SIGMA), pentagastrin (SIGMA), bethanechol (SIGMA), were all reagent grade (p.a.). Thiopental (Thiopentax – Cristália, São Paulo, Brazil).

2.3. Surgical procedure

The animals were fasted for 12 hours before surgery, but with access to water *ad libitum*. All animals were anesthetized with sodium thiopental (50 mg/Kg, i.p.) and trichotomized in the abdominal region. After incision in the epigastric region of approximately 1.5 cm to expose the stomach, we performed the pylorus ligation as described by Shay et al., 1945. We then performed intra-duodenal administration of cimetidine in groups 1 and 2. The surgical wounds were then sutured with non-absorbable, sterile, suture (Brasuture NPA345 nylon monofilament 2.0). Groups 2 and 4 were kept under anesthesia throughout the experiment (240 minutes), while groups 1 and 3 did not receive another dose of anesthetic in the postoperative period, as described in the original Shay et al. (1945) protocol. To assess stimulated gastric acid secretion, the animals were subcutaneously injected with secretagogue (chemical, source and address) corresponding to three dose levels one hour after surgery. Four hours after surgery, all animals were euthanized by overdose of sodium thiopental anesthesia.

2.4. Experimental Delimitation

Thirty-two experimental groups, consisting of six to eight animals each, were included in the study.

Evaluation of secretion: four experimental groups (n= 6-8 rats/group) were included in the assessment of secretion. Two were negative controls (groups 1 and 2), which were intraduodenally injected with 0.9% NaCl solution (2 mL/Kg; pH = 7.0), immediately after the pylorus ligation, and two, positive control groups (groups 3 and 4), which were intraduodenally injected with cimetidine (100 mg/Kg dose; 2 mL/Kg) immediately after the pylorus ligation. Groups 2 and 4 were kept under anesthesia throughout the experiment, while groups 1 and 3 did not receive another dose of anesthetic, therefore remaining awake for the entire period of latency. In this step, two latency times were evaluated, namely, 2 and 4 hours after surgery after which the animals were sacrificed by overdose of sodium thiopental.

Assessment of histamine-stimulated secretion: 1 h after the intraduodenal administration of 0.9% NaCl solution, eight experimental groups (n= 6-8 rats/group) were included in the assessment of gastric secretion after stimulation by histamine, with two negative control groups (groups 1 and 2), as indicators of basal secretion, and six groups that were subcutaneously injected with histamine at the interscapular region (3 mg/Kg for groups 3 and 4; 10 mg/Kg, groups 5 and 6; 30 mg/Kg, groups 7 and 8, respectively; dose volume 2.5 mL/Kg). Groups 2, 4, 6 and 8 were kept under anesthesia throughout the experiment, while groups 1, 3,

5 and 7 did not receive another dose of anesthetic, therefore remaining awake for the entire period of latency until killed at the end of the experiment by overdose of sodium thiopental.

Assessment of pentagastrin-stimulated secretion: 1 h after the intraduodenal administration of 0.9% NaCl solution, eight experimental groups (n= 6-8 rats/group) were included in the assessment of the gastric secretion after stimulation by pentagastrin, with two negative control groups, (groups 1 and 2), as indicators of "basal" secretion, and six groups that were subcutaneously injected with pentagastrin at the interscapular region (0.1 mg/Kg for groups 3 and 4; 0.3 mg/Kg, groups 5 and 6; 1 mg/Kg, groups 7 and 8, respectively; dose volume 2.5 mL/Kg). Groups 2, 4, 6 and 8 were kept under anesthesia throughout the experiment, while groups 1, 3, 5 and 7 did not receive another dose of anesthetic, therefore remaining awake for the entire period of latency until sacrificed by overdose of sodium thiopental.

Assessment of bethanechol-stimulated secretion: 1 h after the intraduodenal administration of 0.9% NaCl solution, eight experimental groups (n= 6-8 rats/group) were included in the assessment of the gastric secretion after stimulation by bethanechol, with two negative control groups (groups 1 and 2), as indicators of "basal" secretion, and six groups that were subcutaneously injected with bethanechol at the interscapular region (1 mg/Kg for groups 3 and 4; 5 mg/Kg, groups 5 and 6; 25 mg/Kg, groups 7 and 8, respectively dose volume 2.5 mL/Kg). Groups 2, 4, 6 and 8 were kept under anesthesia throughout the experiment, while groups 1, 3, 5 and 7 did not receive another dose of anesthetic, therefore remaining awake for the entire period of latency until sacrificed by overdose of sodium thiopental.

Second bethanechol dose-response curve: 1 h hour after the intraduodenal administration of 0.9% NaCl solution, four experimental groups (n=6-8 rats/group) were included in the second assessment of the gastric secretion after stimulation by bethanechol, with one "basal" secretion control group (group 1), which did not receive secretagogue, and three experimental groups that were subcutaneously injected with 0.125, 0.25 and 0.5 mg/Kg of bethanechol (dose volume 2.5 mL/Kg), respectively. In this experiment, all experimental animals were kept anesthetized until the time of euthanasia when they were sacrificed with overdose of sodium thiopental.

The monitoring of the anesthesia was performed by evaluating the signs of alertness, such as reflex of the tail, palpebral and corneal reflex, as well as loss of righting reflex.

2.5. Analysis of stomach contents

Four hours after pylorus ligation, all animals were euthanized by overdose of anesthesia. Removal of the stomach was performed after clamping the esophagus to avoid loss of stomach contents. The serosal surface of the stomach was washed with distilled water, dried with gauze and opened along the lesser curvature. The mucosa was washed with 2 ml of distilled water (pH 7.0), collecting the gastric contents into test tubes for centrifugation (2500 rpm for 10 minutes). After centrifugation, the supernatant was transferred to a glass beaker (15 mL) for the quantification of gastric secretion volume. The final volume was then normalized to 10mL with distilled water, and pH measured with a pH meter (Micronal mod. B474, Micronal S/A, São Paulo, SP) to evaluate free acidity. The determination of total acidity (mEq[H+]/mL/4h) was performed by simple titration with 0.1N NaOH, using 2% phenolphthalein as acid-base indicator (Lapa, Souccar, Lima-Ladman & Lima, 2002).

2.6. Statistical analysis

The determination of differences between experimental groups for all parameters studied was performed by analysis of variance (ANOVA) with Duncan post-test, assuming the probability of error of 5% ($p<0.05$).

3. RESULTS

3.1. Latency time

Animals allowed to recover from the anesthetic showed no difference between saline and cimetidine treatments in terms of free acidity, volume of gastric secretion, or in pH (Fig. 1a-c).

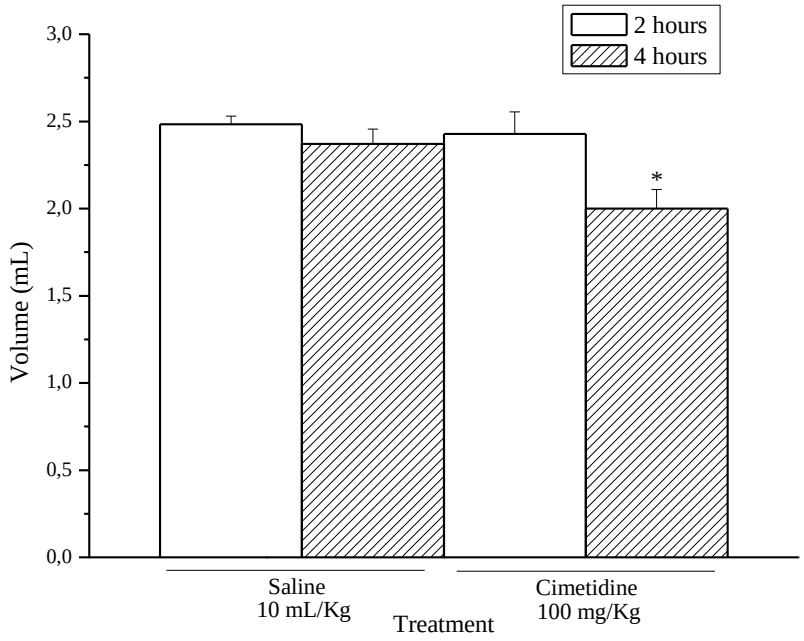
When kept under anesthesia throughout the postoperative period, there was a statistically significant decrease from the negative control groups (0.9% NaCl solution) in those rats given cimetidine at 100 mg/Kg in relation to free acidity ($p<0.05$) (Figure 1c). Additionally, there was a statistically significant decrease in the volume of gastric secretion and a statistically significant increase in the pH but only after 4 h of pylorus ligation (Fig. 1a and b).

3.2.Gastric secretion

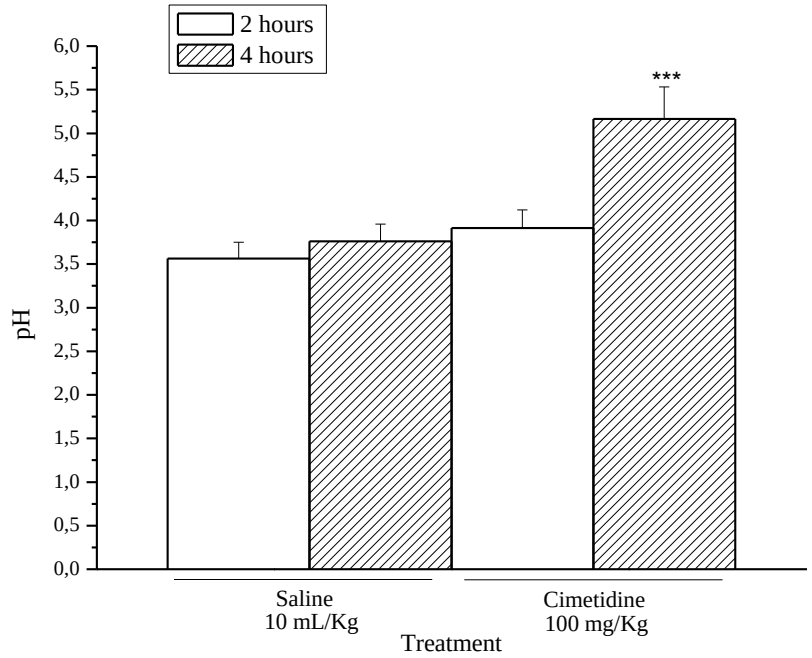
Rats maintained under anesthesia throughout the experiment and given saline showed statistically lower values for free acid (Fig. 2c), and volume of gastric secretion (Fig. 2a) from rats allowed to recover following duodenal ligation (Table 1).

Rats allowed to recover following duodenal ligation showed a statistically significant decrease in gastric free acid and a statistically significant increase in pH whereas rats kept anesthetized throughout the experiment showed lower volumes of gastric secretion when given saline than the unanesthetized rats, and following cimetidine the volume fell even lower (Fig. 2b; Table 1). A similar result was also seen with free acid where a statistically significant cimetidine-induced depression was observed following lower basal levels. While there was no statistically significant difference in basal levels of pH between anesthetized and recovered rats, in the former group there was a statistically significant increase in pH following cimetidine treatment which was greater than that seen in the unanesthetized rats (Fig. 2b; Table 1).

After 4 h of latency, the animals kept under anesthesia until the time of euthanasia showed more homogeneous values (lower standard deviation), in the three parameters analyzed, when compared with those who were only anesthetized during surgery (Fig. 2 and Table 1).



a)



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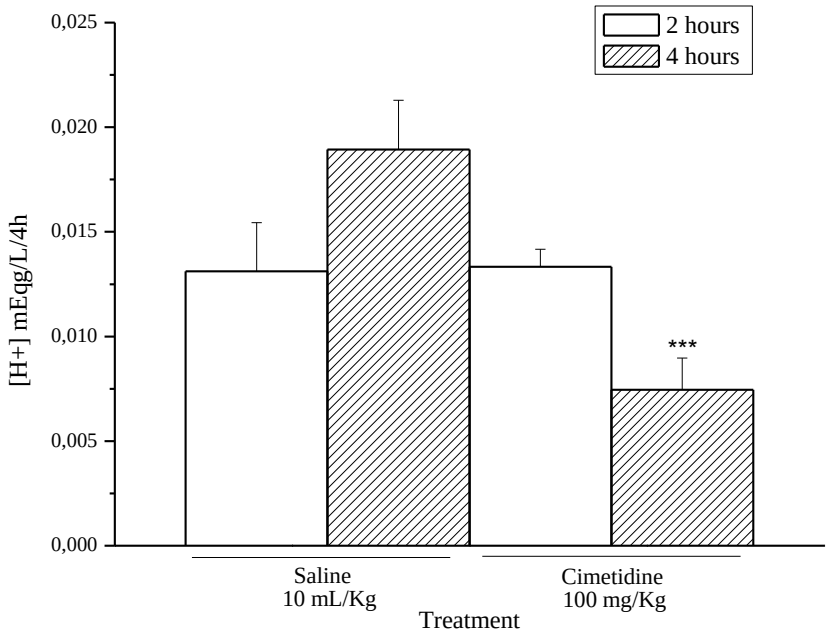


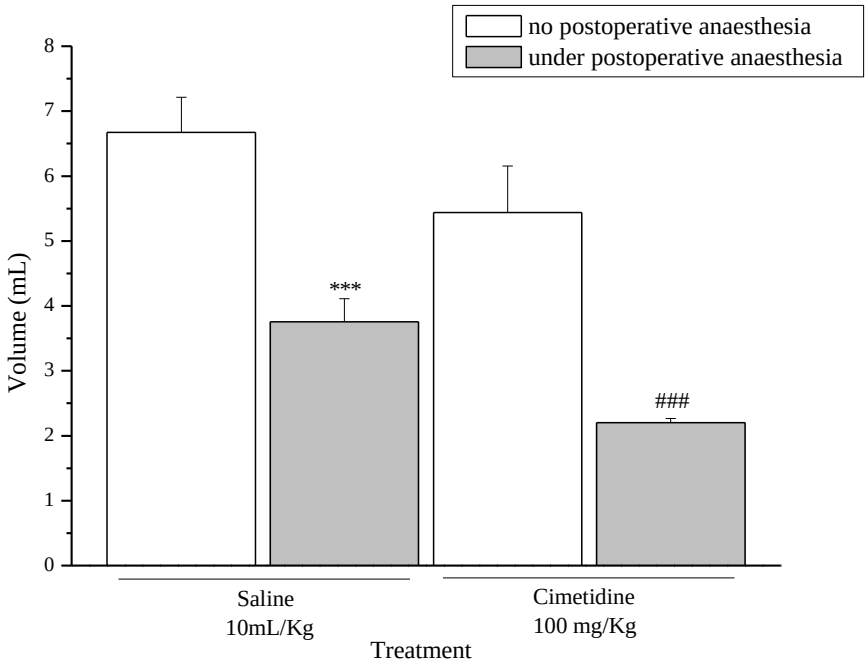
c)

Fig. 1. Comparison between different latency times in pylorus ligation experiment in anesthetized male HanUnib:WH (Wistar/Uni) rats. Parameters analyzed: volume of gastric secretion (a), pH (b) and free acidity (c). Open bars = rats allowed to recover from anesthetic; hatched bars = anesthetized rats. Values are expressed as mean ± error. ($ANOVA_{(volume)}$ $p<0.05$; $ANOVA_{(pH)}$ $p<0.001$; $ANOVA_{([H+])}$ $p<0.05$ Duncan's Test $*p<0.05$ $***p<0.001$ when compared to saline 4h).

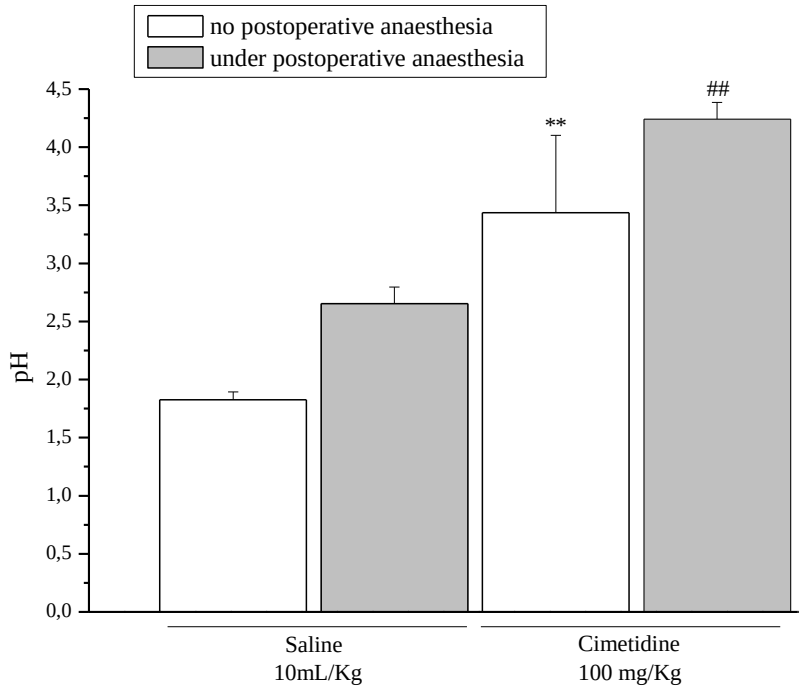
Table 1. Effect of postoperative pain on volume (pH), pH (b), and [H+] (c) of gastric secretion of HanUnib:WH (Wistar/Uni) rats in pylorus ligation experiment.

Experimental group	Parameter analyzed		
	Volume (mL)	**pH**	**[H+] (mEqg/L/4h)**
Negative control without anesthesia	6,675 ± 1,525	1,825 ± 0,197	0,057 ± 0,016
Negative control with anesthesia	3,757 ± 0,931***	2,653 ± 0,379	0,021± 0,007***
Positive control without anesthesia	5,437 ± 2,030	3,436 ± 1,886**	0,029 ± 0,018***
Positive control with anesthesia	2,200 ± 0,193***	4,242 ± 0,405***	0,006 ± 0,002***

Values are expressed as mean ± error. ($ANOVA_{(volume)}$: $p<0,001$; $ANOVA_{(pH)}$: $p<0,001$; $ANOVA_{([H+])}$: $p<0,001$. Duncan's test ***$p<0,001$).



a)



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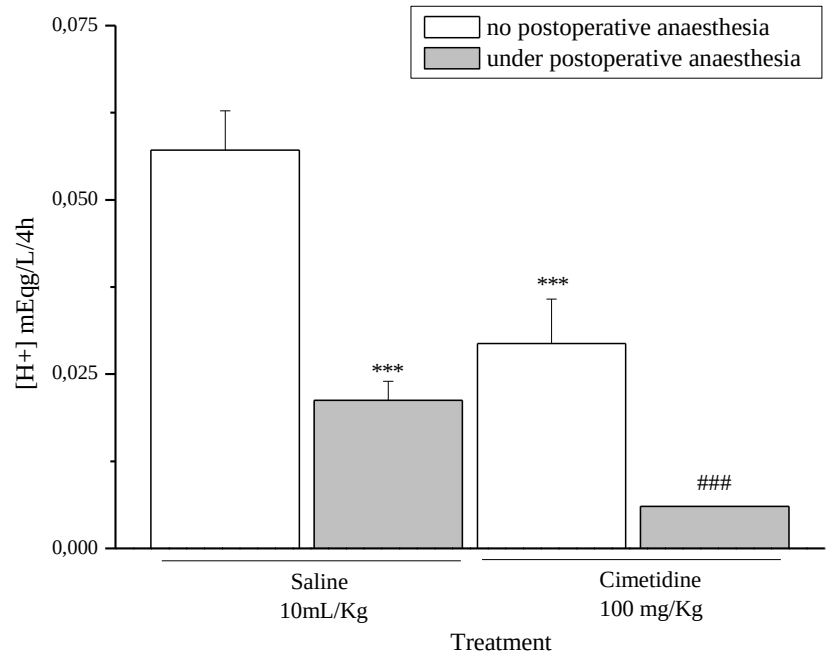


c)

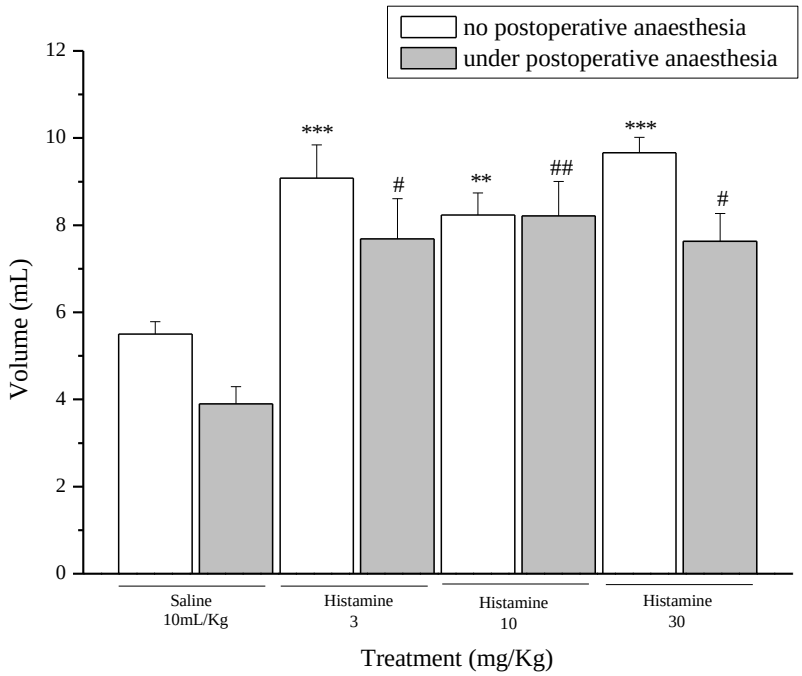
Fig. 2. Effect of postoperative pain on volume (a), pH (b), and [H+] (c) of gastric secretion of male HanUnib:WH (Wistar/Uni) rats in pylorus ligation experiment. Values are expressed as mean ± error. ($ANOVA_{(volume)}$: $p<0.001$; $ANOVA_{(pH)}$: $p<0.001$; $ANOVA_{([H+])}$: $p<0.001$. Duncan's test $^{**}p<0.01$ $^{***}p<0.001$ when compared to saline with no postoperative anesthesia; $^{\#\#}p<0.01$ $^{\#\#\#}p<0.001$ when compared to saline under postoperative anesthesia).

3.3. Stimulated secretion

To standardize the doses of secretagogue in the new model for evaluation in the study, we performed dose-response curves using the dose established in the original published protocol (Shay et al., 1945).

3.3.1. Histamine

The stimulation of gastric acid secretion was achieved after administration of subcutaneous histamine at three dose levels (3 mg/Kg, 10 mg/Kg and 30 mg/Kg) and increases were observed both in animals that remained awake in the postoperative period, as well as in those maintained under anesthesia throughout the period of latency. In the three parameters analyzed (volume, pH and [H+]), the lowest dose of histamine used (3 mg/Kg) was still capable of stimulating gastric acid secretion which was statistically significant for all three parameters (Fig. 3a-c) when compared to the negative control group given 0.9% NaCl solution.



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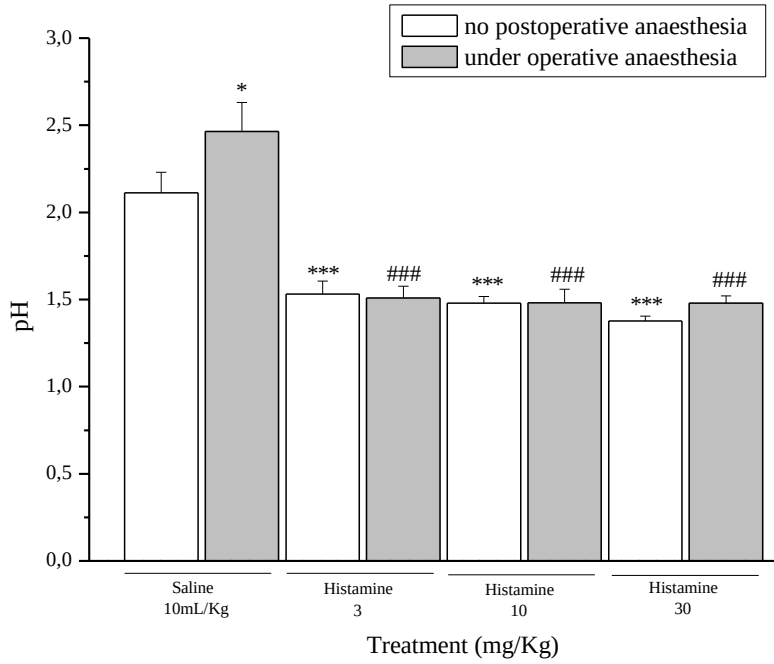

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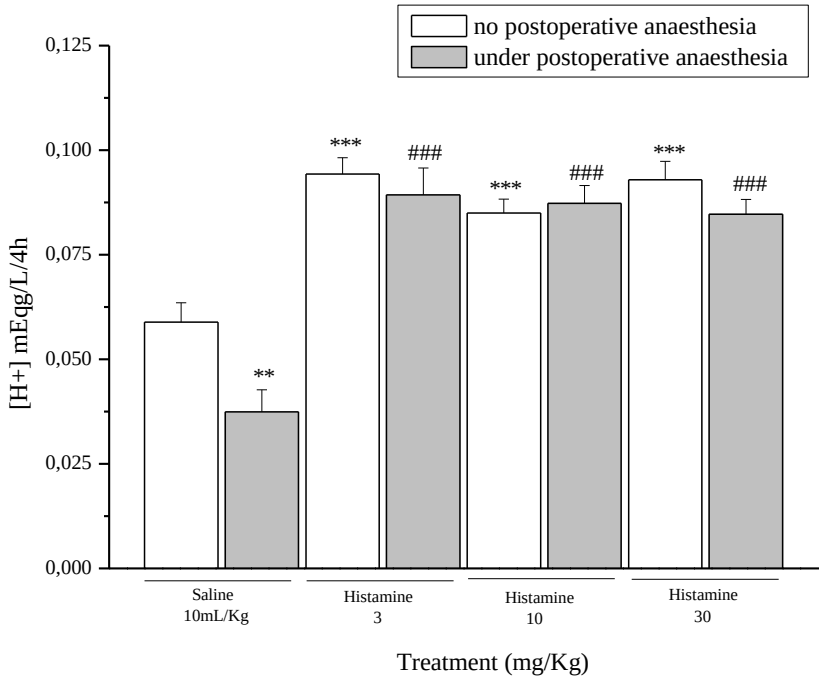
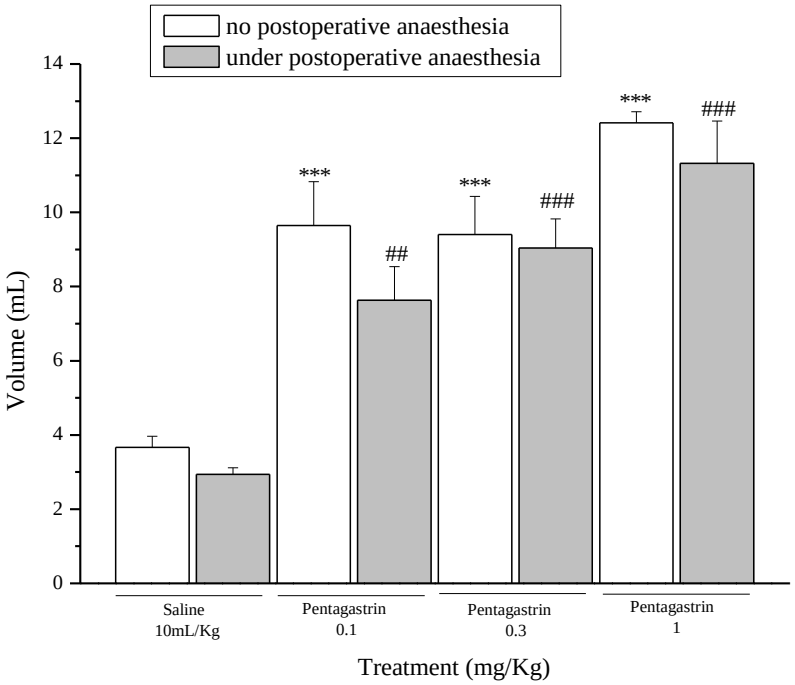


c)

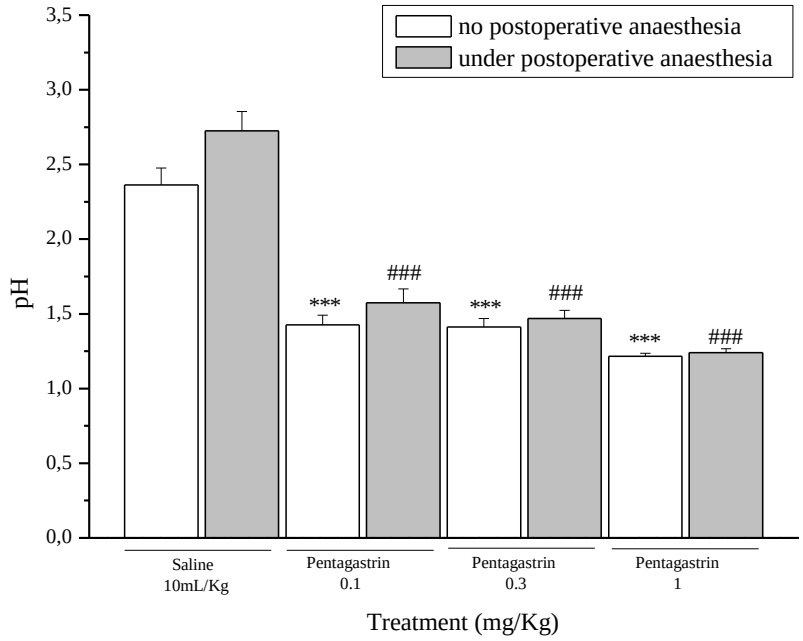
Fig. 3. Effect of postoperative pain on volume (a), pH (b), and [H+] (c) of gastric secretion of HanUnib:WH (Wistar/Uni) rats in pylorus ligation experiment under histamine stimulation. Values are expressed as mean ± error. ($ANOVA_{(volume)}$: $p<0.001$; $ANOVA_{(pH)}$: $p<0.001$; $ANOVA_{([H+])}$: $p<0.001$. Duncan's test $^{*}p<0.05$ $^{**}p<0.01$ $^{***}p<0.001$ when compared to saline with no postoperative anesthesia; $^{\#}p<0.05$ $^{\#\#}p<0.01$ $^{\#\#\#}p<0.001$ when compared to saline under postoperative anesthesia).

3.3.2. Pentagastrin

The stimulation of gastric acid secretion after administration of pentagastrin at three dose levels (0.1 mg/Kg, 0.3 mg/Kg and 1 mg/Kg) was observed in the animals that remained awake during the post-surgery period, as well as in those animals kept under anesthesia throughout the period of latency. In the three parameters analyzed (volume, pH and [H+]), the lowest dose of pentagastrin (0.1 mg/Kg) was capable of stimulating gastric acid secretion, and all three values achieved statistically significance (Fig. 4) when compared to the negative control group (0.9% NaCl solution). Hence there was no difference in the effect of pentagastrin on gastric acid secretion between those rats kept under anesthesia and those allowed to recover.



a)



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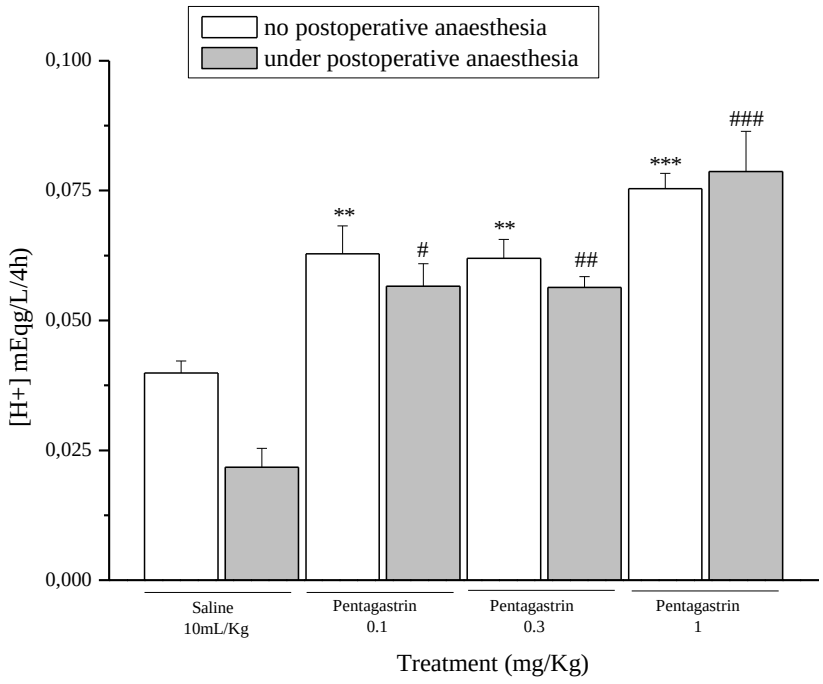


c)

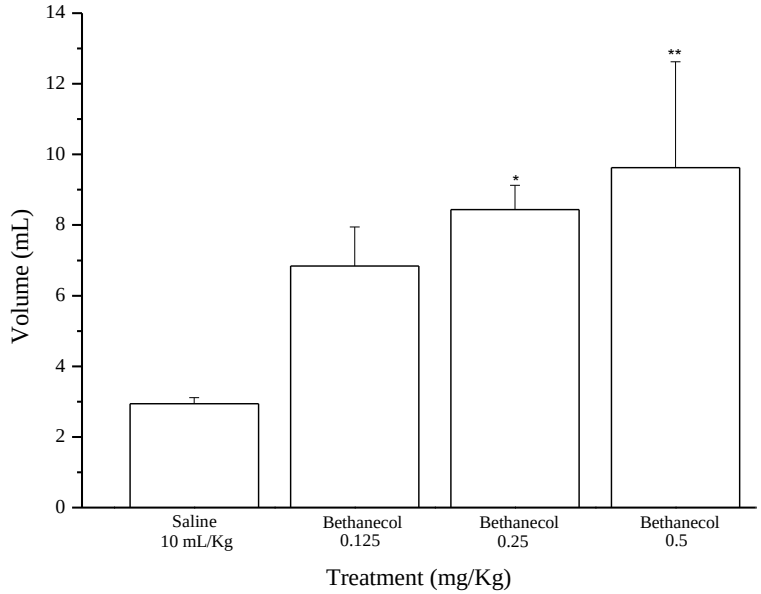
Fig. 4. Effect of postoperative pain on volume (a), pH (b), and [H+] (c) of gastric secretion of HanUnib:WH (Wistar/Uni) male rats in pylorus ligation experiment under pentagastrin stimulation. Values are expressed as mean ± error. ($ANOVA_{(volume)}$: $p<0.001$; $ANOVA_{(pH)}$: $p<0.001$; $ANOVA_{([H+])}$: $p<0.001$. Duncan's test $^{**}p<0.01$ $^{***}p<0.001$ when compared to saline with no postoperative anesthesia; $^{\#}p<0.05$ $^{\#\#}p<0.01$ $^{\#\#\#}p<0.001$ when compared to saline under postoperative anesthesia).

3.3.3. Bethanechol

The stimulation of gastric acid secretion after administration of bethanechol at three dose levels (1 mg/Kg, 5 mg/Kg and 25 mg/Kg) was observed in the animals that remained awake during the post-surgery period. However, even at the lowest dose of bethanechol used (1 mg/Kg) we observed severe drug interaction between the secretagogue and the anesthetic in the rats kept anesthetized throughout the procedure which was not observed in the rats allowed to recover from the anesthesia. Within ten to thirty minutes after the administration of the secretagogue to the anesthetized animals, there was intense salivation, dyspnea, ocular discharge and death of all animals. These effects are characteristic of intense muscarinic stimulation and did not allow the quantification of volume or pH of the gastric fluid secreted, and forced us to carry out a new dose-effect curve at doses much lower than those used in conscious animals.

The new dose-response curve was performed at three dose levels (0.125 mg/Kg, 0.25 mg/Kg and 0.5 mg/Kg) only in animals kept under anesthesia throughout the experimental period. In the three parameters analyzed (volume, pH and [H+]), the 0.25 mg/Kg dose of bethanechol and the 0.5 mg/Kg dose level was able to stimulate gastric acid secretion in animals kept under anesthesia, and statistically significant increases in gastric volume and free acid and statistically significant decreases in pH (Fig. 5) were observed when compared to the negative control group (0.9% NaCl solution).

Hence the sensitivity of response was considerably greater in the anesthetized rats than it was in those rats allowed to recover following the procedure.



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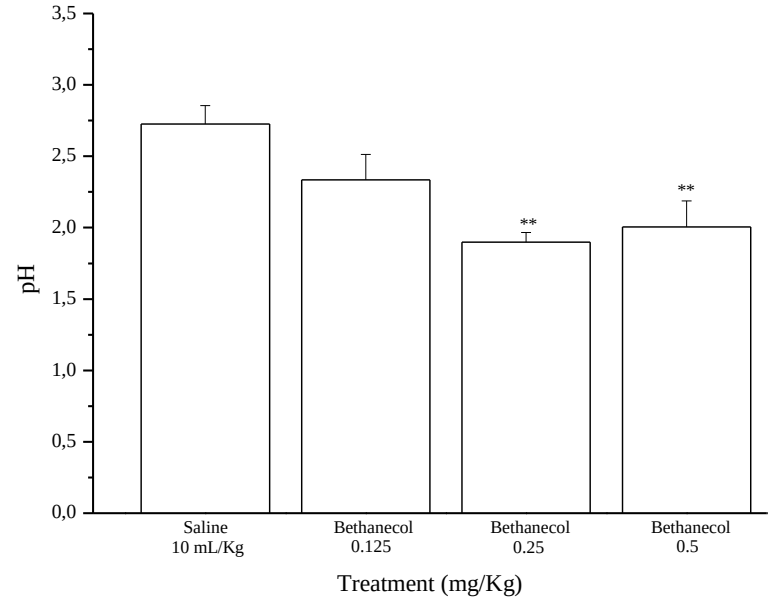

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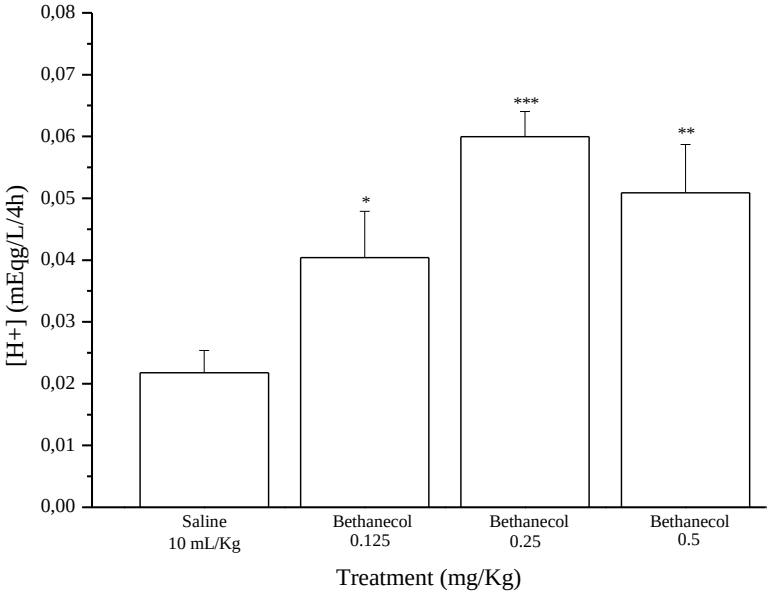

c)

Fig. 5. Effect of stimulation by bethanechol on volume (a), pH (b), and [H+] (c) of gastric secretion of HanUnib:WH (Wistar/Uni) male rats in pylorus ligation experiment kept under anesthesia throughout the entire latency time. Values are expressed as mean ± error. ($ANOVA_{(volume)}$: $p<0.001$; $ANOVA_{(pH)}$: $p<0.001$; $ANOVA_{([H+])}$: $p<0.001$. Duncan's test *$p<0.05$ **$p<0.01$ ***$p<0.001$ when compared to saline).

4. DISCUSSION

The use of animals in biomedical research has raised important questions about the influence of stress factors, including simple laboratory procedures, in various physiological parameters (Gärtner et al., 1980), often questioning the reliability of experimental results (Lave, Ennever, Rosenkranz & Omenn, 1988.) On the other hand, even if the increasingly available alternative "*in silico*", "*in chemico*" or "*in vitro*" models are getting more refined, data regarding the pharmacokinetics, pharmacodynamics and toxicology obtained in living systems cannot yet be abolished. Thus, in situations where animal testing is absolutely essential, the researcher should seek appropriate methods of refinement to provide better conditions for animals and to obtain more reliable results, this way eliminating important variables in the test and minimizing unnecessary discomfort to the animals (Flecknell, 1994; Madden et al., 2012).

Stress can be described as the body's response to environmental stimuli that could threaten its internal balance (homeostasis), thus activating a cascade of events initiated by the activation of the sympathetic system and the hypothalamic-pituitary-adrenal (HPA) axis, as well as triggering proinflammatory processes involving corticotropin-releasing factors (CRF) (Black, 2003; Pavel et al., 2008).

In this study, the largest volume of gastric secretion, lowest pH and highest acidity ([H+]) present in the groups exposed to post-surgical pain corroborate the relationship between stress caused by visceral pain and acid secretion. Both in mammals and humans, the activation of CRF-1 receptors, as a consequence of stresses caused by different types of pain, explain the sequence of events relating to pain stimulus arising from the tissue damage, with increasing acid secretion and the development of gastric ulcer (Hummel et al., 2010; Padol et al., 2012; Warzecha et al., 2011). Besides the alteration of the secretory response, observed with the suppression of pain, we have also shown a greater homogeneity in the experimental parameters analyzed (volume secretion, pH and free acidity) in the groups kept under anesthesia throughout the postoperative period. This fact can be explained by the variability in the individual animal's response to painful stimuli, as well as stress, already largely proven (Grégoire, Michaud, Chapuy, Eschalier & Ardid, 2012; Kabbaj, Devine, Savage & Akil, 2000; Wachulec, Peloso & Satinoff, 1997).

In an attempt to reduce the experimental period, we compared gastric secretion in animals kept under anesthesia, two and four hours after pylorus ligation. The elimination of pain was also beneficial in this respect, since after two hours we could already observe statistical differences in one of the three parameters, confirming literature data that indicated the stability

of gastric acid secretion in rats after two hours in experiments with isolated organs (Huang, Chang, Ho, Lu & Tsai, 2011). However, to perform the assessment of the secretion in the three parameters, our data suggests that the latency time for animals kept under anesthesia, after pylorus ligation, is better kept at 4 h.

Gastric secretion is regulated by processes involving neural, endocrine and paracrine mechanisms acting on the cells of the stomach, resulting in the final release of H+ and Cl- ions and water through the apical membrane of the parietal cells. In this process, three endogenous chemical mediators are involved, acting directly on receptors on the parietal cells: histamine (H_2), pentagastrin (CCK_2) and acetylcholine (M_3), or through the activation of the antral G cells (Schubert, 2011). Histamine, present in enterochromaffin cells, is released through a paracrine mechanism, stimulating acid secretion by interacting with H_2 and causing the release of cyclic AMP (Bighetti, Antônio & Carvalho, 2002).

Pentagastrin, the second secretagogue evaluated, is released by G cells as a result of stimulation by the presence of proteins in food, by Ca^{2+}, Mg^{2+}, and Al^{3+} ions by vagal stimulation, and by alkalinization of the antrum (Dickinson, 2004; Prinz et al., 1992). In the current new pylorus ligation model, the maintenance of experimental animals under anesthesia showed a more sensitive response to the administration of pentagastrin and histamine secretagogues, as statistical difference in the three parameters evaluated were obtained with smaller doses of histamine and pentagastrin tested, namely 3 mg/Kg and 0.1 mg/Kg, respectively than were necessary to elicit response in the non-anesthetized rats.

Acetylcholine is secreted by vagal efferent neurons, initially as a result of stimulation by olfaction, vision, taste, or by chewing and, as the presence of food stretches the stomach wall, local neurons present within the mucosa stimulate such secretions (Nakano, Kitano, Nanri & Kiniwa, 2011; Padol et al., 2012). In the experiment where the stimulation of gastric secretion was induced by subcutaneous administration of bethanechol, we observed an enhanced response to the secretagogue through a pharmacological interaction between the anesthetic and the secretagogue that resulted in the death of all anesthetized rats, even though the non-anesthetized rats showed a normal response. The signs of exacerbation of the parasympathetic system as pupillary contraction, tearing, increased salivation, bradycardia, increased intestinal motility and relaxation of sphincters (Yellamma, Saraswathamma & Kumari, 2010) were observed minutes after the administration of bethanechol in anesthetized rats at doses commonly used in the classic pylorus ligation protocol. Barbiturates such as thiopental and pentobarbital are commonly used for general anesthesia in laboratory animals,

despite the hemodynamic and metabolic effects that result from reduced activity of the sympathetic nervous system (Nishiyama, Misawa, Yokoyama & Hanaoka, 2002). However, the exact mechanism of toxicity of the thiopental-bethanechol association (doses greater than 1mg/Kg) cannot be defined as a predominance of the parasympathetic system, since recent studies suggest that the parasympathetic effects on the heart are not mediated exclusively by direct action over cardiac muscarinic receptors, but through various indirect mechanisms (Olshansky, Sabbah, Hauptman & Colucci, 2008). Considering such an interaction, a safe bethanechol dose, in the new model proposed, is 0.25 mg/Kg to stimulate gastric acid secretion in rats kept under anesthesia. Once again the data shows that the anesthetized rat model is appreciably more sensitive than the conventional rat model and should allow the detection of lower potency candidate drugs or the use of lower doses of high potency drugs, with no loss of sensitivity for detection.

It is also worth noting that there are other mechanisms of gastric secretion regulation, such as ghrelin, a peptide present in the "A-like" cells located in the basal part of oxyntic glands, which is able to increase appetite, stimulates motility, and stimulates gastric emptying and gastric secretion, especially during fasting and at night (Yakabi, Kawashima & Kato 2008). Finally, the peptide activator of the adenylate cyclase enzyme (Pituitary adenylate cyclase-activating peptide - PACAP), present in gastric intramural neurons, can also inhibit or stimulate gastric acid secretion (Pisegna et al., 2000).

Regarding the surgical procedure for pylorus ligation, a disadvantage of the proposed method is the increased bleeding observed during anesthesia with thiopental in our assays, corroborated by studies using normotensive rats, anesthetized by this barbiturate, as a consequence of vasodilation and heart rate reduction (Brookes, Reilly & Brown, 2004). However, the use of animals in the weight range between 200 and 300g enables a good view of the cavity and improved access into the stomach, as there is less abdominal adipose tissue, allowing for easier control of hemorrhagic foci.

The pharmacological model of pylorus ligation developed by Shay et al. has been undergoing minor modifications since it was proposed in 1945. However, recent studies still allow experimental animals to be awakened from the anesthesia, and to be kept awake for the latency period of 3 to 4 hours (Chaleek, Kermani, Eliassi & Haghparast, 2012; Cosmo, Mayer, Freitas, Baggio & Marques, 2007; Padol et al., 2012; Pinelli, Trivulzio, Pojaga & Rossoni, 1996). From the results that compare the effect of stress from post-surgical pain in the experimental protocol of gastric acid secretion evaluation in rats, we conclude that the use of general anesthetic as a tool for pain suppression is beneficial, reduces animal suffering,

increases sensitivity and improves the homogeneity of the data produced, and reduces the number of people required to conduct the experiment, thus allowing the pharmacological study of the activity on gastric secretion with minimal animal suffering.

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CAPÍTULO II

PAW EDEMA TEST IN RATS: DOES IT ALWAYS HAVE TO HURT?

Manuscrito em preparação para ser submetido à revista *Journal of Pharmacological and Toxicological Methods*

ABSTRACT

Introduction: The recognition of the harmful effects of pain upon laboratory animal physiology demands a continuous search for experimental refinement techniques. Yet, the concern with possible pharmacological interactions remains as they may represent important bias. On one side, ethics committee members suggesting the use of drugs to minimize pain, on the other side, researchers equally concerned with animal's welfare, but seriously preoccupied with a possible undesirable interference between painkillers and test substances. Herein we propose a modification in the carrageenan paw edema experimental model in rats in cases where only the anti-inflammatory – and not the antinociceptive - activity of the test substance is under evaluation.

Methods: To minimize animal suffering, either general or local anesthesia was performed prior to the induction of inflammation.

Results: In our refined model, the sciatic nerve blocked with mepivacaine immediately before the induction of the inflammatory response minimized the intense pain caused by the intraplantar administration of carrageenan without compromising the development of paw edema. Local anesthesia did not impair the histopathological characteristics of the inflamed paw tissue after four hours of induction.

Discussion: Through this refined technique, it was possible to stablish a new experimental protocol, with higher statistical significance between control groups ($p < 0.001$), bigger homogeneity among individuals of a same group, as well as a better handling of the animal at the moment of the carrageenan injection and along the subsequent evaluation in plethysmometer, when compared to the original protocol.

Keywords: carrageenan, local anesthesia, methods, inflammation, pain, paw edema, refinement, rats, pharmacology

1. Introduction

The carrageenan-induced paw edema protocol was first proposed by Winter in the sixties (Winter et al., 1963) and, since then, has been used as one of the main experimental models in the study of anti-inflammatory substances. Carrageenans are a group of polysaccharides obtained from a red alga, *Chondrus crispus*, with gel characteristics and able to induce an intense inflammatory response. After being subcutaneously injected into the rat's hind paw, it is possible to observe the cardinal signs of inflammation: gradual increase in volume (edema), redness (erythema), heat (hyperthermia) and pain (hyperalgesia) due to the release of proinflammatory chemical mediators (bradykinin, histamine, tachykinins, complement, reactive oxygen, and nitrogen species) (Morris, 2003). In rats, approximately five hours after the injection of the phlogistic agent, the increase in paw volume reaches its maximum, followed by a stepwise reduction (Morris, 2003). The temporal evaluation of paw volume allows the study of different mediators involved in the local process, in contrast with the first experimental models in which the anti-inflammatory activity of a test substance was evaluated through the inhibition of some cardinal signs: edema, erythema, hyperthermia and hyperalgesia as a consequence of the introduction of a cotton pellet into the subcutaneous tissue of the rodent (Meier et al., 1950).

There are numerous experimental models that cause intense inflammatory response in rodents frequently used to evaluate nociception (acetic acid-induced writhing test and mouse paw formalin test, for example). In these situations, it is possible to evaluate both inflammatory pain as well as other signs, such as edema, leucocyte migration and/or cytokines (carrageenan-induced hind paw test, cotton pellet-induced granuloma, [12-O-tetradecanoylphorbol-13-acetate] TPA-induced edema test and Evans blue vital dye – pleurisy) (Lopes et al., 2013; Spindola et al., 2010; Cavaller-Machado et al., 2008). Understanding the anti-inflammatory mechanisms of action of a test substance through several experimental models is extremely important as a wide range of inflammatory mediators play vital roles in other diseases such as cancer (Vendramini-Costa & Carvalho, 2012).

Ever since the scientific demonstration of the anti-inflammatory properties of indomethacin (Winter et al., 1963), the paw edema experimental model has been widely used for screening as well as studying mechanisms of action (Tavares et al., 2013). There are situations, however, where the carrageenan-induced edema model is also used as a tool for the investigation of possible anti-nociceptive properties of a test substance ("mechanical hyperalgesia induced by carrageenan") (Servat et al., 2012). For obvious reasons, in such cases, it is not possible to use pain-relieving agents as a refinement tool and other forms of minimizing

suffering must be considered. Nevertheless, in some instances where there will be no evaluation of possible antinociception effect, pain represents a by-product that should be avoided not only for ethical reasons, but also for the quality of the results (Kidd and Urban, 2001).

The negative impact of both pain and stress on laboratory animal's physiology has been widely discussed and undoubtedly proven (Luo et al., 2013; Monteiro et al., 2013; Pekow, 2005; Flecknell, 1994). On one side, ethics committee members suggesting the use of pain-relieving drugs and adequate supportive veterinary care; on the other side, researchers equally concerned with animal's welfare, but seriously preoccupied with an undesirable interaction between painkillers and test substances (Gnadt and Leland, 2002). Thus, it is imperative that pain-relieving agents be tested in order to guarantee, as desired, less suffering without compromising the pharmacological response. With this dilemma in mind, the present paper aims to identify, and test, the best pain-relieving pharmacological option to refine the carrageenan paw edema experimental model in rats.

In the present work, two different approaches were tested to evaluate the effect of the reduction of the intense pain due to the carrageenan injection towards the inflammatory response in the paw edema model: general anesthesia and local nerve block. In both studies, the gradual increase of the hind paw volume was measured with the use of a digital plethysmometer.

2. Methods

2.1. Animals

Adult male HanUnib:WH (Wistar) rats weighing 160-200g were obtained from the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (CEMIB), State University of Campinas (UNICAMP, São Paulo, Brazil). During the maintenance period (at least seven days prior to the beginning of the study), animals were group housed in polypropylene cages (five individuals per cage; length 49 cm, width 34 cm, depth 16 cm). Sterile soft wood bedding (*Pinus sp.*) was changed twice a week and clean water as well as pelletized commercial food (Biobase, *Biotécnicas Indústria e Comércio Ltda*., Brazil) provided *ad libitum*. Room temperature was kept constant (22 ± 2°C) on a 12 h light-dark cycle, with lights off at 18:00 h.

Animals' welfare guidelines were adopted for the studies in accordance with both the International Guiding Principles for Biomedical Research Involving Animals (CIOMS-ICLAS, December 2012) as well as the Brazilian Guideline for the Care and Use of Animals

for Scientific and Didactic Purposes (CONCEA, 2013). Experimental protocols were approved by the University's Committee for Ethics in Animal Use (CEUA-UNICAMP, protocol number 3318-1).

2.2. Materials

Barbiturate: 3% sodium pentobarbital, 80 mg kg^{-1}; intraperitoneal route; (Hypnol®, *Rhobifarma Indústria Farmacêutica Ltda*., Brazil). Dissociative anesthetic: ketamine chloridrate; 80 mg kg^{-1}; intraperitoneal route; (Dopalen®, *Sespo Indústria e Comércio Ltda*., Brazil) together with an α_2 agonist: xylazine chloridrate; 8 mg kg^{-1}; intraperitoneal route; (Xilazin®, *Rhobifarma Indústria Farmacêutica Ltda*., Brazil). Non-steroidal anti-inflammatory drug: indomethacin chloridrate (5 mg kg^{-1}; administered volume 10mL kg^{-1}, Indocid®, *Merck Sharp & Dohme Farmacêutica Ltda*., Brazil). Local anesthetic: mepivacaine hydrochloride; 135 mg kg^{-1}; 1,8 mL cartridge (MEPISV®, *Nova DFL Produtos Odontológicos Ltda*., Brazil). Ultra-sharp lancet point needle: 30G Terumo® Dental Needle; Terumo Corporation, Japan. Phlogistic agent: carrageenan (3% solution; 100 µL; prepared immediately before inoculation; SIGMA). Fixation buffer solution for further histological analysis: 10% formaldehyde (*CHEMCO Indústria e Comércio de Produtos Químicos Ltda*., Brazil). Mieloperoxidase dosage: Potassium phosphate solution 50mM (pH 6.0); hexadecyltrimethyl ammonium bromide buffer 0,5% (pH 5.4; SIGMA) and hydrogen peroxide 0.3 mM (*CETUS Indústria e Comércio de Produtos Químicos Ltda*., Brazil).

2.3. Refinement proposal

The first attempt to minimize animal suffering was performed by anesthetizing the experimental animals after the oral treatment of the test substances, but prior to the induction of inflammation. Two types of general anesthesia were tested, using a barbiturate (3% sodium pentobarbital, 80 mg kg^{-1}) as well as a common choice of dissociative anesthetic and an α_2 agonist (ketamine chloridrate; 80 mg kg^{-1} and xylazine chloridrate; 8 mg kg^{-1}, respectively). In both cases, the injection of the carrageenan solution in the right hind paw of the rats was carried out only after complete loss of all reflexes. Monitoring of anesthetic depth, heart and respiratory rates and observation of mucus membrane color were conducted throughout the experimental period (6 hours). Whenever necessary, new doses of the general anesthetic were administered in order to maintain plane 3 of anesthesia (deep anesthesia).

The second attempt to reduce animal suffering was performed through the regional anesthesia of the hind paw also after the oral treatment of the test substances, but right before the carrageenan injection. Under gentle mechanic restraint, 0.9 mlL of mepivacaine hydrochloride (135 mg kg^{-1}) was injected using a dentist syringe and an ultra-sharp lancet point needle (Figure 1a). Two minutes after the perineural injection, it was possible to confirm the interruption of the nervous stimulus through the loss of the hind paw movement (Figure 1b). Five minutes later, the animals were submitted to the subcutaneous injection of carrageenan on the ventral region of the hind paw. Throughout the experimental period (6 hours), new doses of local anesthetic were administered whenever the animal started to recover movement of the hind paw.

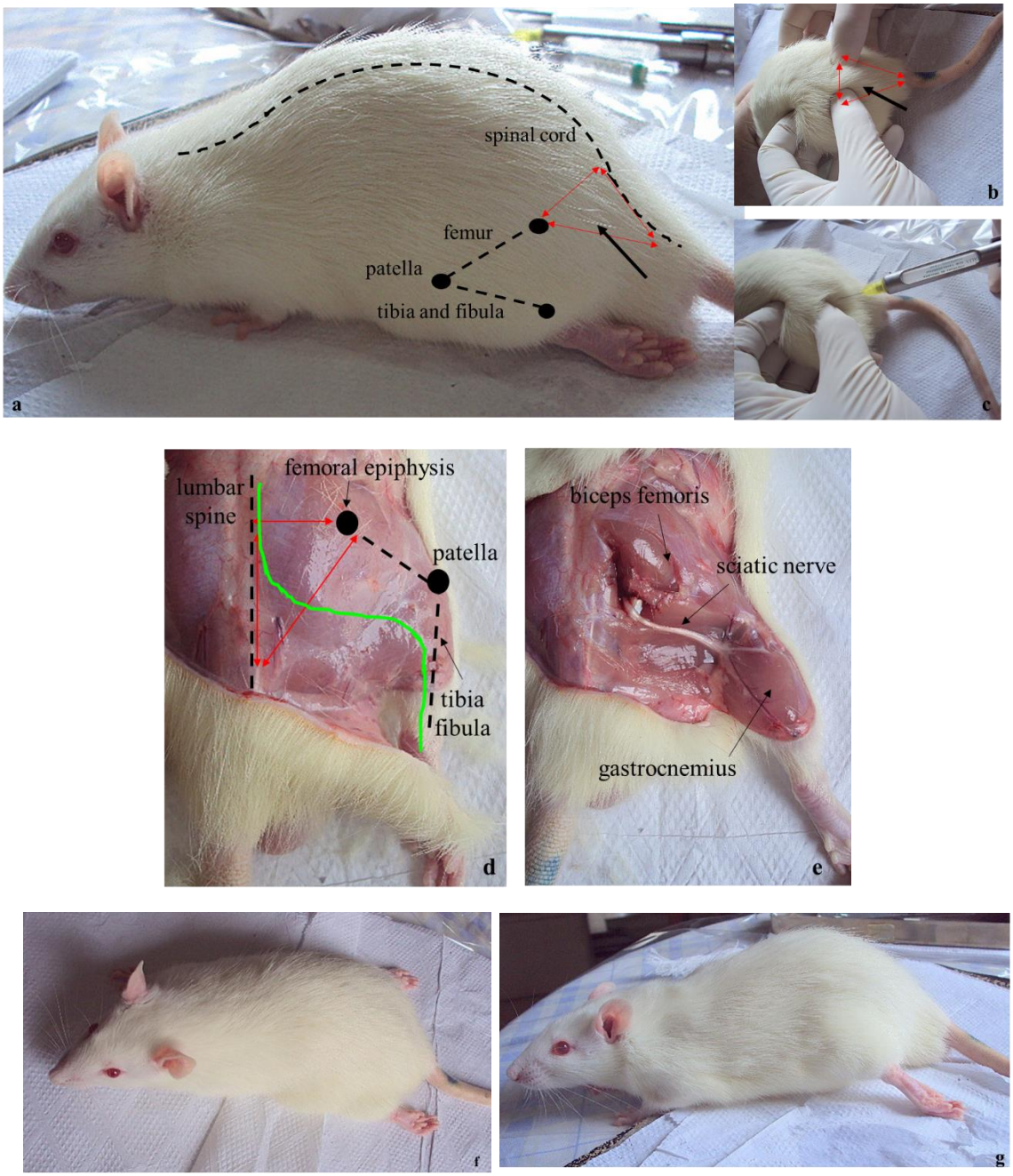

Figure 1. a-e) Demonstration of local anesthetic injection using both the capital femoral epiphysis and the lumbar spine as anatomic references. f-g) Five minutes after the nerve block, it is possible to observe the loss of right hind paw movement.

2.4. Experimental procedures

2.4.1. General anesthesia assay

After a 7-day acclimation period, all animals were submitted to a 12-hour fasting period to ensure a proper oral uptake of the NSAID. Rats were, then, weighed and randomly assigned to six different experimental groups, composed of six animals each: (G1) “standard

negative control" orally treated with phosphate buffered saline solution (pH 7.0; 10 mL kg^{-1}) and maintained "awake" during the entire experimental period, according to the original protocol; (G2) "pentobarbital negative control" orally treated with phosphate buffered saline solution (pH 7.0; 10 mL kg^{-1}), submitted to general anesthesia before the carrageenan injection and maintained anesthetized during the entire experimental period; (G3) "ketamine/xylazine negative control" orally treated with phosphate buffered saline solution (pH 7.0; 10 mL kg^{-1}), submitted to general anesthesia before the carrageenan injection and maintained anesthetized during the entire experimental period; (G5) "standard positive control" orally treated with indomethacin chloridrate (5 mg kg^{-1}; administered volume 10mL kg^{-1}) and maintained "awake" during the entire experimental period, according to the original protocol; (G6) "pentobarbital positive control" orally treated with indomethacin chloridrate (same as previously described), submitted to general anesthesia before the carrageenan injection and maintained anesthetized during the entire experimental period and, finally (G7) "ketamine/xylazine positive control" orally treated indomethacin chloridrate (same as previously described), submitted to general anesthesia before the carrageenan injection and maintained anesthetized during the entire experimental period.

A baseline measurement of the hind paws was carried out using a plethysmometer (Cat. No. 37140, Ugo Basile, Italy). Immediately after registering the basal volume of the hind paws, all animals were submitted to oral treatment according to their experimental group (Table 1). Thirty minutes after dosing, animals belonging to the groups of pentobarbital as well as ketamine/xylazine (both negative and positive control ones) were submitted to a gentle intraperitoneal administration (0,45 x 13 mm / 26 G x ½" needle) of the general anesthesia, respectively. Thirty minutes later (one hour after the oral treatments), all animals were submitted to a subcutaneous injection of carrageenan (3% solution; 100 µL; prepared immediately before inoculation) on the ventral region of the right hind paw. Sixty minutes after the induction of the inflammation, the measuring of the paw volume was carried out and, thereafter, hourly measurements, for the next six hours. At the end of the experimental period, all animals were submitted to euthanasia by anesthesia overdose (Figure 2).

Table 1. Distribution of animals into experimental groups in the paw edema model in rats.

Group number	Carrageenan injection	Test substance (*per os*)	Anesthesia
1	yes	Saline solution 0.9%	None (original protocol)
2	yes	Saline solution 0.9%	Pentobarbital
3	yes	Saline solution 0.9%	Ketamine / Xylazine
4	yes	Saline solution 0.9%	Mepivacaine
5	yes	Indomethacin	None (original protocol)
6	yes	Indomethacin	Pentobarbital
7	yes	Indomethacin	Ketamine / Xilazine
8	yes	Indomethacin	Mepivacaine

2.4.2. Regional anesthesia assay

Similarly, after a 7-day acclimation period and 12 hour fasting period, rats were weighed and randomly divided into four groups, composed of six animals each: (G1) "standard negative control" orally treated with phosphate buffered saline solution (pH 7.0; 10 mL kg^{-1}); (G4) "no pain negative control" orally treated with phosphate buffered saline solution (pH 7.0; 10 mL kg^{-1}), submitted to regional anesthesia (sciatic block) before the carrageenan injection; (G5) "standard positive control" orally treated with indomethacin chloridrate (5 mg kg^{-1}; administered volume 10 mL kg^{-1}) and, finally, (G8) "no pain positive control" orally treated with indomethacin chloridrate (same as previously described), submitted to regional anesthesia before the carrageenan injection.

As mentioned before, a baseline measurement of the hind paws was carried out using the same plethysmometer. Immediately after registering the basal volume of the hind paws, all animals were submitted to oral treatment according to their experimental group. Fifty minutes after dosing, using a dentist syringe with mepivacaine hydrochloride (135 mg kg^{-1}) animals belonging to the "no pain" groups (both negative and positive controls) received 0.9 mL. Ultra-sharp lancet point needle was used to minimize the injection discomfort (one per animal) as well as avoid breakage even if the animal moved violently during the procedure. Two minutes after the perineural injection, it was possible to confirm the interruption of the nervous stimulus through the loss of the hind paw movement (Figure 1b). Eight minutes later (and one hour after the oral treatments), all animals were submitted to a intraplantar injection of carrageenan (3% solution; 100 µL; prepared immediately before inoculation) on the ventral region of the right hind paw.

As in the previous experiment, sixty minutes after the induction of the inflammation, the measuring of the paw volume was carried out and, thereafter, hourly measurements, for the next six hours. Similarly, at the end of the experimental period, all animals were submitted to euthanasia by anesthesia overdose. A second experiment was performed, this time with fewer animals, to compare the histological pattern of the inflammatory tissue with and without nerve block. To do so, one rat of each experimental group was submitted to euthanasia after each paw volume evaluation. Such animals had two 0.5 cm^2 fragments of their inflamed paw removed with the help of a scalpel. Both fragments of each animal were immediately fixed in 10% formaldehyde buffer for further histological analysis.

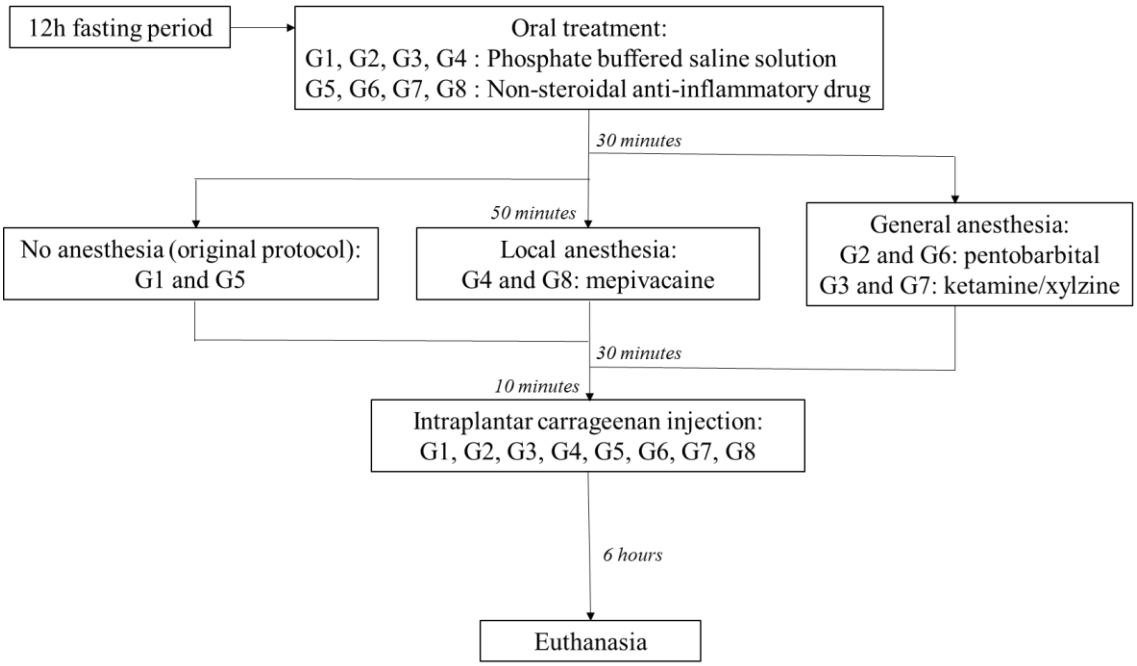

Figure 2. Paw edema original protocol together with the refined technique. G1 to G4 represent negative control groups, receiving phosphate buffered saline solution (pH 7.0) as oral treatment; G5 to G8 represent positive control groups, receiving indomethacin solution (30 mg kg^{-1} b.w.) as oral treatment. Winter's (1962) original experimental protocol was applied for G1 and G5, whereas G4 and G8 were submitted to local anesthesia before carrageenan injection. Groups G2, G6, G3 and G7 remained unconscious for the entire experimental period, under general anesthesia.

2.5. Evaluation of the inflammatory response

2.5.1. Paw volume / edema

Before carrageenan injection, baseline records of the right hind paw volume of all animals were carried out and identified as “time zero”. Increase in volume caused by the inflammatory reaction of the animals’ tissue was calculated by the difference between the value recorded by the plethysmometer and the one registered at “time zero”. For six hours, it was possible to monitor the increase in the paw volume of all experimental groups.

2.5.2. Histological analysis

Immediately after euthanasia, fragments of the ventral region of the hind paw were removed and immersed in a buffered formaldehyde solution. After dehydration, fragments were embedded in paraffin prior to the 5 µm microtome sections. Histological sections were stained with hematoxylin-eosin in glass slide. Blind analysis was carried out through random selection of microscope fields in the subplantar paw region at 400x magnification.

2.6. Statistical analysis

Results are expressed by mean ± standard error of the mean (SEM). We assessed carrageenan-induced paw edema inflammation by two-way analysis of variance (ANOVA). All significant differences were examined by Tukey post-test. Group comparisons were analyzed by Student unpaired *t* test. Values at $p < 0.05$ were considered statistically different.

3. Results

3.1.Paw volume

Animals submitted to general anesthesia, either with pentobarbital or ketamine/xylazine association, did not present an increase in the hind paw volume in contrast with the non-anesthetized group. Unfortunately, it was only possible to observe statistical difference between non-anesthetized control groups (Figure 3). On the other hand, the sciatic block immediately before the carrageenan injection did not interfere either with the inflammatory response or the anti-inflammatory effect of the NSAID used as positive control. Figure 4 presents the comparison between the inflammatory responses with and without the

sciatic block revealing a smaller standard err when the pain-relieving agent was used. It is important to point out that the negative control group presents lower paw volumes under the effect of regional anesthesia. However, statistical differences between negative and positive control values were maintained and, sometimes, showed even better significance ($p < 0.001$).

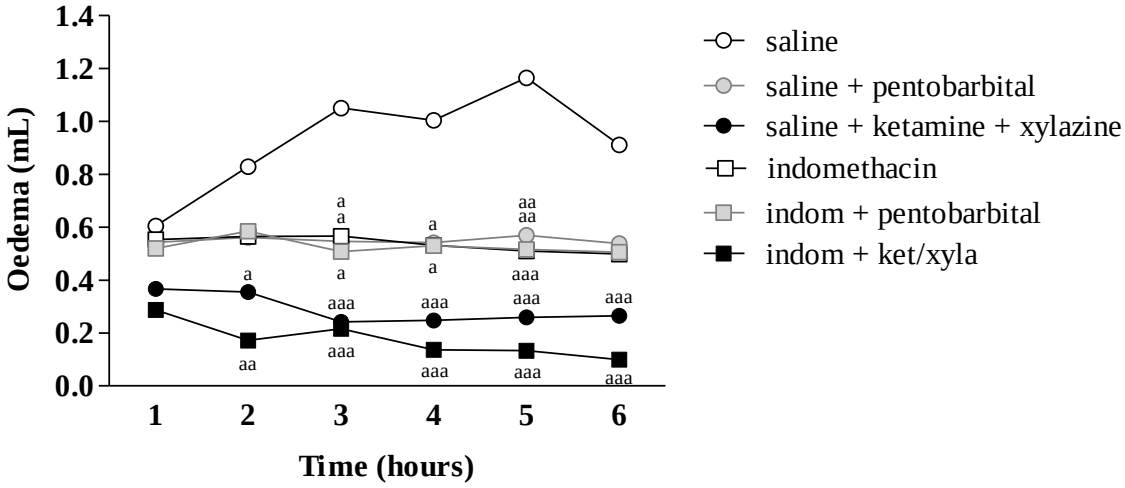

Figure 3. Effect of general anesthesia with pentobarbital or ketamine/xylazine association on rat paw edema induced by carrageenan (3%; 100 μL/paw). Indomethacin (30 mg kg^{-1} p.o.) was used as a positive control. Values are expressed as mean ± SEM. Statistical significance: ^{a} $p<0.05$, ^{aa} $p<0.01$, ^{aaa} $p<0.001$ in comparison to saline.

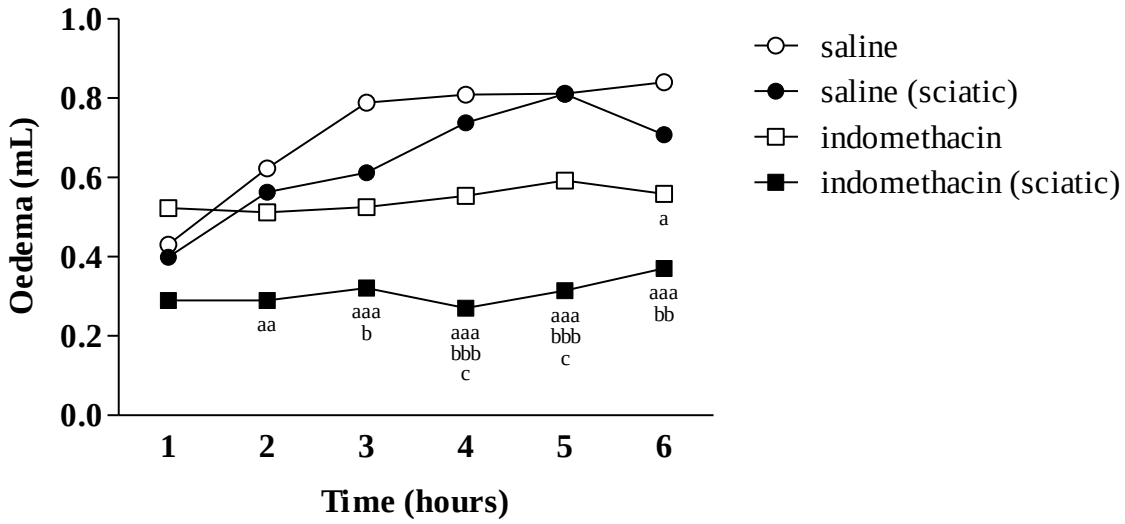

Figure 4. Effect of local anesthesia with mepivacaine hydrochloride on rat paw edema induced by carrageenan (3%; 100μL/paw). Indomethacin (30 mg kg^{-1} p.o.) was used as a positive control. Values are expressed as mean ± SEM. Statistical significance: ^{a} $p<0.05$, ^{aa} $p<0.01$, ^{aaa} $p<0.001$ in comparison to saline; ^{b} $p<0.05$, ^{bb} $p<0.01$, ^{bbb} $p<0.001$ in comparison to saline (sciatic); ^{c} $p<0.05$ in comparison to indomethacin.

Histological analysis

Histopathological analysis of both dorsal and ventral fragments of the inflamed paws revealed moderate edema in both control groups in accordance with the observed volumes registered by plethysmometer. (Figure 5).

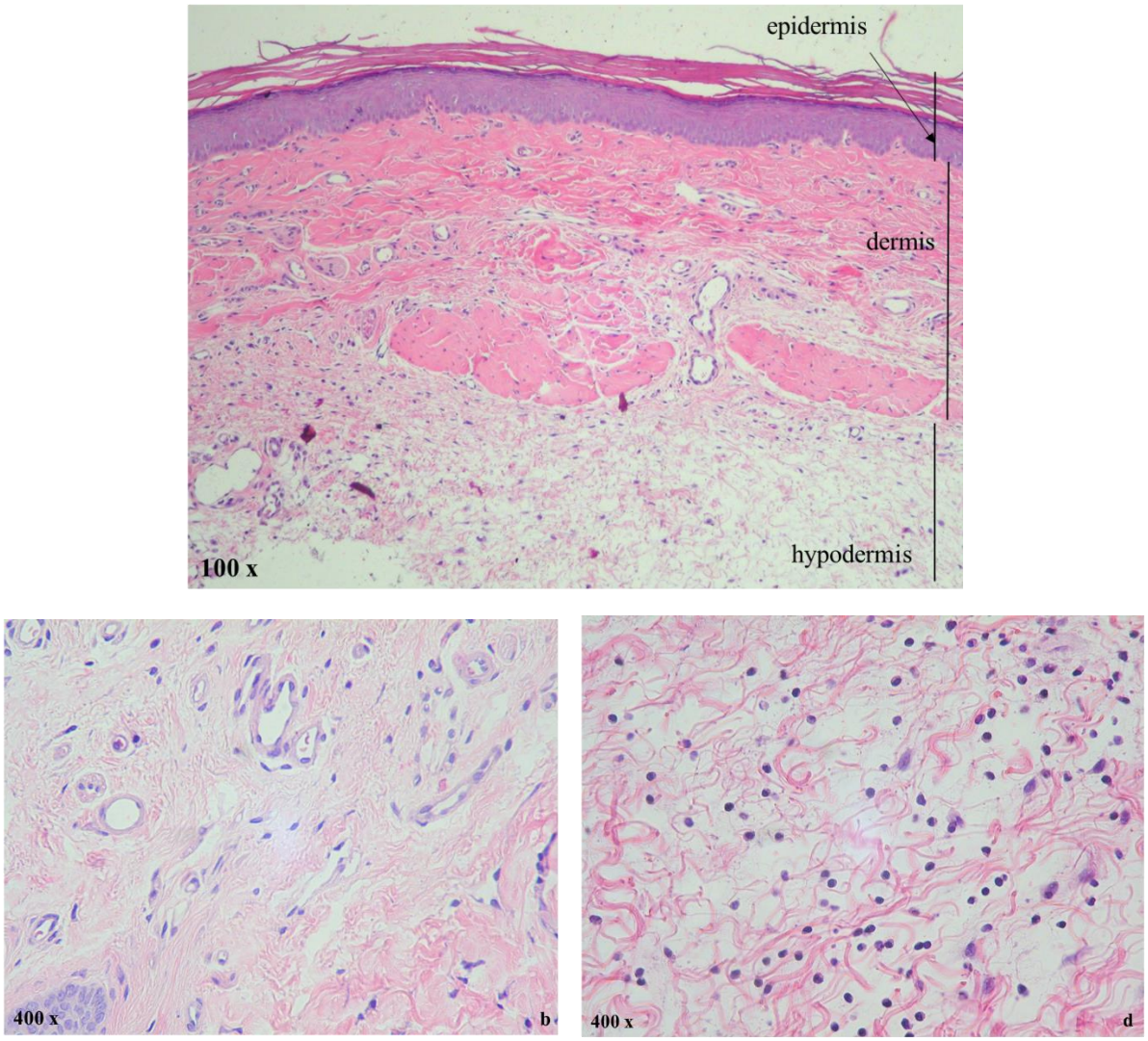

Figure 5. (a) Histological appearance of rat hind paw ventral skin presenting moderate edema photomicrograph H&E stained (G1-saline-original protocol). (b) Mild edema with both lymphocytic and neutrophilic inflammatory infiltrate (G8-indomethacin-mepivacaine). (c) Moderate edema with both lymphocytic and neutrophilic infiltrate (G5-saline-mepivacaine).

4. Discussion

Our findings are coherent with the use of barbiturates to induce coma in cases of cranial trauma, in which the main objective is to decelerate the brain metabolism and blood flow, therefore maintaining cerebral edema under control (Morrow & Pearson, 2010). The use of general anesthesia in the carrageenan paw edema model does reduce animal suffering; however, the depressing effects of both pentobarbital and ketamine/xylazine association equally reduce the inflammatory response, making its use unfeasible as a refinement technique for this experimental model in rats.

On the other hand, the possibility of interrupting the noxious stimulus through sciatic nerve block proves to be beneficial in many aspects. First and foremost, the use of an ultra-fine needle to perform the regional anesthesia reduces considerably the discomfort of the puncture, especially if the researcher injects a small volume of mepivacaine and waits a few seconds to finish the administration in an already desensitized area. In a couple of minutes, a slight mechanical contention is sufficient to allow the inoculation of the phlogistic agent. Not experiencing the intense neuropathic pain caused by the perfusion of the 3% carrageenan solution in the plantar subcutaneous tissue, animals tend to offer less resistance to restraint, contributing to a more precise administration and, consequently, reducing one of the possible bias related to the induction of the inflammatory cascade. Another aspect associated with the accuracy of the inoculation procedure is the researcher's "mental condition"; the negative psychological effect of performing a painful maneuver may be stressful especially for unexperienced individuals and, somehow, jeopardize the technique itself. In addition to that, as both animal and researcher are exposed to less stressful situations, chances of accidents are reduced, such as bites or breaking needles at the moment of carrageenan inoculation as well as during the subsequent paw volume measurements, especially around the fifth hour, when edema reaches its peak.

As far as paw volume is concerned, the present study demonstrated that elimination of pain using mepivacaine with no vasoconstrictor did not impair the evolution of edema after carrageenan injection. Other studies presented a possible anti-inflammatory effect of local anesthesia (bupivacaine), emphasizing the decrease in TNFα and IL1-β (but not IL-10) in cultures of circulating blood cells after lipopolysaccharide stimulation in rats receiving hind paw carrageenan injection (Beloeil et al., 2006). The use of 0.5% bupivacaine associated with epinephrine decreased the inflammatory response only when maintained for 6 hours, with a catheter technique, after the inoculation of carrageenan (Gentili et al., 1999). It is worth mentioning that the antinociceptive effects of a bupivacaine polymer system lasted 2 days, indicating that the reduction of edema, including the reduction of local tissue levels of immunoreactive P substance (iSP) as well as bradykinin (iBK) after the sciatic injection of a polymer/bupivacaine system in CFA-induced edema model in rats is related to an extended release of the local anesthetic (Garry et al., 1999). In principle, even though peripheral nerve block may interfere with the neurogenic component of inflammation, it does not appear to compromise the evolution of the onset of the inflammatory cascade, unless there is a persistent local anesthetic effect (over 6 hours).

Another aspect to be considered is the frequent association with epinephrine or other vasoconstrictors aiming at a longer antinociceptive effect at the site of injection, which might explain, at least partially, the reduction of edema in those cases. In addition, there seems to be no consensus on the best route of administration to achieve a possible anti-inflammatory effect. Intravenous administration of LAs in clinical trials have been widely discussed, especially as a "protective" agent in inflammatory responses (Hollmann & Durieux, 2000). Leduc et al. (2002) report that local anesthetics may present an anti-inflammatory effect both injected as a sciatic nerve block or intraperitoneally whereas Beloeil et al. (2006) discarded any possible systemic effect in rats. Taken together, further studies are essential to elucidate the relation between local anesthetics and other biological systems, not only as sodium channel blockers, but also with several cellular targets and mediators.

In the meantime, the natural variability of the individual to pain and stress remains, in some of our experimental protocols, hampering efforts to reduce the number of animals used. Previous studies have demonstrated that, excluding the anti-nociceptive assays, pain represents a non-desirable by-product, increasing standard deviation of data (Monteiro et al., 2013). From the acute nociceptive stimulus sensitizing the hind paw numerous peripheral nerve endings to the subsequent inflammatory hyperalgesia, carrageenan-induced paw edema animals are required to respond to both physical (paw volume or circumference) and/or molecular aspects of inflammation. In many situations, the effect of a given test substance on the inflammatory pain is not under evaluation.

One potential deficiency of the present refinement proposal is that only volume and histological features were evaluated; specific serum or local inflammatory mediators were not compared after the sciatic block using mepivacaine, considering that some of them, such as bradykinin, are closely related to the inflammatory pain. However, times have changed. Reproducing a 50-year old experimental protocol without applying new data such as the severe effects of stress over the laboratory animals' physiology and, consequently, pharmacological response, probably compromises scientific data. Our results show that on the third hour, there was a significant decrease in the paw volume of the negative control group under sciatic block when compared to the one under the original protocol. As observed in other refined models using pain suppressors (Monteiro et al., 2013), we suggest the use of a "pain-free" control groups (both negative and positive), excluding the animals' response to stress in such a way that the comparison between tested groups and control ones have this bias neglected.

We understand that many aspects are yet to be cleared; conflicting results regarding the effect of the sciatic block may raise the questioning whether the stressful effects of pain

over the laboratory animals justify the use of anesthetics that may offer a possible non-desirable pharmacological interaction. However, the amount of information available on the impact of stress over experimental results mainly due to the consequent central neural plasticity as well as hormonal alterations should not be disregarded (Iarkov et al., 2016; Slater & Cao, 2015; Cao et al., 2010; Hummel et al., 2010; May, 2008; Thaker et al., 2006; Brown et al., 2003; Melzack et al., 2001; Coderre et al., 1993; Ben-Eliyahu et al., 1991).

Very frequently, refining any experimental protocol offers the dilemma: reaching a point where the pain-relieving agent does not interfere adversely with the pharmacological issue under investigation. Yet, minimum animal suffering leads to better quality science (Monteiro et al., 2013; Wolfensohn et al., 2013). As far as the paw edema model is concerned, researchers should ask themselves what seems to be a more important bias in their results: the intense pain caused by the carrageenan injection and the variability of the individual response to such a stressful situation, or a possible – not yet proven – residual anti-inflammatory effect of a local anesthetic. The significant reduction in the standard err observed in the animals submitted to a regional anesthesia should not be neglected. In times where the physiological alterations produced by animal suffering are considered a key component to the study of pharmacological properties of new test substances, old experimental protocols should be reviewed. Further studies are necessary to confirm whether the same modification may be applied to the carrageenan-induced paw edema model in mice, since it is known that this species presents a two-phase inflammation pattern, allowing the experiment to last up to 72 hours. In the meantime, we propose that mepivacaine, with no vasoconstrictor added, be used as a refinement of the carrageenan-induced paw edema model in rats.

In conclusion, our results showed that the use of mepivacaine hydrochloride for sciatic nerve block in the carrageenan paw edema model in rats reduces animal suffering, enables more accuracy in carrageenan injection and paw volume measuring, minimizes psychological stress of performing a painful procedure and reduces the risk of accidents. Finally, the present study aims to raise the question of updating a few pharmacological protocols, and, at the same time, suggest future studies to identify situations where it is feasible to include a pain suppressor without compromising scientific data.

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CAPÍTULO III

EHRLICH SOLID CANCER MODEL AND LABORATORY ANIMAL SUFFERING: CAN WE GET A BETTER RESPONSE FROM OUR MICE?

Manuscrito em preparação para ser submetido à revista *Laboratory Animals*

ABSTRACT

Numerous syngeneic and xenograft solid tumor models are available for the study of cancer biology. However, they invariably involve painful syndromes which may cause intense stress to our laboratory animals. The Ehrlich carcinoma syngeneic model is still widely used due to the "undemanding profile" of its cell in culture, easy reproduction and, finally, the chance to be developed as either ascitic or solid form, depending on the inoculation site. The present study assesses the possibility of refining Ehrlich solid experimental protocols either by including PVC tubes as environmental enrichment in the Ehrlich solid tumor model on flank or reducing oncologic pain by daily treatment with opioids in the Ehrlich solid hind paw tumor model. Adult female Balb/cAnUnib mice were submitted to subcutaneous injection of Ehrlich carcinoma cells either on the flank or on the hind paw and monitored for 16 and 12 days, respectively. Environmental enrichment did not impair the animals' response to doxorubicin in the positive control group or the tumor development in the negative control one. However, the reduction of intense local pain caused by the development of hind paw Ehrlich solid tumor had a positive impact on the clinical evaluation of the animals as well as on tumor size of 5-FU treated ones, reducing tumor volume when compared to the "non-refined 5-FU" group. Diminishing laboratory animal suffering, mainly those submitted to experimental solid tumor models, is not only humanitarian, but minimizes important bias in pharmacological response.

Keywords: Cancer models. Refinement. Cancer pain. Animal suffering.

Introduction

Despite the considerable variety of *ex-vivo, in silico* and *in vitro* models available, the search for new anticancer substances must involve the use of laboratory animals. Cell culture panels, for example, are very useful for the screening of small molecules allowing for the study of multiple cell target interactions (Carvalho et al., 2014). Strong criticism points out the differences between species and, therefore, distinct pharmacological responses related to metabolism and transformation of test compounds. However, animal models still provide valuable data as far as drug efficacy and toxicity are concerned. It is no novelty to affirm that animal cancer models, either spontaneous or genetically modified ones, negative or orphan models (pathologies not described in human beings), are miles away from perfection; however, they represent, with all peculiarities, useful tools for the comprehension of a variety of complex diseases such as cancer. The numerous experimental solid tumor models available for the study of cancer biology invariably involve painful syndromes: bone invasion, peripheral nerve compression, spinal canal infiltration, obstruction of hollow viscera, infiltration of parenchymal viscera causing ischemia or capsule distention, invasion and occlusion of blood vessels causing edema and vascular congestion, infiltration of mucosa and/or skin, besides chemotherapy-related disorders.

The Ehrlich carcinoma murine model was first described in the beginning of the 20th century (Murphy, 1913) and remains being explored especially due to the "undemanding profile" of its cell in culture. Besides, it is simple to be reproduced, and offers the possibility of either an ascitic or solid form, depending on the inoculation site on the animals. It is a syngeneic experimental model, which allows the study of a test substance in the presence of the host's functional immune system (Kapoor et al., 2016). Obviously, there are disadvantages as well as important limitations, but the great number of papers using such animal model in the last 10 years demonstrates the even "old" experimental models, when properly assessed, provide relevant data on the tumor cell at a molecular level (Frajacomo et al., 2016; Dubey et al., 2015; Kabel et al., 2015; Oliveira et al., 2015).

The comparison between preclinical studies of new anticancer drugs and phase II trials reveals, at least, one important difference: a combination of a pharmacologic and non-pharmacologic approach using in supportive care of oncologic patients. Aside from the new chemotherapeutic agent being evaluated in a clinical trial, the necessary supportive care protocol remains unaltered in most cases. Antibiotics, corticoids, analgesics, antiemetics, blood transfusions, psychotherapeutic support and others are particularly relevant, even in a clinical

trial, to prevent the adverse effects of the disease and treatment (Li & Li, 2016; Pearson et al., 2016), which may compromise the patient's response to the anticancer agents and prognosis (Jawed et al., 2014; Scotté, 2012; Ströhle et al., 2010).

Aiming to reduce laboratory animal suffering and, concomitantly, to alleviate some of the negative consequences of both cancer development and chemotherapeutic treatment, the present study assessed the effect of environmental enrichment as well as the use of an opioid analgesic in the solid Ehrlich tumor model on flank and hind paw, respectively.

Materials and Methods

1. Cell suspension preparation

Ehrlich carcinoma cells were maintained in liquid nitrogen. After thawing and verifying cell viability through trypan blue exclusion method in Neubauer chamber (Strober, 2015), 1 x 10^5 cells were intraperitoneally (i.p.) injected into two "donor" mice. After five consecutive days, animals were euthanized and ascitic fluid aseptically collected. After centrifuging (200 g; 5 min) and resuspending in phosphate buffer sterile solution (PBS; pH 7), cell viability test was performed and the same cell density was i.p. injected to two additional "donor" mice. Five days after the second *in vivo* transfer, under general anesthesia (100 mg Kg^{-1} ketamine combined with 10 mg kg^{-1} xylazine; i.p.), both Ehrlich ascitic tumor bearing mice were submitted to an i.p. administration of PBS to wash the abdominal cavity and ease the collection of the ascitic fluid. Centrifuging and cell viability test were, again, performed and the inoculation cell density adjusted to each experiment.

2. Animals

Adult female Balb/cAnUnib mice weighing 18-25g were obtained from the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (CEMIB), State University of Campinas (UNICAMP, São Paulo, Brazil). At least seven days before the beginning of the experiment (maintenance period), animals were group housed in polypropylene cages (7-10 individuals per cage; length 49 cm, width 34 cm, depth 16 cm) with sterile soft wood bedding (*Pinus sp.*). Pelletized commercial diet (Biobase, *Biotécnicas Indústria e Comércio Ltda*., Brazil) and clean water offered *ad libitum*. Room temperature was maintained constant (22 ± 2°C) on a 12 h light-dark cycle, with lights off at 18:00 h.

Experimental protocols were approved by the University's Committee for Ethics in Animal Use (CEUA-UNICAMP, protocol number 3978-1). Animals' welfare guidelines were in accordance with both the International Guiding Principles for Biomedical Research Involving Animals (CIOMS-ICLAS, December 2012) as well as the Brazilian Guideline for the Care and Use of Animals for Scientific and Didactic Purposes (CONCEA, 2013).

3. Experimental procedures

3.1. Ehrlich solid tumor on flank

3.1.1. Original protocol

After acclimatization period, 20 adult Balb/cAnUnib female mice were submitted to trichotomy of the flank region under general anesthesia (ketamine-xylazine mix) followed by a subcutaneous injection of a suspension with 5 x 10^6 Ehrlich cells in 60 µL of PBS solution. Three days later, they were randomly divided into two experimental groups: "positive control" submitted to chemotherapy (Doxorubicin hydrochloride; 3 mg kg^{-1}; 5 mL kg^{-1}; i.p. every three days; Doxolem®, injectable lyophilized powder, *Zodiac Produtos Farmacêuticos S/A*, Pindamonhangaba, SP); "negative control" submitted to intraperitoneal administration of sterile 0.9% NaCl saline solution (5 mL kg^{-1}; every three days). A "non-tumor-bearing" group ("satellite") of 7 animals were included to provide with healthy reference clinical values (Table 1). Throughout the test period (around 16 days), to avoid excessive suffering, individual clinical evaluation was performed daily using a body condition index (Lloyd & Wolfenson, 2003), which scored from 0-4, piloerection, ocular and nasal discharges, body weight loss, changes in normal exploratory behavior, hunched up posture and stool consistency. Food and body weight were measured every 3 days. At the end of the experiment, animals were anesthetized, peripheral blood collected (femoral vein) and euthanized (general anesthesia overdose followed by cervical dislocation). Animals' carcasses as well as vital organs were weighed. Tumor mass was excised, weighed and fixed in buffered formalin solution for histological analysis. For tumor weight analysis, only the non-ulcerated masses were considered. After 24 h in formalin solution, tumor samples were transferred to 70% ethanol, before routine histological processing. Tumor sections (3 µm) were embedded in paraffin wax and stained (Hematoxylin & Eosin) for light microscopic analysis.

3.1.2. Refinement proposal

In order to assess the effect of stress reduction over the experimental animals' general condition and, eventually, over tumor development, three additional groups were added to the ones previously described. All three extra groups ("enriched positive control", "enriched negative control" and "enriched satellite") had access to clean polyvinyl chloride (PVC) tubes as a form of environmental enrichment. All experimental procedures were carried out as described in the original protocol (Table 1).

3.2. Hind paw Ehrlich solid tumor

3.2.1. Original protocol

After acclimatization period, 20 adult Balb/cAnUnib female mice were submitted to intraplantar inoculation of a suspension with 2.5 x 10^6 Ehrlich cells in 20 µL of PBS solution under mechanical restrain. Three days after cell inoculation, 20 animals were randomly divided into two "treated" groups. However, before treatment, a baseline measurement of the hind paw volume was performed using a plethysmometer (Cat. No. 37140, Ugo Basile, Italy). Following the basal volume record, intraperitoneal administration was carried out: "positive control" submitted to chemotherapy (5-fluorouracil; 10 mg kg^{-1}; 5 mL kg^{-1}; i.p.; daily; *Eurofarma Laboratórios Ltda*., São Paulo, SP); "negative control" submitted to intraperitoneal administration of sterile 0.9% NaCl saline solution (5 mL kg^{-1}; daily). A "non-tumor-bearing" group ("satellite") of 7 animals were included to provide with healthy reference clinical values (Table 1). As described in the ES on flank experiment, throughout the test period (12 days), daily individual clinical evaluation was performed daily. Hind paw volume measurement, body weight and food intake was monitored every three days. At the end of the experiment, animals were anesthetized, peripheral blood collected (femoral vein), and euthanized. Excision and weighing of both hind paws (left and right) were performed. Finally, animals' carcasses as well as vital organs were weighed.

3.2.2. Refinement proposal

In order to assess the effect of pain reduction over the experimental animals' general condition and, eventually, over tumor development, three additional groups were added to the ones previously described. All three extra groups ("no pain positive control", "no pain negative control" and "no pain satellite") were submitted to daily intraperitoneal administration of

morphine sulphate (10 mg kg^{-1}; 5 mL kg^{-1}; Dimorf®, Cristália, Brasil). All experimental procedures were carried out as described in the original protocol (Table 1).

Table 1. Distribution of experimental animals used for the Ehrlich solid tumor assay.

Group	Identification	# of Ehrlich cells/ inoculation site	Refinement	i.p. treatment	Dose/volume/ frequency of treatments	*n*
G 1	"Satellite"	-	none	none	-	7
G 2			PVC tubes			7
G 3	Negative control	1 x 10^6 - flank	none	Sterile NaCl 0.9% saline	5 mL kg^{-1}; every 3 days	10
G 4			PVC tubes			10
G 5	Positive control		none	Doxorubicin	3 mg kg^{-1}; 5 mL kg^{-1}; every 3 days	10
G 6			PVC tubes			10
G 1	"Satellite"	-	none	none	-	7
G 2			Morphine 10 mg kg^{-1}			7
G 3	Negative control	2.5 x 10^6 – hind paw	none	Sterile NaCl 0.9% saline	5 mL kg^{-1}; daily	10
G 4			Morphine 10 mg kg^{-1}			10
G 5	Positive control		none	5-Fluorouracil	10 mg kg^{-1}; 5 mL kg^{-1}; daily	10
G 6			Morphine 10 mg kg^{-1}			10

3.3. Statistical analysis

Experimental data was expressed as mean ± SEM (standard error of mean), using analysis of variance (ANOVA) followed by Tukey test or Student unpaired *t* test for individual comparisons. P values lower than 0.05 ($p<0.05$) were considered statistically significant and represented by $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. Statistical software Graph-Pad Prism version 5.0 (San Diego, CA, USA) was used for all calculations.

Results

Diet intake and clinical scoring

Since mice were group housed, diet intake was estimated by the number of animals per cage divided by the time span. In the Ehrlich solid on flank experiment, no differences were observed among all groups (with or without tumor) up to the 9th day. However, from 12th to 15th day both doxorubicin-treated groups showed 10-16% reduction in food intake compared to the saline-treated ones. The comparison between "enriched" and "not enriched" groups revealed no differences (data not shown). In the hind paw solid tumor, the reduction in food consumption of 5-fluorouracil-treated groups was observed from the 5th day onwards compared to the saline-treated ones. Also at this point, it was possible to observe a reduction in diet intake on the tumor-bearing groups (G3-paw to G6-paw). Daily administration of morphine had no influence on this parameter (data not shown).

On day 1, during the first five minutes after the introduction of the PVC tubes, mice kept a certain distance from the new objects in their cages (Fig. 1b). After that, however, they remained most of the day period inside the tubes (Fig.1c). The ones without any enrichment in their cages had no choice but explore their environment with no hiding options, thus, spending most of the day period near the cage walls (Fig 1d). From day 12 until the end of experiment (day 16), doxorubicin-treated animals presented (G5-flank and G6-flank) piloerection, isolated behavior, diarrhea (Fig. 1a) and, in many cases, first tumor ulceration. Due to the severity of clinical scoring, the endpoint of the experiment was anticipated to the 16th day, instead of the 20th day, as originally planned.

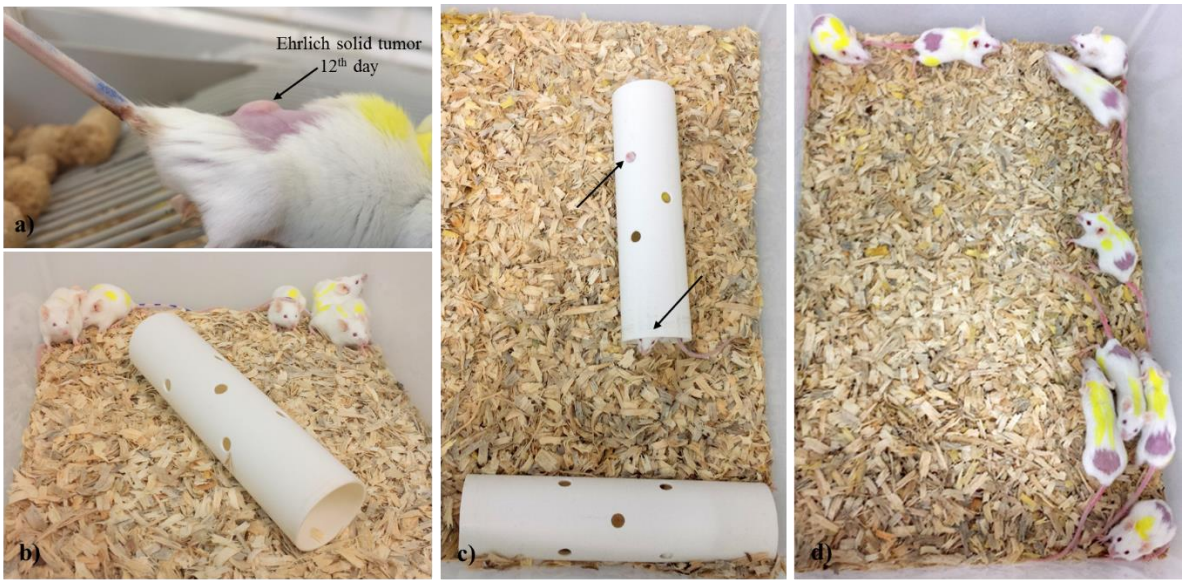

Figure 1. (a) Piloerection and diarrhea presented by doxorubicin-treated mouse. (b) Enriched cage immediately after the placing of two PVC tubes ("novelty effect"). (c) The same cage, afive minutes later; animals remained inside the tubes most of the daily period. (d) Non-enriched cage.

Clinical evaluation of hind paw Ehrlich tumor animals, mainly during the last five days of experiment, revealed important differences in behavior among groups: mice treated only with 0.9% NaCl saline solution (G3-paw) showed signs of intense pain, such as isolation, hunching up, lack of movement, piloerection and ptosis. On the other hand, "enriched negative control" group (G4-paw) showed normal mobility despite their hind paw size. Positive control groups (G5-paw and G6-paw) presented at the same period, piloerection, isolated behavior and severe diarrhea. Daily administration of morphine to "satellite" group (G2-paw) did not seem to affect the animals' behavior compared to G1-paw (data not shown).

Hematological analysis

Environmental refinement did not influence hematological values of either "satellite" groups (G1-flank and G2-flank) ($p>0.05$), negative control groups (G3-flank and G4-flank) ($p>0.05$), or positive control groups (G5-flank and G6-flank) ($p>0.05$). On the other hand, as expected, chemotherapy-treated animals (G5-flank and G6-flank) showed decreased in white blood cell count ($p<0.001$), red blood cell count ($p<0.001$; $p<0.05$), hemoglobin ($p<0.001$; $p<0.01$; $p<0.05$), hematocrit ($p<0.001$; $p<0.01$) and increase in both mean corpuscular hemoglobin concentration ($p<0.001$) and platelet count ($p<0.001$) when compared to negative control groups. Only G6-flank group presented reduced values of mean corpuscular volume ($p<0.01$) when compared to G4-flank group (Table 2).

Between non-bearing animals, daily administration of morphine (G2-paw) led to a decrease in white blood cells count ($p<0.001$) and mean corpuscular hemoglobin ($p<0.05$). Similarly, between negative control groups, morphine (G4-paw) led to a decrease in WBC ($p<0.001$). However, between chemotherapy-treated groups, morphine did not interfere in any hematological parameter. Chemotherapy-treated animals (G5-paw and G6-paw) showed decreased in white blood cell count ($p<0.001$), red blood cell count ($p<0.001$; $p<0.05$), hemoglobin ($p<0.01$; $p<0.05$), mean corpuscular value ($p<0.01$) and increase in both mean corpuscular hemoglobin concentration ($p<0.001$) and platelet count ($p<0.001$) when compared to negative control groups (Table 3). However, daily i.p. administration of morphine did not alter hematological values of 5-FU-treated animals (G6-paw) when compared to the non-refined 5-FU-treated group (G5-paw) ($p>0.05$).

Table 2. Effect of environmental enrichment on hematological parameters of healthy and ES tumor-bearing mice (flank).

	"Satellite" – no tumor		**0.9% Saline solution-treated**		**Doxorubicin-treated**	
	G1 – original (a)	G2 – PVC (b)	G3 – original (c)	G4 – PVC (d)	G5 – original (e)	G6 – PVC (f)
WBC (x 10^3 / µL)	10.89 ± 0.47	11.34 ± 0.67	8.88 ± 0.78	9.28 ± 0.81	3.31 ± 0.38^{cccddd}	2.66 ± 0.23^{cccddd}
RBC (x 10^6 / µL)	10.52 ± 0.11	10.36 ± 0.18	10.41 ± 0.17	9.92 ± 0.23	9.17 ± 0.17^{cccd}	9.06 ± 0.18^{cccd}
HGB (g / dL)	14.66 ± 0.11	14.37 ± 0.28	14.19 ± 0.34	13.71 ± 0.29	12.71 ± 0.18^{cc}	12.48 ± 0.28^{cccd}
HCT (%)	51.69 ± 0.48	50.70 ± 0.94	50.31 ± 1.13	48.36 ± 1.12	43.60 ± 0.64^{cccdd}	42.3 ± 1.00^{cccdd}
MCV (fL)	49.18 ± 0.17	48.92 ± 0.24	48.28 ± 0.38	48.74 ± 0.22	47.62 ± 0.33	47.16 ± 0.26^{dd}
MCH (pg)	13.95 ± 0.08	13.86 ± 0.11	13.59 ± 0.15	13.82 ± 0.07	13.87 ± 0.11	13.78 ± 0.09
MCHC (g / dL)	28.34 ± 0.10	28.33 ± 0.09	28.19 ± 0.08	28.38 ± 0.10	29.17 ± 0.11^{cccddd}	29.20 ± 0.09^{cccddd}
PLT (x 10^3 / µL)	1330 ± 42.92	1239 ± 58.40	1500 ± 57.25	1429 ± 46.18	2186 ± 87.84^{cccddd}	2122 ± 109.30^{cccddd}

Table 3. Effect of morphine daily i.p. treatment on hematological parameters of healthy and ES tumor-bearing mice (hind paw).

	"Satellite" – no tumor		**0.9% Saline solution-treated**		**5-Fluorouracil-treated**	
	G1 – original (a)	G2 – morphine (b)	G3 – original (c)	G4 – morphine (d)	G5 – original (e)	G6 – morphine (f)
WBC (x 10^3 / µL)	12.06 ± 0.58	6.77 ± 0.56^{aaa}	8.87 ± 0.49	4.56 ± 0.25^{ccc}	0.95 ± 0.14^{cccddd}	0.77 ± 0.06^{cccddd}
RBC (x 10^6 / µL)	10.60 ± 0.10	10.58 ± 0.20	10.76 ± 0.10	11.16 ± 0.17	11.98 ± 0.45^{c}	12.69 ± 0.23^{cccddd}
HGB (g / dL)	15.06 ± 0.10	14.57 ± 0.26	14.82 ± 0.13	15.29 ± 0.24	16.30 ± 0.61^{c}	17.52 ± 0.33^{cccddd}
HCT (%)	52.17 ± 0.42	51.43 ± 0.96	52.95 ± 0.52	54.88 ± 0.88	57.14 ± 6.14	60.72 ± 1.04
MCV (fL)	49.21 ± 0.12	48.61 ± 0.23	49.20 ± 0.17	49.17 ± 0.18	47.73 ± 0.37^{cccddd}	47.49 ± 0.17^{cccddd}
MCH (pg)	14.17 ± 0.08	13.77 ± 0.07^{a}	13.77 ± 0.06	13.70 ± 0.08	13.62 ± 0.15	13.70 ± 0.08
MCHC (g / dL)	28.87 ± 0.11	28.33 ± 0.04	27.99 ± 0.09	27.86 ± 0.09	28.51 ± 0.15^{ccddd}	28.86 ± 0.09^{cccddd}
PLT (x10^3 / µL)	1408 ± 58.95	1593 ± 56.40	1370 ± 32.94	1452 ± 36.32	798.80±70.93^{cccddd}	911.90 ± 91.40^{cccddd}

WBC (white blood cell count); RBC (red blood cell count); HGB (hemoglobin); HCT (hematocrit); MCV (mean corpuscular volume); MCH (mean corpuscular hemoglobin); MCHC (mean corpuscular hemoglobin concentration); PLT (platelet count). Values are expressed as mean ± SEM. Statistical significance: aaa $p<0.001$; aa $p<0.01$; a $p<0.05$ when compared to G1; (b) when compared to G2; (c) when compared to G3; (d) when compared to G4; (e) when compared to G5 and (f) when compared to G6.

Carcass weight data and relative organ weight

In order to distinguish tumor mass development from body weight gain/loss, at the end of each experiment the carcasses were weighed immediately after removal of solid tumor. In both experiments, chemotherapy-treated animals showed a significant body weight loss (Fig.2a-b). Among non-bearing animals ("satellite" groups), environmental enriched led to a better weight gain (final b.w. – initial b.w.) in comparison to the ones with no PVC tubes in their cages ($p<0.05$) (Fig. 1c).

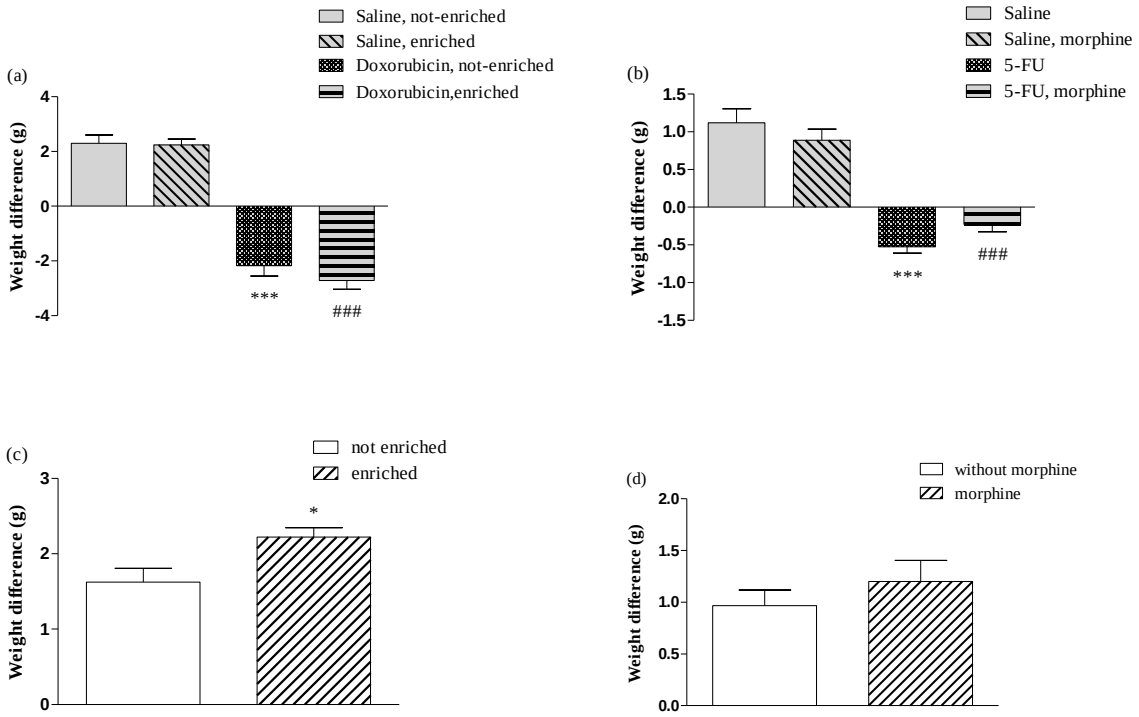

Figure 2. (a) Effect of environmental enrichment on body weight difference of flank Ehrlich solid tumor-bearing Balb/cAnUnib female adult mice after 16 days. (b) Effect of morphine daily treatment (i.p.) on body weight difference of hind paw Ehrlich solid tumor-bearing Balb/cAnUnib female adult mice after 12 days. (c) Effect of environmental enrichment (PVC tubes) on body weight gain of non-bearing tumor Balb/cAnUnib female adult mice after 16 days. (d) Effect of morphine daily treatment (i.p.) on body weight gain of non-bearing tumor Balb/cAnUnib female adult mice after 12 days. Data are expressed as mean ± SEM. Significance ($p<0.05$) is indicated by * in comparison with non-refined negative control group and # in comparison with refined negative control group.

Gross necropsy and relative organ weight

Both “satellite” as well as “saline solution-treated” groups revealed no macroscopic changes in gross necropsy in both experiments. Doxorubicin and 5-FU-treated animals, however, showed pale liver and kidneys. Both organs weights were statistically different ($p<0.001$) from negative control groups. Both flank and hind paw tumor-bearing animals (“saline solution-treated”) showed significant increase in spleen weight in comparison to “satellite” group. Adrenal gland weights were not statistically different among all groups (data not shown).

Histological analysis

Histological features of non-bearing skin as well as tumor mass are illustrated in figure 3. Typical histology of mouse skin in the flank reveals *stratum corneum*, epithelial cells, dermis, sebaceous glands, hair follicles, adipose cells in hypodermis and muscular layer. Ehrlich tumor parenchyma consisting of epithelioid cells with clear cytoplasm, enlarged and pleomorphic nuclei with prominent nucleoli and connective tissue. Necrotic areas are frequently observed.

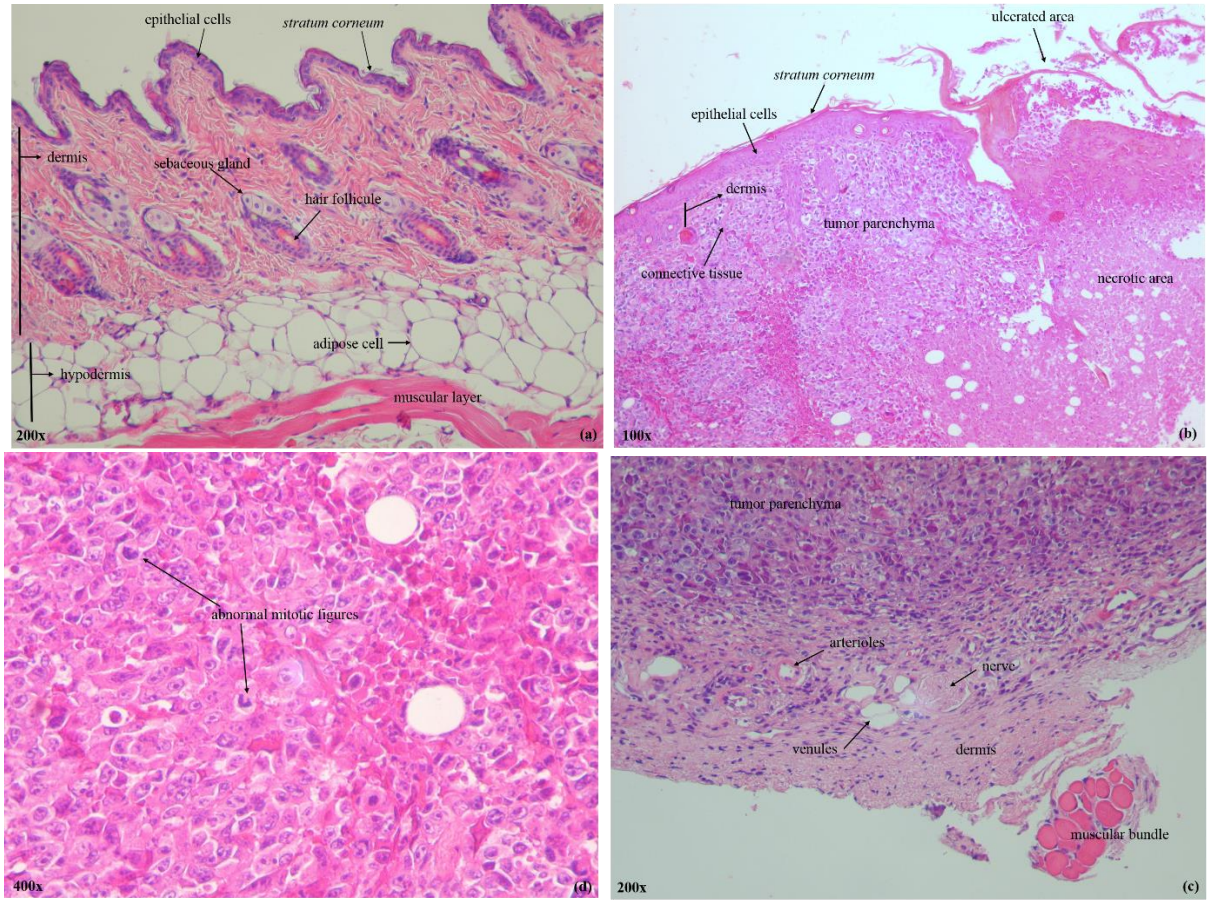

Figure 3. (a) Healthy mice skin photomicrograph H&E stained (G1-flank and G2-flank). (b-c) Cross-sectional cut of Ehrlich tumor on flank: animal treated with doxorubicin (3 mg kg^{-1}; i.p. every 3 days) and animal treated with NaCl saline solution (5 mL kg^{-1}; i.p.; every 3 days), respectively. Environmental enrichment did not alter Ehrlich tumor histological characteristics: epithelioid cells with clear cytoplasm, enlarged and pleomorphic nuclei with prominent nucleoli and conjunctive trabeculae (d).

Tumor development

Doxorubicin intraperitoneal treatment was effective in reducing Ehrlich tumor mass in both enriched and non-enriched experimental groups (Fig. 4a). The cut surface of tumor mass presented a whitish to yellow surface and firm texture, with a higher incidence of ulceration in the chemotherapy-treated groups. It is worth mentioning that, due to the loss of tumor parenchyma (necrotic area) adjacent to ulcerated surfaces, only non-ulcerated tumor masses were considered in the final tumor growth assessment.

On the other hand, morphine treatment did alter Ehrlich hind paw tumor development as shown in Figure 4b. Negative control group not submitted to the opioid daily

treatment (G3-paw) presented higher hind paw volumes when compared to the morphine-treated one (G4-paw). It was possible to observe that the use of morphine associated with the chemotherapy treatment with 5-FU resulted in a smaller hind paw volume. Worth to emphasize that statistical difference between positive and negative control groups were already observed on the first volume measurement ($p<0.01$; $p<0.001$; $p<0.001$ and $p<0.01$) between morphine-treated animals (G4-paw and G6-paw) whereas non-refined control groups (G3-paw and G5-paw) only presented statistical difference on the last two assessments ($p>0.05$; $p>0.05$; $p<0.001$ and $p<0.01$) (Fig. 4).

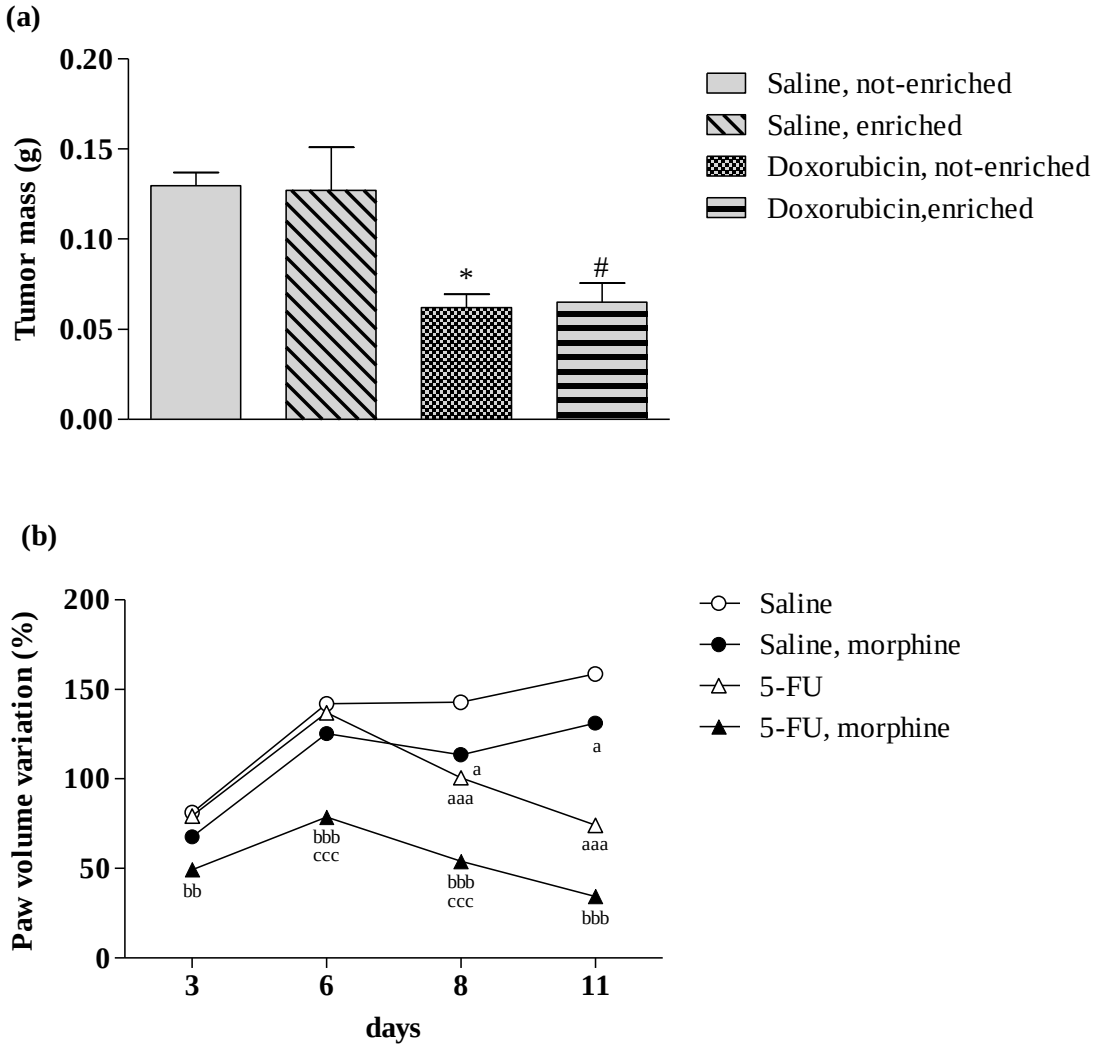

Figure 4. (a) Effect of environmental enrichment on Ehrlich tumor development in NaCl saline solution treated animals and doxorubicin-treated animals. (b) Effect of morphine intraperitoneal treatment on Ehrlich hind paw tumor development in NaCl saline solution treated animals and

5-FU-treated animals (b). Values are expressed as mean ± SEM. Statistical significance: * $p<0.05$ in comparison to G3-flank; $^{\#}$ $p<0.05$ in comparison to G4-flank; ^{a} $p<0.05$, ^{aa} $p<0.01$, ^{aaa} $p<0.001$ in comparison to G3-paw; ^{bb} $p<0.01$, ^{bbb} $p<0.001$ in comparison to G4-paw; ^{ccc} $p<0.001$ in comparison to G5-paw.

Discussion

Despite the undoubted benefits of environmental enrichment, our data demonstrated that it altered neither tumor development nor the animals' pharmacological response to doxorubicin as far as body weight loss and hematological parameters are concerned. Considering that non-bearing animals exposed to the PVC tubes presented a greater body weight gain at the end of the experimental period, our negative results in the Ehrlich solid tumor on flank suggest that the systemic changes induced by tumor growth and/or chemotherapy may have overlapped the possible benefits resulted from the inclusion of an environmental enrichment item.

Another possible explanation is related to the type of enrichment offered; combined forms of cage enrichment such as toys, exercise wheels, shelters, nesting material and shredded paper are proven to reduce the negative effects of stress and even alter the production and signaling of neurochemicals and hormones involved in neuronal plasticity (Ronzoni et al., 2016; Sale et al., 2014; Lambert et al., 2005). The lack of diversity of stimulus, since different types of objects used as enrichment simultaneously may stimulate different areas of the mice brain, altering the animals' neurobiology (Oliva et al., 2010; Lambert et al., 2005). Nonetheless, before selection of the object to be introduced as a form of enrichment, such as animal species, experimental design and future changes in the animal facility's routine (Baumans & Van Loo, 2013) must be considered. Whenever possible, it is essential that veterinarians instruct both animal caretakers and researchers about the benefits of introducing environmental enrichment not only during the animals' maintenance period, but also throughout a pharmacological and/or toxicological test.

Regarding chemotherapy, the severe side effects of anthracyclines such as gastrointestinal disorders, inappetence and general uneasiness generally observed in human patients (Chabner et al., 2012) were probably responsible for the food intake reduction, piloerection, diarrhea and lethargy of doxorubicin-treated animals from the 12th day forward.

Lower liver weight may be the result of hepatic parenchyma damage caused by repeated administration of this anticancer agent. Other animal studies report high serum values of both alanine aminotransferase and aspartate aminotransferase after a single intravenous dose of doxorubicin (Saad et al., 2001), which may reflect leakage of either hepatic or cardiac enzymes, both as consequences of cell damage (Xin et al., 2011; Abboud & Kaplowitz, 2007). Moreover, higher kidney weight also observed in the chemotherapy-treated groups may suggest the adaptation of tubular epithelium partially injured due to the exposure of toxic substances, resulting proliferation and compensatory hypertrophy (Kays & Schnellmann, 1995). Finally, the observed leukopenia and anemia (reduction in hemoglobin, hematocrit and red blood cell count) of animals have been widely described as doxorubicin-induced toxic effect due to a pronounced myelosuppression (Wang et al., 2016; Chabner et al., 2012).

The lower body weight gains of healthy animals not exposed to PVC tubes corroborates studies that point to the importance of "hiding places" for mice, especially when maintained in large cages (Olsson & Dahlborn, 2002). As preys, mice seek for protection or escape at first sign of threat such as handling or removal of cage cover (Apfelbach, 2005; Dickman, 1992). The resulting stress functions as a stimulus to prefrontal and infralimbic cortex, activating the hypothalamus-pituitary-adrenal axis leading to an increase of blood levels of corticosteroids (Ronzoni et al., 2016). Even though there are some factors other than stress may alter circulating glucocorticoids together with the controversy surrounding regulation of lipid and protein metabolism (John et al., 2016), the association between high corticoid blood concentrations, increased immune function and impaired feed efficiency in cattle has long been identified (Foote et al., 2016). Lower body weight gains in rats exposed to either single or repeated stressful situations have also been observed (Renata-Márquez et al., 2003). Moreover, studies reveal that lack of stimulus may lead animals to spend most of the dark cycle asleep when, in natural conditions, they should be active, feeding and exploring their environment (Nevison et al., 1999).

The second aspect assessed in our work was the effect of severe cancer pain – or the reduction of it – on the animals' clinical condition, response to treatment and tumor development. Ehrlich hind paw solid tumor has recently been described as a suitable model for the study of antinociceptive compounds due to the intense muscular, nerve, bone, vascular and cartilaginous tissue invasion in peritumoral area (Calixto-Campos et al., 2013). Skeletal hyperalgesia represents an important cause of animal suffering while tumor mass expansion

provokes periosteum distention, bone invasion and fracture (Shenoy et al., 2016; Simmons et al., 2015). Compression, traction and/or destruction of nerve endings, abundant in fore and hind paws, together with local inflammation are responsible for both mechanical and thermal hyperalgesia experienced by bone cancer patients. In addition to that, the activation of NMDA (N-methyl-D-aspartate) receptors by external stimuli is associated with neural synaptic plasticity and, therefore, production and maintenance of central sensitization (Wang & Yu, 2016). From the 5th day on, the assessment of clinical condition of hind paw tumor bearing animals not submitted to the daily opioid treatment revealed poor grooming, decrease in locomotion and, later, piloerection and lethargy.

The significant lower paw volume of morphine-treated animals (3 mg kg^{-1}, daily, for 8 days) raises the discussion of possible mechanisms of action of opioids in cancer experimental models. Both *in vivo* and *in vitro* immunomodulatory effect of morphine and other opioids have been discussed even though the exact mechanisms remain unclear. The presence of opioid receptors (or the expression of mRNA for these receptors) in mature and immature B cells, T cells and macrophages might induce leukocyte reduction or proliferation (Eisenstein, 2011). It is worth noticing that both healthy (G2-paw) and tumor-bearing groups (G4-paw) presented significant decrease (56.14% and 51.41%, respectively) in WBC count ($p<0.001$) in relation to the non-morphine-treated ones. Prospective study of patients receiving morphine either intravenously or infused into the spinal canal showed that both groups developed lymphopenia and humoral immune response suppression in peripheral blood, but not in the blood from surgical drains (local). However, significantly lower CD4+ blood cell counts were detected only after spinal morphine treatment; also, no differences were observed in the local leukocyte count of either group, suggesting different mechanisms of central modulation of the inflammatory response to stress (Longás Valién et al., 2005). Assessment of cellular immune, plasma cytokine and proteome responses after morphine administration in nonhuman primates revealed a reduction in lymph node and peripheral blood T-cell activation probably due to a decrease in energy metabolism proteins measured in those tissues. In the same study, however, proteomic analysis of other samples, such as colon and cerebrospinal fluid (CSF), showed distinct responses to morphine (Brown et al., 2012). Macrophage function has also been related to both administration of morphine and withdrawal from it in C3HeB/FeJ female mice (Rahim et al., 2003; Rahim et al., 2004). In contrast, studies with transgenic sickle mice demonstrated the proneuroinflammatory activity of morphine, stimulating mast cell activation (Vincent et al.,

2013). Besides its analgesic effect via m-opioid receptors in the central nervous cells as well as anti/pro-inflammatory ones, some authors include other non-neural morphine-responsive cells, such as endothelial, tumor and mast cells (Nguyen et al., 2014). All these findings highlight the need for critical evaluation before selecting the proper pain suppressor agent in a solid tumor experimental protocol, considering their effect in the parameters to be analyzed. Eventually, the use of morphine analogs may represent an alternative for those protocols, even though, in our study, morphine did not alter significantly WBC counts in 5-FU treated group (G6-paw) in comparison to the non-refined one (G5-paw).

Though it might be relevant to find out the exact mechanism(s) through which morphine-treated tumor-bearing animals presented lower hind paw volume, the nociceptive effect of this drug may not be disregarded. Neuropathic pain caused by vast muscular, venous, bone and cartilage damage in the Ehrlich hind paw tumor model represents an important experiment variable, considering the individuality of pain perception (Burton et al., 2016). Every time a given pharmacological model aims to evaluate an anticancer activity and no antinociceptive effect will be measured, the intense pain caused by tissue destruction becomes a useless by-product. In such cases, the heterogeneous reactions to nociception will probably contribute to a larger standard deviation in the pharmacological response to the test substance, as observed in previous studies (Monteiro et al., 2013).

Unfortunately, aside from the distress caused by the disease itself, oncologic patients – including laboratory animals submitted to experimental cancer models – must face the severe side effects of chemotherapy (Scotté, 2012). Mucositis, nausea, vomiting, diarrhea, febrile neutropenia (Lee et al., 2016), severe fatigue (Williams et al., 2016), depression, cognitive impairment (Iarkov et al., 2016) and chemotherapy-induced peripheral neuropathy (Iżycki et al., 2016; Schloss et al., 2016) are considered of utmost relevance to the patient's prognosis. Even disregarding the psychological aspects which affect human cancer patients (emotional suffering, mutilation, depression, fear of death) for the veterinary ones remains intense painful processes caused either by the disease or treatment-related. Our data demonstrated that animals daily treated with morphine combined with 5-FU showed smaller hind paw volume.

Finally, as far as experimental refinement is concerned, ulceration is also an important parameter for clinical evaluation of solid tumors. Scoring ulcerated area, depth of ulceration, aspect of wounded area in association with general body condition assessment may

help researchers anticipate experiment endpoint, avoiding excessive animal distress as well as loss of biological material (Wolfensohn & Lloyd, 2003).

It is well known that the inclusion of a pain relieving agent in cancer models as part of our "patient's" supportive care is controversial until proven, at a molecular level, that it does not compromise the pharmacological experimental model per se. Nonetheless, the large amount of scientific data regarding the adverse effects of pain and distress on our laboratory animals' physiology as well as the uniqueness of an individual's response to pain significantly increasing the standard deviation of many parameters should induce us to question some of our conventional cancer models, where the oncologic "patient" does not benefit from any supportive care and the researcher disregards one high-impact variable in his/her experimental results: cancer pain.

Conclusion

Reducing laboratory animal suffering, mainly those submitted to experimental solid tumor models, is not only humanitarian, but minimizes important bias in pharmacological response. A case-by-case assessment of the proper refinement measure to reduce the stressful effect of the possible painful syndromes for each pharmacological cancer model is needed. Improving our experimental animals' quality of life with an adequate supportive care will probably provide us with better quality data.

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DISCUSSÃO GERAL

A utilização de animais na pesquisa científica desperta nos seres humanos reações distintas, uma vez que ao fazer o seu julgamento de valor o indivíduo se baseia em aspectos culturais, emocionais, religiosos e morais. Há quase oito décadas, a discussão envolvendo ética na experimentação animal tem impulsionado avanços na área de criação e manutenção dos animais de laboratório, visando o bem-estar e melhores condições de higiene. Ao contrário do que se pode imaginar, o movimento em direção ao tratamento humanitário na pesquisa pré-clínica não foi iniciado por grupos de "proteção animal", mas por profissionais envolvidos diretamente na rotina dos biotérios: médicos veterinários, pesquisadores e bioteristas (Haynes, 2010).

Desde então, as áreas de criação e manutenção de animais de laboratório têm avançado sobremaneira. No aspecto sanitário, partimos das caixas de madeira que acomodavam ratos e camundongos nos anos 50 e evoluímos para a histerectomia asséptica e rederivação por transferência de embriões para controle de colônias, chegando até a identificação e erradicação de patógenos responsáveis por infecções subclínicas, transgênese e controle genético (Fox et al., 2015; Suckow et al., 2006). Outros aspectos relacionados a exigências nutricionais das espécies, faixas desejáveis de temperatura e umidade, luminosidade, trocas de ar, índice de ruídos, inserção ou não de enriquecimento ambiental têm sido discutidos, há muito, visando atingir tanto macro como microambientes ideais para a criação e manejo dos animais de laboratório, sempre que possível. Todo este conhecimento, respaldado por estudos científicos, comprova a influência direta de tais fatores na fisiologia animal e, consequentemente, na qualidade dos resultados experimentais (Beck & Gobatto, 2016; Langgartner et al., 2016; Thériault et al., 2016; Wren-Dail et al., 2016; Rosenwasser et al., 2015; Söderlund et al., 2015; Yoshida et al., 2015; Jackson et al., 2014; Bogdanova et al., 2013; Klöting et al., 2013; Costa et al., 2012; Flanagan et al., 2011).

Por outro lado, tanto a Farmacologia como a Toxicologia experimentais têm oferecido maior resistência no que diz respeito às modificações relacionadas ao conceito de refinamento, especialmente quando envolvem a inclusão de agentes farmacológicos com o objetivo de reduzir o sofrimento animal. Tanto assim que, frequentemente, a justificativa para a não inclusão de analgésicos ou anestésicos apresentada pelo pesquisador ao elaborar seu protocolo de pesquisa para submissão às Comissões de Ética é a "possibilidade de interferência

nos resultados experimentais, seja por interação com a substância-teste ou influência sobre a fisiologia animal, comprometendo o resultado da pesquisa". Sendo assim, neste ponto enfrentamos um verdadeiro impasse: seguir a "cartilha" dos protocolos experimentais já consagrados, há muito descritos e validados, sem questionar os efeitos de procedimentos dolorosos e estressantes sobre os animais de laboratório ou buscar novas bases científicas para propor atualizações de protocolos antigos, levando em conta toda a informação disponível sobre os efeitos do estresse e da dor sobre a fisiologia animal. É bem verdade que, muitas vezes, os mesmos agentes que poderiam minimizar o sofrimento animal alteram ou inviabilizam a pesquisa, como em experimentos onde a dor ou o estresse propriamente dito constituem o objeto do estudo.

É possível afirmar, contudo, que em diversas situações, o sofrimento infringido aos indivíduos representa um subproduto do procedimento experimental, que não será avaliado. Nestes casos, conhecedor dos efeitos deletérios do estresse aliado à individualidade da resposta à dor, o pesquisador deve buscar possibilidades de refinamento em seu procedimento e, ao discutir seus resultados, justificar a eventual modificação do método.

Os dados obtidos no presente projeto de pesquisa, aliados à grande quantidade de informação acerca das alterações fisiológicas promovidas pelo estresse, nos induzem a avaliar de forma mais cuidadosa este importante viés na pesquisa. A participação dos hormônios relacionados às situações de estresse agudo ou crônico tem sido descrita nos mais variados estudos, tais como: alterando parâmetros hematológicos, peso de órgãos e aumentando a incidência de úlcera gástrica em ratos (Sato et al., 2010); como agentes indutores de lesão gástrica experimental (Landeira-Fernandez, 2015; Aboubakr et al., 2013; Liu et al., 2012); elevando índice de metástase, em alguns casos por ineficiência da resposta imune (Chang et al., 2016; Le et al., 2016; Levi et al., 2016), bem como da incidência de adenocarcinoma mamário em ratas (Nakamura et al., 2011); aumentando a progressão tumoral em resposta à dor em modelo de câncer de próstata em camundongos (Thompson et al., 2015); alterando os níveis de opióides endógenos (Guerrero-Alba et al., 2016); elevando a taxa de endometriose induzida em camundongas (Long et al., 2016), entre tantos outros estudos. Contudo, os mecanismos envolvidos na influência da ativação do eixo hipotálamo-hipófise-adrenal nas alterações fisiológicas que possam comprometer os resultados experimentais nem sempre estão completamente elucidados.

Nos modelos experimentais avaliados neste projeto, a manutenção dos animais sob anestesia geral não só durante o procedimento cirúrgico para ligadura de piloro como previsto

pelo protocolo original, mas durante todo o período de latência foi capaz de reduzir de forma significativa a variação dos resultados intragrupo, permitindo, inclusive, a redução do número de indivíduos necessários para a análise estatística dos parâmetros de secreção ácida gástrica. Por sua vez, o bloqueio do nervo ciático em modelo de edema de pata por carragenina pareceu não comprometer a avaliação da atividade anti-inflamatória no que diz respeito à mensuração do volume da pata, bem como da análise histopatológica dos componentes celulares da inflamação. Entretanto, estudos adicionais são necessários para investigar a influência do uso de anestesia local sobre a dosagem de citocinas inflamatórias teciduais e séricas no referido modelo. Em experimento de tumor sólido de Ehrlich em flanco, observou-se que a avaliação da atividade anticâncer não foi comprometida pela inclusão de tubos de PVC nas caixas de animais portadores de tumor, corroborando a tendência do uso de enriquecimento ambiental como item básico, reduzindo a monotonia dos animais, sem prejuízo para o bom andamento da pesquisa. Finalmente, o uso diário de analgésico opióide em teste de tumor de Ehrlich em pata, como forma de reduzir o sofrimento gerado pela intensa dor neuropática e inflamatória decorrentes da progressão tumoral, pareceu não apenas melhorar o estado clínico dos animais, mas também atuar de maneira sinérgica com o quimioterápico usado como controle positivo do experimento.

É importante ressaltar a participação do médico veterinário, com a devida especialização em Ciência de Animal de Laboratório, como profissional responsável não apenas por assegurar as melhores condições ambientais e de manejo em biotérios de produção e manutenção de animais de laboratório, mas, principalmente, em biotérios e laboratórios de experimentação, como profissional prescritor, capaz de identificar, nos modelos farmacológicos e toxicológicos, situações e estados clínicos que possam prejudicar não só a integridade do animal como, também, a qualidade do resultado gerado. Soma-se a este raciocínio a necessidade de se estabelecer um paralelo entre as pesquisas clínica e pré-clínica, seguindo as diretrizes do Código de Nuremberg e da Declaração de Helsinque, nos quais são asseguradas as melhores condições para o bem-estar dos indivíduos, discutindo-se continuamente as questões éticas particulares a cada modelo experimental (World Medical Association, 2013; Mitscherlich & Mielke, 1949).

Como conclusão, antes de ser uma atitude humanitária, a redução do sofrimento animal, especialmente em protocolos que envolvem dor de grande intensidade, elimina importante viés na resposta farmacológica. É necessário, entretanto, um estudo caso a caso da medida adequada de refinamento para cada modelo em questão. Estar atento às condições de

bem-estar dos animais utilizados na pesquisa biomédica resulta em dados experimentais mais precisos e de melhor qualidade científica.

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ANEXO I. Certificados de aprovação – CEUA / UNICAMP

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

CERTIFICADO

Certificamos que o projeto **"Refinamento do modelo experimental de edema de pata em ratos"** (protocolo nº **3318-1**), sob a responsabilidade de **Prof. Dr. João Ernesto de Carvalho / Karin Maia Monteiro**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA, SISBIO** ou **CIBio**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **10 de março de 2014**.

Campinas, 10 de março de 2014.

Prof. Dr. Alexandre Leite Rodrigues de Oliveira
Presidente

Fátima Alonso
Secretária Executiva

CEUA/UNICAMP
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UNICAMP

CEUA/UNICAMP

CERTIFICADO

Certificamos que o projeto intitulado **"Refinamento do estudo de atividade anticâncer em modelo experimental de Ehrlich sólido e ascítico em camundongos"**, protocolo nº **3978-1**, sob a responsabilidade de **Prof. Dr. João Ernesto de Carvalho / Karin Maia Monteiro**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica ou ensino, encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais e do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal - CONCEA**, e foi aprovado pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP,** em **10 de setembro de 2015**.

Vigência do projeto: 10/2015-03/2017
Espécie/Linhagem: Camundongo isogênico / Balb/c
No. de animais: 170
Peso/Idade: 04 semanas / 20g
Sexo: fêmeas
Origem: CEMIB/UNICAMP

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

Campinas, 10 de setembro de 2015.

Profa. Dra. Liana Maria Cardoso Verinaud
Presidente

Fátima Alonso
Secretária Executiva

ANEXO II. Artigos publicados durante o doutoramento

1. Refinamento do modelo experimental de ligadura de piloro. (Monteiro et al., 2013)

Journal of Pharmacological and Toxicological Methods 67 (2013) 121–128

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Original article

Characterization of a refinement of the "pylorus ligation" model of rat gastric ulceration resulting in "no pain" and a more specific pharmacological response

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ABSTRACT

Introduction: The pharmacological assessment of the factors for gastric protection of a test substance should involve experimental models that can determine the involvement of cytoprotective factors, as well as their influence on the secretion of hydrochloric acid. The original protocol of pylorus ligation in rats proposed by Shay et al. in 1945, still in use today, provides a latency time of 240 min without considering the effect of postoperative pain in the mechanisms of peptic ulcer. This paper proposes a modification of this experimental protocol by eliminating the pain throughout the postoperative period, as a refinement of the test with consequent improvement of the pharmacological response. **Methods:** Adult male Wistar/Uni rats underwent surgical ligation of the pylorus and were kept anesthetized throughout the experimental period (4 h) in contrast to the other experimental groups that followed the original protocol proposed by Shay et al., 1945. **Results:** We were able to determine effective doses for a positive control, as well as of a variety of secretagogues in the new experimental protocol proposed. **Discussion:** The suppression of post-surgical pain, through the use of anesthesia throughout the experimental period, brought several benefits for the study of gastric acid secretion, rendering a more homogeneous pharmacologic response in non-inbred animals, thus being an effective experimental procedure.

2. Trabalho em colaboração com o Instituto de Química, UNICAMP. (Vendramini-Costa et al., 2014)

Chemico-Biological Interactions 224 (2014) 206–212

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Chemico-Biological Interactions

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Gastroprotective effects of goniothalamin against ethanol and indomethacin-induced gastric lesions in rats: Role of prostaglandins, nitric oxide and sulfhydryl compounds

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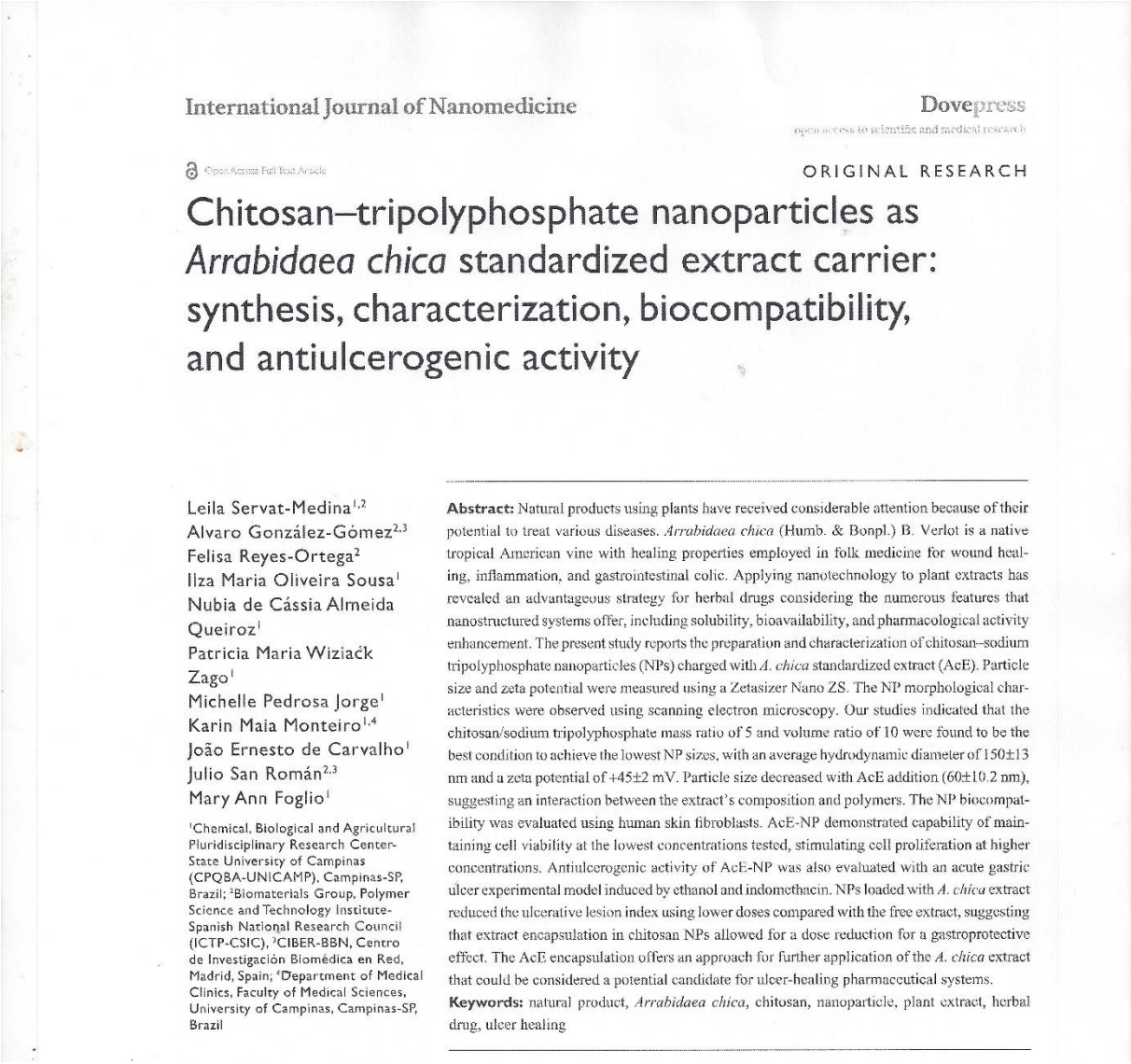
Rats

ABSTRACT

Goniothalamin (GTN), a styryl-lactone, is a secondary metabolite naturally found in its enantiomeric form (*R*) in plants of the genus *Goniothalamus* (Annonaceae). The antiproliferative activity against human tumor cell lines reported in several studies suggest that the α,β-unsaturated δ-lactone moiety emerges as a key Michael acceptor for cysteine residues or other nucleophilic biological molecules. Our group reported on the *in vivo* activity of (*R*)- and (*S*)-GTN as well as its racemic form (*rac*-GTN) in both Ehrlich solid tumor and carrageenan-induced paw edema in mice, without side effects in the effective doses. Despite the rich body of data on the *in vitro* GTN biological activity, much less is known about its *in vivo* pharmacological action. Herein we describe the gastroprotective activity of *rac*-GTN on chemical-induced gastric ulcers models in rats. GTN has a potent gastroprotective effect on ethanol-induced ulcers (effective $dose_{50}$ = 18 mg/kg) and this activity is dependent on sulfhydryl compounds and prostaglandins generation, but independent of nitric oxide (NO), gastric secretion and mucus production. We hypothesize that goniothalamin may act as a mild irritant, inducing the production of sulfhydryl compounds and prostaglandins, in a process known as adaptive cytoprotection. This hypothesis is supported by the fact that Michael acceptors are the most potent inducers of antioxidant response (as activation of Nrf2 pathway) through generation of mild oxidative stress and that gastroprotective activity of goniothalamin is inhibited after pre-treatment with NEM (*N*-ethylmaleimide) and NSAID (non-steroidal anti-inflammatory drugs), highlighting the importance of sulfhydryl compounds and prostaglandins on GTN activity.

3. Trabalho em colaboração com *Polymer Science and Technology Institute – Spanish National Research Council (ICTP-CSIC).* (Servat-Medina et al., 2015)



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ORIGINAL RESEARCH

Chitosan–tripolyphosphate nanoparticles as *Arrabidaea chica* standardized extract carrier: synthesis, characterization, biocompatibility, and antiulcerogenic activity

Leila Servat-Medina[1,2]
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Abstract: Natural products using plants have received considerable attention because of their potential to treat various diseases. *Arrabidaea chica* (Humb. & Bonpl.) B. Verlot is a native tropical American vine with healing properties employed in folk medicine for wound healing, inflammation, and gastrointestinal colic. Applying nanotechnology to plant extracts has revealed an advantageous strategy for herbal drugs considering the numerous features that nanostructured systems offer, including solubility, bioavailability, and pharmacological activity enhancement. The present study reports the preparation and characterization of chitosan–sodium tripolyphosphate nanoparticles (NPs) charged with *A. chica* standardized extract (AcE). Particle size and zeta potential were measured using a Zetasizer Nano ZS. The NP morphological characteristics were observed using scanning electron microscopy. Our studies indicated that the chitosan/sodium tripolyphosphate mass ratio of 5 and volume ratio of 10 were found to be the best condition to achieve the lowest NP sizes, with an average hydrodynamic diameter of 150±13 nm and a zeta potential of +45±2 mV. Particle size decreased with AcE addition (60±10.2 nm), suggesting an interaction between the extract's composition and polymers. The NP biocompatibility was evaluated using human skin fibroblasts. AcE-NP demonstrated capability of maintaining cell viability at the lowest concentrations tested, stimulating cell proliferation at higher concentrations. Antiulcerogenic activity of AcE-NP was also evaluated with an acute gastric ulcer experimental model induced by ethanol and indomethacin. NPs loaded with *A. chica* extract reduced the ulcerative lesion index using lower doses compared with the free extract, suggesting that extract encapsulation in chitosan NPs allowed for a dose reduction for a gastroprotective effect. The AcE encapsulation offers an approach for further application of the *A. chica* extract that could be considered a potential candidate for ulcer-healing pharmaceutical systems.

Keywords: natural product, *Arrabidaea chica*, chitosan, nanoparticle, plant extract, herbal drug, ulcer healing

4. Trabalho em colaboração com a Faculdade de Engenharia de Alimentos, UNICAMP. (Breda et al., 2016)

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ORIGINAL ARTICLE

Phytochemical Analysis and Antifungal Activity of Extracts from Leaves and Fruit Residues of Brazilian Savanna Plants Aiming Its Use as Safe Fungicides

Caroline Alves Breda · Alessandra Marcon Gasperini · Vera Lucia Garcia · Karin Maia Monteiro · Giovana Anceski Bataglion · Marcos Nogueira Eberlin · Marta Cristina Teixeira Duarte

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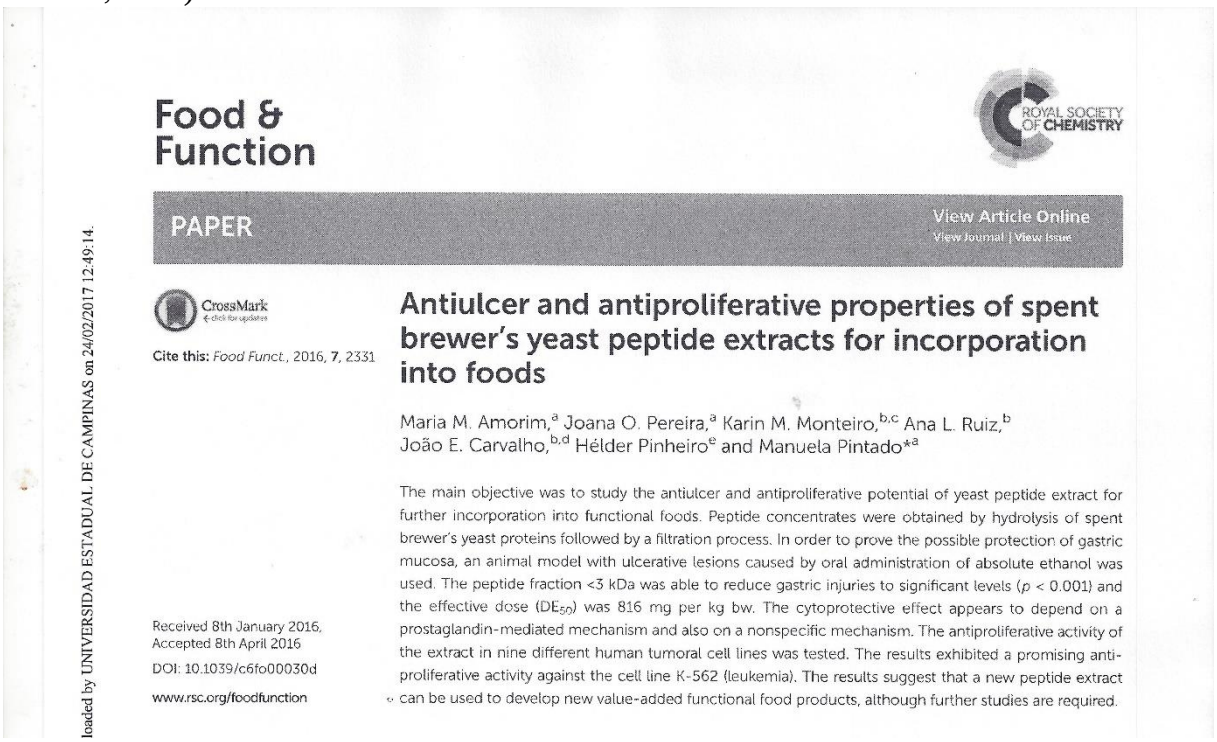
Abstract The increasing demand for safe food without preservatives or pesticides residues has encouraged several studies on natural products with antifungal activity and low toxicity. In this study, ethanolic extracts from leaves and fruit residues (peel and seeds) of three Brazilian savanna species (Acrocomia aculeata, Campomanesia adamantium and Caryocar brasiliense) were evaluated against phytopathogenic fungi. Additionally, the most active extract was chemically characterized by ESI-MS and its oral acute toxicity was evaluated. Extracts from C. brasiliense (pequi) peel and leaves were active against Alternaria alternata, Alternaria solani and Venturia pirina with minimal inhibitory concentrations between 350 and 1000 l g/mL. When incorporated in solid media, these extracts extended the lag phase of A. alternata and A. solani and reduced the growth rate of A. solani. Pequi peel extract showed better antifungal activity and their ESI-MS analysis revealed the presence of substances widely reported as antifungal such as gallic acid, quinic acid, ellagic acid, glucogalin and corilagin. The oral acute toxicity was relatively low, being considered safe for use as a potential natural fungicide.

5. Trabalho em colaboração com o Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa (Amorim et al., 2016)



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Antiulcer and antiproliferative properties of spent brewer's yeast peptide extracts for incorporation into foods

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The main objective was to study the antiulcer and antiproliferative potential of yeast peptide extract for further incorporation into functional foods. Peptide concentrates were obtained by hydrolysis of spent brewer's yeast proteins followed by a filtration process. In order to prove the possible protection of gastric mucosa, an animal model with ulcerative lesions caused by oral administration of absolute ethanol was used. The peptide fraction <3 kDa was able to reduce gastric injuries to significant levels ($p < 0.001$) and the effective dose (DE_{50}) was 816 mg per kg bw. The cytoprotective effect appears to depend on a prostaglandin-mediated mechanism and also on a nonspecific mechanism. The antiproliferative activity of the extract in nine different human tumoral cell lines was tested. The results exhibited a promising anti-proliferative activity against the cell line K-562 (leukemia). The results suggest that a new peptide extract can be used to develop new value-added functional food products, although further studies are required.

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6. Trabalho em colaboração com *Institute of Food Science Research – Group of food lipids biomarkers and health, Universidad Autónoma de Madrid* (Castro-Gómez et al., 2016)

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Antiproliferative activity of buttermilk lipid fractions isolated using food grade and non-food grade solvents on human cancer cell lines

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ABSTRACT

Buttermilk is a dairy by-product with a high content of milk fat globule membranes (MFGMs), whose protein constituents are reported to be antiproliferative. Lipids represent about half of the composition of MFGM. The aim of this study was to isolate buttermilk lipid fractions and evaluate their potential antiproliferative effect. Selective extraction with food grade or non-food grade solvents was performed. Antiproliferative effectiveness of lipid extracts and their neutral and polar fractions was evaluated on nine human cancer cell lines. Fractions obtained using food grade ethanol gave a higher yield than those obtained using non-food grade solvents, and they effectively inhibited cell viability of the cancer cell lines investigated. These fractions, rich in phospho- and sphingolipids, were strongly antiproliferative against human ovary and colon cancer cells. This observation allowed us to hypothesize further analyses aimed at promoting the use of buttermilk polar lipid fractions as functional food additives.