UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

NANOCÁPSULAS CONTENDO INDOMETACINA: AVALIAÇÃO DOS EFEITOS ANTITUMORAL, NEUROPROTETOR E ANTI-INFLAMATÓRIO

Andressa Bernardi

Orientadora: Dra. Ana Maria Oliveira Battastini
Co-Orientadora: Dra. Sílvia Stanisçuaski Guterres

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Tese apresentada ao curso de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do grau de **Doutor em Bioquímica**.

Porto Alegre
2009

DEDICO

Àqueles que sempre estiveram ao meu lado:
meus pais, Maria e Ademir,
meu irmão, Fabiano e
meu noivo, Rudimar.

“Só a especulação ousada pode nos levar adiante, e não o acúmulo de fatos.”

Albert Einstein

AGRADECIMENTOS

À Ana, primeiramente por ter aceitado me orientar em um projeto ousado e fora de sua habitual linha de pesquisa. Pela confiança em mim depositada e pela oportunidade concedida de trabalhar ao seu lado por todos estes anos. Por ter apoiado as minhas decisões, compartilhando seus conhecimentos e experiências profissionais e, acima de tudo, pela amizade.

À Profa. Sílvia Guterres, pela co-orientação que foi fundamental para a realização deste trabalho. Pela valiosa oportunidade de colaboração, por toda confiança e liberdade sempre oferecidas. Por toda a atenção e disponibilidade dispensadas ao longo deste trabalho e pelo exemplo de dedicação aos alunos. Pelo apoio e incentivo que foi fundamental principalmente nesta fase final do doutorado.

À Profa. Adriana Pohlmann, pela valiosa oportunidade de colaboração, por toda confiança e liberdade sempre oferecidas. Por toda ajuda e disponibilidade. Pela fundamental contribuição prestada durante a discussão dos resultados obtidos.

Às Profas. Maria Martha Campos e Fernada Morrone, por todo o carinho e por terem me acolhido tão bem em seus laboratórios possibilitando que os experimentos pudessem ser realizados. Por toda contribuição dada ao trabalho.

À Profa. Christianne Salbego, pela oportunidade de colaboração, pela disposição e atenção dadas sempre que necessário e por todos os momentos compartilhados.

À Profa. Maria Isabel Edelweiss, pelo auxílio fundamental nas incansáveis análises patológicas que tanto contribuíram para que esse trabalho pudesse se realizar.

À Neiva e ao Jorge do Laboratório de Pesquisa em Patologia do HCPA, pelo auxílio fundamental no preparo das lâminas e por estarem sempre dispostos em ajudar.

À Eliz, querida amiga e parceira de todos os momentos. Pelo espírito colaborativo e por nunca ter medido esforços para me ajudar nos experimentos. Por todas as nossas conversas, troca de experiências e apoio durante todo esse tempo de convivência. Pela grande amiga que tu és!

Aos queridos bolsistas com os quais tive a oportunidade de conviver e aprender nesse período: Fabrício e Ana Carolina (Cacá). Pela dedicação e seriedade com que me auxiliaram nos experimentos e pela amizade.

À Carol, por ter me ensinado os primeiros passos no laboratório, por todos os ensinamentos durante iniciação científica, que tanto contribuíram para a minha formação profissional. Pela amizade que mantemos até hoje apesar da distância.

A todos os colegas e amigos que ainda estão ou que um dia passaram pelo Laboratório 22 do Departamento de Bioquímica: Alessandra (Japa), Álvaro, Ana Lucília, Andrés, Angélica, Bernardo, Christine, Cristiane, Daiane, Elizandra, Fabrícia, Fabrício, Fernanda Morrone, Fernanda, Gabi, Isabeli, Joséli, Leandre, Letícia, Lila, Luci, Luis Felipe, Márcia, Rafael, Patrícia, Paula, Spiller, Vanessa.

A todos os colegas e amigos do Laboratório de Sistemas Nanoestruturados para a Administração de Fármacos (Lab 405) da Faculdade de Farmácia e do Laboratório K204 do Instituto de Química. Em especial, ao Eliézer pela atenção dispensada ao longo deste trabalho no preparo das formulações e pela parceria nos experimentos.

Ao pessoal do Laboratório 37 (atual Laboratório 23) do Departamento de Bioquímica, por toda ajuda, pela amizade e por todos os momentos compartilhados.

A todos os amigos com os quais eu convivi durante esses dez anos no Departamento de Bioquímica e que estão distribuídos pelos diversos laboratórios. Agradeço pelo espírito de colaboração e pela amizade.

Ao Departamento de Bioquímica, aos professores e funcionários que, de alguma forma, contribuíram para a realização deste trabalho.

À UFRGS pela oportunidade de realizar meus estudos desde a graduação até o Doutorado.

À Capes, pela bolsa nos três primeiros anos de Doutorado e ao CNPq, pela bolsa neste último ano de Doutorado.

À IBRO *(International Brain Research Organization)* pelo *"Travel Grant"* concedido, o qual me possibilitou participar do "*Third Joint Italian-German Purine Club Meeting - Purinergic Receptors: New Frontiers for Novel Therapies"* na Itália.

Aos meus pais, que souberam compreender todos os meus momentos de ausência. Por não terem medido esforços para que eu e meu irmão pudéssemos estudar. Pelo constante incentivo e por terem me apoiado em todas as decisões.

Ao meu irmão, por todos os momentos compartilhados desde a nossa graduação, mestrados, até os nossos doutorados, pela amizade e apoio.

Ao meu noivo Rudi, por ter participado comigo de todas as etapas desse doutorado, incluindo experimentos e discussões científicas. Pelo companheirismo e apoio, permanecendo sempre ao meu lado, mesmo nos momentos mais difíceis. Pela dedicação, ajuda, compreensão e paciência em todos os momentos. Por todo amor e por me fazer cada dia mais feliz!

À Deus, por ter me iluminado em todas as decisões e ter me dado força e coragem nos momentos mais difíceis.

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RESUMO

Nanopartículas de polímeros biodegradáveis têm atraído um intenso interesse nos últimos anos, pois esses sistemas podem prover vetorização de forma sustentada, controlada e atuar como carreadores de fármacos aumentando a eficácia terapêutica e diminuindo os efeitos adversos. Doenças degenerativas do sistema nervoso central têm sido vistas como um problema pela complexa patogênese e pela dificuldade na vetorização de fármacos. Dentre essas, estão os gliomas e os insultos isquêmicos, os quais são pobremente responsivos às intervenções terapêuticas. Atualmente, o tratamento com indometacina em doenças inflamatórias tem sido relacionado com diversos efeitos adversos gastrointestinais. Neste contexto, o presente estudo avaliou os efeitos do tratamento com indometacina em nanocápsulas em modelos experimentais de gliomas, isquemia cerebral, inflamações aguda e crônica em ratos. O tratamento com indometacina em nanocápsulas foi mais potente do que indometacina em solução em diminuir a viabilidade e a proliferação celular de linhagens de gliomas. Esse efeito citotóxico foi seletivo para as células tumorais. Adicionalmente, nós observamos em um modelo *in vivo* de gliomas que nanocápsulas poliméricas foram capazes de vetorizar a indometacina ao cérebro. Essa vetorização reduziu o crescimento de glioblastoma e aumentou a sobrevida dos animais. Estes efeitos foram mediados, pelo menos em parte, por mecanismos antiproliferativos e anti-angiogêncos. Além disso, o tratamento com indometacina em nanocápsulas apresentou efeitos neuroprotetores em culturas organotípicas de hipocampo expostas à privação de oxigênio e glicose. Esses efeitos foram mediados pela redução dos níveis de fosforilação de ERK1/2 e JNK, redução na iNOS e na ativação glial. Adicionalmente, a indometacina em nanocápsulas reduziu os níveis de citocinas pró-inflamatórias, sugerindo que o bloqueio da neuroinflamação está envolvido no efeito neuroprotetor observado. Em modelos de inflamação crônica em ratos (modelo de artrite), o tratamento sistêmico com indometacina em nanocápsulas produziu simultaneamente uma redução nos níveis de citocinas pró-inflamatórias e um aumento da citocina anti-inflamatória IL-10. A maior eficácia anti-inflamatória foi associada a uma redução da toxicidade gastrointestinal. Juntos, nossos resultados sugerem que a indometacina em nanocápsulas pode ser considerada uma alternativa terapêutica promissora para o tratamento de gliomas, de isquemia cerebral e de inflamação crônica.

ABSTRACT

Nanoparticles of biodegradable polymers have attracted intensive interest in recent years because these systems can provide a sustained, controlled, and targeted delivery acting as drug carriers thus leading to high therapeutic efficiency and low side effects. Degenerative diseases of the central nervous system have long been viewed as a problem due to the complex pathogenesis of these disorders and the difficulty in drug delivery. Among these diseases, are the gliomas and the ischemic insults, which are poorly responsive to therapeutic interventions. Actually, indomethacin treatments for inflammatory diseases are related with several gastrointestinal side effects. Within this context, the present study was designed to evaluate the effects of indomethacin-loaded nanocapsules treatment in experimental models of gliomas, cerebral ischemia, acute and chronic inflammation in rats. Indomethacin-loaded nanocapsules treatment was more potent that indomethacin in solution in decreasing the viability and the cell proliferation of glioma lines. This cytotoxic effect was selective for tumoral cells. In addition, we have observed in an *in vivo* model of gliomas that polymeric nanocapsules are able to successfully carry indomethacin into the brain tumor. Local delivery of indomethacin reduced glioblastoma growth and improved the animals' survival. These effects were mediated, at least in part, by antiproliferative and antiangiogenic mechanisms of indomethacin-loaded nanocapsules. Also, indomethacin-loaded nanocapsules treatment presented neuroprotective effects in organotypic hippocampal cultures exposed to oxygen-glucose deprivation. These effects were mediated by the reduction in the levels of ERK1/2 and JNK phosphorylation, reduction in iNOS and glial activation. Additionally, indomethacin-loaded nanocapsules decreased the levels of the pro-inflammatory cytokines, suggesting that the blockage of neuroinflammation is involved in the neuroprotective effect observed. In models of chronic inflammatory in rats (arthritis model), the systemic treatment with indomethacin loaded nanocapsules produced simultaneity a reduction of the levels of pro-inflammatory cytokines and an increased in the levels of anti-inflammatory cytokine IL-10. The anti-inflammatory efficacy increase was allied to an improved gastrointestinal safety. Taken together, our results imply that nanocapsule formulations containing indomethacin might be considered as promising alternative therapeutic for gliomas, cerebral ischemia and chronic inflammation treatment.

APRESENTAÇÃO

Esta tese está organizada em seções dispostas da seguite maneira: Introdução, Objetivos, Artigos Científicos publicados e/ou submetidos, Discussão, Conclusões, Perspectivas, Referências Bibliográficas e Anexos.

A **Introdução** apresenta o embasamento teórico que nos levou a formular a proposta do trabalho. Os **Materiais, Métodos e Resultados,** assim como as **Referências Bibliográficas** específicas encontram-se no corpo de cada trabalho, os quais estão apresentados na forma de **Artigos Científicos**, denominados Capítulos 1, 2, 3 e 4.

A seção **Discussão** contém uma interpretação geral dos resultados obtidos nos diferentes trabalhos.

A seção **Conclusões** aborda as conclusões gerais obtidas na tese.

A seção **Perspectivas** aborda as possibilidades de desenvolvimento de projetos a partir dos resultados obtidos, dando continuidade a essa linha de pesquisa.

A seção **Referências Bibliográficas** lista as referências utilizadas na Introdução e Discussão da tese.

A seção **Anexo** contém uma lista de outros trabalhos realizados em co-autoria durante todo o período de desenvolvimento do doutorado.

LISTA DE ABREVIATURAS

AAS - **á**cido **a**cetil**s**alicílico *(acetylsalicylic acid)*

AINEs - **a**nti-**i**nflamatórios **n**ão-**e**steróides

AKT/PKB - proteína cinase B *(**P**rotein **K**inase **B**)*

ATCC - ***A**merican **T**ype **C**ulture **C**ollection*

ATP - adenosina trifosfato *(**A**denosine **Tri**p**h**osphate)*

BBB - barreira hemato-encefálica *(**B**lood-**B**rain **B**arrier)*

BHE - **B**arreira **H**emato-**E**ncefálica

CDK4 - ciclina dependente de cinase 4 *(**C**yclin-**D**ependent **K**inase **4**)*

CDK6 - ciclina dependente de cinase 6 *(**C**yclin-**D**ependent **K**inase **6**)*

CFA - adjuvante completo de Freund (***C**omplete **F**reund's **A**djuvant)*

CNS - sistema nervoso central *(**C**entral **N**ervous **S**ystem)*

COX - ciclooxigenase (**C***yclo**o**x**ygenase*)

COX-1 - ciclooxigenase-1 (**C***yclo**o**x**ygenase**-1***)

COX-2 - ciclooxigenase-2 (**C***yclo**o**x**ygenase**-2**)*

COX-3 - ciclooxigenase-3 (***C**yclo**o**x**ygenase**-3**)*

DAB - 3,3'-tetra-hidrocloreto de diaminobenzidina *(3,3'-**d**i**a**mino**b**enzidina tetrahydrochloride)*

DMEM - meio de cultivo celular de Eagles modificado por Dulbecco *(**D**ulbecco´s **M**odified **E**agle´s **M**edium)*

DMSO - dimetilsulfóxido *(**Dim**ethyl**s**ulph**o**xide)*

EGFR - receptor de fator de crescimento endothelial *(**E**ndothelial **G**rowth **F**actor **R**eceptor)*

EPR - efeito de retenção e permeabilidade *(**E**ffect of **P**ermeability **R**etention)*

ERK - proteína cinase regulada por sinais extracelulares *(**E**xtracellular Signal-**R**egulated **K**inase)*

FBS - soro fetal bovino *(**F**etal **B**ovine **S**erum)*

FDA - ***F**ood and **D**rug **A**dministration*

GBM - Glio**b**lastoma **M**ultiforme

GFAP - proteína glial fibrilar ácida (***G**lial **F**ibrillary **A**cidic **P**rotein*)

HBSS - solução salina balenceada de Hank's *(**H**ank's **B**alanced **S**alt **S**olution)*

H&E - hematoxilina e eosina *(**H**aematoxylin and **E**osin)*

HPLC - cromatografia líquida de alta eficiência *(**H**igh-**P**erformance **L**iquid **C**hromatography)*

IB_4 - isolectina B_4 *(**I**solectin **B_4**)*

Il-6 - interleucina-6 *(**I**nter**l**eukin**-6**)*

IL-10 - interleucina-10 *(**I**nter**l**eukin**-10**)*

IL-18 - interleucina-18 *(**I**nter**l**eukin**-18**)*

IL-1β - interleucina -1 beta *(**I**nter**l**eukin**-1beta**)*

IndOH - indometacina *(**Ind**omethacin)*

IndOH-NC - nanocápsulas contendo indometacina *(**Ind**omethacin-loaded **N**ano**c**apsules)*

IndOEt - éster etílico da indometacina *(**Ind**omethacin **Et**hyl Ester)*

IndOEt-NC - nanocápsulas contendo éster etílico da indometacina *(**Ind**omethacin **Et**hyl Ester-loaded **N**ano**c**apsules)*

iNOS – óxido nitrico sintase induzível *(**i**nducible **N**itric **O**xide **S**ynthase)*

JNK - proteína cinase c-Jun N-terminal *(c-**J**un **N**-terminal **K**inase)*

LDL - lipoproteínas de baixa-densidade *(**L**ow **D**ensity **L**ipoprotein)*

LI - índice lesional *(**L**esional **I**ndex)*

MAPK - proteína cinase ativada por mitógenos (***M**itogen-**A**ctivated **P**rotein **K**inase*)

MDR - multi-resistência a drogas *(**M**ullti-**D**rug **R**esistance)*

MEK - protaína cinase ativada por mitógenos ativadora de ERK *(**M**itogen-Activated **E**RK-activating **K**inase)*

MEM - meio essencial mínimo *(**M**inimum **E**ssential **M**edium)*

NSAIDs - anti-inflamatórios não-esteróides *(**N**onsteroidal **A**nti-**I**nflammatory **D**rug**s**)*

MTIC - metil-triaceno-imidazol-carboxamida *(**M**ethyl-**T**riazeno-**I**midazole-**C**arboxamide)*

MTT - *([3-(4,5-di**m**ethyl**t**hiazol-2-yl)-2,5-diphenyl**t**etrazolium bromide])*

NC - nanocápsulas sem droga *(drug-unloaded **N**ano**c**apsules)*

NO - óxido nítrico *(**N**itric **O**xide)*

OGD - privação de oxigênio e glicose *(**O**xygen-**G**lucose **D**eprivation)*

OMS - **O**rganização **M**undial da **S**aúde

PBS - tampão fosfato *(**P**hosphate **B**uffered **S**aline)*

PCL - polímero poli ε-caprolactona *[**P**oly(ε-**c**apro**l**actone)]*

PEG - **p**oli**e**tileno**g**licol

PGP - glicoproteína P *(**P**-**g**lico**p**rotein)*

POG - **p**rivação de **o**xigênio e **g**licose

PI - iodeto de propídeo *(**P**ropidium **I**odide)*

PI3K - fosfatidilinositol 3-cinase *(**P**hospho**i**nositide 3-**k**inase)*

PTEN - homólogo fosfatase e tensina deletado do cromossomo 10 *(**P**hosphatase and **ten**sin homologue deleted from chromossome 10)*

SNC- **S**istema **N**ervoso **C**entral

TGF-β - fator de crescimento tumoral- beta *(**T**umoral **G**rowth **F**actor **beta**)*

TNF-α - fator de necrose tumoral- alfa *(**T**umor **N**ecrosis **F**actor **alpha**)*

VEGF - fator de crescimento vascular endotelial *(**V**ascular **E**ndothelial **G**rowth **F**actor)*

INTRODUÇÃO

1. Gliomas

1.1. Aspectos Gerais

Dentre os vários tipos de tumores do SNC, os mais freqüentes e devastadores são os gliomas, contabilizando cerca de 78% de todos os tumores primários do SNC (Sathornsumetee et al*.,* 2007). Os gliomas são atualmente um dos grandes desafios da oncologia (Rich & Bigner, 2004). Existem aproximadamente 25.000 novos casos diagnosticados a cada ano no continente Americano (He & Sun, 2007). De acordo com a Organização Mundial da Saúde (OMS), nos Estados Unidos são estimados 18.500 novos casos de tumores cerebrais primários por ano com aproximadamente 13.000 mortes. Os fatores de risco mais relacionados a estes tumores são radiação ionizante, mutações gênicas e histórico familiar. Os índices de incidência têm aumentado 0,9% ao ano, provavelmente devido à crescente melhoria da qualidade do diagnóstico por imagem (Schwartzbaum et al., 2006).

Uma das características mais marcantes dos gliomas é o seu elevado grau de proliferação e invasividade. Como consequência, pacientes com esse tipo de tumor apresentam um grande comprometimento do tecido nervoso periférico ao tumor com o desenvolvimento de sintomas que incluem cefaléia, mudanças cognitivas, papiloedema, disfasia e hemiparesia progressiva (Girolami, 2000).

O termo glioma refere-se a todas as neoplasias originadas de células gliais ou precursores gliais (Holland et al., 2001), incluindo tumores constituídos de células com características de astrócitos (astrocitomas), oligodendrócitos (oligodendrogliomas), células ependimais (ependimomas) e mistura de vários tipos

de céluals gliais (oligoastrocitomas). Embora a transformação neoplásica de células gliais diferenciadas seja tradicionalmente assumida como um mecanismo da gliomagênese, recentemente foi demostrado que células-tronco podem estar envolvidas neste processo (Kondo et al., 2006). As células-tronco neurais são reguladas pelas mesmas vias de sinalização celular que são ativas em muitos tumores cerebrais. Consequentemente elas são capazes de exibir comportamentos característicos de gliomas, incluindo elevada habilidade proliferativa, elevada motilidade, associação com vasos sanguíneos, desenvolvimento de fenótipos antigênicos imaturos como a expressão de nestina e do marcador de células-tronco neurais CD133, além da ativação de vias sinalizadoras de crescimento e proliferação celular (Sanai et al., 2005). Dessa forma, os gliomas são constituídos por populações de células heterogêneas contendo uma maior população de células diferenciadas e uma minoria de células tumorigênicas indiferenciadas multipotentes. Embora ambas contenham mutações oncogênicas que poderão resultar na tumorigênese, somente as células-tronco neurais têm capacidade de se autorenovar gerando assim a propagação tumoral (Fomchenko & Holland, 2006).

1.2. Glioblastoma Multiforme

O sistema de classificação de tumores cerebrais da OMS utiliza quatro graus (I a IV) para descrever os gliomas, baseados nas características histopatológicas (Kleihues et al., 1993; Chintala et al., 1999). Dentre os gliomas, o glioblastoma multiforme (GBM) é o tumor cerebral primário mais comum e letal (grau IV) (Laws & Shaffrey et al., 1999). Os GBMs se dividem em dois subtipos, primários e secundários. Os GBMs primários, tipicamente surgem em pacientes idosos, como um tumor agressivo e altamente invasivo, usualmente sem qualquer evidência

clínica prévia da doença. Já os GBMs secundários são observados em pacientes mais jovens, que inicialmente apresentaram astrocitomas de baixo grau (Maher et al., 2001). Uma vez estabelecidos, glioblastomas primários e secundários são indistinguíveis clinicamente (Maher et al., 2001; Ohgaki & Kleihues, 2007).

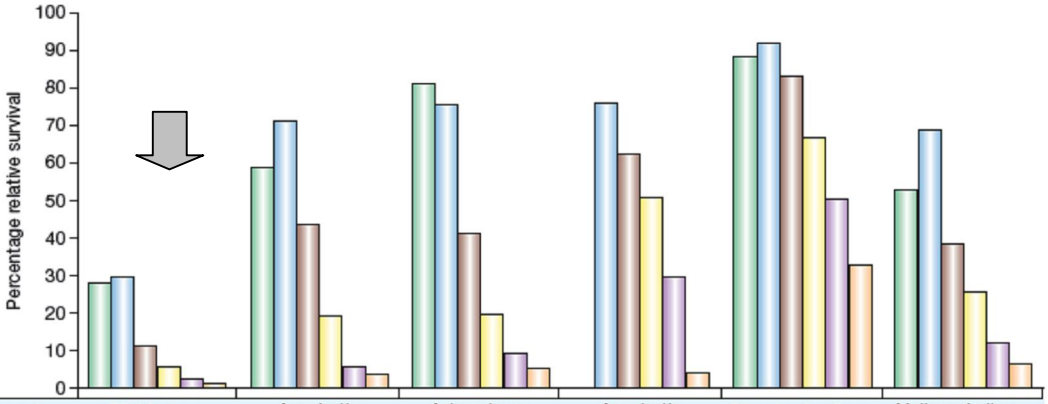
Os GBMs são formados por uma massa intraparenquimal heterogênea apresentando áreas de necrose e hemorragia (Holland et al., 2000). Microscopicamente a massa tumoral consiste em diferentes tipos celulares: células próprias do glioma, células endoteliais hiperproliferativas, macrófagos e células normais de áreas do cérebro que estão sendo invadidas pelo glioma. Dentre as características histológicas mais comuns, incluem-se regiões de necrose, células dispostas em pseudopaliçada, vasos sanguíneos hipertrofiados no interior e em áreas adjacentes ao tumor, proliferação microvascular, pleomorfismo celular e nuclear, além da intensa capacidade proliferativa (Dai & Holland et al., 2001).

Os GBMs são caracterizados por uma grande infiltração no tecido cerebral normal circundante (Rao et al., 2001). Devido ao rápido crescimento, provocam extensas áreas de destruição do tecido nervoso, causando edema e necrose (Laws et al., 1999). O crescimento dos GBMs pode matar as células neuronais do tecido circundante através da liberação de glutamato (Takano et al., 2001). Essa liberação de glutamato pelas células tumorais em concentrações neurotóxicas pode ser responsável, pelo menos em parte, pelas convulsões apresentadas pelos pacientes com este tipo de tumor (Ye & Sontheimer, 1999). Além disso, dados do nosso grupo de pesquisa, têm demonstrado o envolvimento do ATP e de seus produtos de degradação no meio extracelular no crescimento dos GBMs, sendo a sinalização purinérgica um potencial alvo terapêutico no tratamento destes tumores (Morrone et al., 2006; Braganhol et al., 2007., Bernardi et al., 2007.; Braganhol et al., 2009).

Estas características patológicas heterogêneas são um dos maiores obstáculos para o manejo efetivo dos GBMs (Holland, 2001).

1.2.1. Prognóstico e Terapêutica

O prognóstico para pacientes com GBM é ruim, apesar de intensos esforços em desenvolver novas terapias agentes efetivos ainda não estão disponíveis (Konopka & Bonni, 2003). O tempo médio de sobrevida é relacionado com a idade do paciente no momento do diagnóstico bem como com o grau histológico do tumor (Schwartzbaum et al., 2006). Na Figura 1 estão descritos os tipos histológicos dos tumores cerebrais e a percentagem relativa de sobrevida dos pacientes. Apesar dos grande avanços em neuroradiologia, neurocirurgia, radioterapia e quimioterapia, a sobrevida média dos pacientes com GBM pouco se alterou nos últimos 30 anos. A sobrevida média é de 9 a 12 meses, sendo que a maioria vai ao óbito no período de 2 anos e menos de 5% sobrevive 5 anos (Bondy et al., 2008).



Age at diagnosis	Glioblastoma	Anaplastic astrocytoma	Astrocytoma, NOS	Anaplastic oligodendroglioma	Oligodendroglioma	Malignant glioma, NOS
<20 years	27.9%	59%	81.8%		88.8%	53.1%
20–44 years	29.8%	71.4%	76%	76.5%	92.3%	68.6%
45–54 years	11.6%	44%	41.3%	62.8%	83.7%	38.6%
55–64 years	6%	19.4%	20%	51.2%	67.1%	26%
65–74 years	2.7%	6.4%	9.1%	29.5%	50.9%	12.7%
75+ years	1.4%	4.1%	5.8%	4.9%	33.3%	6.9%

Histologic type

Figura 1. Relação entre a idade de ocorrência dos gliomas, o grau histológico e a sobrevida dos pacientes. Adaptado de Schwartzbaum et al., 2006.

A cirurgia representa o tratamento de primeira escolha para os pacientes com gliomas, entretanto a ressecção completa do tumor é limitada pela alta invasividade das células tumorais nos tecidos normais circundantes (Behin et al., 2003). Dessa forma, a recorrência do tumor é quase inevitável, uma vez que a remoção cirúrgica do tumor invariavelmente deixa no tecido normal uma população de células tumorais (Rooprai et al., 1999). Portanto, a maioria dos pacientes é tratada com radioterapia e/ou quimioterapia pós-operatória (Mazeron & Kantor, 1998). Contudo, estes agentes demonstram eficácia limitada, principalmente pela ausência de especificidade terapêutica das drogas citotóxicas contra tais neoplasias, além da quimiorresistência intrínseca destes tumores e da baixa tolerância do tecido normal aos efeitos tóxicos decorrentes a terapia. Além disso, a BHE limita a entrada dos quimioterápicos no SNC, de modo que apenas fármacos altamente lipofílicos, como temozolomida e nitrosuréias, podem ser utilizados (Mousseau et al, 1993). No tratamento quimioterápico, a temozolomida é atualmente o fármaco de escolha para o tratamento de GBM, embora apresente algumas limitações como mielosupressão, leucopenia e trombocitopenia nos pacientes (Friedman et al., 2000; Villano et al., 2009). Esse fármaco é um antineoplásico da classe dos agentes alquilantes, sendo que na circulação sistêmica em pH fisiológico forma o composto ativo MTIC (metil-triaceno-imidazol-carboxamida). O principal objetivo do tratamento com temozolomida é o aumento da sobrevida dos pacientes (Yun et al., 2000; Bondy et al., 2008).

Dessa forma, a pobre responsividade dos tumores primários do SNC à quimoterapia, bem como o sucesso limitado da cirurgia e radioterapia pós-operatória justificam os esforços dedicados no desenvolvimento e aprimoramento de novas modalidades terapêuticas.

1.2.2. Alterações Moleculares

A tumorigênese envolve, em essência, o descontrole das funções de proto-oncogenes e genes supressores tumorais. Tais genes codificam proteínas reguladoras de complexas rotas de transdução de sinais para funções celulares essenciais, tais como: proliferação, diferenciação, apoptose, adesão, migração e angiogênese (Weinberg, 1995; Collins et al., 2002). Dessa forma, a maioria das alterações genéticas identificadas nos GBMs resulta em ativação anormal de vias de sinalização a partir de receptores tirosina-cinase e/ou a partir de perda do controle do ciclo celular (Ohgaki & Kleihues, 2009).

Pode-se afirmar que, além da heterogeneidade patológica, os GBMs apresentam uma heterogeneidade molecular. O processo de transformação de uma célula normal em uma célula tumoral é extenso e pode prosseguir por vários caminhos. Acredita-se que somente alguns dos processos moleculares são compartilhados por um determinado tipo de neoplasia, de modo que cada tumor possui uma característica genética e epigenética única.

Os GBMs desenvolveram a capacidade de expressar seus próprios fatores de crescimento junto aos seus respectivos receptores, resultando na possibilidade de estimulação autócrina (Feldkamp et al., 1997; Tang et al., 1997). Nesse contexto, o fator de crescimento vascular endotelial (VEGF) é considerado o mais proeminente fator de crescimento angiogênico, sendo superexpresso em 40-60% de todos os GBMs diagnosticados (Wong et al., 2009). Além disso, os receptores para VEGF são altamente expressos em células endoteliais vasculares e predominantemene em vasos nas proximidades do tumor (Libermann et al., 1984; Ekstrand et al., 1991) de modo que, as células tumorais podem migrar e alcançar a circulação através desses novos vasos permeáveis. Apesar da intensa

neovascularização capilar, a circulação sanguínea muito frequentemente não é suficiente para áreas tumorais com grande proliferação celular, sendo esse um fator limitante para o crescimento tumoral o que leva ao desenvolvimento de necrose intratumoral (Schaper & Buschmann, 1999). Como consequência dessa superexpressão de receptores e ligantes, há um aumento na sinalização, que leva à ativação descontrolada de várias vias de proliferação celular, como por exemplo, a via das MAPKs (*Mitogen-Activated Protein Kinase*) (Ras→ Raf→ MEK→ ERK) e a via da PI3K/AKT, dentre outras. A Figura 2 apresenta, de forma simplificada e esquemática, algumas das principais vias relacionadas à proliferação dos gliomas.

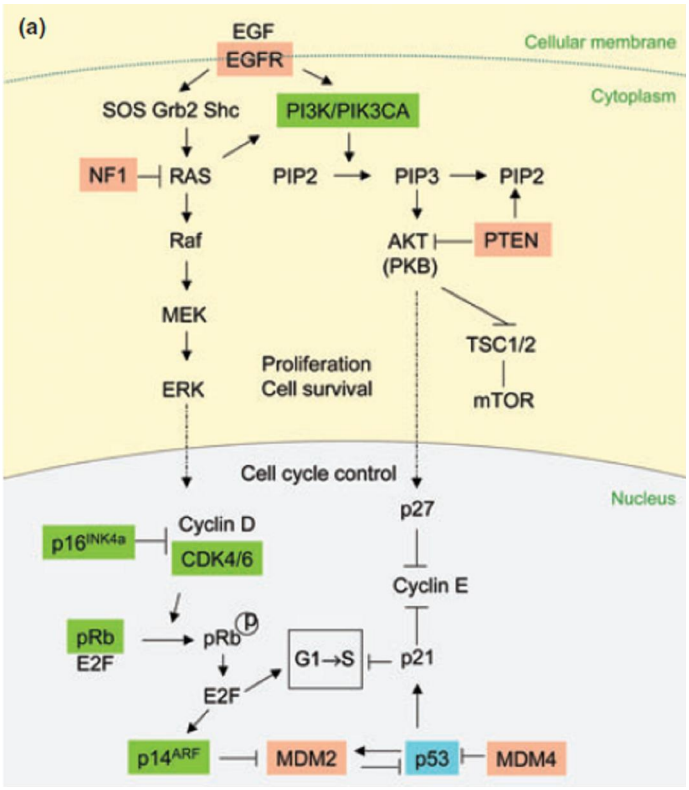


Figura 2. Principais vias de sinalização envolvidas na patogênese dos glioblastomas multiforme. Adaptado de Ohgaki & Kleihues, 2009.

Outra característica importante que os GBMs apresentam, é a perda da região 10q22-25, porção cromossomal que carrega importantes genes supressores tumorais, entre os quais a PTEN (*Phosphatase and tensin homologue deleted from*

chromossome 10). A perda da expressão da PTEN resulta em ativação da AKT, uma das vias centrais de sobrevivência celular, que dessa forma torna-se constitutivamente ativa, impedindo que as células sejam sinalizadas à morte celular apoptótica. O nível de expressão da PTEN está inversamente relacionado com a malignidade dos gliomas: quanto maior a expressão desta proteína, menos maligno é o tumor, sendo que GBMs apresentam PTEN mutada ou deletada, o que sugere que PTEN ocupa um papel decisivo na gliomagênese (Koul et al., 2008). O outro grande grupo de alterações resulta da interrupção nas vias de parada do ciclo celular, como a deleção de INK4-ARF que codifica proteínas $p16^{INK4A}$ e $p14^{ARF}$ (Nakamura et al., 2001), perdas das funções de p53 (Watanabe et a., 2001), amplificação de CDK4, CDK6 e ciclina D1 (Giani & Finocchiaro, 1994; Sherr et al., 1999). A soma de todas essas alterações contribui para a principal característica dos GBMs, a proliferação descontrolada. A Figura 3 apresenta as principais alterações genéticas dos gliomas de diferentes graus.

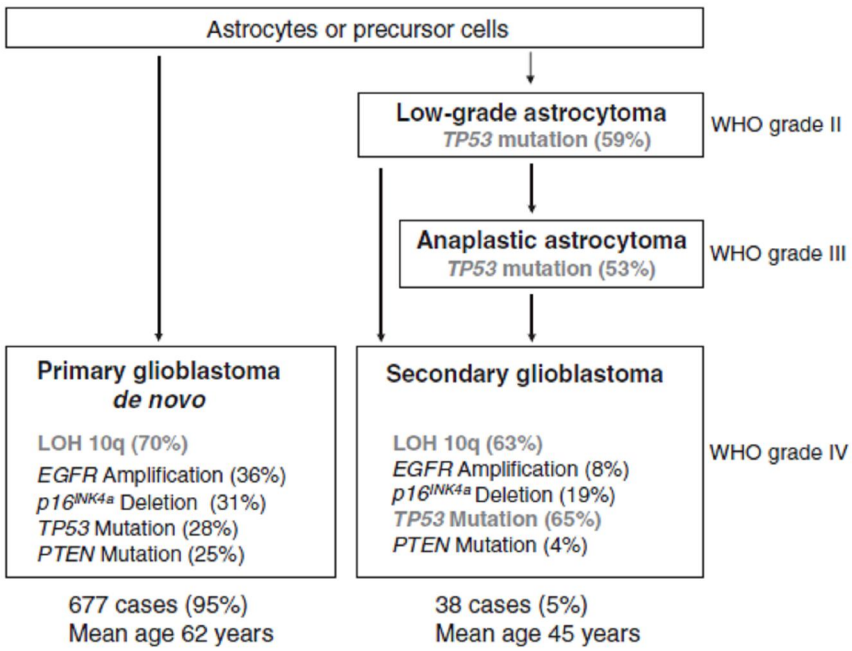


Figura 3. Principais alterações genéticas nos gliomas de diferentes graus. Adaptado de Ohgaki, 2005.

Além disso, dados do nosso grupo de pesquisa demonstraram que o metabolismo extracelular das purinas encontra-se alterado em células de GBM, sugerindo que alterações no sistema purinérgico podem ser uma característica dos gliomas que podem contribuir para o seu fenótipo de malignidade (Wink et al., 2003; Bavaresco et al., 2008; Braganhol et al., 2009).

2. Isquemia Cerebral

2.1. Aspectos gerais

A isquemia constitui uma importante condição fisiopatológica de lesão cerebral e tem sido alvo de intensa investigação. A isquemia cerebral pode ser definida como uma redução severa ou um completo bloqueio do fluxo sanguíneo, que resulta em várias alterações celulares que clinicamente causam distúrbios comportamentais e patológicos (Taylor et al., 1996; Mehta et al., 2007). A isquemia cerebral está intimamente ligada a uma variedade de fatores de risco como hipertensão, hipercolesterolemia e diabetes (Price, 1999). É uma das principais causas de morbidade e mortalidade entre adultos e idosos. Apresenta elevada freqüência e enorme repercussão econômica, pois na maioria das vezes, leva à incapacidade física e/ou mental (Price, 1999; Wang et al., 2007). Dados do Ministério da Saúde mostram que em 2005 as doenças cerebrovasculares foram as maiores causas de morte no Brasil, com um número de óbitos ultrapassando os 90.000, correspondendo a 10% da mortalidade do país. Outro dado importante é que doenças circulatórias (aqui incluídas a isquemia cerebral e a isquemia cardíaca) são a principal causa de morte em todas as regiões brasileiras, tanto para homens quanto para mulheres acima dos 40 anos (Ministério da Saúde, 2009).

O metabolismo energético normal do cérebro apresenta algumas características especiais que incluem uma taxa metabólica elevada, estoques de energia limitados e uma alta dependência do metabolismo aeróbico de glicose. Por esses motivos, o cérebro é mais vulnerável a um insulto isquêmico do que outros tecidos. O tipo mais frequente de isquemia cerebral em humanos é a isquemia focal, que é definida como a interrupção do fluxo sanguíneo para uma determinada região do cérebro, afetando apenas as regiões circundantes à área não irrigada. Outro tipo é a isquemia global, que resulta da interrupção transitória do fluxo sanguíneo para todo o cérebro, o que ocorre durante uma parada cardíaca, por exemplo (Lipton, 1999). Além dos danos causados pela falta de oxigênio e metabólitos durante a isquemia, culminando em morte celular, a volta da circulação sanguínea, chamada de reperfusão, pode aumentar ainda mais a morte neuronal, especificamente nas áreas mais vulneráveis do cérebro, como a região CA1 do hipocampo (Schmidt-Kastner & Freund, 1991).

A terapêutica indicada para a isquemia cerebral é a terapia trombolítica, entretanto os critérios de exclusão dos pacientes são tantos que poucos podem receber esse tratamento (Chopp & Li, 2002; Gilman, 2006). Estratégias de proteção das áreas afetadas ou de resgate das populações de células que estão morrendo tornam-se necessárias na tentativa de diminuir a morbidade e as sequelas dos pacientes vítimas de isquemia cerebral. Até o momento não existe um protocolo efetivo que melhore as condições de vida desses pacientes. Muitas substâncias neuroprotetoras já foram caracterizadas e mostraram-se eficazes em pesquisas pré-clínicas, mas infelizmente não apresentaram o mesmo efeito quando utilizadas em pacientes (De Keyser et al., 1999; Ginsberg, 2008).

2.2. Fisiopatologia

A isquemia cerebral resulta em várias alterações hemodinâmicas, bioquímicas e neurofisiológicas que levam à morte celular. A morte de neurônios após a isquemia pode ser imediata ou tardia (Graham & Chen, 2001; Mehta et al., 2007). A morte imediata é causada pelo mecanismo necrótico e acontece na região central do insulto isquêmico, enquanto que a morte por apoptose ocorre na região de penumbra ou pré-infarto podendo acontecer até 3 ou 4 dias após a isquemia (Kirino, 2000; Mehta et al., 2007). Assim, o período imediatamente após a isquemia é crucial para possíveis intervenções terapêuticas, objetivando a prevenção da morte neuronal tardia. É importante ressaltar que a intensidade e duração do evento isquêmico são fundamentais para determinar o tipo de morte celular. O fluxo sanguíneo cerebral durante uma isquemia focal decresce gradualmente de uma pequena redução na periferia (zona de penumbra) até uma perda total na zona central (núcleo) da isquemia (Mergenthaler et al., 2004). A Figura 4 apresenta de forma esquemática as regiões de um insulto isquêmico, bem como os tipos de morte predominates em cada região.

Estudos experimentais identificaram quatro processos dominantes que, sozinhos ou combinados, levam ao dano neuronal pós-isquêmico: aumento de cálcio intracelular, neurotoxicidade mediada por receptores glutamatérgicos, formação de espécies reativas de oxigênio e inflamação (White et al., 2000; Endres et al., 2002).

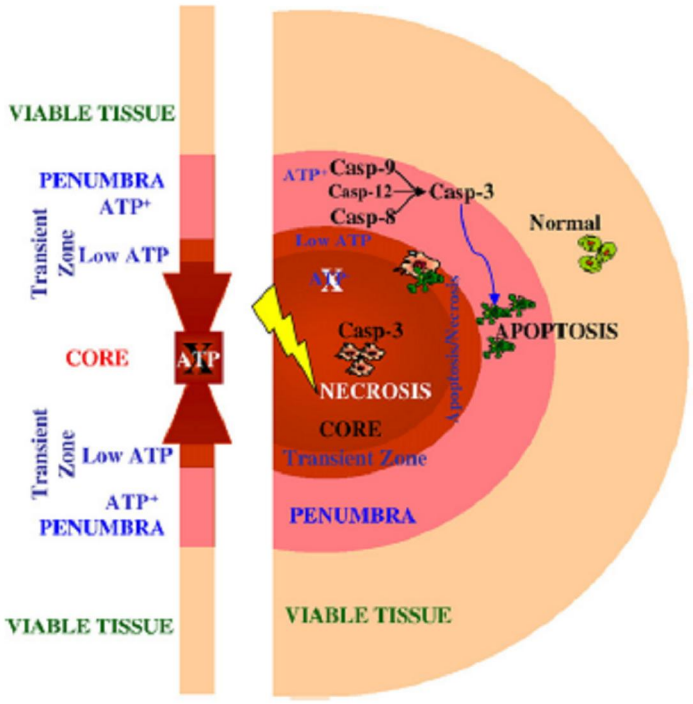


Figura 4. Representação esquemática das regiões afetadas e dos tipos de morte celular durante um insulto isquêmico. A região central do insulto isquêmico é circundada pela área de penumbra, na qual ocore a morte celular por apoptose dependendo da disponibilidade de ATP no local. Adaptado de Mehta et al., 2007.

Com a depleção dos estoques energéticos, o potencial de membrana é perdido e os neurônios despolarizam-se. Consequentemente, os canais de Ca^{2+} dependentes de voltagem são ativados e aminoácidos excitatórios, particularmente o glutamato, são liberados no espaço extracelular (De Keyser et al, 1999). Ao mesmo tempo, os processos dependentes de energia, como a captação de glutamato, são bloqueados, levando ao acúmulo deste no espaço extracelular. A ativação de receptores glutamatérgicos ocasiona o aumento do Ca^{2+} intracelular pela abertura direta de canais de Ca^{2+} (receptores ionotrópicos) e pela ligação aos receptores ligados a proteínas G (receptores metabotrópicos). Como resultado da ativação glutamatérgica, há um aumento do influxo de Na^{+} e Cl^{-} nos neurônios, juntamente com água, ocasionando o edema celular, que leva à necrose (Dirnagl et al., 1999). O aumento no Ca^{2+} intracelular pode também desencadear uma série de

eventos citoplasmáticos e nucleares, causando danos no tecido através da ativação de enzimas proteolíticas, endonucleases e lipases, contribuindo para a morte celular (Mitani et al., 1993). A ativação da fosfolipase A_2 e da COX-2 gera espécies reativas de oxigênio acima da capacidade dos mecanismos antioxidantes endógenos, produzindo peroxidação lipídica e dano à membrana citoplasmática (Mergenthaler et al., 2004). A membrana mitocondrial interna também é afetada pelo distúrbio mediado pelas espécies reativas de oxigênio, formando poros que pode culminar em apoptose (Fujimura et al., 1998).

Embora todos esses mecanismos estejam envolvidos na patogênese da isquemia cerebral e contribuam para a excitotoxicidade, crescentes evidências mostram que a inflamação possui papel fundamental (Hallenbeck et al., 2002; Emsley et al., 2008). Após o insulto isquêmico, quando o fluxo sanguíneo é restaurado (reperfusão), a disponibilidade de oxigênio aumenta, bem como as reações bioquímicas que levam ao aumento da formação de espécies reativas de oxigênio (Aschener, 1998). Dessa forma, ocorre a ativação de cascatas inflamatórias nas células endoteliais, astrócitos e microglia que secretam citocinas, moléculas de adesão, dentre outras substâncias, as quais contribuem para o dano irreversível (Mehta et al., 2007). Essas substâncias podem alterar a permeabilidade da BHE, de modo que essa alteração contribui para o dano que é secundário à isquemia. Esse dano secundário é decorrente principalmente de uma robusta reação inflamatória caracterizada pelo influxo de leucócitos periféricos no parênquima cerebral e a ativação da microglia (Zheng & Yenari, 2004).

3. Inflamação

O processo inflamatório está presente na maioria das patologias. Apresenta-se como um mecanismo de defesa do organismo, cujo objetivo é a eliminação da causa inicial da lesão celular, provocada por patógenos ou por ação de agentes físicos. Dessa forma, o processo inflamatório pode ser definido como uma sequência de eventos que ocorrem em resposta a estímulo nocivo, trauma ou infecção. Essa resposta envolve uma ação coordenada entre os mediadores da inflamação e as células inflamatórias (Sacca et al., 1997).

O processo inflamatório é desencadeado pela liberação de mediadores químicos originados nos tecidos lesionados e pelas células migratórias. Dentre os principais mediadores figuram a histamina, as proteases plasmáticas, os metabólitos do ácido araquidônico (prostaglandinas, tromboxanos e leucotrienos), o fator ativador de plaquetas, as interleucinas, o óxido nítrico, os constituintes lisossômicos dos leucócitos e as espécies reativas de oxigênio (Calixto et al., 2000). As citocinas representam um grupo de substâncias multifuncionais que estão envolvidas nos principais pontos da resposta inflamatória. Até o momento, mais de 100 membros das famílias das citocinas e seus respectivos receptores foram identificados (Barnes, 2001; Haddad, 2002). De uma forma geral, citocinas podem ser classificadas como pró- ou anti-inflamatórias, dependendo do momento da inflamação que irão interferir (Hopkins, 2003). Numa visão simplificada, citocinas pró-inflamatórias, como IL-1β, TNF-α, Il-6 e IL-18, estão envolvidas na iniciação e amplificação do processo inflamatório, enquanto citocinas anti-inflamatórias, como IL-10 e TGF-β, modulam negativamente esses eventos (Dinarello, 2000; Opal et al., 2000).

Na literatura são relatados diversos modelos experimentais amplamente utilizados para avaliação da atividade anti-inflamatória, como o edema de pata de rato induzido por carragenina, CFA (*Complete Freund's Adjuvant*), bradicinina ou

histamina, a pleurisia induzida por carragenina, dentre outros (Stein et al., 1988; Tratsk et al., 1997; Rocha et al., 2006; Quintão et al., 2008).

Dentre esses métodos citados, o edema de pata de rato induzido por carragenina é um dos modelos mais comumente empregados (Tratsk et al., 1997; Quintão et al., 2008). A carragenina causa edema com consequente aumento do volume da pata e uma exacerbada sensibilidade a estímulos mecânicos e térmicos, conhecida como hiperalgesia (Nantel et al., 1999). Essa técnica baseia-se na medida da capacidade de uma substância em inibir o edema produzido na pata do animal por um agente flogístico, neste caso, a carragenina. Esse modelo é amplamente utilizado com o objetivo de investigar a eficácia anti-inflamatória de substâncias, bem como os mecanismos de ação de anti-inflamatórios esteróides e não-esteróides (Valo et al., 1973; Kawamura et al., 2000).

O modelo de artrite induzido por CFA em ratos é um modelo amplamente utilizado na investigação de novas terapias para a artrite reumatóide (Joe & Wilder, 1999). Esse modelo permite avaliar parâmetros da resposta inflamatória, bem como da resposta imunológica, uma vez que o desenvolvimento dos sintomas que caracterizam a artrite inicia-se de 4 a 7 dias após a injeção do CFA (Yue et al., 2004; Granado et al., 2005; Caparroz-Assef et al., 2007). O CFA, reagente que contém antígenos do *Mycobacterium tuberculosis*, atua como um estímulo de respostas imunológicas, incluindo a imunidade celular e o aumento da produção de certas imunoglobulinas, levando ao desenvolvimento de uma reação articular inflamatória intensa dependente de células T. Ocorre o aumento persistente da produção local e sistêmica de TNF-α, IL-1β e IL-6 (Joe & Wilder, 1999). A produção de citocinas com predomínio de citocinas pró-inflamatórias tem papel fundamental na iniciação e propagação da inflamação crônica da membrana sinovial (Klimiuk et al., 1999;

McInnes et al., 2007). Por apresentar características inflamatórias, incluindo alterações nos níveis de citocinas e resposta variável a diferentes drogas imunossupressoras, o modelo de artrite induzido por CFA tem se apresentado útil nos estudos de mecanismos imunopatogênicos da artrite reumatóide e no estudo pré-clínico de novas terapias (Inglis et al., 2005).

4. Anti-inflamatórios não-esteróides

Os anti-inflamatórios não-esteróides (AINEs) são fármacos amplamente prescritos, existindo atualmente mais de 50 compostos deste grupo disponíveis no mercado. Os AINEs são fármacos com propriedades analgésicas, antipiréticas e anti-inflamatórias, sendo que sua principal vantagem é a ausência de dependência física ou psíquica com o uso prolongado, quando comparados aos opióides (Alsalameh et al., 2003). A ação anti-inflamatória dos AINEs decorre principalmente da inibição da inibição da enzima ciclooxigenase (COX) impedindo, dessa forma, a formação de prostaglandinas e de tromboxanos, mediadores do processo inflamatório (Burian & Geisslinger, 2005).

Os principais usos clínicos dos AINEs são para tratamento de doenças articulares como artrite reumatóide, artrite gotosa, osteoartrite, tendinite, além de outros processos inflamatórios em geral como lesões músculo-esqueléticas e inflamações oftálmicas (Vane & Botting, 1996). O uso de AINEs costuma ser indicado nos casos em que a morbidade da reação inflamatória supera os benefícios de regeneração tecidual causados pela inflamação. Quando utilizados por períodos prolongados, como em doenças inflamatórias crônicas, os AINEs geralmente causam efeitos adversos como toxicidade gástrica, disfunção hepato-real e reações cutâneas (Guterres et al., 2000; Guterres et al., 2001; Tomisato et al.,

2004). Os AINEs são classificados de acordo com suas funções químicas. A maioria são ácidos orgânicos e atuam como inibidores competitivos reversíveis da atividade da COX, enquanto que o ácido acetilsalicílico (AAS) é o único AINE que atua de forma a modificar covalentemente a COX, resultando na inibição irreversível da atividade da enzima (Botting et al., 2006).

Atualmente são conhecidas três isoformas da enzima ciclooxigenase: COX-1, COX-2 e COX-3. A isoforma COX-1 é expressa constitutivamente na maioria dos tecidos, incluindo plaquetas e estômago e está envolvida na sinalização entre células e na homeostasia tecidual. A isoforma COX-2 é induzida principalmente nas células inflamatórias, quando estas são ativadas durante a inflamação a COX-2 tende a facilitar a resposta inflamatória. Entretanto, no cérebro, rins e alguns outros tecidos, a COX-2 é expressa constitutivamente (Deninger & Schluesener, 1999). Por sua vez, a isoforma COX-3 é uma variante do gene da COX-1, através da ocorrência de um *splicing* alternativo. Sabe-se que esta isoforma é mais abundante no coração e no córtex cerebral, porém esforços vêm sendo realizados a fim de se obter maiores informações a respeito de sua função e modulação (Cui et al., 2004; Botting et al., 2006).

Embora todos os AINEs sejam antipiréticos, analgésicos e anti-inflamatórios, existem diferenças em suas atividades individuais. Os motivos para estas diferenças entre os AINEs não estão totalmente esclarecidos, mas provavelmente estão relacionados às suas diferenças de especificidade sobre as isoformas de COX, bem como pelos mecanismos de ação secundários ainda desconhecidos. Além disso, recentes estudos com linhagens de células tumorais, estudos clínicos e epidemiológicos têm demonstrado a potencial utilização dos AINEs como agentes farmacológicos no tratamento e prevenção de vários tipos de câncer (Toomey et al.,

2009; Harris, 2009; Hyde & Missailidis, 2009). Sugere-se que a expressão de COX-2 possa ser constitutiva em vários tipos de tumores, sendo que a superexpressão dessa enzima está associada com gliomas clinicamente mais agressivos e de pior prognóstico para o paciente (Shono et al., 2001). Apesar de os mecanismos moleculares envolvidos não estarem ainda bem definidos, acredita-se que a COX contribua para produção de fatores pró-angiogênicos e que o aumento de sua expressão durante a tumorigênese seja provavelmente conseqüência de múltiplos efeitos (Howe et al., 2001).

4.1. Indometacina

A indometacina é um fármaco derivado do ácido indolacético, de nome químico [1-(4-clorobenzoil)-5-metóxi-2-metilindol-3-il]acético (Figura 5). Apresenta-se como pó cristalino, inodoro, de coloração branco-amarelada (Reynolds, 1993).

A indometacina pertence à classe dos AINEs, apresentando potente ação anti-inflamatória além de ação analgésica e antipirética. Foi introduzida na terapêutica para o tratamento de doenças inflamatórias reumáticas, principalmente artrite reumatóide, osteoartrite e espondilite anquilosante. A dose comumente empregada por via oral situa-se entre 25 a 50 mg (o que corresponde a faixa de 5 - 10 mg/Kg em animais), com intervalos de 8 horas, sendo a dose máxima diária de 200 mg. A indometacina também tem sido usada em recém nascidos por via intravenosa nas doses de 0,1 a 0,2 mg/Kg a cada 12 horas com a finalidade de fechamento do ducto arterioso persistente (Wannamacher e Ferreira, 2004).

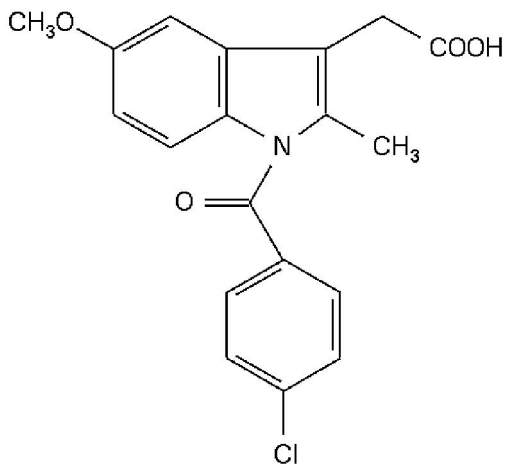


Figura 5. Estrutura química da indometacina

A absorção da indometacina após administração oral é rápida, sendo o pico de concentração plasmática alcançado após aproximadamente 2 horas. A biodisponibilidade cerebral da indometacina é baixa, pois aproximadamente 90% da indometacina administrada liga-se a proteínas plasmáticas, o que reduz a fração livre do fármaco na circulação e dificulta a passagem pela BHE (Parepally et al., 2006).

A atividade anti-inflamatória da indometacina é mediada tanto pela inibição de COX-1 e COX-2, quanto pela inibição da migração dos neutrófilos (Jacobs & Bijlsma, 1997; Peskar, 2001). Em função de seu efeito inibitório sobre a COX-1, a indometacina apresenta importantes efeitos adversos, que são dose-dependente e, portanto, restringem sua utilização apenas a doenças inflamatórias graves, onde outras alternativas terapêuticas não são efetivas. Sua toxicidade está relacionada principalmente ao trato gastrointestinal, onde pode provocar ulcerações e até mesmo perfurações e hemorragias. Tem sido descrito que não apenas esse efeito sistêmico de inibição das COXs, mas também um efeito citotóxico pelo contato direto do fármaco à mucosa gástrica seria o reponsável pelas lesões

gastrointestinais. A irritação local causada pelo contato direto dos AINEs à mucosa gástrica está relacionada com a inonização do fármaco. A indometacina é um ácido orgânico fraco e em pH baixo está na forma não ionizada, o que possibilita uma alta solubilidade em lipídeos, comprometendo a integridade da mucosa pela redução da hidrofobicidade da superfície. Ao penetrar na membrana celular e acumular-se no epitélio da mucosa gástrica, onde o pH local é 7,4, o fármaco apresenta-se na forma ionizada sendo absorvido (Reynolds, 1993; Fiorucci & Antonelli, 2001). Além disso, a inibição de prostaglandinas renais protetoras dos efeitos vasoconstritores de angiotensina e catecolaminas pode agravar disfunções renais pré-existentes e causar insuficiência renal, tornando necessária a interrupção do tratamento para a reversão do quadro clínico (Tomisato et al., 2004). Dessa forma, a principal limitação do emprego terapêutico da indometacina são os efeitos adversos que ela causa.

Com a finalidade de reduzir os efeitos tóxicos da indometacina, tem sido proposta a esterificação ou amidação de seu grupamento ácido com o objetivo de reduzir a irritação da mucosa gástrica por ação local do fármaco (Bonina et al., 1997; Kalgutkar et al., 2000; Cruz et al. 2006). Outro artefato é a introdução do fármaco em sistemas carreadores, como lipossomas (Srinath et al., 2000), microesferas lipídicas (Srinath e Diwan, 1998) ou nanopartículas (Ammoury et al., 1991; Calvo et al., 1996; Kim et al., 2001; Pohlmann et al., 2002). Esses sistemas possibilitam a redução dos efeitos adversos, tanto por minimizar o contato do fármaco com a mucosa gástrica, como por proporcionar uma vetorização do fármaco para o local de ação, além de promover, em certos casos, o aumento da absorção no trato gastrointestinal (Fawaz et al., 1996; Srinath & Diwan, 1998).

Recentemente, estudos experimentais têm sugerido novas aplicações terapêuticas para a indometacina. Demonstrou-se que, em células de glioma, a indometacina modula a expressão de proteínas da matriz extracelular (Ishibashi, et al., 2005) e diminui a capacidade de invasão das células (Wang et al., 2005). Além disso, resultados do nosso grupo de pesquisa demostraram que a indometacina exerce efeito antiproliferativo em linhagens celulares de gliomas por induzir uma parada na progressão do ciclo celular (Bernardi et al., 2006) e por modular a atividade e expressão de enzimas do sistema purinérgico (Bernardi et al., 2007).

5. Nanopartículas

5.1. Aspectos gerais da Nanobiotecnologia

A nanobiotecnologia refere-se à fusão de duas abordagens tecnológicas recentes, a biotecnologia e a nanotecnologia, apresentando enormes inovações e potencialidades. A nanobiotecnologia pode ser, portanto, definida como o estudo, processamento, fabricação e desenho de dispositivos orgânicos, nanomateriais para atuação biológica ou biomateriais, nos quais pelo menos um componente funcional possui tamanho nanométrico. Áreas importantes da nanobiotecnologia incluem a nanomedicina (biologia molecular e genética), a física-médica (diagnóstico), o desenvolvimento de nanofármacos (fármacos encapsulados), além da nanocosmecêutica (cosméticos com efeitos farmacológicos consideráveis). Estima-se que a nanobiotecnologia possibilitará meios para o aprimoramento do diagnóstico prematuro de diversas doenças, levando a abertura de novas estratégias terapêuticas. De forma bastante relevante, a nanobiotecnologia se mostra promissora em aumentar a eficiência do processo de desenvolvimento de fármacos.

5.2. Sistemas de nanoencapsulamento de fármacos

A nanotecnologia farmacêutica teve início em meados da década de 1970 com os lipossomas. Historicamente, as primeiras nanopartículas propostas para uma aplicação terapêutica eram compostas de gelatina ligada à albumina (Scheffel et al., 1972; Marty et al., 1978). Na sequência, foram desenvolvidas as nanopartículas poliméricas empregando principalmente polímeros biodegradáveis sintéticos e as nanopartículas lipídicas sólidas (Couvreur et al., 1979). A partir de 1986, houve uma aceleração no desenvolvimento de novas metodologias para a preparação de vários tipos de nanopartículas (Fattal & Vauthier, 2002). A tabela 1 apresenta os sistemas nanoparticulados para vetorização de fármacos que até o presente momento foram aprovados pelo FDA (*Food and Drug Administration*) para *Trials* ou uso clínico.

Tabela 1. Sistemas nanoparticulados para vetorização de fármacos que foram aprovados pelo FDA para uso clínico ou *Trials.* Adaptado de Faraji et al., 2009.

Agente Terapêutico	Indicação	Referência
Anfotericina B em lipossomas	Infecções fúngicas, Leishmaniose	Alder-Moore, 1994
Adenosina deaminase - PEG	Desordens de imunodeficiência	Bory et al., 1991
Doxorrubicina em lipossomas	Sarcoma de Káposi, câncer de ovário	Northfelt et al., 1996
Citosina Arabinosina em lipossomas	Meningite linfomatosa ou neoplásica	Glantz et al.,1999a, 1999b
Interferon α-2b - PEG	Hepatite C	Glue et al., 2000
Interleucina 2 com toxina diftérica	Linfoma cutâneo de células T	Olsen et al., 2001
Verteporfina em lipossomas	Degeneração macular	Bressler et al., 2001
Fator estimulador de granulócitos - PEG	Quimioterapia associada a neutropenia	Siena et al., 2003
L-asparaginase - PEG	Leucemia linfocítica aguda	Rosen et al., 2003
Paclitaxel ligado à albumina	Câncer de mama metastático	Nyman et al., 2005
Aptanibe - PEG	Degeneração macular	Lee et al., 2005a, 2005b
Pemetrexed	Mesotelioma maligno pleural	Ceresoli et al., 2006

Carreadores coloidais de fármacos, incluindo nanoemulsões, nanoesferas, nanocápsulas, lipossomas e complexos lipídicos, apresentam-se como veículos

promissores para administração intravenosa de fármacos lipofílicos, bem como para administração nas vias oral, ocular, cutânea e endovenosa possibilitando a otimização da velocidade de cedência e do regime de dosagem das substâncias (Alvarez-Róman et al., 2001; Fattal & Vauthier, 2002; Couvreur et al., 2002). Cada um dos diferentes sistemas nanoparticulados apresentam peculiaridades que devem ser consideradas de acordo com sua finalidade. De uma forma geral, estes sistemas nanométricos apresentam elevada área superficial, sendo considerados vetores para a administração de substâncias lipofílicas, possibilitando uma liberação homogênea e, muitas vezes, controlada de fármacos, aumentando assim a resposta terapêutica no sítio de ação por tempo prolongado. A vetorização em órgãos, tecidos ou células específicas também apresenta como vantagem a diminuição de efeitos adversos apresentados sistemicamente pelo fármaco não vetorizado (Guterres et al., 2001). Outro aspecto importante é a possibilidade de diminuição das doses e do número de aplicações, de modo que os pacientes apresentam maior adesão ao tratamento. Além disso, em alguns casos, o nanoencapsulamento pode ter como objetivo a melhora da biodisponibilidade de um fármaco através do aumento da estabilidade desse fármaco nos fluídos biológicos ou ainda o aumento de estabilidade da própria formulação farmacêutica (Couvreur et al., 2002; Couvreur & Vauthier, 2006). Sistemas de nanoencapsulamento de fármacos têm sido propostos principalmente para compostos antitumorais, peptídeos, proteínas, vacinas, oligonucleotídeos e agentes anti-infecciosos (Couvreur & Vauthier, 2006). A Figura 6 apresenta alguns sistemas que podem ser utilizados como carreadores de fármacos.

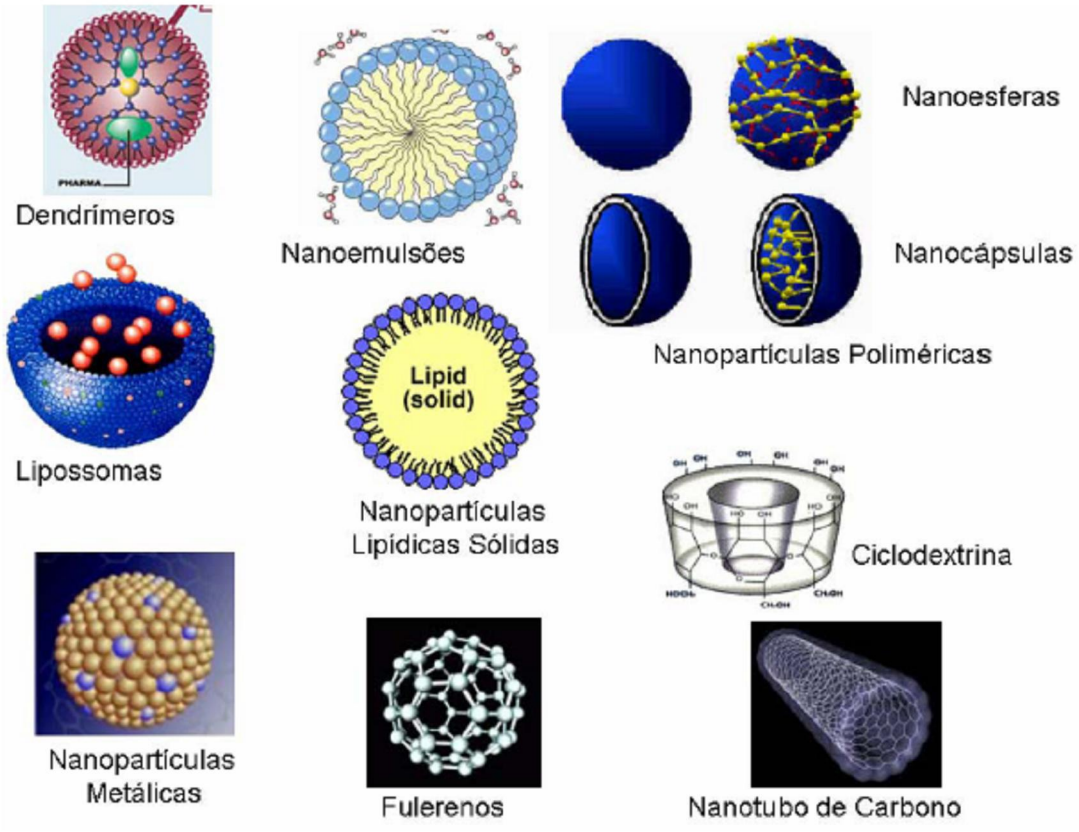


Figura 6. Sistemas nanométricos que podem ser utilizados para a vetorização de fármacos. Adaptado de Suh et al., 2009 e de Faraji et al., 2009.

5.2.1. Nanopartículas poliméricas

As nanopartículas poliméricas são sistemas carreadores de fármacos que apresentam diâmetros inferiores a 1μm. Dependendo do processo de preparação das nanopartículas podem-se obter nanocápsulas ou nanoesferas que diferem entre si segundo sua composição e organização estrutural (Soppimath et al., 2001). As nanoesferas não apresentam óleo em sua composição e são formadas por um núcleo sólido e por uma rede polimérica possuindo uma estrutura matricial onde o fármaco pode ficar retido ou adsorvido (Schaffazick et al., 2003). As nanocápsulas são formadas por um invólucro polimérico disposto ao redor de um núcleo oleoso, podendo o fármaco estar dissolvido neste núcleo e/ou adsorvido à parede

polimérica, dependendo das propriedades físico-químicas e da composição das nanocápsulas. Dessa forma, nanocápsulas podem ser consideradas um sistema "reservatório" (Couvreur et al., 2002).

A utilização de materiais poliméricos para a encapsulação de fármacos ou ativos cosméticos é uma alternativa para mascarar as propriedades físico-químicas intrínsecas da substância, melhorar sua interação com membranas, bem como o transporte através delas e facilitar sua absorção ou penetração cutâneas (Couvreur & Vauthier, 2006). Além disso, as nanopartículas poliméricas permitem modificações químicas na sua superfície, possibilitando um controle farmacocinético na vetorização de agentes terapêuticos (Faraji et al., 2009). Comparado com outros carreadores coloidais, as nanopartículas poliméricas apresentam maior estabilidade quando estão em contato com fluídos biológicos (Roney et al., 2005). Além disso, elas podem ser administradas por via endovenosa sem causar embolia (Couvreur et al., 2002). Inicialmente nanopartículas convencionais apresentaram limitações para vetorização de fármacos quando administradas intravenosamente, pois foram fagocitadas pelo sistema fagocitário mononuclear. Mais recentemente, desenvolveram-se nanopartículas chamadas de "invisíveis", pois são caracterizadas por permanecerem longo tempo na circulação sem serem fagocitadas (Brigger et al., 2002).

Dentre os polímeros utilizados a poli(ε-caprolactona) se destaca devido sua biocompatibilidade, biodegradabilidade e propriedades mecânicas, pois é um polímero semi-cristalino que apresenta degradação mais lenta quando comparado aos polímeros amorfos (Guterres et al., 2007). A poli(ε-caprolactona) é um dos mais importantes polímeros biodegradáveis na medicina, sendo amplamente empregado em suturas (Lu & Chen, 2004). Além disso, poliésteres como a poli(ε-caprolactona)

apresentam propriedades bioadesivas, característica bastante relevante pois a bioadesividade confere um acréscimo na deposição das partículas em regiões do trato gastrointestinal, aumentando assim a absorção sistêmica de fármacos (Lamprecht et al., 2000a). Vários trabalhos têm demonstrado a importância do emprego deste material polimérico no desenvolvimento de sistemas carreadores de fármacos nanoparticulados (Lamprecht et al., 2000a; 2000b; Sinha et al., 2004; Lu & Chen, 2004; Fattal et al., 2007), sendo empregados inclusive para uso intravenoso (Ravi Kumar, 2000).

Existem vários métodos descritos na literatura para preparação de nanopartículas poliméricas, dentre os quais se destaca o método de deposição interfacial de polímeros pré-formados proposto por Fessi e colaboradores (1989), no qual o polímero biodegradável é dissolvido em um solvente orgânico juntamente com o componente oleoso, o tensoativo lipofílico e o fármaco ou ativo a ser encapsulado. Esta fase oleosa é vertida, sob agitação moderada, sobre a fase aquosa, a qual é composta de água e tensoativo hidrofílico. Esta mistura origina, de forma espontânea, as nanocápsulas, com diâmetros médios situados entre 200 e 500 nm. A vantagem de utilização deste método é a obtenção espontânea, simples, eficiente e reprodutível de pequenas partículas em escala nanométrica com elevada capacidade de encapsulação de fármacos (Fessi et al., 1989; Schaffazick et al., 2003; Guterres et al., 2007). A obtenção de partículas com diferentes diâmetros é dependente de vários fatores, tais como: concentração do polímero e fármaco, quantidade de tensoativo, concentração de óleo na fase orgânica e velocidade de difusão da fase orgânica aquosa (Couvreur et al., 2002). Todos esses parâmetros são fundamentais na caracterização de uma formulação nanoencapsulada.

5.3. Vetorização de fármacos antitumorais

Considerando todas as vantagens que as nanopartículas apresentam, a vetorização de fármacos antitumorais é uma das mais promissoras aplicações biológicas dessas formulações. Dentre todas as formas de malignidade, os tumores sólidos apresentam um maior desafio para a quimioterapia convencional (Wong et al., 2007). Esses tumores apresentam várias particularidades que se tornam obstáculos para uma terapia efetiva. Pode-se citar mecanismos chamados de "celulares" que incluem alterações bioquímicas, alterações nas vias de sinalização para apoptose e proliferação celular e o sistema de efluxo da glicoproteína P (Pgp) que é responsável por resistência múltipla a drogas (MDR - *mullti-drug resistance*) (Krishna & Mayer, 2000). Em adição a esses mecanismos "celulares", os tumores sólidos são mais resistentes à quimioterapia devido a mecanismos "não celulares" que são responsáveis pela dificuldade do fármaco em atingir concentrações farmacologicamente ativas na massa intratumoral. Tumores sólidos apresentam uma vascularização irregular e tortuosa em algumas regiões, além da aumentada pressão intersticial e baixa pressão microvascular que impede ou retarda o extravasamento de moléculas (Krishna & Mayer, 2000; Brigger et al., 2002). Dessa forma, o sistema linfático é ineficaz nos tumores sólidos (Wong et al., 2007). Assim, o racional de se utilizar nanopartículas poliméricas para vetorização de fármacos em tumores sólidos se deve principalmente a duas propriedades desses sistemas: 1) devido ao tamanho reduzido, nanopartículas são capazes de penetrar em pequenos capilares, aumentando eficientemente o acúmulo de fármaco na massa tumoral, apresentando o denominado "efeito de aumento de retenção e permeabilidade" (EPR); 2) o uso de polímeros biodegradéveis no preparo de nanopartículas, permite uma liberação sustentada do fármaco no sítio de ação por

um período de dias ou semanas após a administração (Vinogradov et al., 2002). Além disso, é importante considerar que o processo inflamatório presente nos tecidos adjacentes ao tumor também contribui para alterações de permeabilidade microvascular, facilitando dessa forma o direcionamento das nanopartículas para o tecido tumoral. A Figura 7 representa de forma esquemática o acúmulo de nanopartículas no compartimento intersticial do tumor.

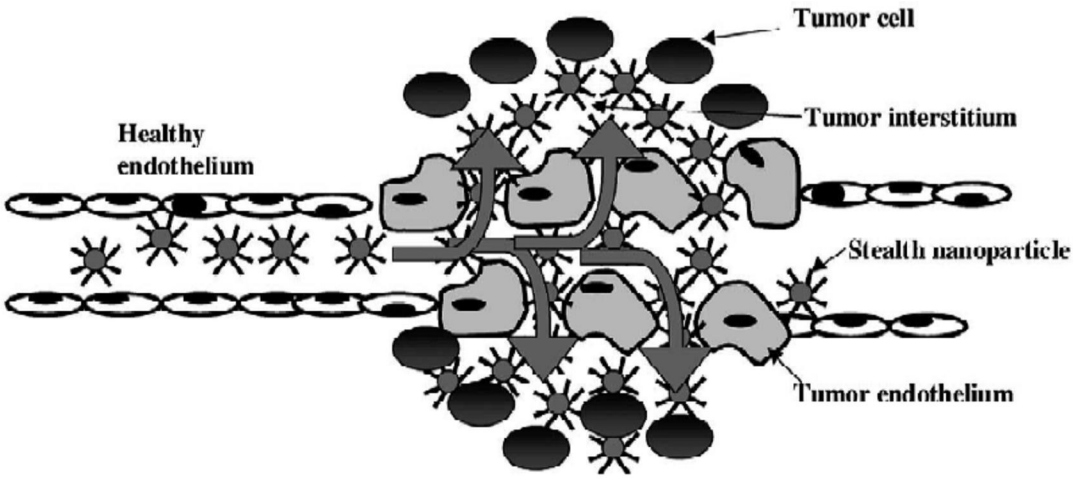


Figura 7. Acúmulo de nanopartículas pelo efeito de aumento de retenção e permeabilidade no interstício tumoral. Adaptado de Brigger et al., 2002.

5.4. Vetorização de fármacos ao Sistema Nervoso Central

O tratamento de doenças neurodegenerativas e de tumores cerebrais é desafiador devido às limitações impostas pela barreira hematoencefálica (BHE). A BHE é uma monocamada de células endoteliais associadas à pericitos e astrócitos, que separam o parênquima cerebral do sistema circulatório, prevenindo assim a penetração de toxinas, patógenos e também de potenciais agentes terapêuticos (Béduneau et al., 2007). Apenas moléculas pequenas com tamanho menor de 5.000 Daltons, lipofílicas e eletricamente neutras conseguem difundir passivamente

através da BHE (Abraham et al., 1994). As células endoteliais da BHE apresentam *tight junctions* entre as células, ausência de fenestrações e atividade pinocítica 100x reduzida quando comparada ao endotélio periférico, o que contribui para restringir a passagem de compostos para o sistema nervoso central (SNC) (Garcia-Garcia et al., 2005). A Figura 8 apresenta de forma esquemática a anatomia da BHE.

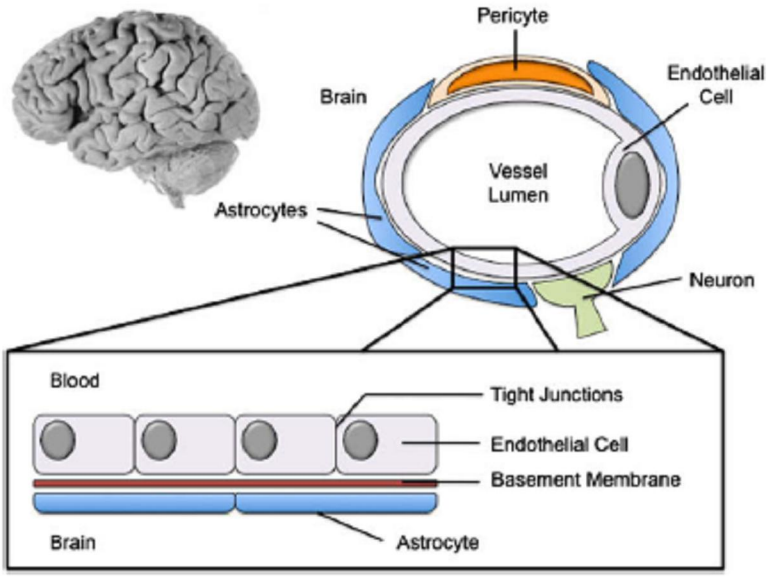


Figure 8. Anatomia da barreira hematoencefálica. Adaptado de Faraji et al., 2009.

Extensivas pesquisas têm sido direcionadas para explorar a aplicabilidade de sistemas nanoparticulados para a vetorização de fármacos ao SNC (Juillerat-Jeanneret, 2008). Os principais nanocarreadores descritos pela literatura para a vetorização cerebral são lipossomas, micelas poliméricas e nanopartículas lipídicas ou poliméricas (Béduneau et al., 2007). Esses sistemas apresentam vantagens quando comparados às terapias convencionais, pois além de serem mais eficazes, podem ser administrados de forma não-invasiva melhorando a qualidade de vida dos pacientes (Garcia-Garcia et al., 2005). Os mecanismos pelos quais as

nanopartículas são capazes de atravessar a BHE ainda não são completamente compreendidos. Tem sido proposto que o tamanho da partícula, o tipo de polímero, bem como as características físico-químicas da superfície são fundamentais para induzir uma estabilização estérica, aumentando assim o tempo de circulação, o que favorece a interação e penetração nas células endoteliais da BHE (Garcia-Garcia et al., 2005). Recentes estudos mostram que alterações na superfície das nanopartículas podem aumentar o transporte de fármacos através da BHE. Nanopartículas de doxorrubicina revestidas com polietilenoglicol (PEG) foram mais eficazes em reduzir o tamanho tumoral em modelo de tumor cerebral quando comparado à doxorrubicina na forma convencinal (solução) ou em nanopartículas não-revestidas (Petri et al., 2007). Além disso, o revestimento das nanopartículas poliméricas com polisorbato 80 (Tween 80®) foi relatado pela literatura como fundamental para a vetorização cerebral (Koziara et al., 2003; Ambruosi et al., 2006; Zhang et al., 2006). Isso poderia ser explicado pelo fato do polisorbato 80 seletivamente promover a adsorção de certas proteínas plasmáticas, como a apolipoproteína E, na superfície da nanopartículas. Assim, as nanopartículas com apolipoproteína E adsorvida seriam reconhecidas como lipoproteínas de baixa-densidade (LDL), sendo transportadas pelas células endoteliais da BHE através de endocitose receptor-mediada (Calvo et al., 2001, Kreuter et al., 2002). As células endoteliais da BHE teriam um papel fundamental na vetorização cerebral através da adesão das nanopartículas com posterior endocitose, transcitose, modulação das *tight junction* e inibição da glicoproteína P (Kreuter et al., 1995; Kreuter et al., 2003; Vauthier et al., 2003).

OBJETIVOS

Objetivo geral:

Investigar os efeitos antitumoral, neuroprotetor e anti-inflamatório da indometacina em nanocápsulas.

Objetivos específicos:

- ✓ Avaliar o efeito citotóxico da indometacina em nanocápsulas em linhagens celulares de gliomas e em tecido neural não-tumoral (Capítulo 1).

- ✓ Investigar o efeito do tratamento sistêmico com indometacina em nanocápsulas sobre o crescimento de gliomas implantados em cérebro de ratos (Capítulo 2).

- ✓ Investigar o potencial efeito neuroprotetor da indometacina em nanocápsulas em culturas organotípicas de hipocampo de ratos submetidas à privação de oxigênio e glicose (Capítulo 3).

- ✓ Verificar a eficácia anti-inflamatória do tratamento sistêmico com indometacina em nanocápsulas em modelos de inflamação aguda e crônica em ratos (Capítulo 4).

CAPÍTULO 1

SELECTIVE CYTOTOXICITY OF INDOMETHACIN AND INDOMETHACIN ETHYL ESTER-LOADED NANOCAPSULES AGAINST GLIOMA CELL LINES: AN *IN VITRO* STUDY

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Periódico: ***European Journal of Pharmacology***

Status: **Publicado**

Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmacology 586 (2008) 24–34
www.elsevier.com/locate/ejphar

Selective cytotoxicity of indomethacin and indomethacin ethyl ester-loaded nanocapsules against glioma cell lines: An *in vitro* study

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Received 25 September 2007; received in revised form 29 January 2008; accepted 13 February 2008
Available online 19 February 2008

Abstract

Gliomas are the most common and devastating tumors of the central nervous system. Several studies have suggested that nonsteroidal anti-inflammatory drugs (NSAIDs) are promising anticancer agents. Biodegradable nanoparticulate systems have received considerable attention as potential drug delivery vehicles. The aim of this study was to evaluate the effects of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules on glioma cell lines. In addition, the effect of these formulations on normal neural tissue was also evaluated. In order to investigate this, glioma cell lines (U138-MG and C6) and hippocampal organotypic cultures were used. The main finding of the present study is that indomethacin-loaded nanocapsules formulation was more potent than a solution of indomethacin in decreasing the viability and cell proliferation of glioma lines. Indomethacin and indomethacin ethyl ester associated together in the same nanocapsule formulation caused a synergic effect decreasing glioma cell proliferation. In addition, when the glioma cells were exposed to 25 μM of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules, a necrotic cell death was observed. Interestingly, 5 μM of indomethacin-loaded nanocapsules was able to cause an antiproliferative effect without promoting necrosis in glioma cells. Another important finding was that the cytotoxic effect induced by 25 μM or 50 μM of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules, in glioma cells was not observed in the organotypic cultures, indicating selective cytotoxicity of those formulations for tumoral cells. Further investigations using *in vivo* glioma model should be helpful to confirm the distinct effects of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules, in normal versus tumoral cells.

Keywords: Glioma cell; Hippocampal organotypic culture; Cell death; Indomethacin; Indomethacin ethyl ester; Polymeric nanocapsule

1. Introduction

Malignant gliomas are the most common primary brain tumors, representing 50–60% of this type of tumor (Bigner et al., 1988; Holland, 2001). Glioblastomas, malignant glioma of grade IV, are poorly responsive to multimodaly therapeutic interventions, including surgery, radiotherapy, and chemotherapy (Deen et al., 1993). Despite treatment, malignant gliomas recur early, leading to a mean survival of less than 12 months (Holland, 2001). The highly lethal nature of this tumor results from the acquisition of an invasive phenotype that allows the glioblastoma cells to infiltrate surrounding brain tissue (Greemberg et al., 1993). Anticancer drugs are toxic to both tumoral and normal cells and the efficacy of chemotherapy is often limited by important side effects (Brigger et al., 2002a).

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doi:10.1016/j.ejphar.2008.02.026

Besides, the presence of the blood-brain barrier limits the penetration of antineoplasic drugs into brain tumors (Gelperina et al., 2002). An alternative approach for the treatment of gliomas is the employment of a drug delivery system, as nanocarriers, which are able to improve or even target delivery of the anti-tumoral agents to the brain (Gelperina et al., 2002).

Nanoparticles are submicronic (<1 μm) systems generally, but not necessarily, made of polymers, which have been widely studied as drug delivery systems (Soppimath et al., 2001; Brigger et al., 2002a, Schaffazick et al., 2003; Garcia-Garcia et al., 2005; Wong et al., 2007). Generally, nanoparticles have a size around 200 nm and the drugs or other molecules may be dissolved, entrapped, encapsulated, adsorbed and/or attached to the nanoparticles (Brigger et al., 2002a). Applying the nanoprecipitation and the interfacial deposition techniques (Fessi et al., 1989), nanostructured systems such as nanospheres or nanocapsules can be obtained, according to the formulation composition. Nanocapsules are vesicular systems in which the drug can be confined to an aqueous or oily cavity surrounded by a single polymeric wall (Jäger et al., 2007). Nanocapsules may, thus, be considered as a 'reservoir' system (Brigger et al., 2002a). The most promising application of polymeric nanoparticles is their use as carriers for anticancer drugs (Kim and Lee , 2001). It has been recently reported that novel nanoparticles could be used as potential drug carriers across the blood-brain barrier (Lockman et al., 2003; Koziara et al., 2003). Additionally, it has been found that the polymer-anticancer drug conjugates in comparison with low-molecular-weight anticancer drugs were accumulated more in the tumor tissues than in the normal tissues due to the enhanced permeability and retention effect (Kim and Lee , 2001). The literature also reported that polymer-anticancer drug conjugates could prolong the antitumoral activity by releasing the drug at a controlled rate (Alonso, 1996; Seijo et al., 1990; Vinogradov et al., 2002). The approach of treating glioma cells by liposomal carriers in order to obtain an increased efficiency or reduced side effects has been reported (Hu et al., 1995; Koukourakis et al., 2000). Moreover, nanoparticles of biodegradable polymers coated with polysorbate 80 (Tween 80®) were reported in the literature for cerebral delivery of several substances (McCarthy et al., 2005; Zhang and Feng , 2006; Brioschi et al., 2007).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most widely used drugs for the treatment of autoimmune and chronic inflammatory diseases (Baek et al., 2002). Numerous experimental, epidemiological, and clinical studies suggest that NSAIDs are promising anticancer agents (Thun et al., 2002). Recent studies have provided evidence of an additional role for NSAIDs in preventing growth of cancer cells by inhibiting cyclooxygenase (COX) enzymes (Grubbs et al., 2000; Williams et al., 2000). Other studies revealed that the antiproliferative effect of NSAIDs may be, in part, independent of cyclooxygenase inhibition (Baek et al., 2002). The exact mechanisms by which NSAIDs contribute to the antitumor activity remain controversial and it is peculiar for each tumor (Thun et al., 2002). The data from the literature suggest the involvement of these drugs on the induction of apoptosis, on the control of cell proliferation and invasion and/or on the inhibition of angiogenesis (Tegeder et al., 2001).

We have recently shown that indomethacin, a powerful NSAID derived from indolacetic acid, causes antiproliferative effects in glioma cell lines due to an arrest of cell cycle progression (Bernardi et al., 2006). In addition, we demonstrated that these antiproliferative effects of indomethacin on glioma cells are mediated, at least in part, by increasing the catabolism of extracellular purines (Bernardi et al., 2007). Considering these effects recently demonstrated by our group (Bernardi et al., 2006; Bernardi et al., 2007), the aim of the present study was to investigate the effect of indomethacin-loaded nanocapsules on the glioma cell lines. Additionally, the effects of nanoencapsulated indomethacin ethyl ester, a selective inhibitor of cyclooxygenase-2 (COX-2) (Kalgutkar et al., 2000), were also evaluated.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM), HBSS, horse serum, Fungizone®, penicillin/streptomycin, 0.25% trypsin/EDTA solution were obtained from Gibco (Gibco BRL, Carlsbad, CA, USA). Fetal bovine serum was obtained from Cultilab (Cultilab, Campinas, SP, Brazil). Gentamicin was obtained from Schering do Brazil (Rio de Janeiro, RJ, Brazil). Indomethacin, dimethylsulphoxide, propidium iodide (PI) and MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) were obtained from Sigma (St. Louis, USA). Poly(ε-caprolactone) (PCL) (M_w=65,000) was supplied by Aldrich (Strasbourg, France). Caprilic/capric triglyceride mixture was delivered from Brasquim (Porto Alegre, Brazil). Span 60® (sorbitan monostearate) and Tween 80® (polysorbate 80) were obtained from Delaware (Porto Alegre, Brazil). Indomethacin ethyl ester was synthesized by our group (Cruz et al., 2006) after adapting the methodology described by Kalgutkar et al. (2000). All other chemicals and solvents used were of analytical or pharmaceutical grade.

2.2. Preparation of nanocapsules

Nanocapsule suspensions were prepared by interfacial deposition (Fessi et al., 1989). At 40 °C, indomethacin or indomethacin ethyl ester (0.010 g), poly(ε-caprolactone) (0.100 g), capric/caprylic triglyceride (0.33 ml) and sorbitan monostearate (0.077 g) were dissolved in acetone (27 ml). In a separate flask, polysorbate 80 (0.077 g) was added into 53 ml of water. The organic solution was poured into the aqueous phase under magnetic stirring at room temperature. After 10 min, the acetone was eliminated and the suspensions concentrated under reduced pressure. The final volume was adjusted to 10 ml. Control formulations (drug-unloaded nanocapsules) were prepared omitting the drug (indomethacin or indomethacin ethyl ester). Additionally, a suspension containing both indomethacin (0.0054 g) and indomethacin ethyl ester (0.0046 g) was also prepared.

2.3. Characterization of nanocapsules

After preparation, the pH values of nanocapsule suspensions were determined using a potentiometer (Micronal B-474). The particle size, polydispersity and zeta potential of the systems were determined using a Zetasizer®nano-ZS ZEN 3600 model (Malvern, UK). The samples were diluted with water (MilliQ®) (particle size) or in 10 mM NaCl aqueous solution (zeta potential). The measurements were made in triplicate. The total concentrations of indomethacin or indomethacin ethyl ester in the formulations were measured by HPLC (Perkin-Elmer S-200, with injector S-200, detector UV–vis, a guard-column and a column Lichrospher 100 RP-18, 250 mm, 4 mm, 5 μm, Merck). The mobile phase (1.2 ml/min) consisted of acetonitrile/water (70:30, v/v) adjusted to apparent pH 5.0±0.5 with 10% (v/v) acetic acid. Each suspension (100 μl) was treated with acetonitrile (10 ml), the solution was filtered (Millipore 0.45 μm) and injected (20 μl). The HPLC method was validated following the ICH (1996). Linear calibration curves for indomethacin ester and for indomethacin were obtained in the range of 1.00–25.00 μg/ml presenting correlation coefficients higher than 0.9994 (indomethacin ethyl ester) and 0.9992 (indomethacin).

2.4. Maintenance of cell lines

The human glioblastoma cell line U138-MG (derived from spontaneously occurring human malignant gliomas) and the rat glioma cell line C6 (derived from *N*-nitrosomethylurea-induced glioma in rat) were obtained from American Type Culture Collection (ATCC) (Rockville, Maryland, USA). The cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) containing antibiotics penicillin/streptomycin 0.5 U/ml, and supplemented with 5% (C6) or 15% (U138-MG) (v/v) fetal bovine serum (FBS). Cells were kept at a temperature of 37 °C, a minimum relative humidity of 95%, and an atmosphere of 5% CO_2 in air. All the experiments throughout this study were conducted in serum supplemented DMEM.

2.5. Drug exposure

Indomethacin or indomethacin ethyl ester in solution was dissolved in cell culture-grade dimethylsulphoxide (DMSO; Sigma). The indomethacin-loaded nanocapsules, the indomethacin ethyl ester-loaded nanocapsules and drug-unloaded nanocapsules were prepared as described above. The glioma cells were seeded according each experiment and after reaching subconfluence the cultures were exposed for 1, 3, 24 or 48 h to formulations: indomethacin, indomethacin ethyl ester, indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules (5, 10, 25, 50 or 100 μM). Control cells were treated with vehicle, i.e., 1.0% of DMSO or with drug-unloaded nanocapsules. In experiments made in hippocampal organotypic cultures, the cultures received 25 or 50 μM of indomethacin-loaded nanocapsules, indomethacin ethyl ester-loaded nanocapsules or drug-unloaded nanocapsules for 24 h. Control cultures were performed without nanocapsules.

2.6. Assessment of glioma cell viability

The method MTT provides a quantitative measure of the number cells with metabolically active mitochondria and it is based on the mitochondrial reduction of a tetrazolium bromide salt, MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]), to a chromophore, formazan product, whose absorbance can be determined by spectrophotometric measurement. Glioma cells were plated in a 96-well plate at 10^3 per well and, after reaching semi-confluence, the cultures were treated with 5, 10, 25, 50 or 100 μM of indomethacin, indomethacin-loaded nanocapsules, indomethacin ethyl ester or indomethacin ethyl ester-loaded nanocapsules for 48 h. Control cultures were also treated with DMSO or drug-unloaded nanocapsules. After 48 h of treatment, each culture medium containing the drug was removed and the cells were washed twice with 100 μl of PBS. After removing the PBS, 90 μl of culture medium and 10 μl of MTT were added to each of the wells. The cells were incubated for 3 h and the solution was then removed from the precipitate. A total of 100 μl of DMSO were added to the wells and the level of absorbance was read by an ELISA plate reader at 490 nm. This absorbance was linearly proportional to the number of live cells with active mitochondria. The cell viability was calculated using Eq. (1):

$$\text{Cell viability}\,(\%) = (\text{Abs}_s/\text{Abs}_{\text{control}})100 \quad (1)$$

where Abs_s is the absorbance of cells treated with different formulations and $\text{Abs}_{\text{control}}$ is the absorbance of control cells (incubated with cell culture medium only).

2.7. Cell counting

The human glioma cells (U138-MG) were seeded at 1×10^4 cells per well in DMEM/15% FBS in 24-well plates, and allowed to grown for 24 h. After reaching semi-confluence, glioma cells were treated with 5, 10, 25, 50 or 100 μM of indomethacin, indomethacin-loaded nanocapsules, indomethacin ethyl ester or indomethacin ethyl ester-loaded nanocapsules for 1, 3, 24 or 48 h. Control cultures were treated with DMSO or drug-unloaded nanocapsules. At the end of the treatment, the medium was removed. Cells were washed with phosphate buffered saline (PBS) and 200 μl of 0.25% trypsin/EDTA solution was added to detach the cells, which were counted immediately in a hemocytometer. The procedure was the same for the rat glioma cells (C6) except that they were seeded at 5×10^3 cells per well in DMEM/5% FBS.

2.8. Propidium iodide assay

Glioma cell lines were treated with 5, 10, 25, 50 or 100 μM of indomethacin, indomethacin-loaded nanocapsules, indomethacin ethyl ester or indomethacin ethyl ester-loaded nanocapsules for 24 h. Control cultures were also treated with DMSO or drug-unloaded nanocapsules. After the end of treatment, glioma cells were incubated with 5 μM of propidium iodide (PI) (Sigma Chemical) for 1 h. PI is excluded from healthy cells, but following loss of membrane integrity this molecule enters cells,

binds to DNA and becomes highly fluorescent (Macklis and Madison, 1990). PI fluorescence was excited at 515–560 nm using an inverted microscope (Nikon Eclipse TE300) fitted with a standard rhodamine filter. Images were captured using a digital camera connected to the microscope.

2.9. Organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared according to the method of Stoppini et al. (1991). Briefly, 400-μm-thick hippocampal slices were prepared from 6- to 8-day-old male *Wistar* rats using a Mcllwain tissue chopper (all animal use procedures were approved by local Animal Care Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals) and separated in ice-cold Hank's balanced salt solution (HBSS) composed of (mM): glucose 36, $CaCl_2$ 1.26, KCl 5.36, NaCl 136.89, KH_2PO_4 0.44, Na_2HPO_4 0.34, $MgCl_2$ 0.49, $MgSO_4$ 0.44, HEPES 25; Fungizone® 1% (Gibco, Grand Island, NY,USA) and gentamicine 36 μl/100 ml (Schering do Brasil, Rio de Janeiro, RJ, Brazil); pH 7.2. The slices were placed on Millicell culture insert (Millicell®-CM, 0.4 μm, Millipore®, Bedford, MA, USA) and the inserts were transferred to a 6-well culture plate (Cell Culture Cluster, Costar®, New York, NY, USA). Each well contained 1 ml of tissue culture medium consisting of 50% minimum essential medium (MEM) (Gibco), 25% HBSS (Gibco), 25% heat inactivated horse serum (Gibco) supplemented with (mM, final concentration): glucose 36, HEPES 25 and $NaHCO_3$ 4; Fungizone® 1% and gentamicine 36 μl/100 ml; pH 7.3. Organotypic cultures were maintained in a humidified incubator gasified with 5% CO_2 atmosphere at 37°C. The medium was changed every 3 days and experiments were carried out after 14 days *in vitro* when the cells received 25 or 50 μM of indomethacin-loaded nanocapsules, indomethacin ethyl ester-loaded nanocapsules or drug-unloaded nanocapsules for 24 h. Control cultures were performed without nanocapsule formulations.

2.10. Quantification of cellular death in organotypic hippocampal cultures

Cell death was assessed by fluorescent image analysis of propidium iodide (IP) uptake (Noraberg et al., 1999). After a period of 22 h in the presence of the different formulations, 5 μM PI was added to the cultures and incubated for 2 h. Cultures were observed with an inverted microscope (Nikon Eclipse TE300) using a standard rhodamine filter set. Images were captured and then analyzed using Scion Image software (http://www.scioncorp.com). The area where PI fluorescence was determined using the "density slice" option of Scion Image software and compared to the total hippocampus area to obtain the percentage of damage (Valentin et al., 2003).

2.11. Statistical analysis

Data are expressed as mean±S.E.M. and analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by *post-hoc* for multiple comparisons (Tukey test) using an Instat software package (GraphPad Software, San Diego, CA, USA). Differences between mean values were considered significant when $P<0.05$.

IC_{50} values were calculated by linear regression analysis of $\log_{10}$ cell viability versus $\log_{10}$ concentration. The values were compared using one-way analysis and Tukey test for simultaneous comparisons between groups.

3. Results

3.1. Physico-chemical characterization of nanocapsule formulations

All nanocapsule formulations were prepared by interfacial deposition of polymer without the need of any subsequent step

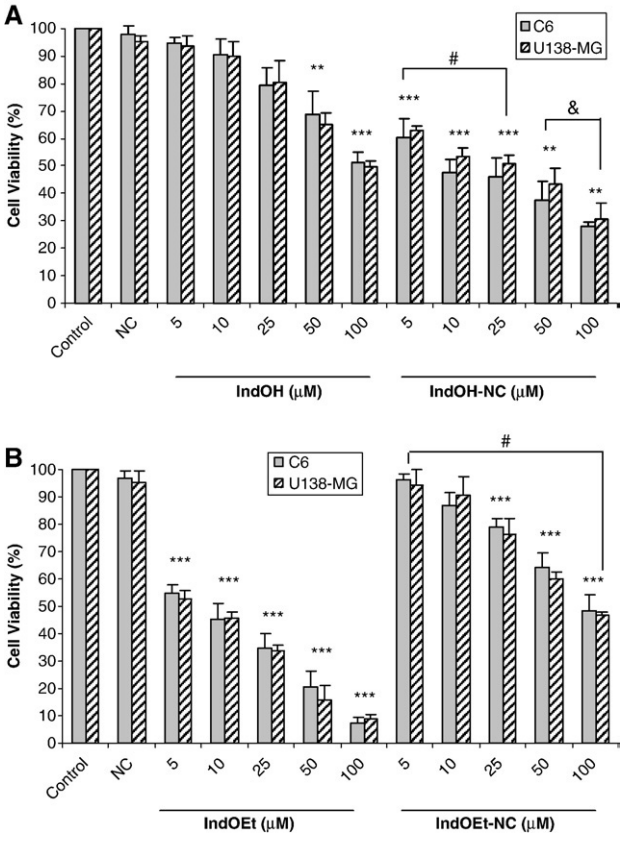

Fig. 1. Effect of indomethacin and indomethacin ethyl ester in solution or in nanocapsule formulations on cell viability of glioma cell lines. U138-MG and C6 glioma cell lines were grown on a 96-well plate and, after reaching approx. 80% confluence, the cultures were treated with 5, 10, 25, 50 or 100 μM of (A) indomethacin (IndOH) or indomethacin-loaded nanocapsules (IndOH-NC) and (B) indomethacin ethyl ester (IndOEt) or indomethacin ethyl ester-loaded nanocapsules (IndOEt-NC) for 48 h. Cultures were also treated with drug-unloaded nanocapsules (NC). After 48 h of treatment, the cell viability was evaluated by MTT assay, as described in Materials and methods. The cell viability was represented in relation of control cells (100% of cell viability). The values are represented as means±S.E.M. of six independent experiments made in quadruplicate. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). **Significantly different from the control group ($P<0.01$); ***Significantly different from the control group ($P<0.001$); #Significantly different from the respective group treated with drug in solution ($P<0.001$); &Significantly different from the respective group treated with drug in solution ($P<0.01$).

of purification. Indomethacin-loaded nanocapsules, indomethacin ethyl ester-loaded nanocapsules and drug-unloaded nanocapsules presented macroscopic homogeneous aspect like milky white bluish opalescent liquids. After preparation, the particle sizes were 240 nm (Indomethacin-loaded nanocapsules), 234 nm (indomethacin ethyl ester-loaded nanocapsules) and 226 nm (drug-unloaded nanocapsules). The suspensions showed monomodal size distributions and polydispersity indexes lower than 0.19, indicating narrow distributions. The pH values were 5.95 (Indomethacin-loaded nanocapsules), 6.14 (indomethacin ethyl ester-loaded nanocapsules) and 6.05 (drug-unloaded nanocapsules). The zeta potential values were −6.9, −7.4 and −7.3 mV, respectively. The drug contents were 0.991 ± 0.012 mg/ml (Indomethacin-loaded nanocapsules) and 1.003 ± 0.017 (indomethacin ethyl ester-loaded nanocapsules) and the encapsulation efficiencies were close to 100% for both formulations.

3.2. Indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules decrease cell viability in human and rat glioma cells

To investigate whether indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules affect the cell viability, we used an MTT assay that measures the mitochondrial activity and, indirectly, the cell viability of cells treated with these formulations. Cultured cells were treated with several concentrations (5, 10, 25, 50 or 100 μM) of indomethacin or indomethacin ethyl ester in solution or in nanocapsule formulations for 48 h. Analysis of MTT assay showed that, in solution, the effect of indomethacin ethyl ester was more pronounced than that of indomethacin. All the concentrations of indomethacin-loaded nanocapsules caused a significant reduction in cell viability compared to the control culture cells (Fig. 1A). An important finding of the present study is that the effect of indomethacin-loaded nanocapsules was more pronounced than indomethacin in solution at the same tested concentrations (Fig. 1A). The IC_{50} values showed that indomethacin-loaded nanocapsules increases 2.5 fold the cytotoxicity for C6 and 2.0 fold for U138-MG, when compared to indomethacin in solution for same cell lines (Table 1). A significant reduction on cell viability was also observed after the treatment with indomethacin ethyl ester-loaded nanocapsules at the concentrations of 25, 50 or 100 μM (Fig. 1B). However, on the contrary to indomethacin, the indomethacin ethyl ester in solution was more efficient than its nanocapsule formulations, in all tested concentrations (Fig. 1B). These effects were similar in human and rat glioma cell lines and the treatment of drug-unloaded nanocapsules did not show significant alterations in cell viability (Fig. 1).

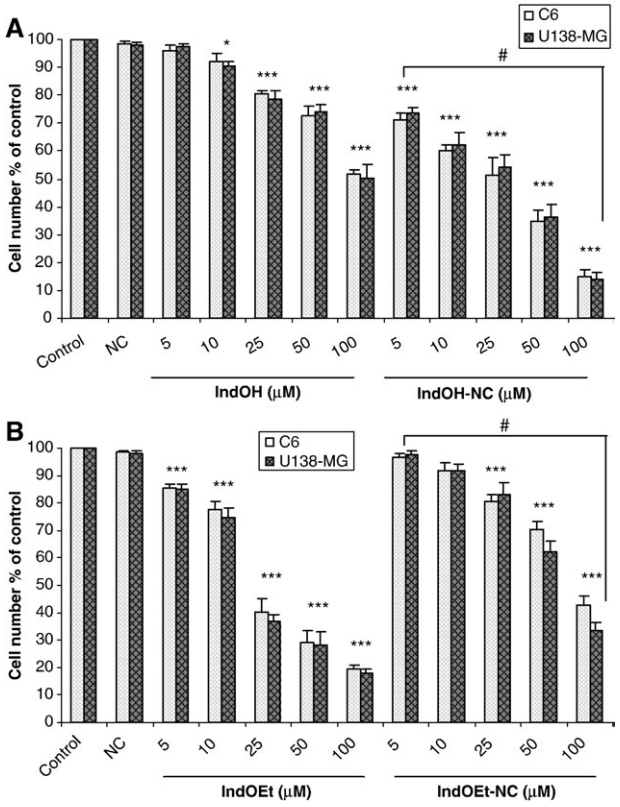

Fig. 2. Effect of indomethacin and indomethacin ethyl ester in drug solution or nanocapsule formulations on glioma cell proliferation. Semi-confluent cultures of glioma cells were treated with 5, 10, 25, 50 or 100 μM of (A) indomethacin (IndOH) or indomethacin-loaded nanocapsules (IndOH-NC) and (B) indomethacin ethyl ester (IndOEt) or indomethacin ethyl ester-loaded nanocapsules (IndOEt-NC) for 48 h. Cultures were also treated with drug-unloaded nanocapsules (NC). After 48 h of treatment, the cells were detached with 0.25% trypsin-EDTA and counted in hemocytometer. The values are represented as means ± S.E.M. of four independent experiments made in triplicate. The average number of cells in untreated controls (100%) was 110,000 ± 5600 for U138-MG and 232,000 ± 9800 for C6 glioma cells. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). *Significantly different from the control group ($P<0.05$); ***Significantly different from the control group ($P<0.001$); #Significantly different from the respective group treated with drug in solution ($P<0.001$).

Table 1
IC_{50} values of indomethacin and indomethacin ethyl ester in solution or in nanocapsules formulations in glioma cell lines

	C6	U138-MG
IndOH	97.08 ± 2.379	92.15 ± 2.269
IndOH-NC	38.44 ± 4.723 [a]	45.9 ± 4.376 [a]
IndOEt	22.60 ± 5.346	20.88 ± 5.642
IndOEt-NC	89.99 ± 2.125 [a]	84.43 ± 1.975 [a]

Mean (S.E.M.) IC_{50} values (μM) for 48 h of treatment with Indomethacin (IndOH), indomethacin-loaded nanocapsules (IndOH-NC), indomethacin ethyl ester (IndOEt) or indomethacin ethyl ester-loaded nanocapsules (IndOEt) by MTT assay, as described in Materials and methods. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test).

[a] Significantly different from the group treated with respective drug in solution for same cell line ($P<0.001$).

3.3. Indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules inhibit growth of the glioma cell lines

In order to investigate the effect of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules

on cell growth, we studied the effect of these formulations in the proliferation of glioma cell lines. Treatment of glioma cells with indomethacin-loaded nanocapsules (5, 10, 25, 50 or 100 μM) for 48 h resulted in significant decrease in cell number when compared to the control cultures (Fig. 2A). Again, indomethacin-loaded nanocapsules were more potent to reduce the cell proliferation in comparison with indomethacin in solution (Fig. 2A). It is important to note that the concentration of 5 μM indomethacin-loaded nanocapsules is already sufficient to cause a significative antiproliferative effect in glioma cells (Fig. 2A). On the order hand, indomethacin ethyl ester in nanocapsule formulations was able to significantly decrease the cell proliferation only in the concentrations of 25, 50 or 100 μM, being more efficient in solution (Fig. 2B). The treatment of drug-unloaded nanocapsules did not show significant alterations in cell proliferation (Fig. 2). These effects were observed in human and rat glioma cell lines and are in agreement with the results observed in MTT assay (Fig. 1).

To address the effect of short exposure to nanocapsule formulations, we treated U138-MG glioma cells with 25 μM of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules for 1 or 3 h. After 1 or 3 h of exposure with these formulations, the medium (DMEM) was changed to DMEM formulations-free. After 48 h in culture, the assay of cell proliferation was performed. Our results showed that the short exposure of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules is enough to cause a significant decrease in cell proliferation of U138-MG glioma cells (Fig. 3). In 3 h and 48 h of exposure, the effects were significantly similar (Fig. 3). Similar effects were observed in C6 glioma cell line (data not shown).

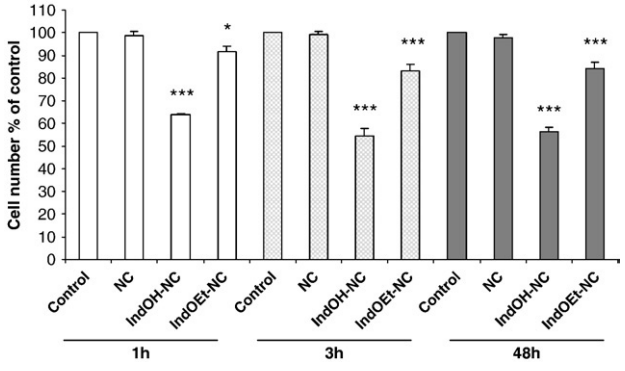

Fig. 3. Effect of short exposure to indomethacin and indomethacin ethyl ester in nanocapsule formulations on glioma cell proliferation. U138-MG glioma cells were exposed to 25 μM of indomethacin-loaded nanocapsules (IndOH-NC) or 25 μM of indomethacin ethyl ester-loaded nanocapsules (IndOEt-NC) for 1, 3 or 48 h. Cultures also were treated with drug-unloaded nanocapsules (NC). After 1 or 3 h of exposure with formulations, the medium (DMEM) was changed to DMEM formulations-free. After 48 h in culture, the cells were detached with 0.25% trypsin-EDTA and counted in hemocytometer. The values are represented as means±S.E.M. of four independent experiments made in triplicate. The average number of cells in untreated controls (100%) was 116,000±4200. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). *Significantly different from the respective control group ($P<0.05$); ***Significantly different from the respective control group ($P<0.001$).

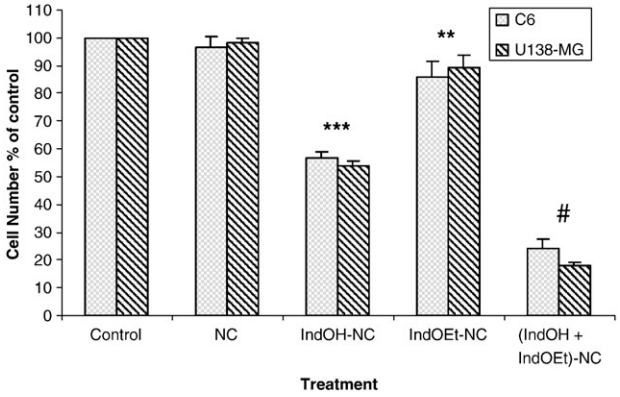

Fig. 4. Synergic effect of indomethacin and indomethacin ethyl ester in nanocapsule formulations on glioma cell proliferation. Semi-confluent cultures of glioma cells were treated for 48 h with 25 μM of nanocapsule formulations containing simultaneously indomethacin and indomethacin ethyl ester [(IndOH+IndOEt) −NC]. Cultures also were treated with drug-unloaded nanocapsules (NC). After 48 h of treatment, the cells were detached with 0.25% trypsin-EDTA and counted in hemocytometer. The values were represented as means±S.E.M. of four independent experiments made in triplicate. The average number of cells in untreated controls (100%) is 108,000±3800 for U138-MG and 225,000±7200 for C6 glioma cells. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). ***Significantly different from the control group ($P<0.001$); **Significantly different from the control group ($P<0.01$); #Significantly different from the control, indomethacin-loaded nanocapsules (IndOH-NC) and indomethacin ethyl ester-loaded nanocapsules (IndOEt-NC) groups ($P<0.001$).

3.4. Indomethacin and indomethacin ethyl ester in the same nanocapsule formulation cause synergic effect in decreasing cell growth of glioma cells

Considering the antiproliferative effect observed when the glioma cell line was exposed to indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules, we studied the effect on cell proliferation after the treatment with nanocapsule formulation containing simultaneously 25 μM of both indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules. Treatment for 48 h of culture cells with indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules caused a significant inhibition on cell proliferation when compared to the control cultures (Fig. 4). Interestingly, the antiproliferative effects of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules treatment (76% for C6 and 82% for U138-MG) were higher than the effects observed for each nanocapsule formulation containing indomethacin (44% for C6 and 47% for U138-MG) or indomethacin ethyl ester (15% for C6 and 17% for U138-MG) (Fig. 4). These results suggest a synergic effect between indomethacin and indomethacin ethyl ester in decreasing cell growth of C6 and U138-MG glioma cell lines.

3.5. Cell death induced by Indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules in glioma cell lines

To verify whether indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules could induce

necrosis cell death, glioma cells were treated with several concentrations (5, 10, 25, 50 or 100 μM) of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules for 24 h and then analyzed for cell death. Fig. 5 shows representative pictures of U138-MG glioma cells treated with 5 or 25 μM of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules. As observed, the glioma cells treated with 5 μM of indomethacin-loaded nanocapsules presented a reduction in the cell number with no significant PI incorporation (Fig. 5D). Similar results were obtained with 10 μM (data not shown). When the cells were treated with 25 μM of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules it was observed a significant PI incorporation, indicating an intense cell necrosis (Fig. 5E and F). Similar results were obtained with 50 and 100 μM (data not shown). The treatment of drug-unloaded nanocapsules did not show significant alterations in PI incorporation (Fig. 5B).

3.6. Indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules did not cause cytotoxic effect on organotypic hippocampal slice cultures

To evaluate the effect of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules on normal neural cells, organotypic hippocampal slice cultures were used as model. These cultures provide an excellent *in vitro* model system to study physiological factors, cellular and molecular mechanisms of neural death, and pharmacological compounds to neural survival (Holopainen, 2005). After 14 days in culture, the organotypic cultures were treated with 25 or 50 μM of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules for 24 h and the cell death was analyzed by PI uptake (Fig. 6). As a positive control of cell damage we used organotypic hippocampal slice cultures exposed to oxygen and glucose deprivation (OGD), which showed significant cell damage (approximately 50%) (Fig. 6). It is important to note that either indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules did not promote organotypic hippocampal culture damage (25 μM: 1.9% for indomethacin ethyl ester-loaded nanocapsules and 3.1% for indomethacin-loaded nanocapsules with 4.8% of PI incorporation in control cultures; 50 μM: 2.7% for indomethacin ethyl ester-loaded nanocapsules and 2.9% for indomethacin-loaded nanocapsules with 3.3% of PI incorporation in control cultures) (Fig. 6). Taken together, these results showed that the cell death induced by 25 μM indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules in glioma

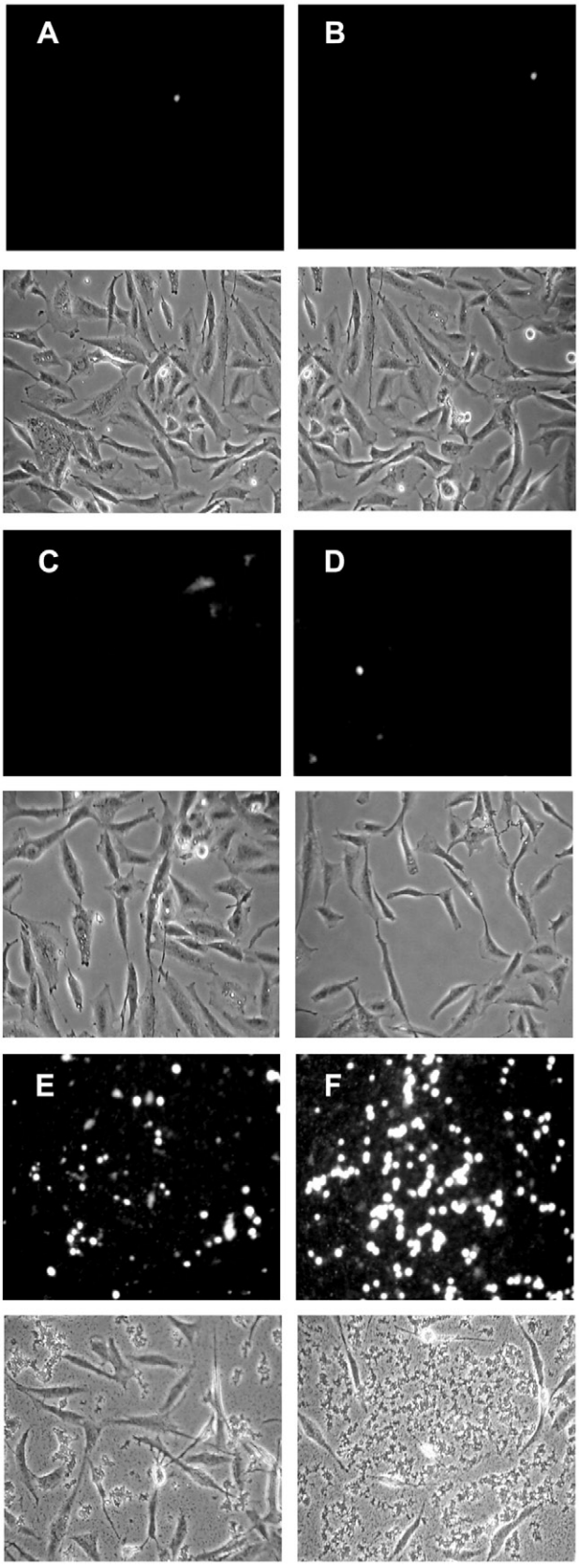

Fig. 5. Effect of indomethacin and indomethacin ethyl ester in nanocapsule formulations on cell death in U138-MG glioma cells by propidium iodide incorporation. Representative pictures of: control cultures (A); cells treated with drug-unloaded nanocapsules (NC) (B); cells treated with 5 μM (C) or 25 μM (E) of indomethacin ethyl ester-loaded nanocapsules (IndOEt-NC), 5 μM (D) or 25 μM (F) of indomethacin-loaded nanocapsules (IndOH-NC). After 24 h of treatment, glioma cells were incubated with 5 μM of propidium iodide (PI). Cellular death was analyzed by PI incorporation that was visualized using a Nikon inverted microscope. Panels below A, B, C and D are correspondent contrast phase photomicrographs. The data are representative of three different experiments.

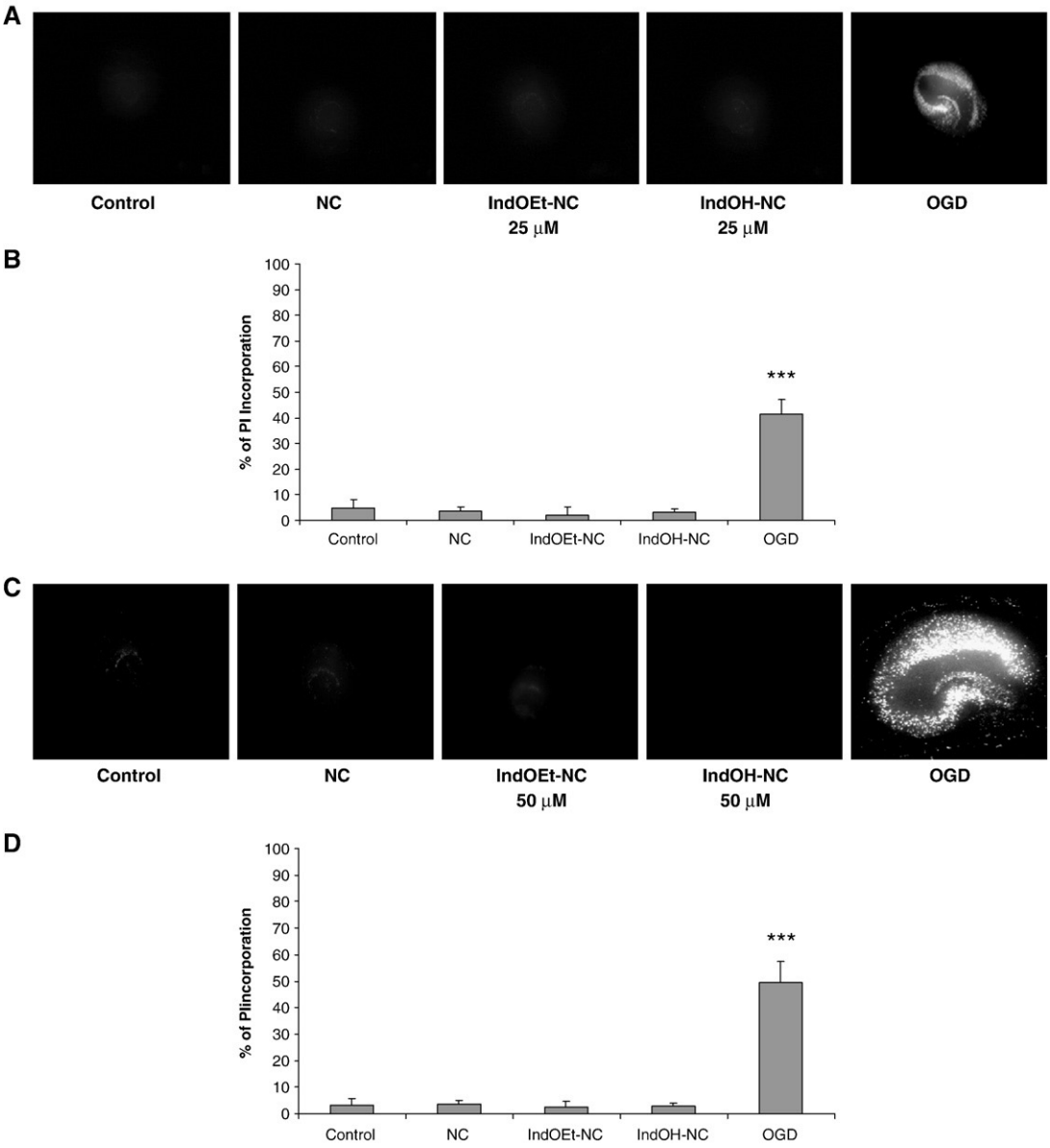

Fig. 6. Effect of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules on organotypic hippocampal slice cultures. (A) and (C) Representative pictures of organotypic hippocampal cultures. (B) and (D) Quantitative analysis of hippocampus damage after treatment of formulations. Organotypic hippocampal slices at 14 days were treated with drug-unloaded nanocapsules (NC), 25 or 50 μM of indomethacin-loaded nanocapsules (IndOH-NC), 25 or 50 μM of indomethacin ethyl ester-loaded nanocapsules (IndOEt-NC) for 24 h. (A) and (B): treatment with 25 μM of formulations, (C) and (D): treatment with 50 μM of formulations; Control: organotypic cultures not exposed to formulations; OGD: neural cultures had been exposed to oxygen glucose deprivation and then used as a positive control of cell damage. Cellular death was analyzed by propidium iodide (PI) incorporation, which was visualized using a Nikon inverted microscope (at 40× magnification). Data represent the means ± S.E.M. of nine independent experiments made in duplicate. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey test). ***Significantly different from the control group ($P<0.001$).

cultures was not observed in the organotypic cultures treated with 25 or even 50 μM.

4. Discussion

Gliomas present a particular therapeutic challenge because of their poor response to chemotherapy. Standard chemotherapy for brain tumors includes highly lipophilic drugs (nitrosurea, temozolomide) able to cross the blood-brain barrier (Brigger et al., 2004). The blood-brain barrier represents an insurmountable obstacle for a large number of drugs, including antibiotics, antineoplasic agents and a variety of central nervous system (CNS)-active drugs (Aktas et al., 2005). One of the possibilities to overcome this barrier is the use of nanoparticles in order to target the drug to the brain (Calvo et al., 2001; Brigger et al., 2002b). Several authors showed very encouraging results obtained at the cellular level with nanoparticles: efficient drug protection (Chavany et al., 1994; Raffin et al., 2006), cell internalization (Couvreur et al., 1977), drug release and drug transport (Beck et al., 2007), controlled release or reversion of the multidrug resistance (MDR) (Bennis et al., 1994; Colin de Verdiere et al., 1997; Koziara et al., 2004).

The mechanisms of encapsulation and release for several drugs in nanoparticulated systems have been extensively studied by our group (Guterres et al., 2000; Schaffazick et al., 2003; Pohlmann et al., 2004; Beck et al., 2007; Schaffazick et al., 2007; Poletto et al., 2007). In the present study, we investigated the cytotoxic effects of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules in U138-MG and C6 glioma cell lines. Previously we have showed that indomethacin caused antiproliferative effects in glioma cell lines due to an inhibition of cell cycle progression and by increasing the catabolism of extracellular purines (Bernardi et al., 2006; Bernardi et al., 2007). Within this context, we hypothesize that NSAIDs in nanocapsule formulations could be more cytotoxic for glioma cells than the respective drugs in solution. Our results show that indomethacin-loaded nanocapsules were at least 2 folds more cytotoxic than indomethacin in solution for both glioma cell lines (Table 1). On the order hand, indomethacin ethyl ester-loaded nanocapsules formulation was less efficient in reducing the cell viability in comparison with indomethacin ethyl ester in solution (Fig. 1, Table 1; Fig. 2). One plausible explanation for these effects is related to the mechanism of encapsulation of each drug in the nanocapsules. Indomethacin is adsorbed at the particle/water interface, while its ethyl ester is predominantly entrapped within the nanocapsules (Pohlmann et al., 2004; Cruz et al., 2006). Thus, different magnitudes of time are required for the total release of indomethacin or its ester from the nanocapsules (Cruz et al., 2006). Furthermore, these differences in the mechanism of drug encapsulation could explain, at least in part, the synergic effect observed in cell proliferation when the glioma cells were treated with nanocapsule formulations containing simultaneously indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules (Fig. 4). Probably the indomethacin causes an initial antiproliferative effect, which is maintained and sustained for indomethacin ethyl ester released from nanocapsules.

Another interesting finding of the present study was that the short exposures (1 and 3 h) of indomethacin-loaded nanocapsules or indomethacin ethyl ester-loaded nanocapsules were enough to cause significant decreases in cell proliferation after 48 h of culture (Fig. 3). We suggest that the antiproliferative effect of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules is not a temporal response. According to literature, the higher cytotoxic effect of nanocapsules in glioma cells may be triggered by a higher potential cell division activity or an increased endocytotic activity resulting in enhanced nanocarrier uptake (Lamprecht and Benoit, 2006). Thus, in our experiment the cell uptake of drug-loaded nanocapsules was probably sufficient to achieve a high intracellular concentration of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules, and the subsequent intracellular release of drug led to the glioma cell death. Further studies, however, are necessary to give support to this hypothesis.

Necrosis is the pathological process, which occurs when cells are exposed to a serious physical or chemical insult. The literature reports that compounds that can induce antiproliferative effects in tumor cells without promoting a necrotic cell death are considered as being good candidate as antitumor drugs. In the present study, we observed that even though 5 μM of indomethacin-loaded nanocapsules is sufficient to cause antiproliferative effects in glioma cell lines, this drug concentration in nanocapsules did not cause necrotic cell death, which was observed with concentrations above 25 μM (Fig. 5). Considering that 400 μM of indomethacin in solution is necessary to cause a necrotic death in glioma cell lines (Bernardi et al., 2006), the results presented here, confirm that nanocapsule formulations are more efficient for glioma cell lines compared to the respective drugs in solution despite a cytotoxic effect was observed.

A major problem of drugs used in cancer chemotherapy is nonspecific toxicity against normal cells and tumoral cells. Such toxic action to normal cells limits the dose of the anticancer drugs to be administered to patients (Kim and Lee, 2001). Thus, to evaluate the selective cytotoxic effect of indomethacin-loaded nanocapsules and indomethacin ethyl ester-loaded nanocapsules on glioma cell cultures, hippocampal organotypic cultures were used as model of normal neural cells. Thus, an important outcome of the present study is that, even though 5 μM of indomethacin-loaded nanocapsules is sufficient to cause cell viability and cell proliferation inhibition (Figs. 1A and 2A) without necrosis in glioma cell lines (Fig. 6), over 10-fold concentration of this formulation (50 μM) did not cause cell death in the organotypic cultures. These results indicate a selective cytotoxicity of these formulations for the tumor cells, which could be explained, at least in part, by the high metabolism of glioma cells that have a potential higher cell division activity and an increased endocytotic activity, enhancing the uptake of nanocapsules.

Although indomethacin is not an agent used in the treatment of brain tumors, our results imply that indomethacin in nanocapsule formulations may be considered a potential candidate for glioma treatment, being more potent than this drug in solution, without the undesirable side effects of conventional chemotherapy. Moreover, it is important to note that *in vitro* studies are limited and that further investigations using *in vivo* glioma model could be helpful to confirm the distinct effects of indomethacin-loaded nanocapsules in normal versus tumoral cells, as well as to determine the appropriate doses for therapy of gliomas.

Acknowledgments

This study was supported by the Brazilian agencies: CNPq, Rede Brasil-França CNPq/MCT, Rede Nanocosméticos/CNPq-MCT and FINEP/MCT. A. Bernardi was a recipient of Brazilian Capes fellowship.

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CAPÍTULO 2

INDOMETHACIN-LOADED NANOCAPSULES TREATMENT REDUCES *IN VIVO* GLIOBLASTOMA GROWTH IN A RAT GLIOMA MODEL

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Periódico: ***Cancer Letters***

Status: **Publicado**

Cancer Letters 281 (2009) 53–63

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Indomethacin-loaded nanocapsules treatment reduces *in vivo* glioblastoma growth in a rat glioma model

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ARTICLE INFO

Article history:
Received 28 October 2008
Received in revised form 5 February 2009
Accepted 9 February 2009

Keywords:
Glioblastoma multiform
Cell proliferation
Indomethacin
Polymeric nanocapsules
Drug delivery

ABSTRACT

Multimodal combinations of target agents with radiation and chemotherapy may enhance cancer treatment efficacy; however, despite these treatments, gliomas recur early due to their highly proliferative, infiltrative and invasive behaviors. Nanoparticles of biodegradable polymers for anticancer drug delivery have attracted intensive interest in recent years since they may provide a sustained, controlled and targeted delivery. In the present study, we investigated the effect of indomethacin-loaded nanocapsules in an experimental glioma model. The rats treated with indomethacin-loaded nanocapsules demonstrated a significant reduction in tumor size and half of these animals presented just cells with characteristics of a residual tumor, as shown by immunostaining for nestin. Pathological analyses showed that the treated gliomas presented a significant reduction in the mitotic index and other histological characteristics that indicate a less invasive/proliferative tumor. An important finding of the present study is that indomethacin carried by polymeric nanocapsules achieved higher intracerebral drug concentrations than those of indomethacin in solution. Furthermore, indomethacin achieved a greater concentration in the hemisphere where the glioma was implanted, compared with the contralateral healthy hemisphere. Indomethacin-loaded nanocapsule treatment did not cause characteristics of toxicity and increased the survival of animals. Thus, our results show that polymeric nanocapsules are able to increase the intratumoral bioavailability of indomethacin and reduce the growth of implanted gliomas. Data suggest that indomethacin-loaded nanocapsules could offer new and potentially highly effective strategies for the treatment of malignant gliomas.

1. Introduction

Glioblastoma multiform (GBM) is the most common malignant tumor of the central nervous system (CNS) in adults. These tumors show a high proliferation rate, variability in tumor histopathology [1] and diffusely infiltrate adjacent brain tissue [2]. Systemically, chemotherapy has provided minimal benefits to GBM patients and its utility has been questioned [3–5]. The amount of drug that can be administered to patients is limited by its high systemic toxicity and numerous side effects [6]. In addition, an endothelial cell monolayer, associated with pericytes and astrocytes, known as the blood–brain barrier, separates the blood from the cerebral parenchyma and limits the penetration of drugs into the CNS [7]. An alternative approach for the treatment of gliomas is the employment of a drug delivery system, such as nanocarriers, which are

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0304-3835/$ - see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2009.02.018

able either to improve or target the delivery of anti-tumoral agents to the brain [8].

Nanoparticle, a generic term that refers to a colloidal structure of less than 1 μm, has received considerable attention as potential drug delivery vehicles [8,9]. Among the different nanocarrier systems, the biodegradable polymeric nanoparticles have been designed to encapsulate lipophilic drugs [10–12]. The mechanisms of encapsulation and release of several drugs from nanoparticuled systems have been extensively studied by our group [13–16]. The most promising application of polymeric nanoparticles is their use as carriers for anticancer drugs [17], particularly in solid tumors [18]. Several authors have shown very encouraging results, obtained at the cellular level, with nanoparticles; these include, efficient drug protection [19], cell internalization [20], drug release and drug transport [15], controlled release or reversion of multidrug resistance (MDR) [21,22]. Moreover, the literature suggests that novel nanoparticles could be used as potential drug carriers across the blood–brain barrier (BBB) [12,23,24], an important requirement for the development of novel drugs against gliomas.

Recent studies have reported that gastric, colon and other carcinogenic processes could be blocked by nonsteroidal anti-inflammatory drugs (NSAIDs) [25,26]. The exact mechanisms by which NSAIDs contribute to anti-tumoral activity remain controversial and are peculiar for each tumor [27]. The data from the literature suggest the involvement of these drugs in the induction of apoptosis, in the control of cell proliferation and invasion and/or in the inhibition of angiogenesis [25]. In a previous study, we reported that indomethacin, a powerful NSAID derived from indolacetic acid, caused antiproliferative effects on glioma cell lines due to an arrest of cell cycle progression [28,29].

Considering the advantages of nanoparticulated systems, we recently investigated the effect of indomethacin-loaded nanocapsules on glioma cell lines (*in vitro* model) [30]. Our results showed that indomethacin-loaded nanocapsules were more potent than a solution of indomethacin in decreasing the cell proliferation of glioma lines. Furthermore, we observed a selective cytotoxicity of this formulation for tumoral cells [30]. Taking the limited success in glioma therapy and these promising data into account, the aim of the present study was to investigate the effect of the indomethacin-loaded nanocapsule treatment on a rat glioma experimental model that has been extensively used to test antitumoral interventions.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), Fungizone®, penicillin/streptomycin, 0.25% trypsin/ EDTA solution were obtained from Gibco (Gibco BRL, Carlsbad, CA, USA). Fetal bovine serum was obtained from Cultilab (Cultilab, Campinas, SP, Brazil). Indomethacin was obtained from Sigma (St. Louis, USA). Temodal® (temozolomide) was obtained from Schering-Plough Corporation (USA). Poly(ε-caprolactone) (PCL) (M_w = 65,000) was supplied by Aldrich (Strasbourg, France). Caprilic/capric triglyceride mixture was acquired from Brasquim (Porto Alegre, Brazil). Span 60® (sorbitan monostearate) and Tween 80® (polysorbate 80) were obtained from Delaware (Porto Alegre, Brazil). The antibodies anti-Ki67, anti-VEGF and Streptavidin–Avidin–Biotin kit were obtained from Dako (USA) and the anti-nestin was obtained from Chemicon International Inc. (USA & Canada). Acetonitrile was of chromatographic grade. All other chemicals and solvents used were of analytical or pharmaceutical grade.

2.2. Preparation of nanocapsules

Nanocapsule suspensions were prepared by interfacial deposition [31]. At 40 °C, indomethacin (0.010 g), poly(ε-caprolactone) (0.100 g), capric/caprylic triglyceride (0.33 ml) and sorbitan monostearate (0.077 g) were dissolved in acetone (27 ml). In a separate flask, polysorbate 80 (0.077 g) was added to 53 ml of water. The organic solution was injected into the aqueous phase under magnetic stirring at room temperature. After 10 min, the acetone was eliminated and the suspensions concentrated under reduced pressure. The final volume was adjusted to 10 ml. Control formulation (drug-unloaded nanocapsules) was prepared omitting the drug (indomethacin).

2.3. Characterization of nanocapsules

After preparation, the pH values of nanocapsule suspensions were determined using a potentiometer (Micronal B-474). The particle size, polydispersity and zeta potential of the suspensions were determined using a Zetasizer®nano-ZS ZEN 3600 model (Malvern, UK). The samples were diluted with water (MilliQ®) (particle size) or in 10 mM NaCl aqueous solution (zeta potential). The measurements were made in triplicate. The total concentrations of indomethacin in the formulations were measured by reverse phase high-performance liquid chromatography (HPLC) (Perkin–Elmer S-200, with injector S-200, detector UV–vis, a guard-column and a Lichrospher 100 RP-18 column of 250 mm, 4 mm and 5 μm; Merck). The mobile phase consisted of acetonitrile/water (70:30, v/v) adjusted to apparent pH 5.0 ± 0.5 with 10% (v/v) acetic acid. Each suspension (100 μl) was treated with acetonitrile (10 ml), the solution was filtered (Millipore 0.45 μm) and injected (20 μl). The HPLC method was previously validated [14]. Linear calibration curves for indomethacin were obtained in the range of 1.00–25.00 μg/ml, presenting correlation coefficients of higher than 0.9992.

2.4. Cell culture

The C6 rat glioma cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA). Cells at 5–15 passages were grown in culture flasks and maintained in Dulbecco's modified Eagle's medium (DMEM) (pH 7.4) containing 1% DMEM (Gibco BRL), 8.4 mM HEPES, 23.8 mM $NaHCO_3$, 0.1% fungizone, penicillin/streptomycin 0.5 U/ml and supplemented with 5% (v/v) fetal bovine serum (FBS). Cells were kept at a tempera-

ture of 37 °C, a minimum relative humidity of 95%, and an atmosphere of 5% CO_2 in air.

2.5. Glioma implantation

Rat C6 glioma cells at around 80% confluence were trypsinized (0.25% trypsin/EDTA solution), washed once in DMEM/5% FBS, spun down and ressuspended in the same medium. A total of one million cells in a volume of 3 μl were injected using a 5 μl Hamilton microsyringe coupled in the infusion pump (1 μl/min) at a depth of 6.0 mm into the right striatum (coordinates with regard to bregma: 0.5 mm posterior and 3.0 mm lateral) of adult Wistar male rats (8 weeks old, 220–260 g) anesthetized by intraperitoneal (i.p.) administration of ketamine/xylazine [32]. The negative control group (Sham) was underwent the same procedure and received an injection of 3 μl of DMEM in the right striatum. All procedures used in the present study followed the "Principles of Laboratory Animal Care" of the National Institutes of Health (NIH) and were approved by the Ethical Committee of the Hospital de Clínicas de Porto Alegre.

2.6. Treatment of animals

Ten days after glioma implantation, the animals were randomly divided into four groups as follows: (1) untreated (control group); (2) treated with drug-unloaded nanocapsules (NC group); (3) treated with 1 mg/Kg/day of indomethacin in solution (solubilized in 10% DMSO and sonicated with ultra-sound for 20 min) (IndOH group) and (4) treated with 1 mg/Kg/day of indomethacin-loaded nanocapsules (IndOH–NC group). A positive control group was also maintained, these animals were treated with 5 mg/Kg/day of Temozolomide (solubilized in 10% DMSO and sonicated with ultra-sound for 20 min). The formulations were administered intraperitonealy (i.p.) to the animals for 10 consecutive days. After 20 days (10 days for glioma implantation + 10 days for treatment), the rats were decapitated and the entire brain was removed, sectioned and fixed with 10% paraformaldehyde. Blood samples were collected from all animals for posterior enzymatic assays (alkaline phosphatase, γ-glutamyl transpherase, alanine aminitranspherase, aspartate aminotranspherase). Other structures (liver, stomach, kidney, heart and lung) were also removed, sectioned and fixed with 10% paraformaldehyde for posterior pathological analysis.

For body weight analysis, the experimental groups were compared to the control group. For survival experiments, the animals were treated as described above and observed for 60 days after glioma implantation. After 60 days, the rats were decapited and the entire brain was removed and processed as described above.

2.7. Pathological analysis and tumor volume quantification

At least five Hematoxylin and Eosin (H&E) sections (2–3 μm thick, paraffin embedded) from each animal were analyzed by a pathologist, blinded for the experimental data. For tumor size quantification, images were captured using a digital camera connected to a microscope (Nikon Eclipse TE300) and the tumor area (mm^2) was determined using Image Tool Software™. The total volume (mm^3) of the tumor was computed by the multiplication of the slice sections and by summing the segmented areas [33].

2.8. Immunohistochemical staining

Paraffin embedded, 5 μm formalin fixed tissue sections were mounted on microscope slides. Tissue sections were then dried overnight at 60 °C, dived in xylene and rehydrated with distilled water. Endogenous peroxidase was inhibited by 5% H_2O_2 in methanol for 15 min. Incubation with the following antibodies was performed overnight at 4 °C temperature: anti-nestin (1:200), anti-Ki67 (1:200) and anti-VEGF (1:30), followed by incubation with secondary antibody and Streptavidin–Avidin–Biotin. The peroxidase reaction was performed using 3,3′-diaminobenzidina tetrahydrochloride (DAB), according to the manufacturer's specifications. Finally, sections were counterstained with Harris hematoxylin. For each animal, glioma cell proliferation was assessed by counting the percentage of Ki67 positive glioma cell nuclei in 10 independent high-magnification (×200) fields per microscope slide. Sections of rat spleen were used as positive controls.

2.9. Quantification of indomethacin in cerebral tissues

A reverse phase high-performance liquid chromatography (HPLC) protocol was adapted and validated to quantify indomethacin in cerebral tissue of animals. After 20 days (10 days for glioma implantation + 10 days for treatment), after 1 h of the last treatment, the rats were decapited and the entire brain was removed, the hemispheres were separated and homogenized with 5 ml of acetonitrile in order to dissolve all components. The suspensions were centrifuged at 3000 rpm for 10 min and the supernatant was filtered (0.45 μM, Millipore) and injected (20 μl) in HPLC. The total concentrations of indomethacin in cerebral tissue (contralateral and ipsilateral to the tumor) were measured by HPLC (Perkin–Elmer S-200, with injector S-200, detector UV–vis, a guard-column and a Lichrospher 100 RP-18 column of 250 mm, 4 mm and 5 μm; Merck). The mobile phase consisted of acetonitrile/water (70:30, v/v) adjusted to apparent pH 5.0 ± 0.5 with 10% (v/v) acetic acid. Indomethacin was detected at 267 nm with a retention time of 3.45 min. The HPLC method was validated considering the linearity, inter- and intra-day variability, selectivity, accuracy, limit of quantification and recovery [14]. Linear calibration curves for the indomethacin dissolved in acetonitrile were obtained in the range of 1.00–25.00 μg/ml, presenting correlation coefficients of higher than 0.9992. The limit of quantification was 1.00 μg/ml. The area under the peak was calculated using numerical integration.

2.10. Statistical analysis

Data are expressed as mean ± S.D. and analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by post-hoc for multiple comparisons (Tukey's test) using an Instat software package (GraphPad Software, San Diego, CA, USA). Differences between mean

Table 1
Proportion of intracerebral C6 glioma development.

Animal groups	Animals bearing tumor (%)
Control	(10/11) 90
Drug-unloaded nanocapsules (NC)	(10/10) 100
Indomethacin in solution (IndOH)	(10/10) 100
Indomethacin-loaded nanocapsules (IndOH–NC)	(5/10) 50

The proportion of animals that developed a defined tumor mass detectable by the hematoxylin and eosin (H&E) analysis after 20 days of intracerebral C6 implantation. Intracerebral C6 implantation and animal treatments were performed as described in Section 2.

values were considered significant when $p < 0.05$. For the survival experiment, the Chi-Square test was used.

3. Results

3.1. Physico–chemical characterization of nanocapsule formulations

All nanocapsule formulations were prepared by interfacial deposition of polymer without the need of any subsequent step of purification. Indomethacin-loaded nanocapsules and drug-unloaded nanocapsules presented macroscopic homogeneous aspect like milky white bluish opalescent liquids. After preparation, the particle sizes were 240 nm (Indomethacin-loaded nanocapsules) and 226 nm (drug-unloaded nanocapsules). The suspensions showed monomodal size distributions and polydispersity indexes lower than 0.19, indicating narrow distributions. The pH values were 5.95 (Indomethacin-loaded nanocapsules) and 6.05 (drug-unloaded nanocapsules). The zeta potential values were −6.9 and −7.3 mV, respectively. The indomethacin contents were 0.991 ± 0.012 mg/ml and the encapsulation efficiencies were close to 100% for the formulation.

3.2. Indomethacin-loaded nanocapsules treatment reduced tumor size in the rat glioma model

Implanted gliomas were obtained by the injection of C6 glioma cells in the striatum of adult Wistar rats, as described in Section 2. The rat glioma experimental model has been extensively used for a variety of studies, especially for investigations of glial tumor biology, as well as for experimental chemotherapy of brain malignancies [34]. After 10 days of glioma implantation, the animals were treated with indomethacin (IndOH group), indomethacin-loaded nanocapsules (IndOH–NC group) or drug-unloaded nanocapsules (NC group) for 10 days, as described in Section 2. After the different treatments (10 days for glioma implantation + 10 days for treatment), the hematoxylin and eosin (H&E) analysis showed that only 50% of animals treated with indomethacin-loaded nanocapsules presented a defined tumor mass (Table 1). The other 50% of the animals treated with indomethacin-loaded nanocapsules showed

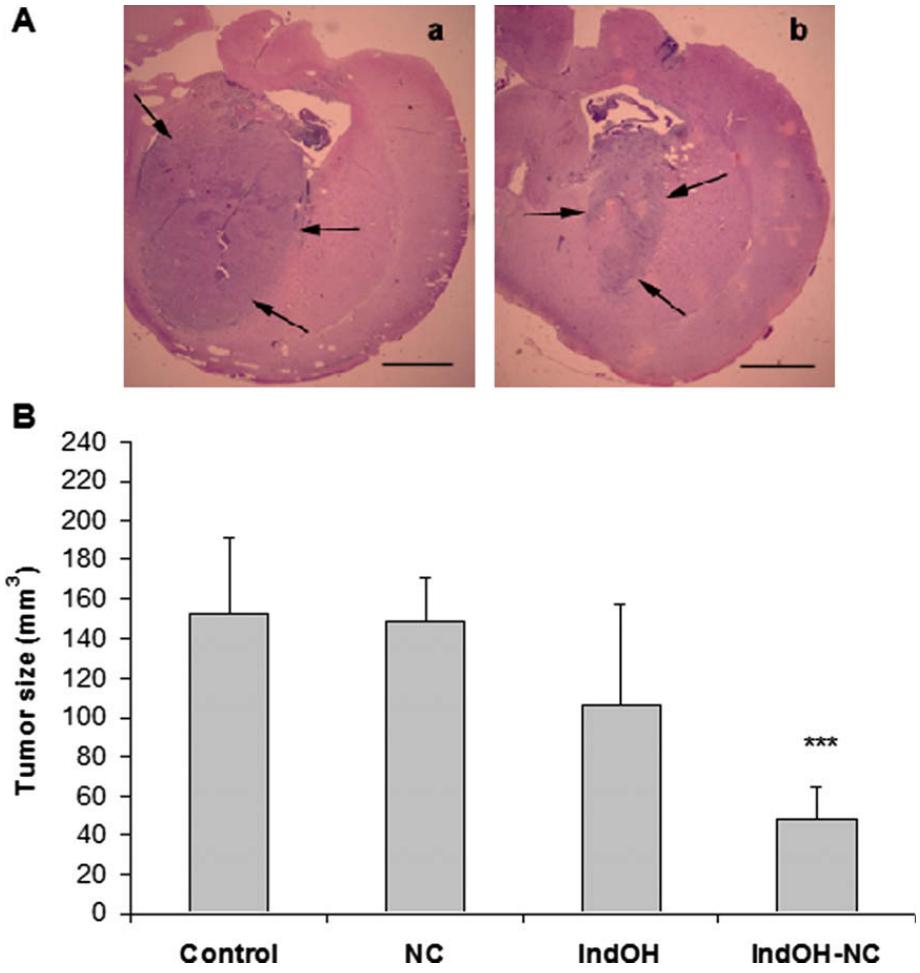

Fig. 1. *Tumor size of implanted gliomas*. Animals were treated, as described in Section 2. Tumor size was measured 20 days after implantation of C6 cells by three hematoxylin and eosin (H&E) sections of each tumor. For tumor size quantification, images were captured using a digital camera connected to a microscope and total volume (mm^3) was determined using Image Tool Software™. (A) Representative tumor images of the control group (a) and the indomethacin-loaded nanocapsules group (b) – Bar: 1 mm; (B) Tumor size quantification of implanted gliomas. The values were represented as means ± S.D. of ten animals per group. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey's test). ***Significantly different from the control, drug-unloaded nanocapsules (NC) and indomethacin (IndOH) groups ($p < 0.001$).

only cells with characteristics of a residual tumor in the site of glioma implantation. Therefore, the analysis of the tumor volume was carried out exclusively in animals presenting a tumoral mass that could be quantified. The results showed that the animals treated with indomethacin-loaded nanocapsules displayed a significant reduction in tumor size (48.32 ± 16.23 mm^3) compared to untreated (152.72 ± 39.26 mm^3), drug-unloaded nanocapsules (148.73 ± 22.38 mm^3) and indomethacin (105.99 ± 51.90 mm^3) groups (Fig. 1). As a positive control group, rats with implanted gliomas were treated with Temozolomide (5 mg/Kg/day) to analyze whether the tumor implanted into host brain was responsive to drugs that are clinically used to treat gliomas. According to previous results published by our group [33], the tumor size of rats treated with Temozolomide was significantly reduced to 52.21 ± 19.18 mm^3.

The indomethacin-loaded nanocapsules treatment either reduced the size of the implanted glioma or hindered tumor implantation. As such, we investigated the presence of gliomas at 10 days after implantation of C6 cells (when the treatment of the animals begun). For this purpose, a group of animals was implanted with C6 glioma cells and after 10 days they were killed and the presence of tumor was evaluated. Hematoxylin and eosin examination showed the presence of implanted gliomas in this group of rats with a tumor size of 84.31 mm^3 ± 10.27. These data indicate that the effect shown in the Fig. 1 is due to the indomethacin-loaded nanocapsules treatment, since gliomas were already implanted on the 10th day, and that indomethacin-loaded nanocapsules treatment is probably responsible for an *in vivo* reduction in glioma growth.

3.3. Histopathological analysis

Implanted tumors have characteristics that are closer to those of human glioblastomas, with C6 cells demonstrated to grow in the intracerebral, intraventricular and intraparenchymal spaces (data not shown). A representative figure of H&E analysis showed that the control group presented a high mitotic index, nuclear pleomorphism, foci of tumor necrosis and vascular proliferation (Fig. 2). Moreover, pathological analysis identified pallisading cells delineating the foci of necrosis and lymphocytic infiltration, with formation of edema fluid and neovascularization, which are characteristics of glioblastoma multiforme in humans (Fig. 2, Table 2). It is important to note that, in addition to the observed reduction in the

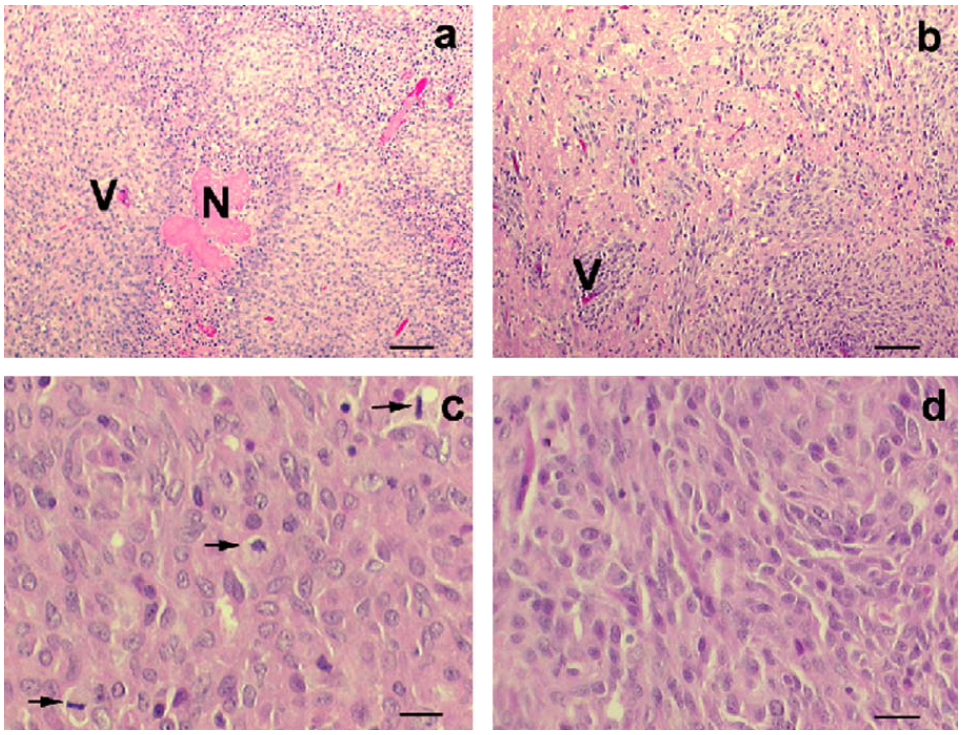

Fig. 2. *Histological analysis of implanted gliomas.* The sections of implanted rat glioma were stained with hematoxylin and eosin (H&E), as described in Section 2. Representative pictures of histological characteristics that define glioblastoma multiform, as seen in rats implanted with gliomas (control group) (a, c) and in rats implanted with gliomas and treated with indomethacin-loaded nanocapsules (IndOH–NC group) (b, d). Necrosis (N), microvascular proliferation (V) and mitosis (arrows) were observed. Scale bars = 100 μM (a, b); 10 μM (c, d).

Table 2
Histological characteristics of implanted gliomas.

	Control (n = 10)	NC (n = 10)	IndOH (n = 10)	IndOH–NC (n = 5)
Coagulative necrosis	7/10 (70%)	8/10 (80%)	6/10 (60%)	0/5 (0%)
Intratumoral hemorrhage	5/10 (50%)	5/10 (50%)	7/10 (70%)	1/5 (20%)
Lymphocytic infiltration	10/10 (100%)	8/10 (80%)	9/10 (90%)	5/5 (100%)
Peritumoral edema	6/10 (60%)	6/10 (60%)	3/10 (30%)	1/5 (20%)
Peripheric pseudopalisading	9/10 (90%)	9/10 (90%)	5/10 (50%)	0/5 (0%)
Vascular proliferation	10/10 (100%)	10/10 (100%)	9/10 (90%)	3/5 (60%)
Mitotic index: mitosis/HPF	15.5 ± 2.03	15.01 ± 1.81	12.14 ± 3.64	5.6 ± 1.72[a]

The histological variables (coagulative necrosis, intratumoral hemorrhage, lymphocytic infiltration, peritumoral edema, peripheric pseudopalisading and vascular proliferation) were regarded as present or absent. Mitosis was counted in ten high power fields (HPF) of the periphery of the tumor, and the average of this counting was used as mitotic index (means ± S.D).

[a] Significantly different from the control, NC and IndOH groups ($p < 0.001$).

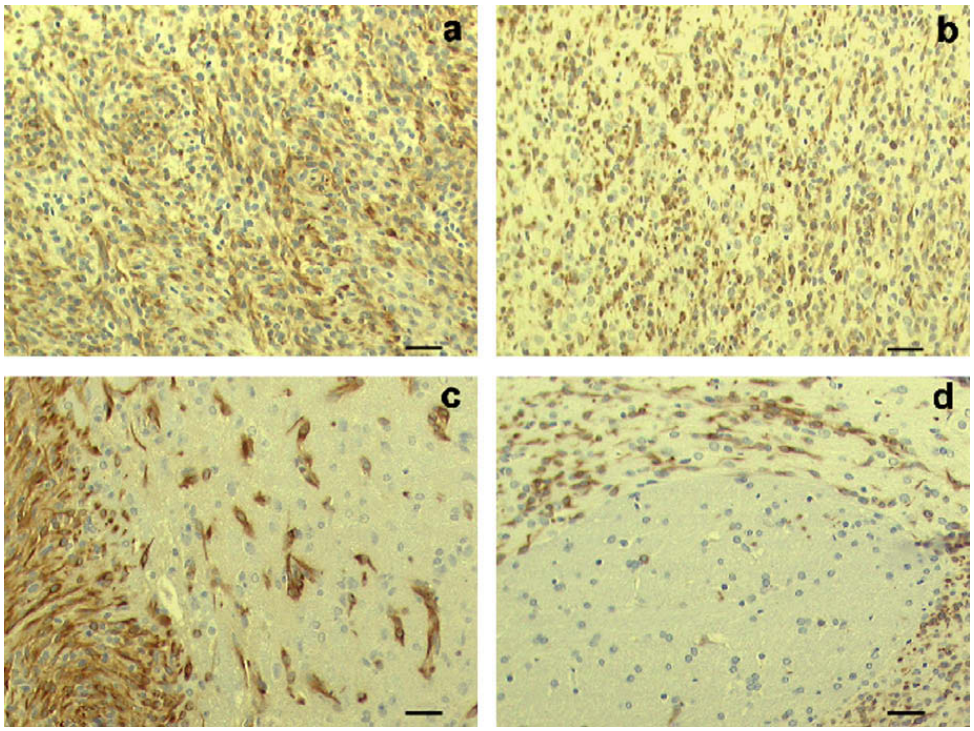

Fig. 3. *Immunohistochemical staining of nestin in implanted gliomas.* The sections of implanted rat glioma were stained with nestin, as described in Section 2. Intense staining of nestin is seen in the cytoplasm of tumor cells. Representative pictures of immunohistochemical analysis in rats implanted with gliomas (control group) (a, c) and in rats implanted with gliomas and treated with indomethacin-loaded nanocapsules (IndOH–NC group) (b, d). Scale bars = 100 μM (a, b); 20 μM (c, d).

tumor volume, the indomethacin-loaded nanocapsules treatment produced gliomas with a significant reduction in mitotic index, as well as less tumor coagulative necrosis, intratumoral hemorrhage, peritumoral edema, peripheric pseudopallisading and vascular proliferation, indicating a less invasive/proliferative tumor (Fig. 2, Table 2). In addition, we observed a significant reduction in the mitotic index in the indomethacin-loaded nanocapsules treated group (Table 2), indicating a reduction in the proliferation of tumoral cells.

3.4. Immunohistochemical analysis

The H&E examination showed that 50% of animals treated with indomethacin-loaded nanocapsules presented cells with characteristics of a residual tumor, without presenting a considerable tumoral mass. To investigate the presence of tumoral cells in the brain area of implanted glioma in these animals, we performed an immunohistochemical analysis for nestin, an intermediate filament protein involved in the organization of the cytoskeleton and expressed in almost all glioblastoma multiforme [35,36]. The implanted tumors of untreated rats (control group) presented an intense staining with nestin antibody (Fig. 3A and C). The cytoplasm of the residual cells was also stained with nestin antibody in the indomethacin-loaded nanocapsules treated group, although this staining was less intense (Fig. 3B and D), indicating the presence of a residual tumor in these animals. The reduction in the mitotic index observed by H&E examination was confirmed by counting the percentage of Ki67 positive glioma cell nuclei, indicating that tumors of rats treated with indomethacin-loaded nanocapsules are less proliferative and, consequently, less invasive (Table 3, Fig. 4A and B).

Since tumor growth depends on the ability to induce angiogenesis, we also performed immunohistochemical experiments to determine vascular endothelial growth factor (VEGF) expression. In addition to the other alterations in histopathological characteristics, our results showed that the tumors of animals treated with indomethacin-loaded nanocapsules presented less VEGF staining, in comparison to the control group (Table 3, Fig. 4C and D).

Table 3
Immunohistochemical analysis of Ki67 and VEGF in rats implanted gliomas.

	Control	NC	IndOH	IndOH–NC
Ki 67 ($n = 10$)	59.5 ± 7.7	58.5 ± 5.8	53.0 ± 7.0	16.5 ± 3.8[a]
VEGF ($n = 10$)	33.0 ± 4.6	31.5 ± 4.1	30.55 ± 5.18	19.5 ± 4.7[b]

For Ki67 the results are expressing by percentage of positive cells.
For VEGF the results are expressing by the number of vessels.
Data are the means ± S.D. ANOVA followed by *post-hoc* comparisons (Tukey's test).
[a] Significantly different from the control, NC and IndOH groups ($p < 0.001$).
[b] Significantly different from the control, NC and IndOH groups ($p < 0.01$).

3.5. Quantification of indomethacin in cerebral tissue

Quantitative analyses were performed to determine the intracerebral bioavailability of indomethacin carried by polymeric nanocapsules, compared to indomethacin in solution, after treatment using an equivalent dosage (1 mg/kg/day). Results showed that, in rats with implanted gliomas, the indomethacin carried by polymeric nanocapsules achieved increased concentrations, compared to indomethacin in solution, which was not found at detectable levels by HPLC analysis (Fig. 5). Additionally, the amount of indomethacin in the brain of rats treated with IndOH–NC was also higher in comparison with healthy or sham-operated rats (Fig. 5). Furthermore, the concentration of indomethacin was significantly higher in the cerebral hemisphere where the tumor was implanted (right hemisphere), compared to the contralateral healthy hemisphere (Fig. 5).

3.6. Investigation of toxicity of indomethacin-loaded nanocapsules treatment in rats with implanted gliomas

The treatment with indomethacin-loaded nanocapsules at a dose of 1 mg/Kg/day for 10 days did not cause mortality and improved the body weight in comparison with the control group, within a 20-day observa-

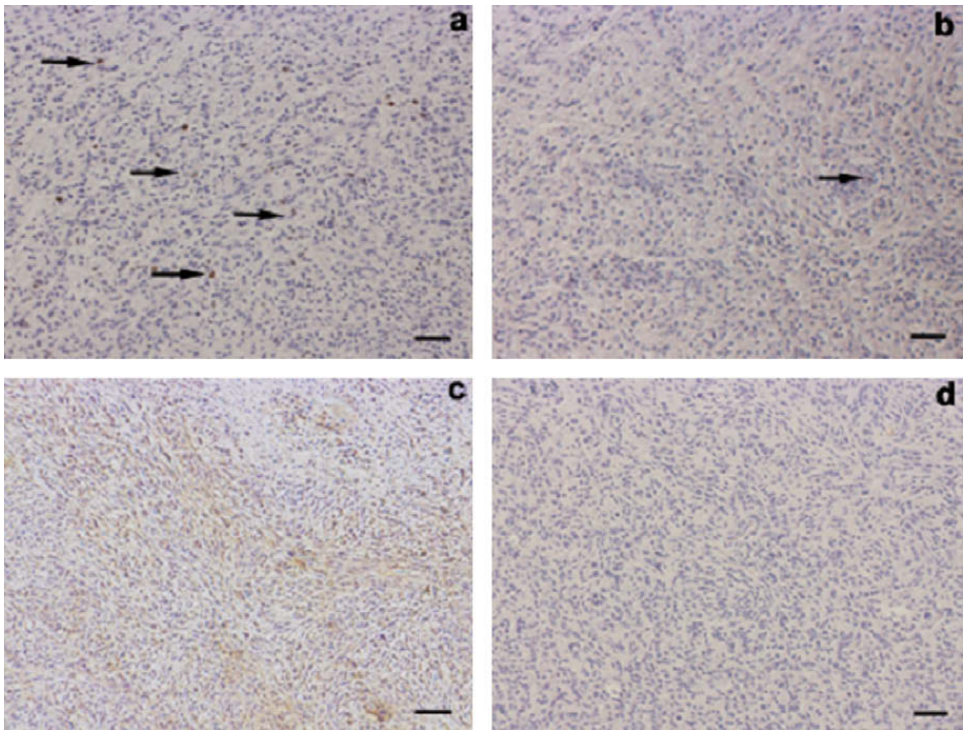

Fig. 4. *Immunohistochemical staining of Ki67 and VEGF in implanted gliomas.* The sections of implanted rat glioma were stained with ki67 or VEGF, as described in Section 2. Glioma cell proliferation was assessed by immunostaining for Ki67 positive glioma cell nuclei (arrows) (a, b) and angiogenesis was assessed by VEGF staining (c, d). Representative pictures of immunohistochemical analysis in rats implanted with gliomas (control group) (a, c) and in rats implanted with gliomas and treated with indomethacin-loaded nanocapsules (IndOH–NC group) (b, d). Scale bars = 20 μM.

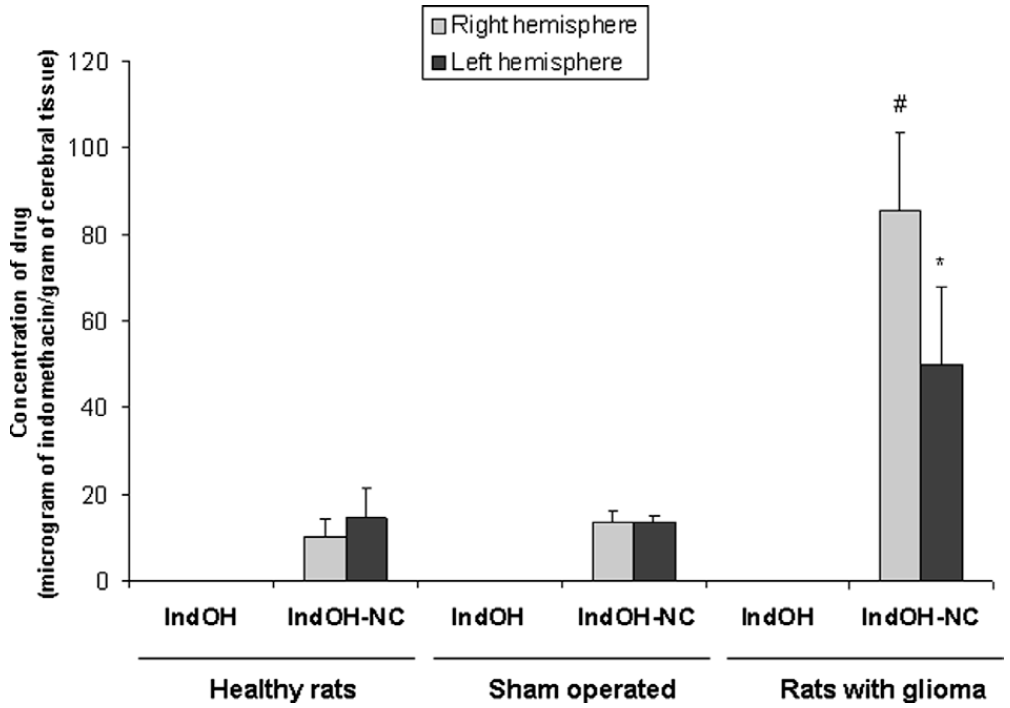

Fig. 5. *Quantification of indomethacin in cerebral tissue.* After 20 days (10 days for glioma implantation + 10 days for treatment), the rats were decapitated and the entire brain was removed, the hemispheres were separated and homogenized with 5 ml of acetonitrile and the total concentrations of indomethacin were measured by HPLC, as described in Section 2. As an esteriotaxic surgical control, a control group underwent the same procedure, although 3 μl of DMEM was injected in the right striatum (Sham operated group). The values are depicted as means ± S.D. of five animals per group. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey's test). $^{\#}$Significantly different from all groups ($p < 0.01$), *Significantly different from all groups ($p < 0.05$).

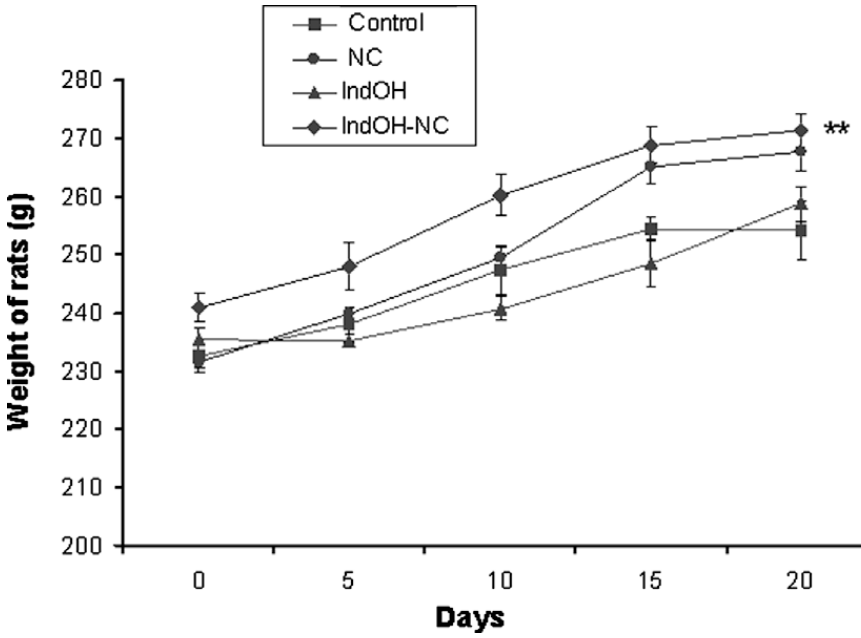

Fig. 6. *Body weight of animals with implanted gliomas.* Animals were treated, as described in Section 2. Weights were observed daily. The values are represented as means ± S.D. of all animals used in this study ($n = 25$ animals per group). Data were analyzed by Repeated Measures Analysis of Variance. **Significantly different from the control group ($p < 0.01$).

tion period (Fig. 6). Necropsy of the animals at the end of the treatments did not show any macroscopic changes in the observed organs (liver, stomach, kidney, heart and lung) and the weights of these organs did not differ from those of the control group (data not shown). Microscopic investigation of these organs by H&E analysis demonstrated absence of toxicity and possible metastasis (data not shown). Moreover, to evaluate the possible liver damage in the rats treated with IndOH–NC, we measured the levels of hepatic enzymes, γ-glutamyl transpherase, alanine aminotransaminase, aspartate aminotransaminase and alkaline phosphatase, in the rat blood serum. None of the treated animals presented significant alterations in the investigated enzymes, discarding hepatic alterations in the animals (data not shown).

3.7. Indomethacin-loaded nanocapsules treatment improved the survival rate of animals with implanted gliomas

To verify whether indomethacin-loaded nanocapsules treatment was able to increase the survival rate of animals with implanted gliomas, the animals were observed for 60 days after glioma implantation and subsequent treatments. Thus, our results showed a consistent improvement in the survival of the animals treated with indomethacin carried by polymeric nanocapsules (Fig. 7).

4. Discussion

The treatment of brain cancer is one of the most difficult challenges in oncology [7]. Drug-loaded nanocarriers capable of crossing brain capillary endothelial cells and recognize cerebral tumoral cells have shown promising potential in oncology [7,37].

Here, we show that the treatment with sub-therapeutic concentrations of indomethacin carried by polymeric nanocapsules reduced the growth of implanted gliomas (Fig. 1). Moreover, pathological analysis demonstrated the lack of some important malignant characteristics typical of glioblastomas in the tumors of rats treated with this formulation (Fig. 2, Table 2). Among these, of note is the reduction in the mitotic index, confirming the potential role of indomethacin as an antiproliferative drug (Table 2, Fig. 4A and B). This result is in accordance with a previous *in vitro* study, where we showed that indomethacin caused antiproliferative effects on glioma cell lines by the inhibition of cell cycle progression [28].

An important finding of the present study was that half of the animals treated with indomethacin-loaded nanocapsules presented cells with characteristics of a residual tumor in the site of glioma implantation, which was confirmed by immunostaining for nestin (Fig. 3, Table 1). Nestin is one marker that has been used to identify neural stem cells, being expressed in almost all glioblastoma multiformes [34,38,39]. This protein is down-regulated in mature cells, being expressed in astrocytes of the adult CNS in response to cellular stress, such as neoplasic transformation [40]. Thus, whilst less intense, staining for nestin in cerebral tissue of treated rats indicates the presence of glioma cells, probably as a result of a tumor involution (Fig. 3). Recently, Stronjinik and collaborators (2007) [41] reported that high levels of nestin expression indicates significantly shorter survival of glioma patients. Moreover, nestin may therefore correlate with invasiveness, being expressed mainly in the tumor periphery in the human brain [41]. It is important to note that we also observed that nestin-positive cells are more abundant at the transition zone of the tumor control (data not shown).

The vascular endothelial growth factor (VEGF) signaling pathway is a critical regulatory mechanism of tumor angiogenesis [42,43]. The majority of glioblastomas exhibit aberrant endothelial growth factor receptor (EGFR) activity. Expression of EGFR increases tumor cell malignancy and decreases patient survival [42,43]. Therefore, antiangiogenic therapies are considered promising. Our results demonstrated a greater VEGF staining in the tumors of the control group in relation to animals that were treated with indomethacin-loaded nanocapsules (Table 3, Fig. 4C

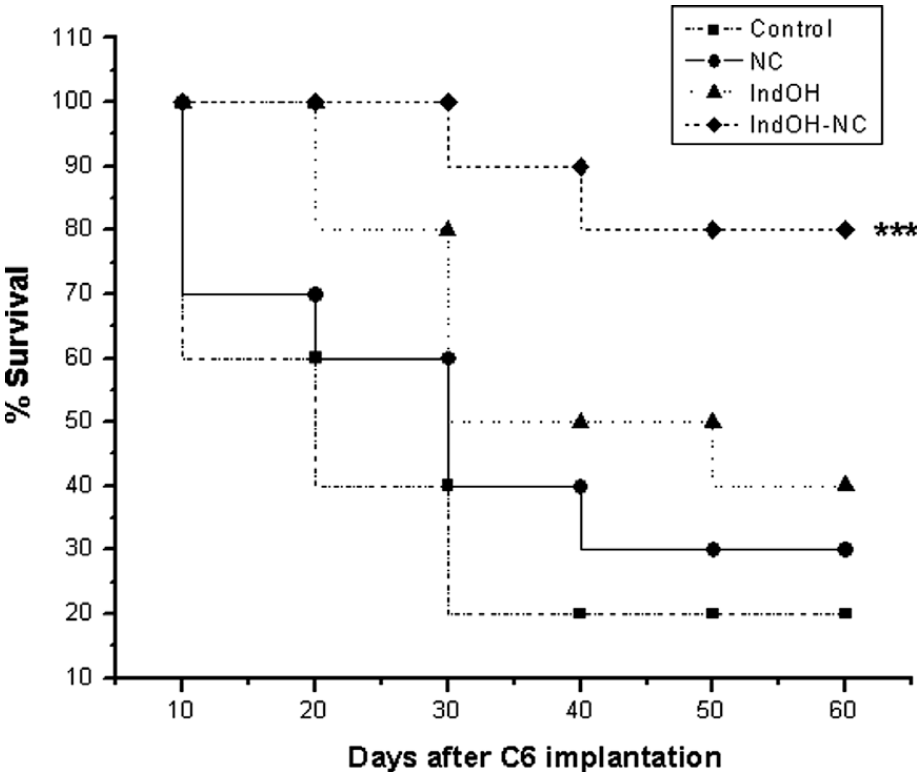

Fig. 7. *Survival rate of animals with implanted gliomas*. For survival experiments, the animals were treated as described in Section 2. The animals were observed for 60 days after glioma implantation ($n = 10$ animals per group). Histopathological examination of tumors was performed in all animals. Data were analyzed by Chi-square test. ***Significantly different from the control, drug-unloaded nanocapsules (NC) and indomethacin (IndOH) groups ($p < 0.001$).

and D). This result together with the reduction in the mitotic index indicates that microvascular proliferation is decreased in the implanted gliomas of rats treated with indomethacin-loaded nanocapsules, with a consequent decrease in the blood flow in tumor areas with high cell proliferation.

The pharmacological response to a drug is directly related to its concentration at the required site of action. However, the distribution of a substance in the body is essentially determined by its physicochemical properties. An unspecific distribution causes great drug concentration in healthy organs, tissues and cells, leading to drug toxicity [9,11]. Considering the important effects observed in present study, where indomethacin-loaded nanocapsules reduced *in vivo* glioblastoma growth, we performed experiments to evaluate the intracerebral bioavailability of indomethacin carried by the polymeric nanocapsules. Our results showed that indomethacin carried by polymeric nanocapsules achieved higher intracerebral concentrations compared to indomethacin in solution, which did not reach detected levels by HPLC (Fig. 5). Furthermore, in rats with implanted glioma, indomethacin achieved a minor concentration in the contralateral healthy hemisphere compared to the hemisphere where the glioma was implanted (Fig. 5). One plausible explanation for these effects is that the proliferation and invasion of glioma cells generally causes a local disruption of the BBB [44]. Cancer cells produce various mediators and proangiogenic factors, including VEGF, that increase the permeability of the capillary endothelium [45]. These capillaries, characterized by frequent fenestrations, also improve the permeability of the blood–tumor interface and, consequently, the penetration of molecules. The mechanisms by which polymeric nanoparticles pass through the BBB are not completely understood. Recent studies attribute a central role to endothelial cells in the process of nanoparticle adhesion (e.g. by recognition of a specific blood protein adsorbed on the particle surface) and subsequent endocytosis, transcytosis, tight junction modulation and P-glycoprotein inhibition [46–48]. It has also been reported that the size of the carriers, polymer type, as well as their surface characteristics could induce steric stabilization of nanoparticles, thus increasing blood circulation time and accumulation in the solid tumor [6,36]. Moreover, nanoparticles of biodegradable polymers coated with polysorbate 80 (Tween 80®) were reported in the literature for cerebral delivery of several substances [49,50]. All these factors probably contribute to the drug delivery observed here.

In chemotherapy, pharmacologically-active concentrations of an anticancer drug in the tumor tissue are often reached at the expense of massive toxicity to the rest of the body [51]. This poor specificity creates a toxicological problem that represents a serious obstacle to effective antitumoral therapy. Thus, an important outcome of the present study is that the treatment with indomethacin-loaded nanocapsules caused neither mortality nor produced characteristics of toxicity, beyond improving the body weight of treated animals, compared to the control group (Fig. 6). It is important to notice that the concentration used in the present study is sub-therapeutic (1 mg/kg/

day) and further studies are necessary to establish a therapeutic dose range, as well as toxicity for this formulation in rats with implanted C6 glioma. Another important finding of the present study was that the rats treated with indomethacin-loaded nanocapsules survive significantly longer than their respective controls (Fig. 7), demonstrating a relative benefit of treatment in increasing the survival of animals with implanted glioma.

In summary, the data reported here clearly demonstrated that polymeric nanocapsules are able to successfully carry indomethacin into the brain tumor. Local delivery of indomethacin reduced *in vivo* glioblastoma growth and improved the survival of animals in a rat glioma model. Although indomethacin is not an agent used in the treatment of brain tumors, our results imply that this drug, in nanocapsule formulation, may be considered a potential candidate for glioma treatment, without the undesirable side effects of conventional chemotherapy.

5. Conflict of interest

We declare that we have no conflict of interest.

Acknowledgments

This study was supported by the Brazilian agencies: Rede Brasil-França CNPq/MCT, FINEP, Rede Nanocosméticos/CNPq-MCT and Fundo de Incentivo à Pesquisa e Eventos (HCPA). A. Bernardi and E. Jäger were recipients of Brazilian Capes fellowships. E. Braganhol was recipient of a Brazilian CNPq fellowship. The authors thank to Neiva Copetti and Jorge A. Lopes for their excellent technical assistance.

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CAPÍTULO 3

PROTECTIVE EFFECTS OF INDOMETHACIN-LOADED NANOCAPSULES AGAINST OXYGEN-GLUCOSE DEPRIVATION IN ORGANOTYPIC HIPPOCAMPAL SLICE CULTURES

Andressa Bernardi, Rudimar L. Frozza, Ana Paula Horn, Maria Martha Campos, João B. Calixto, Christianne Salbego, Adriana R. Pohlmann, Sílvia S. Guterres and Ana Maria O. Battastini

Periódico: ***Neurobiology of Disease***

Status: **Submetido**

Neurobiology of Disease: Submission Confirmation

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Protective effects of indomethacin-loaded nanocapsules against oxygen-glucose deprivation in organotypic hippocampal slice cultures

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Abstract

Here, we investigated the neuroprotective effects of indomethacin-loaded nanocapsules (IndOH-NC) using organotypic hippocampal cultures exposed to oxygen-glucose deprivation (OGD). When the cultures were exposed to 60 min of OGD, about 50% of the hippocampal slices was labeled with propidium iodide. On the other hand, when the cultures were treated with 50 or 100 μM of IndOH-NC the cell death was significantly reduced to 31% and 15%, respectively. Immunoblotting revealed that treatment with 100 μM of IndOH-NC was able to significantly reduce the levels of ERK1/2 and JNK phosphorylation, as well as iNOS activation. Additionally, IndOH-NC prevented glial activation induced by OGD. The treatment with IndOH-NC markedly inhibited the levels of the pro-inflammatory cytokines secreted, while the levels of the anti-inflammatory cytokine IL-10 were significantly increased. Our results clearly demonstrate that IndOH-NC might represent a promising pharmaceutical neuroprotective formulation for cerebral ischemia, most probably by inhibiting the inflammatory cascades.

Keywords: neuroprotection; indomethacin-loaded nanocapsules; organotypic hippocampal culture; oxygen-glucose deprivation; neuroinflammation.

Abbreviations: ERK1/2 - Extracellular signal-regulated kinase 1/2; GFAP - Glial Fibrillary Acid Protein; HBSS - Hank's Balanced Salt Solution; IB_4 - Isolectin B_4; IL-6 – Interleukin-6 ; IL-10 - Interleukin-10; IL-1β - Interleukin-1β; iNOS - Inducible nitric oxide synthase; JNK - *c-Jun* N-terminal kinase; MAPK - Mitogen-activated protein kinase; MEM - Minimal Essential Medium; NO – nitric oxide; OGD - Oxygen-glucose; deprivation; PI - Propidium iodide; TNF-α - Tumor Necrosis Factor α.

Introduction

Cerebral ischemia (stroke) is one of the most frequent causes of death and disability worldwide, and has significant clinical and socio-economic impact (Wang et al., 2007). Although different mechanisms are involved in the pathogenesis of stroke, there is increasing evidence showing that inflammation accounts for its progression, at least acutely (Samson et al., 2005). Stroke-induced activation of inflammatory cascades leads to further cerebral damage. Therefore, injury to any part of the neurovascular unit could permit the extravasation of vascular inflammatory cells and proteins that are injurious to neurons (Huang et al., 2006). The major players in the inflammatory injury are cytokines, adhesion molecules, eicosanoids and nitric oxide, which are produced immediately after the onset of ischemia and contribute to irreversible damage (Mehta et al., 2007). Thus, neuroprotective therapy is essential for the survival and functional recovery of neurons after cerebral ischemia.

Blockade of inflammation has been proven to attenuate cerebral ischemic damage (Nogawa et al., 1997; Phillis et al., 2006). Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most prescribed drugs in the world for the treatment of inflammation, fever, and pain. Among those, indomethacin exerts its pharmacological actions by inhibiting the enzymatic activity of both cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) (Burian and Geisslinger, 2005). Numerous studies have documented the neuroprotective ability of COX inhibitors in different models of experimental brain ischemia (Hurley et al. 2002; Ahmad et al., 2009). For instance, indomethacin was able to enhance poststroke neurogenesis in an *in vivo* model of focal cerebral ischemia (Hoehn et al., 2005).

Another signaling system, which has a crucial role in mediating the cell survival, is the mitogen-activated protein kinase (MAPK) pathway, which plays an

important function in transducing stress-related signals by a cascade of intracellular kinase phosphorylations and transcription factor activation that regulate inflammatory gene production, among other functions (Irving and Bamford, 2002). During cerebral ischemia, the stress-activated protein kinases or *c-Jun* N-terminal kinases (SAPK/JNK) and extracellular signal-regulated kinase-1 and 2 (ERK1/2) pathways are activated playing a role in cell death and/or repair (Runden et al., 1998; Irving and Bamford, 2002).

The blood-brain barrier (BBB) represents a homeostatic defense mechanism of the brain against pathogens and toxins, although it prevents the penetration of a large variety of central nervous system (CNS)-active agents (Carvey et al., 2009; Palmer, 2009). Consequently, the BBB is a limiting factor for developing new drugs and dosage forms for the brain. Nanoparticles are large and complex constructs, which can be made from a variety of chemical constituents and may range up to 1 μm in diameter. Most polymeric nanoparticles are biodegradable, biocompatible and have been adopted as potential carriers for drug delivery to the CNS (Faraji et al., 2009; Gabathuler, 2009). Moreover, nanoparticles can be used to alter the kinetic profiles of drugs, leading to more sustained release, with a reduced requirement for frequent dosing, as well to reduce drug adverse effects (Faraji et al., 2009). In this regard, it was been suggested that NSAIDs-loaded nanocapsules display an increased efficacy in experimental models of peripheral inflammation (Guterres et al., 2001, Bernardi et al., 2009a).

Considering that acute treatments for cerebrovascular disease are very limited, that recent studies clearly indicate an important role for neuroinflammatory events in the evolution of ischemic events, and the advantages of nanoparticulate systems to deliver drugs at the required sites of action, the present study was

designed to investigate the potential neuroprotective effects of indomethacin-loaded nanocapsules in an *in vitro* model of ischemia.

Materials and methods

Preparation of nanocapsules

Nanocapsule suspensions were prepared by interfacial deposition of polymer (Fessi et al., 1989). At 40 °C, indomethacin (0.010 g), poly(ε-caprolactone) (0.100 g), capric/caprylic triglycerides (0.33 ml) and sorbitan monostearate (0.077 g) were dissolved in acetone (27 ml). In a separate flask, polysorbate 80 (0.077 g) was added to 53 ml of water. The organic solution was injected into the aqueous phase under magnetic stirring at room temperature. After 10 min, the acetone was eliminated and the suspensions concentrated under reduced pressure. The final volume was adjusted to 10 ml. Control formulation (drug-unloaded nanocapsules) was prepared, as described above without adding indomethacin.

Characterization of nanocapsules

After preparation, the pH values of nanocapsule suspensions were determined using a potentiometer (Micronal B-474). The particle size, polydispersity index and zeta potential of the suspensions were determined using a Zetasizer®nano-ZS ZEN 3600 model (Malvern, UK). The samples were diluted with water (MilliQ®) (particle size and polydispersity index) or 10 mM NaCl aqueous solution (zeta potential). The measurements were made in triplicate. The total concentrations of indomethacin in the formulations were measured by reverse phase high-performance liquid chromatography (HPLC) (Perkin-Elmer S-200, with injector

S-200, detector UV-vis, a guard-column and a Lichrospher 100 RP-18 column of 250 mm, 4 mm and 5 μm; Merck). The mobile phase consisted of acetonitrile/water (70:30, v/v) adjusted to apparent pH 5.0 ± 0.5 with 10% (v/v) acetic acid. Each suspension (100 μl) was treated with acetonitrile (10 ml), the solution was filtered (Millipore 0.45 μm) and injected (20 μl). The HPLC method was validated (Pohlmann et al., 2004). Linear calibration curves for indomethacin were obtained in the range of 1.00-25.00 μg/ml, presenting correlation coefficients of higher than 0.9992.

Organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared according to the method of Stoppini et al. (1991), with some modifications (Horn et al., 2005; Zamin et al., 2006). Briefly, 400 μm thick hippocampal slices were prepared from 6 to 8-days-old male *Wistar* rats using a McIlwain tissue chopper, and separated in ice-cold Hank´s balanced salt solution (HBSS) composed of (mM): glucose 36, $CaCl_2$ 1.26, KCl 5.36, NaCl 136.89, KH_2HPO_4 0.44, Na_2HPO_4 0.34, $MgCl_2$ 0.49, $MgSO_4$ 0.44, HEPES 25; fungizone 1% and gentamicin 0.100 mg/ml, pH 7.2. The slices were placed on Millicell culture membranes and the inserts were transferred to a 6-well culture plate. Each well contained 1 ml of culture medium consisting of 50% MEM (minimum essential medium), 25% HBSS, 25% heat inactivated horse serum supplemented with (mM, final concentration): glucose 36, HEPES 25 and $NaHCO_3$ 4; fungizone 1% and gentamicin 0.100 mg/ml, pH 7.3. The organotypic cultures were maintained in a humidified incubator gasified with a 5%CO_2/95%O_2 atmosphere at 37 °C for 14 days. Culture medium was changed twice a week. All animal procedures were approved by the local Animal Care Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Drug exposure

After 14 days *in vitro*, the organotypic hippocampal slice cultures received 50 or 100 μM of indomethacin-loaded nanocapsules (IndOH-NC) or drug-unloaded nanocapsules (NC) for 27 h. These formulations were added to the culture medium 2 h before oxygen-glucose deprivation (OGD), maintained during the 60 min of OGD and during the recovery period of 24 h. Control cultures were not exposed to nanocapsules. This protocol represents an acute treatment.

Oxygen-glucose deprivation (OGD)

The induction of OGD was based on the method described by Strasser and Fischer (1995), with some modifications (Valentim et al., 2003). After 14 days *in vitro*, the inserts were transferred to a sterilized 6-well plate and incubated with 1 ml of OGD medium for 15 min to deplete glucose from intracellular stores and extracellular space. The OGD medium is composed of: $CaCl_2$ 1.26 mM, KCl 5.36 mM, NaCl 136.9 mM, H_2PO_4 0.34 mM, $MgCl_2$ 0.49 mM, $MgSO_4$ 0.44 mM, HEPES 25 mM, pH 7.2. After that, the medium was exchanged for one with the same composition, but previously bubbled with N_2 for 30 min, and the plate transferred to an anaerobic chamber at 37 °C with N_2-enriched atmosphere for 60 min. During this process, control slices were maintained in an incubator with 5% CO2 atmosphere at 37 °C. After the OGD period, the slice cultures were carefully washed twice with HBSS, and then incubated in culture medium at 37 °C and 5%CO_2/95%O_2 atmosphere for 24 h, corresponding to the recovery period.

Quantification of cellular death

Cell damage was assessed by fluorescent image analysis of propidium iodide (PI) uptake (Noraberg et al., 1999). PI is a polar compound that is impermeable to the intact cell membrane, but it is able to penetrate damaged cell membranes of dying cells, binding nuclear DNA to generate a bright red fluorescence. PI (5 μM) was added to the culture medium 2 h before the end of the recovery period. PI uptake is indicative of significant membrane injury (Macklis and Madison, 1990). Cultures were observed with an inverted microscope (Nikon Eclipse TE 300) using a standard rhodamine filter set. Images were captured and then analyzed using Scion Image software (http://www.scioncorp.com). The area where PI fluorescence was detectable above background levels was determined using the "density slice" option of Scion Image software and compared to the total slice area to obtain the percentage of damage.

Western blotting analysis

After obtaining fluorescent images, cells were homogenized in lysis buffer (4% sodium dodecylsulfate, 2.1 mM EDTA and 50 mM Tris); aliquots were taken for protein determination and β-mercaptoethanol (Sigma Chemical) was added to a final concentration of 5%. Proteins were resolved (50 μg per lane) on 8% or 12% SDS-PAGE. After electrophoresis, proteins were electro-transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Bio-Rad Trans-Blot SD, Hercules, CA, USA). Membranes were incubated for 2 h at 4 °C in blocking solution (Tris-buffered saline containing 5% powdered milk and 0.1% Tween-20, pH 7.4) and further incubated with the appropriate primary antibody dissolved in the blocking solution overnight at 4 °C. Primary antibodies against pERK1/2 (1:1000; Cell Signaling Technology), ERK1/2 (1:1000; Cell Signaling Technology), JNK (1:1000;

Cell Signaling Technology), pJNK (1:1000; Cell Signaling Technology), GFAP (1:2000; Chemicon International Inc.) and iNOS (1:1000; Abcam) were used. The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (1:1000; Cell Signaling Technology) for 2 h. The chemioluminescence (ECL, Amersham Pharmacia Biotech) was detected using X-ray films that were scanned and analyzed using the Optiquant software (Packard Instruments). For each experiment, the group referred as control cultures (not exposed to OGD) was considered 100% and data are expressed as percentage of control cultures.

Isolectin B_4 reactivity in microglial cells

As described above, the proteins were resolved (50 µg per lane) on 8% SDS-PAGE and, after electrophoresis, they were electro-transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Bio-Rad Trans-Blot SD, Hercules, CA, USA). Membranes were incubated overnight at 4 °C in albumin solution (5% albumin and 2% Tween 80 in PBS, pH 7.4). Isolectin B_4 peroxidase conjugated (Sigma) was incubated in PBS containing 0.05% Tween overnight in a final concentration of 0.125 µg/mL. The chemioluminescence (ECL, Amersham Pharmacia Biotech) was detected using X-ray films that were scanned and analyzed using the Optiquant software (Packard Instruments). For each experiment, the group referred as control cultures (not exposed to OGD) was considered 100% and data are expressed as percentage of control cultures.

Determination of cytokine levels in the culture medium of organotypic cultures

At the end of the recovery period of OGD and treatment with indomethacin-loaded nanocapsules in organotypic hippocampal slice cultures, the culture media was collected, rapidly frozen and stored at -20 °C for later measurement of TNF-α, IL-6, IL-1β and IL-10 levels using specific enzyme-linked immunosorbent assay (ELISA) kits, according to the recommendations of the supplier (R&D Systems).

Statistical analysis

Data are expressed as mean ± S.D. and analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by post-hoc for multiple comparisons (Tukey test) using a *GraphPad Prism Software*. Differences between mean values were considered significant when $p<0.05$.

Results

Physico-chemical characterization of nanocapsule formulations

The nanocapsule formulations were prepared by interfacial deposition of poly(ε-caprolactone) without the need of any subsequent step of purification. Indomethacin-loaded nanocapsules and drug-unloaded nanocapsules showed a macroscopic homogeneous aspect, such as white bluish opalescent liquids. After preparation, the particle sizes (z-average) were 240 nm (indomethacin-loaded nanocapsules) and 226 nm (drug-unloaded nanocapsules). The suspensions showed monomodal size distributions and polydispersity indexes lower than 0.19, indicating narrow size distributions. The pH values were 5.95 (indomethacin-loaded nanocapsules) and 6.05 (drug-unloaded nanocapsules). The zeta potential values

were -6.9 and -7.3 mV, respectively. The indomethacin content was 0.994 ± 0.010 $mg \cdot mL^{-1}$ and the encapsulation efficiency were close to 100% for all batches.

Indomethacin-loaded nanocapsules treatment protects from cell damage induced by OGD

These series of experiments were designed to evaluate whether indomethacin-loaded nanocapsules are able to exert neuroprotective effects against the cell damage induced by OGD. The exposure of cultures to 60 min of OGD, followed by 24 h of recovery, caused a marked fluorescence in the hippocampus, indicating a high incorporation of PI, as presented in the photomicrographs (Fig. 1A and 1C). Quantification of PI fluorescence showed that OGD caused about 50% damage in hippocampus, a significant increase when compared to control cultures (about 2-4% of cellular damage) (Fig. 1B and 1D). It is important to note that no difference was detected among control slices after the treatment, indicating that neither drug-unloaded nanocapsules nor indomethacin-loaded nanocapsules had toxic effects in basal conditions (Fig. 1). The treatment with 50 or 100 μM of indomethacin in solution did not protect from OGD damage (data not shown); however, when the cultures were treated with 50 or 100 μM of indomethacin-loaded nanocapsules, the cell death was significantly decreased to 31% and 15%, respectively (Fig. 1). These results indicate that indomethacin-loaded nanocapsules were able to achieve the organotypic hippocampal slice cultures and protect them against ischemia. To investigate the probable mechanisms by which indomethacin-loaded nanocapsules exert its neuroprotective effect, the therapeutic concentration of 100 μM was chosen.

Indomethacin-loaded nanocapsules treatment prevents OGD-induced ERK1/2 and JNK activation

Since cell survival and differentiation in several cell types are mediated by ERK1/2 and JNK activation, we examined whether the neuroprotection mediated by indomethacin-loaded nanocapsules could involve those pathways. We measured the levels of the immunocontent of ERK1/2 and JNK, as well as its phosphorylation status 24 h after OGD. As observed in Fig. 2 and 3, the exposure of the culture to 60 min of OGD and 24 h of recovery caused a significant increase in ERK1/2 and JNK phosphorylation levels when compared to control cultures not exposed to OGD. The treatment with indomethacin-loaded nanocapsules (100 μM) was able to significantly reduce the levels of ERK1/2 phosphorylation, without modifying the levels of total ERK1/2 in OGD cultures (Fig. 2). Similarly, the treatment with indomethacin-loaded nanocapsules reduced JNK phosphorylation to the levels of control cultures (Fig. 3). Furthermore, no alterations in the total amount of JNK were observed (Fig. 3).

Indomethacin-loaded nanocapsules treatment suppress glial activation induced by OGD

To investigate a possible role of inflammation in the hippocampal slices in response to OGD, we investigated the reactivity of iNOS, GFAP and Isolectin B_4. The exposure of the culture to 60 min of OGD caused an inflammatory reaction, as evidenced by an increase in iNOS (Fig. 4) and GFAP immunocontent (Fig. 5), as well as in the reactivity of Isolectin B_4, a hallmark of microglial activation (Fig. 6). A significant reduction in iNOS and GFAP immunocontent was observed in the cultures treated with 100 μM of indomethacin-loaded nanocapsules (Fig. 4 and 5). As observed for iNOS and GFAP immunodetection, the treatment of cultures with 100

μM of indomethacin-loaded nanocapsules was able to prevent the Isolectin B_4 reactivity induced by 60 min of OGD (Fig. 6).

Indomethacin-loaded nanocapsules treatment prevents the increase in pro-inflammatory cytokine levels induced by OGD

We also investigated some pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, thought to play a central role in self-propagation of neuroinflammation. As observed in Fig. 7, IL-1β, IL-6 and TNF-α levels were highly increased in the culture medium 24 h after exposure of hippocampal culture to 60 min of OGD. The results demonstrated that treatment with 100 μM of indomethacin-loaded nanocapsules induced a significant reduction in the secretion of the pro-inflammatory cytokines in the culture medium (Fig. 7A-C). An important outcome of the present study is that, the same treatment with indomethacin-loaded nanocapsules was able to induce a marked increase of the anti-inflammatory cytokine IL-10 levels (Fig. 7D). Furthermore, control cultures did not show any increase in IL-1β, IL-6 and TNF-α levels after the treatment with drug-unloaded nanocapsules, indicating that the nanostructure carrier itself does not cause inflammatory alterations.

Discussion

In the present study, we tested whether indomethacin, carried by lipid-core polymeric nanocapsules, has a neuroprotective effect in oxygen-glucose deprivation (OGD) injury. We have also proposed an underlying mechanism by which this neuroprotection occurs. For this purpose, we used organotypic hippocampal cultures, which provide a good experimental access to mimic pathophysiological pathways in living tissues and to facilitate design of therapeutic agents (Stoppini et al., 1991;

Holopainen, 2005). Those cultures maintain their cell architecture and interneuron connections, and neurons survive during the long-term culture and physiologically mature over this period, allowing an extended survival study (Muller et al., 1993; Xiang et al., 2000; Frozza et al., 2009).

Ischemic stroke leads to a complex cascade of cell events resulting in extensive cell death. The energy failure or drastic decrease in cellular ATP and glucose levels is responsible for the underlying mechanisms of necrosis in the core of the ischemic region. Moreover, reperfusion also plays a prominent role in damage distribution, as well success of appropriate therapeutic approach to provide neuroprotection lies on the brain tissue, which remains functionally impaired but viable and potentially salvageable (Mehta et al., 2007). Therefore, there is growing evidence that the initial few hours of reversible neuronal injury offers an opportunity for therapeutic intervention. Here, we showed that, on the contrary to indomethacin in solution, the treatment with indomethacin-loaded nanocapsules during the lesion and the recovery period of 24 h is neuroprotective against the cell damage induced by OGD. This result is encouraging considering that in ischemic cerebral injury, the period between the insult and the occurrence of cell death may be clinically observable. It is important to hallmark that we have previously demonstrated that polymeric nanocapsules are able to successfully carry indomethacin into the healthful brain (Bernardi et al., 2009). In addition, considering that brain distribution of indomethacin *in vivo* is limited by plasma protein binding, which reduces the plasma free fraction in the blood circulation by >90% (Parepally et al., 2006), the nanoparticulated systems represent a promising alternative. Furthermore, one plausible explanation for indomethacin-loaded nanocapsules to have been more

potent that indomethacin in solution is that nanoparticles can accumulate in the inflamed tissues, promoting a sustained drug release.

Taking into account that cerebral ischemia activates MAPK pathway signaling and that pharmacological intervention of those pathways may result in neuroprotection, we also investigated the effect of indomethacin-loaded nanocapsules in this cascade. In this context, our results show that indomethacin-loaded nanocapsules treatment was able to significantly reduce the levels of ERK1/2 and JNK phosphorylation. These data are in agreement with other works, which reported that activation of ERK and JNK pathways are involved in neuronal death in neurodegenerative diseases and cerebral ischemia (Park et al., 2004; Borsello et al., 2007). Ferrer and colleagues (2003) have reported the expression of ERK, JNK and their downstream phosphorylated specific substrates in the infarct core and penumbra area in focal cerebral ischemia. Thus, we propose that indomethacin-loaded nanocapsules could, at least in part, mediate its neuroprotective effect by modulating these important signaling pathways implicated in supporting neural survival.

In the attempt to better understand the neuroprotective effects of indomethacin-loaded nanocapsules in our model of ischemia, we also investigated what effect this formulation would have on OGD-mediated inflammation. The extensive cell death observed in cultures submitted to OGD was accompanied by a strong inflammatory reaction that is believed to contribute to brain injury. Inflammatory responses in the brain are associated mainly with microglial and astrocytic activation that accelerates neurodegeneration (Park et al., 2007). Following ischemia, astrocytes are activated resulting in increased glial fibrillary acidic protein (GFAP) expression and so-called “reactive gliosis”, characterized by specific

structural and functional changes (Pekny and Nilsson, 2005). Astrocytes are also capable of secreting inflammatory factors such as cytokines and chemokines, as well to increase the inducible nitric oxide synthase (iNOS) enzyme expression. iNOS is expressed by activated microglia, astrocytes and leukocytes after ischemia, contributing to the inflammatory response of the brain tissue (Jander et al., 2000). In addition, iNOS contributes to nitric oxide (NO) synthesis, stimulating oxygen reactive species formation and promoting neuronal damage after stroke (Dalkara et al., 1998). The high sensitivity of neurons to NO is partly due to NO causing inhibition of respiration, rapid glutamate release from both astrocytes and neurons, and subsequent excitotoxic cell death of the neurons via the NMDA receptor (Brown, 2007). Our results show that indomethacin-loaded nanocapsules enhanced neuroprotection against OGD by reducing glial activation, as observed by a marked decrease in iNOS and GFAP immunocontents. These results corroborate literature data suggesting that iNOS and COX seems to offer important targets, as their blockade even at 6 – 24 h after ischemia appears to be neuroprotective (Zhang et al., 1996; Sugimoto and Ladecola et al., 2003). Additionally, microglial activation, as demonstrated by the increase of Isolectin B_4 reactivity, was prevented by the treatment with indomethacin-loaded nanocapsules. Thus, we hypothesized that prevention of microgilal and glial activation caused by indomethacin-loaded nanocapsules should be due to the inhibition of cytokine production and secretion, culminating in the neuroprotection observed in hippocampal cultures. Next, we sought to determine whether the neuroprotective effects of indomethacin-loaded nanocapsules were associated with changes in cytokine generation by the cultures.

An important finding of the present study was that treatment with indomethacin-loaded nanocapsules produced a significant decrease in the secretion

of the cytokines IL-1β, IL-6 and TNF-α, the most relevant pro-inflammatory cytokines related to inflammation in stroke (Han & Yenari et al., 2003). It was demonstrated that IL-6 mRNA expression is upregulated as early as 3 h after occlusion of the middle cerebral artery, which maximal levels at 12 h, and persisted for at least 24 h (Wang et al., 1995). Clinical studies showed that increased IL-6 plasma concentrations were associated with early neurological worsening (Vila et al., 2001), and high IL-6 levels predicted early neurological deterioration and a poor functional outcome in lacunar infarction (Castellanos et al., 2002). Both IL-1β and TNF-α are increased not only during early reperfusion (1 – 3 h), but also at later times (24 – 36 h), indicating a biphasic expression (Wang et al., 2007). Expression of IL-1β is increased after transient or permanent stroke in microglia by astrocytes and neurons (Buttini et al., 1994). The possible IL-1β mechanisms of action include the release of arachidonic acid, the enhancement of NMDA excitotoxicity and the stimulation of iNOS (Huang et al., 2006). TNF-α expression was initially observed in neurons (Liu et al., 1994), then later in microglia and astrocytes (Uno et al., 1997). Additionally, TNF-α appears to have pleiotropic functions in the ischemic brain (Hallenbeck et al., 2002). In an *in vivo* model, the inhibition of TNF-α was able to reduce the ischemic brain injury (Yang et al., 1998), while the administration of a recombinant TNF-α protein after stroke onset worsens ischemic brain damage (Barone et al., 1997). In organotypical cultures exposed to ischemic conditions, the pre-treatment with TNF-α was protective against neuronal death, while treatment after ischemia was detrimental and increased oxidative stress (Wilde et al., 2000). The accumulation of cytokines in stroke does not appear to be a mere consequence of the degenerative processes, but it appears to play a role in the cascade of events inducing neuronal death by stress-activated signal transduction pathways, as the MAPK cascade (Xie et al., 2004). Therefore, the

role of individual cytokines is more complex than previously anticipated; their concentration and time of activation, and the cross-talk between different signals are essential for the cellular and pathological outcome (Planas et al., 2006).

More relevantly, we demonstrated for the first time, that treatment with indomethacin-loaded nanocapsules was capable of inducing a marked increase in the levels of the anti-inflammatory cytokine IL-10 in cerebral tissues, which is likely associated with beneficial effects in the injuried brain (Planas et al., 2006). IL-10 acts by inhibiting IL-1β and TNF-α actions, and also by suppressing cytokine receptor expression and/or activation. Both exogenous administration and gene transfer of IL-10 in cerebral ischemia models appear to have beneficial effects (Wang et al., 2007). In clinical studies conducted with stroke patients, poor outcome and neurological worsening were predicted by low IL-10 plasma levels (Vila et al., 2003).

In summary, the data reported herein clearly demonstrate that blockage of neuroinflammation is involved in the neuroprotective effect of indomethacin-loaded nanocapsules. Although indomethacin is not an agent currently used in the treatment of cerebral ischemia, our results imply that nanocapsule formulations containing infomethacin might be considered as potential candidates for stroke treatment. Further investigations using *in vivo* models of focal and global ischemia could be helpful to determine the appropriate doses for therapy of stroke.

Acknowledgments

This study was supported by the Brazilian agencies: CNPq, Rede Brasil-França CNPq/MCT, FINEP, Rede Nanocosméticos/CNPq-MCT and Capes. A. Bernardi and R. Frozza were recipients of CNPq PhD fellowships. A.P. Horn was recipient of CNPq Posdoc fellowships.

Legends to the Figures

Figure 1. Effects of indomethacin-loaded nanocapsules (IndOH-NC) treatment on cell damage induced by oxygen-glucose deprivation (OGD) for 60 min in organotypic hippocampal cultures. (A and C) Representative photomicrographs of hippocampal slices stained with PI 24 h after exposure to OGD. (B and D) Quantitative analysis of hippocampal damage 24 h after exposure to OGD. Indomethacin-loaded nanocapsules (IndOH-NC) 50 μM (A and B) or 100 μM (C and D) were added 2 h before of the lesion and maintained during the recovery period for 24 h. Bars represent the mean ± SD, n=9 animals. # Significantly different from the respective control culture; *** Significantly different from the OGD and OGD NC groups (One-way ANOVA followed by Tukey's test, $p<0.001$).

Figure 2. Effects of indomethacin-loaded nanocapsules (IndOH-NC) treatment on the percentage of phosphorylated ERK1/2 in organotypic hippocampal cultures. (A) Representative Western Blotting of phosphorylated ERK1/2 (pERK1/2), ERK1/2 and β-actin immunocontent 24 h after oxygen-glucose deprivation (OGD) and treatment with indomethacin-loaded nanocapsules (IndOH-NC). (B) Histogram representing the quantitative Western Blotting analysis of ERK1/2 phosphorylation state. The densitometric values obtained to phospho- and total-ERK1/2 from treatments were normalized to their respective controls non-exposed to OGD condition (control bar) (100%). IndOH-NC 100 μM were added 2 h before of the lesion and maintained during the recovery period for 24 h. Data are expressed as a ratio of the normalized percentages of pERK1/2 and ERK1/2. Bars represent the mean ± SD, n=6 animals. #Significantly different from the respective control culture; ***

Significantly different from the OGD and OGD NC groups (One-way ANOVA followed by Tukey's test, $p<0.001$).

Figure 3. Effects of indomethacin-loaded nanocapsules (IndOH-NC) treatment on the percentage of phosphorylated JNK in organotypic hippocampal cultures. (A) Representative Western Blotting of phosphorylated JNK (pJNK), JNK and β-actin immunocontent 24 h after oxygen-glucose deprivation (OGD) and treatment with indomethacin-loaded nanocapsules (IndOH-NC). (B) Histogram representing the quantitative Western Blotting analysis of JNK phosphorylation state. The densitometric values obtained to phospho- and total-JNK from treatments were normalized to their respective controls non-exposed to OGD condition (control bar) (100%). IndOH-NC 100 µM were added 2 h before of the lesion and maintained during the recovery period for 24 h. Data are expressed as a ratio of the normalized percentages of pJNK and JNK. Bars represent the mean ± SD, n=6 animals. $^{\#}$Significantly different from the respective control culture; *** Significantly different from the OGD and OGD NC groups (One-way ANOVA followed by Tukey's test, $p<0.001$).

Figure 4. Effects of indomethacin-loaded nanocapsules (IndOH-NC) treatment on the percentage of iNOS in organotypic hippocampal cultures. (A) Representative Western Blotting of iNOS and β-actin immunocontent 24 h after oxygen-glucose deprivation (OGD) and treatment with indomethacin-loaded nanocapsules (IndOH-NC). (B) Histogram representing the quantitative Western Blotting analysis of iNOS. The densitometric values obtained to iNOS immunocontent

from treatments were normalized to their respective controls non-exposed to OGD condition (control bar) (100%). IndOH-NC 100 μM were added 2 h before of the lesion and maintained during the recovery period for 24 h. Data are expressed as a ratio of the normalized percentages of iNOS. Bars represent the mean ± SD, n=6 animals. #Significantly different from the respective control culture; * Significantly different from the OGD and OGD NC groups (One-way ANOVA followed by Tukey's test, $p<0.05$).

Figure 5. Effects of indomethacin-loaded nanocapsules (IndOH-NC) treatment on the percentage of GFAP in organotypic hippocampal cultures. (A) Representative Western Blotting of GFAP and β-actin immunocontent 24 h after oxygen-glucose deprivation (OGD) and treatment with indomethacin-loaded nanocapsules (IndOH-NC). (B) Histogram representing the quantitative Western Blotting analysis of GFAP. The densitometric values obtained to GFAP immunocontent from treatments were normalized to their respective controls non-exposed to OGD condition (control bar) (100%). IndOH-NC 100 μM were added 2 h before of the lesion and maintained during the recovery period for 24 h. Data are expressed as a ratio of the normalized percentages of GFAP. Bars represent the mean ± SD, n=6 animals. # Significantly different from the respective control culture; **Significantly different from the OGD and OGD NC groups (One-way ANOVA followed by Tukey's test, $p<0.01$).

Figure 6. Effects of indomethacin-loaded nanocapsules (IndOH-NC) treatment on Isolectin B_4 reactivity in organotypic hippocampal cultures. Representative

Western blotting of Isolectin B_4 reactivity 24 h after oxygen-glucose deprivation (OGD) and treatment with indomethacin-loaded nanocapsules (IndOH-NC). IndOH-NC 100 µM were added 2 h before of the lesion and maintained during the recovery period for 24 h. Data are representative of n=6 animals.

Figure 7. Effects of indomethacin-loaded nanocapsules (IndOH-NC) treatment on the levels of cytokines in organotypic hippocampal cultures. IL-1β (A), IL-6 (B), TNF-α (C) and IL-10 (D) levels were measured 24 h after exposure of organotypic hippocampal cultures to OGD and treatment with indomethacin-loaded nanocapsules (IndOH-NC). IndOH-NC 100 µM were added 2 h before of the lesion and maintained during the recovery period for 24 h. Bars represent the mean ± SD, n=6 animals. $^{\#}$Significantly different from the respective control culture; * Significantly different from the OGD and OGD NC groups ($p<0.05$); *** Significantly different from the OGD and OGD NC groups ($p<0.001$) (One-way ANOVA followed by Tukey's test).

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Figure 1

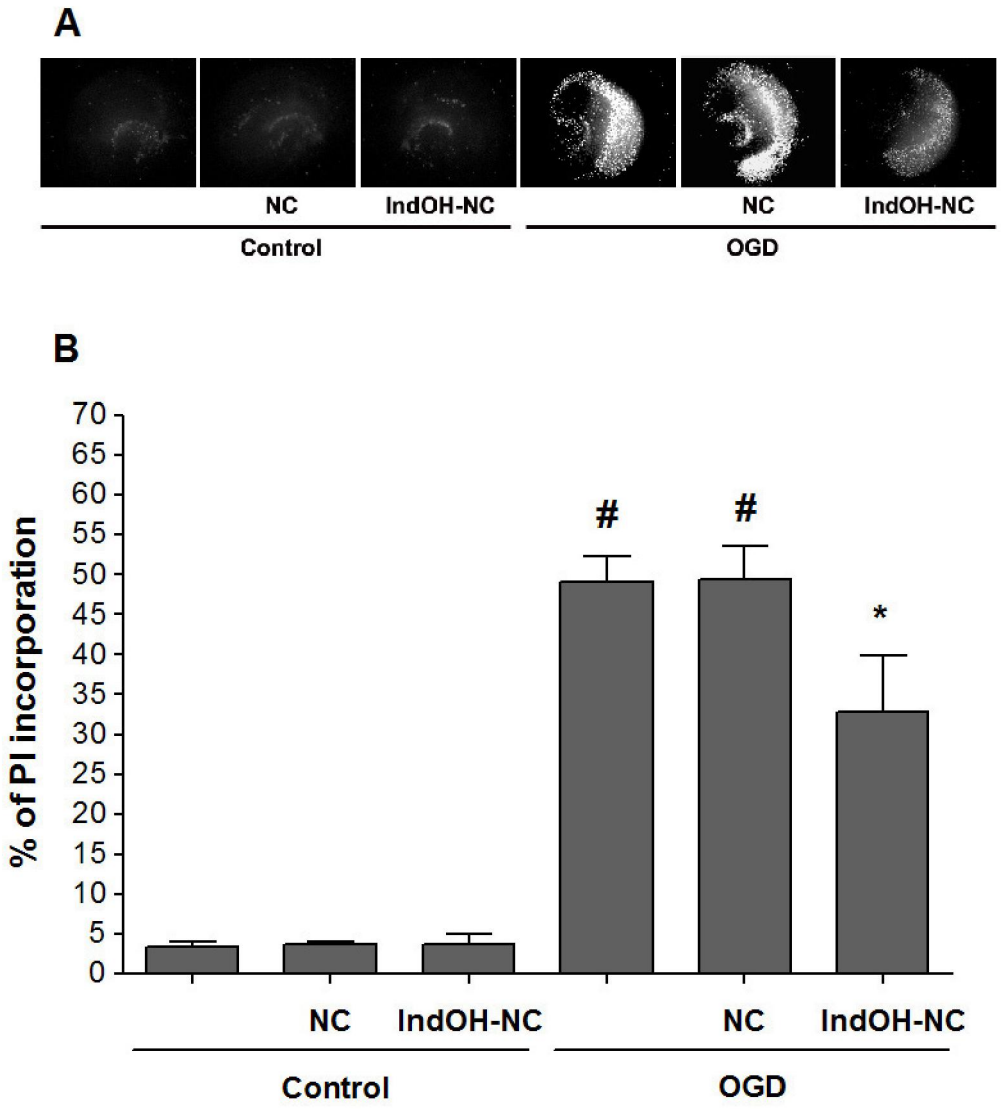

A
NC
IndOH-NC
NC
IndOH-NC
Control
OGD
B
% of PI incorporation
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
#
#
*
NC
IndOH-NC
NC
IndOH-NC
Control
OGD

Figure 1

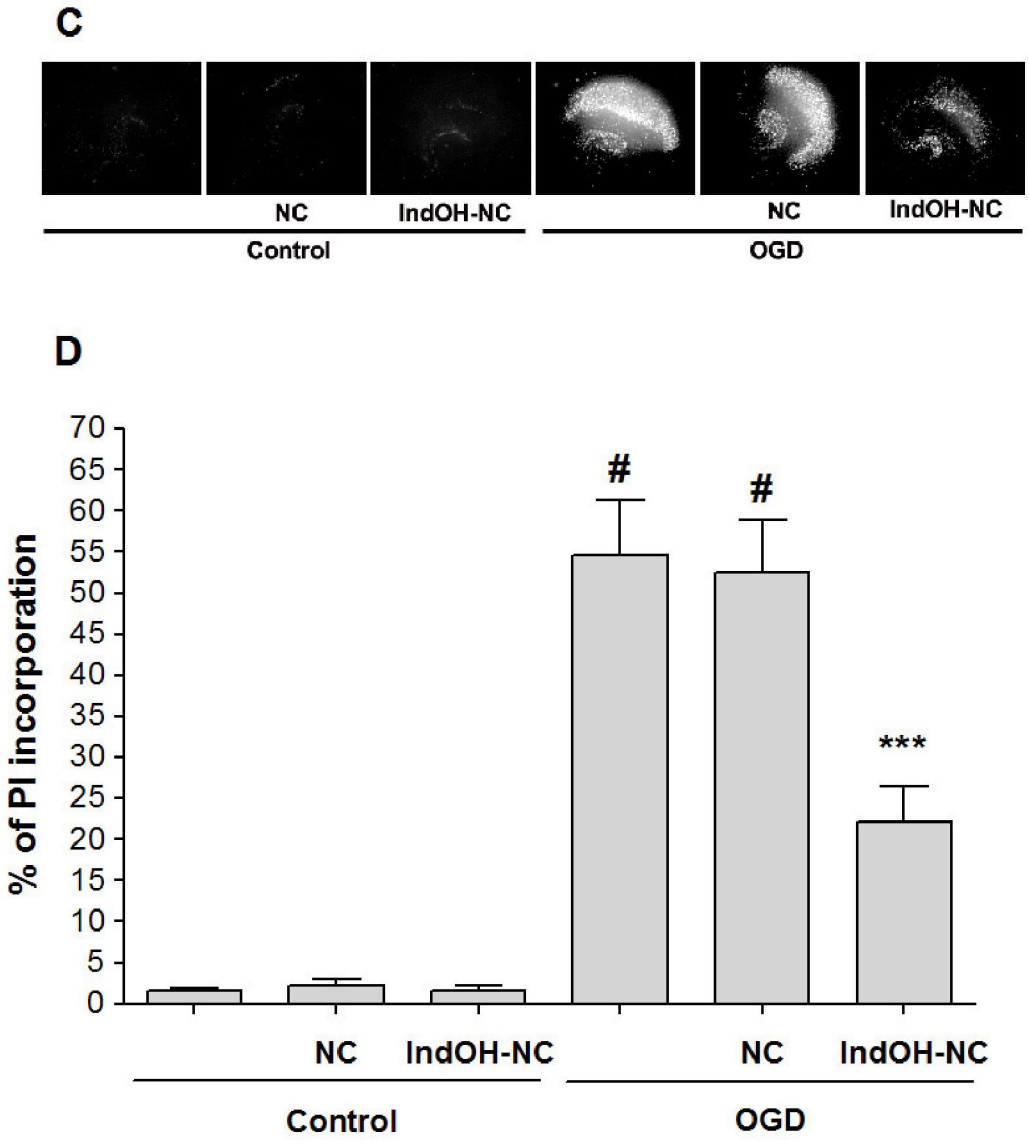
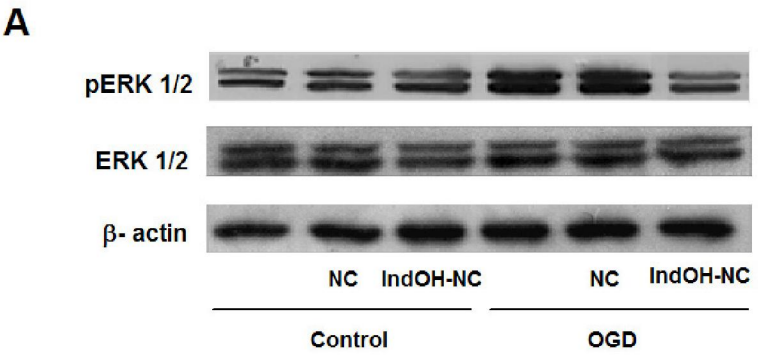

C
NC
IndOH-NC
NC
IndOH-NC
Control
OGD
D
% of PI incorporation
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
#
#
***
NC
IndOH-NC
NC
IndOH-NC
Control
OGD

Figure 2


A
pERK 1/2
ERK 1/2
β- actin
NC
IndOH-NC
NC
IndOH-NC
Control
OGD

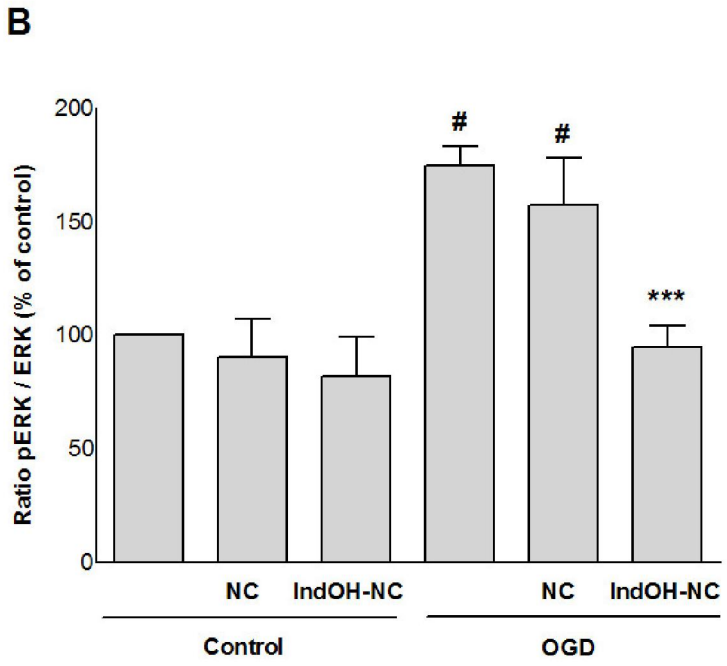
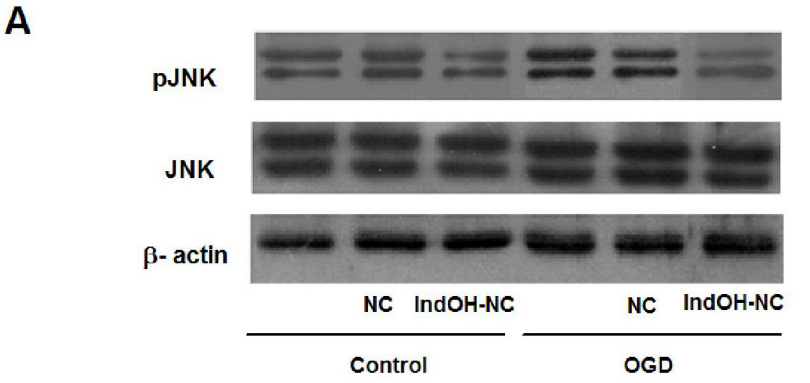

B
Ratio pERK / ERK (% of control)
200
150
100
50
0
#
#
***
NC
IndOH-NC
NC
IndOH-NC
Control
OGD

Figure 3


A
pJNK
JNK
β- actin
NC
IndOH-NC
NC
IndOH-NC
Control
OGD

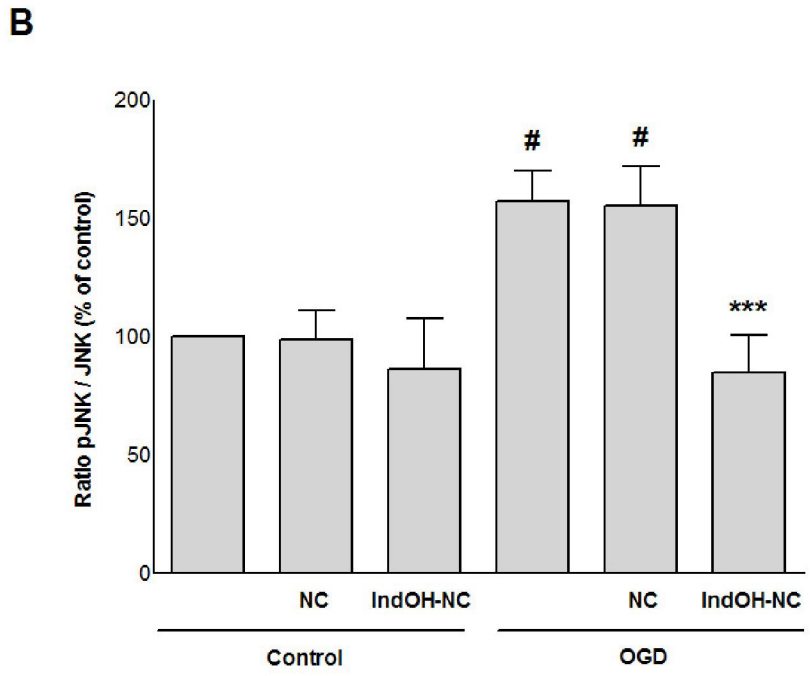
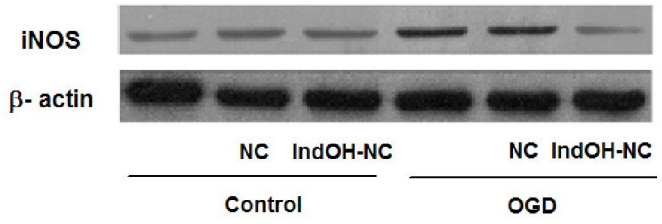

B
Ratio pJNK / JNK (% of control)
200
150
100
50
0
#
#
***
NC
IndOH-NC
NC
IndOH-NC
Control
OGD

Figure 4

A


iNOS
β- actin
NC
IndOH-NC
NC
IndOH-NC
Control
OGD

B

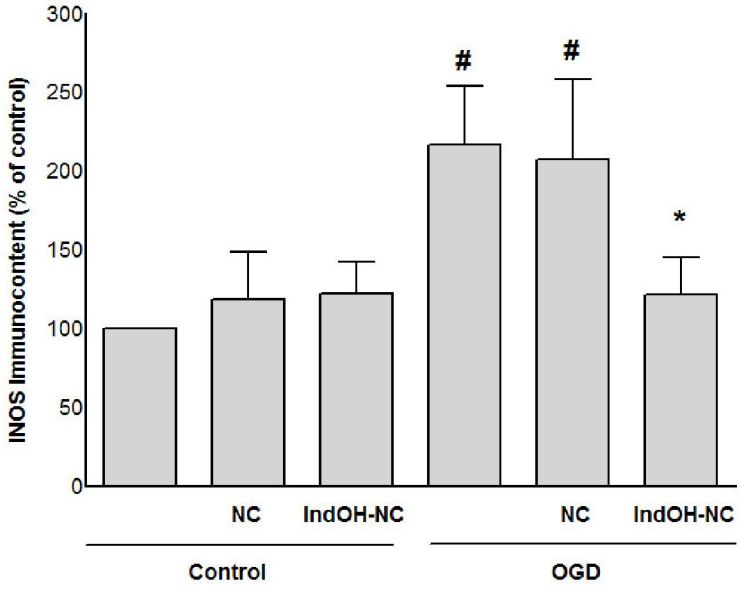
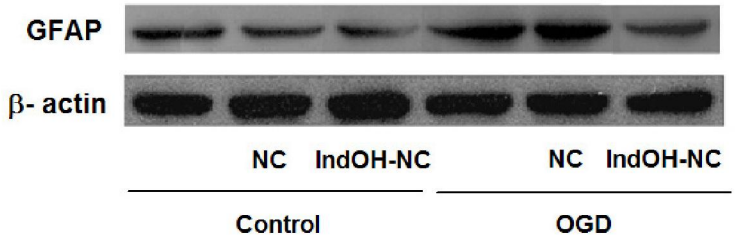

iNOS Immunocontent (% of control)
300
250
200
150
100
50
0
#
#
*
NC
IndOH-NC
NC
IndOH-NC
Control
OGD

Figure 5

A


GFAP
β- actin
NC
IndOH-NC
NC
IndOH-NC
Control
OGD

B

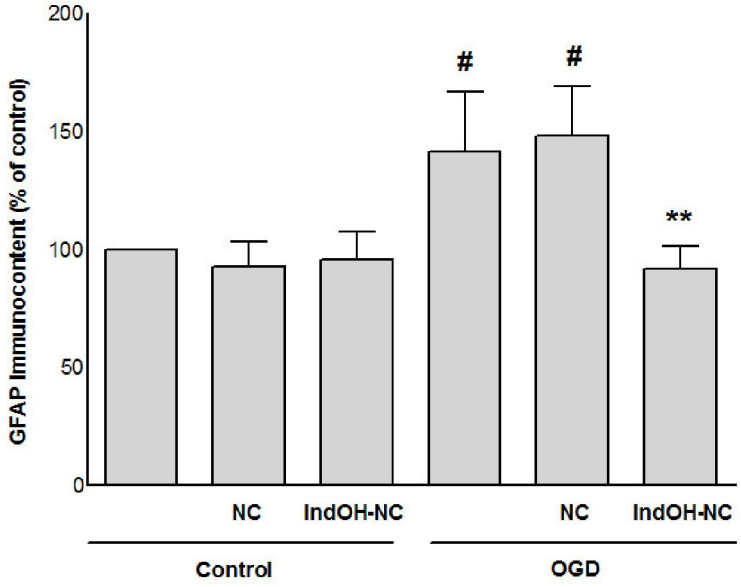

GFAP Immunocontent (% of control)
200
150
100
50
0
#
#
**
NC
IndOH-NC
NC
IndOH-NC
Control
OGD

Figure 6

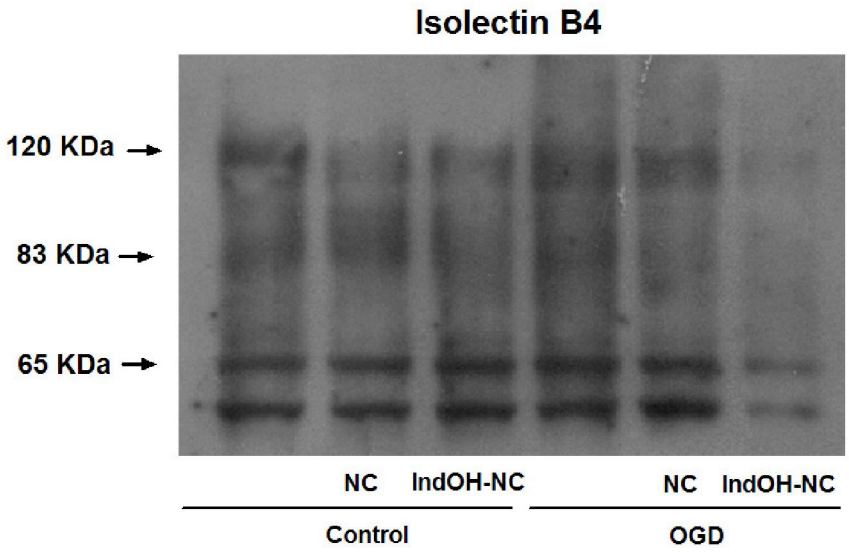

Isolectin B4
120 KDa
83 KDa
65 KDa
NC
IndOH-NC
NC
IndOH-NC
Control
OGD

Figure 7

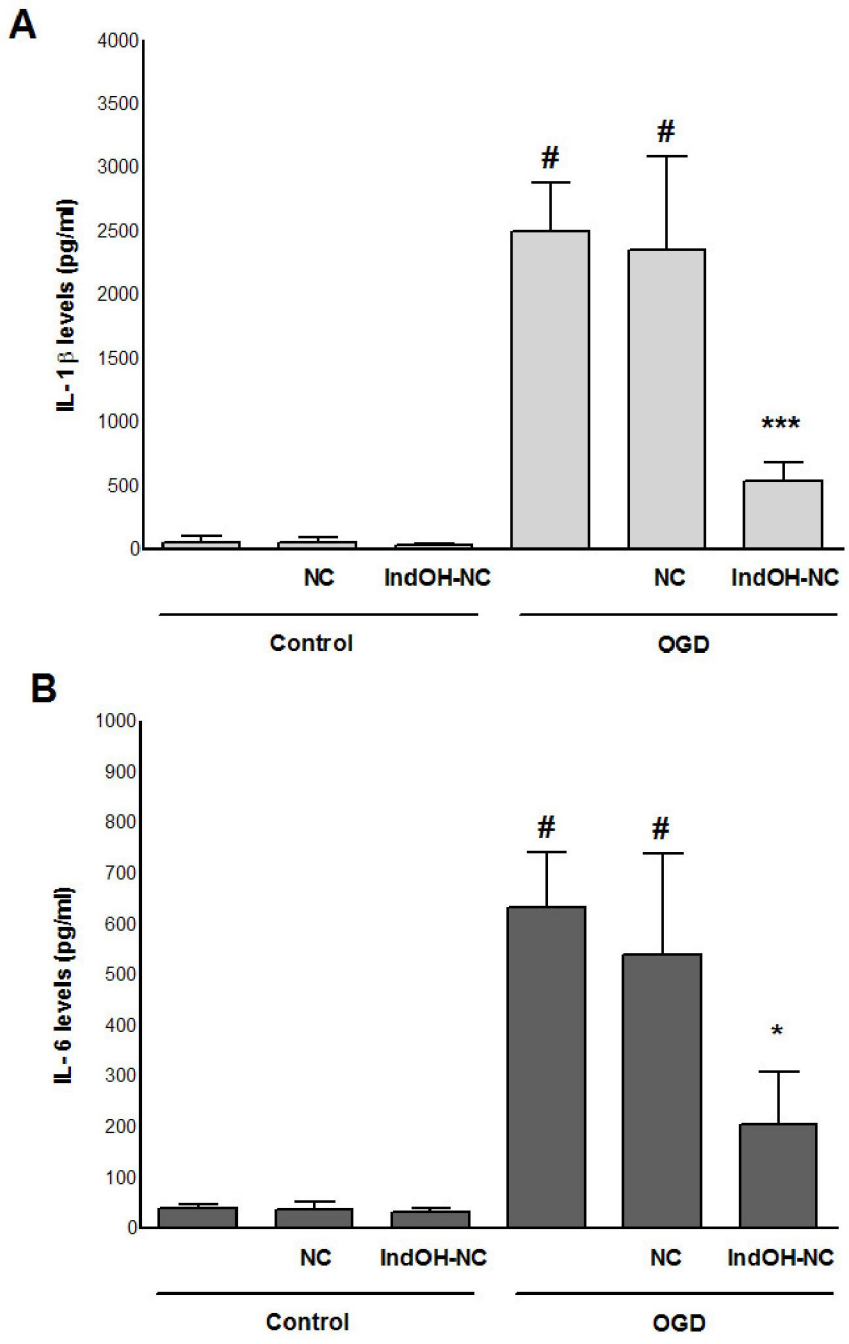
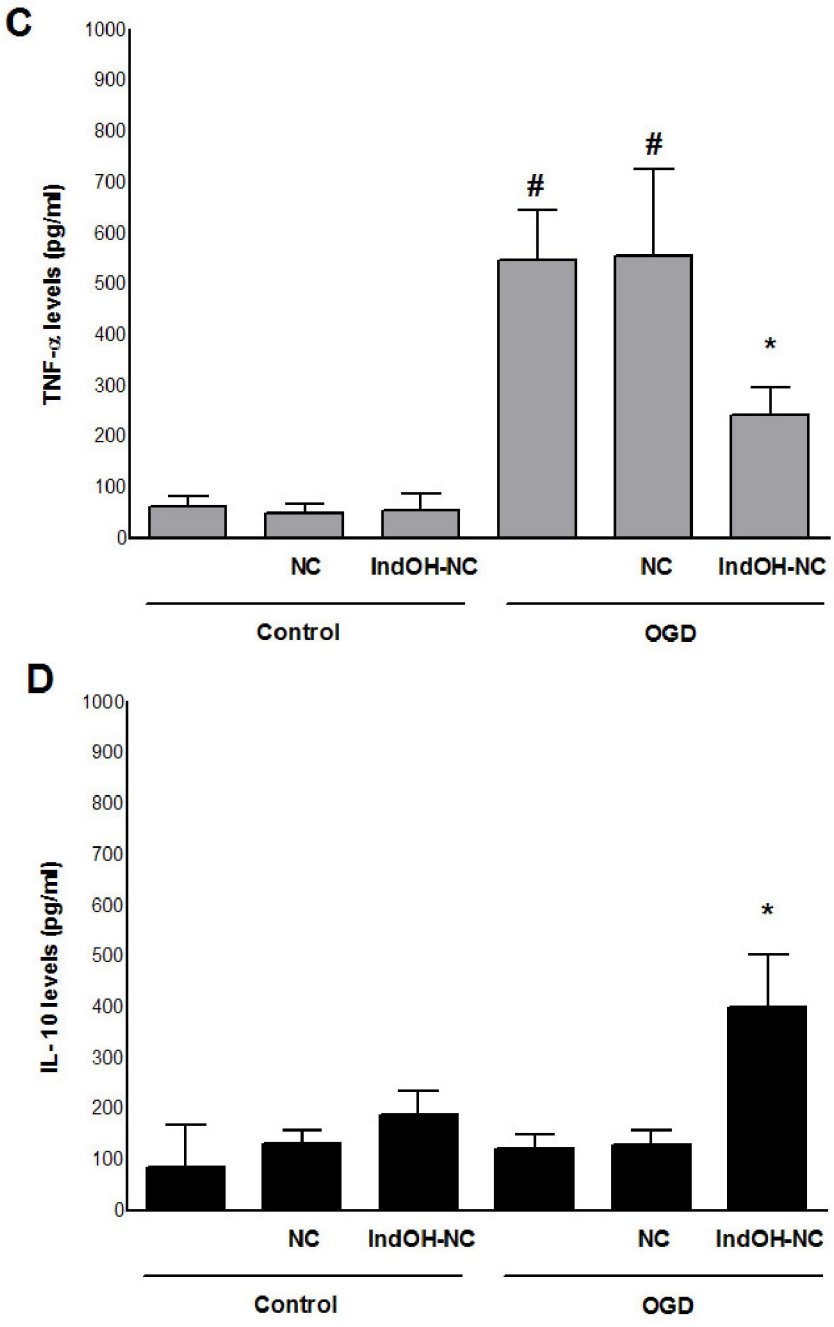

A
IL-1β levels (pg/ml)
4000
3500
3000
2500
2000
1500
1000
500
0
#
#
***
NC
IndOH-NC
NC
IndOH-NC
Control
OGD
B
IL-6 levels (pg/ml)
1000
900
800
700
600
500
400
300
200
100
0
#
#
*
NC
IndOH-NC
NC
IndOH-NC
Control
OGD

Figure 7


C
TNF-α levels (pg/ml)
1000
900
800
700
600
500
400
300
200
100
0
#
#
*
NC
IndOH-NC
NC
IndOH-NC
Control
OGD
D
IL-10 levels (pg/ml)
1000
900
800
700
600
500
400
300
200
100
0
*
NC
IndOH-NC
NC
IndOH-NC
Control
OGD

CAPÍTULO 4

EFFECTS OF INDOMETHACIN-LOADED NANOCAPSULES IN EXPERIMENTAL MODELS OF INFLAMMATION IN RATS

Andressa Bernardi, Ana Carolina Zilberstein, Eliézer Jäger, Maria Martha Campos, Fernanda B. Morrone, João B. Calixto, Adriana R. Pohlmann, Sílvia S. Guterres and Ana Maria O. Battastini

Periódico: ***British Journal of Pharmacology***

Status: **Publicado**

BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2009), 158, 1104–1111

0007-1188/09
www.brjpharmacol.org

THEMED SECTION: MEDIATORS AND RECEPTORS IN THE RESOLUTION OF INFLAMMATION

RESEARCH PAPER

Effects of indomethacin-loaded nanocapsules in experimental models of inflammation in rats

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Background and purpose: The effects of systemic treatment with indomethacin-loaded nanocapsules (IndOH-NC) were compared with those of free indomethacin (IndOH) in rat models of acute and chronic oedema.
Experimental approach: The following models of inflammation were employed: carrageenan-induced acute oedema (measured between 30 min and 4 h), sub-chronic oedema induced by complete Freund's adjuvant (CFA) (determined between 2 h and 72 h), and CFA-induced arthritis (oedema measured between 14 and 21 days).
Key results: IndOH or IndOH-NC produced equal inhibition of carrageenan-elicited oedema. However, IndOH-NC was more effective in both the sub-chronic (33 ± 4% inhibition) and the arthritis (35 ± 2% inhibition) model of oedema evoked by CFA, when compared with IndOH (21 ± 2% and 14 ± 3% inhibition respectively) ($P < 0.01$). In the CFA arthritis model, treatment with IndOH-NC markedly inhibited the serum levels of the pro-inflammatory cytokines tumour necrosis factor α and IL-6 (by 83 ± 8% and 84 ± 11% respectively), while the levels of the anti-inflammatory cytokine IL-10 were significantly increased (196 ± 55%). The indices of gastrointestinal damage in IndOH-NC-treated animals were significantly less that those after IndOH treatment (58 ± 16%, 72 ± 6% and 69 ± 2%, for duodenum, jejunum and ileum respectively).
Conclusions and implications: IndOH-NC produced an increased anti-inflammatory efficacy in long-term models of inflammation, allied to an improved gastrointestinal safety. This formulation might represent a promising alternative for treating chronic inflammatory diseases, with reduced undesirable effects.
British Journal of Pharmacology (2009) **158,** 1104–1111; doi:10.1111/j.1476-5381.2009.00244.x; published online 7 May 2009

This article is part of a themed issue on Mediators and Receptors in the Resolution of Inflammation. To view this issue visit http://www3.interscience.wiley.com/journal/121548564/issueyear?year=2009

Keywords: indomethacin; polymeric nanocapsules; drug delivery; inflammation; gastrointestinal damage

Abbreviations: CFA, complete Freund's adjuvant; COX-1, cyclooxygenase 1; COX-2, cyclooxygenase 2; IL-6, interleukin 6; IL-10, interleukin 10; IndOH-NC, indomethacin-loaded nanocapsules; NC, unloaded nanocapsules; NSAIDs, non-steroidal anti-inflammatory drugs; TNF-α, tumour necrosis factor α

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Received 19 December 2008; revised 5 February 2009; accepted 9 February 2009

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) represent a group of approximately 50 different medicines widely prescribed for the management of pain, which display variable anti-inflammatory, anti-pyretic and analgesic activities. Their

effects are mainly mediated by the inhibition of cyclooxygenases 1 and 2 (COX-1 and COX-2), with a consequent decrease in the formation of central and peripheral prostanoids (Burian and Geisslinger, 2005). Furthermore, most NSAIDs share a number of adverse effects, especially those related to gastrointestinal complications (Kean and Buchanan, 2005). Patients taking systemic NSAIDs for the treatment of chronic inflammatory diseases (such as rheumatoid arthritis and osteoarthritis) show variable relief of painful symptoms and they have increased risk of developing gastric or duodenal ulcers and bleeding, which might preclude their long-term use (Langford *et al.*, 2006; Fiorucci *et al.*, 2007).

The development of new drugs and/or new formulations for treating chronic inflammatory and painful diseases continues to be an issue of high interest. The pharmacological response to a drug is directly related to its concentration at the required site of action. A non-specific distribution leads to high drug concentration in healthy organs, tissues and cells, leading to toxicity (Soppimath *et al.*, 2001; Couvreur *et al.*, 2002). One method of restricting the drug to the required site is to associate it with a carrier system (Couvreur *et al.*, 2002; Vauthier and Couvreur, 2007). Among the different nanocarrier systems, biodegradable nanoparticles have received considerable attention as potential drug delivery vehicles over the last few years. Polymeric nanoparticles are colloidal structures below 1 μm, which have been designed to encapsulate lipophilic drugs in order to target organs or tissues, to avoid drug degradation, to improve its efficacy or to circumvent the toxicity (Allémann *et al.*, 1998; Pinto-Alphandary *et al.*, 2000; Guterres, 2001; Couvreur *et al.*, 2002; Vila *et al.*, 2002). In this regard, it has been suggested that NSAIDs-loaded nanocapsules might display an increased efficacy, associated with a marked reduction of adverse effects (Guterres *et al.*, 2001; Bansal JoshiBansal *et al.*, 2007). A recent publication from our group reinforced this idea, by showing that indomethacin-loaded nanocapsules (IndOH-NC) were more potent than free indomethacin in decreasing the viability and proliferation of glioma cell lines, without exerting significant cytotoxic effects on normal cells (Bernardi *et al.*, 2008).

In order to provide additional evidence on the effects of alternative delivery systems for NSAIDs, the present study was designed to characterize the effects of systemic treatment with IndOH-NC in rat models of acute or chronic inflammation. Attempts have also been made to determine the gastrointestinal effects of IndOH-NC. Additionally, we have aimed to compare both the anti-inflammatory and the adverse effects of IndOH-NC, with those displayed by free indomethacin in solution.

Methods

Preparation of nanocapsules

Nanocapsules were prepared by interfacial deposition of polymer as previously described (Fessi *et al.*, 1989). At 40°C, indomethacin (0.010 g), poly(ε-caprolactone) (0.100 g), capric/caprylic triglyceride (0.33 mL) and sorbitan monostearate (0.077 g) were dissolved in acetone (27 mL). In a separate flask, polysorbate 80 (0.077 g) was added to 53 mL of water. The organic solution was injected into the aqueous phase under magnetic stirring at room temperature. After 10 min, the acetone was evaporated and the suspensions concentrated under reduced pressure. The final volume was adjusted to 10 mL. Control formulation (unloaded nanocapsules) was prepared by omitting the drug (indomethacin).

Characterization of nanocapsules

After preparation, the pH of the suspensions was determined using a potentiometer (Micronal B-474). Particle size, polydispersity and zeta potential of the suspensions were determined using a Zetasizer®nano-ZS ZEN 3600 model (Malvern, UK). The samples were diluted in water (MilliQ®) (particle size) or in 10 mmol·L^{-1} NaCl aqueous solution (zeta potential). The measurements were made in triplicate. The total concentrations of indomethacin in the formulations were measured by reverse phase high-performance liquid chromatography (HPLC) (Perkin-Elmer S-200, with injector S-200, detector UV-Vis, a guard-column and a Lichrospher 100 RP-18 column of 250 mm, 4 mm and 5 μm; Merck). The mobile phase consisted of acetonitrile/water (70:30, v/v) adjusted to apparent pH 5.0 ± 0.5 with 10% (v/v) acetic acid. Each suspension (100 μL) was treated with acetonitrile (10 mL); the solution was filtered (Millipore 0.45 μm) and injected (20 μL). The HPLC method was previously validated (Pohlmann *et al.*, 2004). Linear calibration curves for indomethacin were obtained in the range of 1–25 μg·mL^{-1} presenting correlation coefficients higher than 0.9992.

Animals

All animal care and experimental procedures used in the present study followed the 'Principles of Laboratory Animal Care' from NIH publication No. 85–23 and were approved by the Ethics Committee of Pontifícia Universidade Católica do Rio Grande do Sul. The number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatment. Male Wistar rats (180–200 g) were obtained from the Central Biotery of the Federal University of Pelotas (Brazil). Animals were housed under conditions of optimum light (12:12 h light–dark cycle), temperature (22 ± 1°C) and humidity (50 to 60%), with food and water provided *ad libitum*. For the experiments, the animals were acclimatized to the laboratory for at least 1 h, and they were used only once in each test.

Carrageenan-induced rat paw oedema-acute protocol

The experiments were conducted according to the method described by Tratsk *et al.* (1997). Under light anaesthesia with oxygen (3%) and isoflurane (2%), the animals received an intradermal (i.d.) injection in the right hindpaw of saline (0.9%) containing carrageenan (300 μg per paw; 100 μL). As a control, the contralateral paw (left paw) received 100 μL of saline. Oedema was measured by means of a plethysmometer (Ugo Basile) at several time points after carrageenan injection (30, 60, 120 and 240 min). Oedema is expressed in mL as the difference between the right and left paws.

In this model, two distinct schedules of treatment have been adopted. In the prophylactic scheme, the animals were

pretreated with IndOH-NC group or indomethacin in solution (solubilized in calcium carbonate 3%) (IndOH group) (both at 1 mg·kg^{-1}, i.p.), 30 min before carrageenan injection. In the therapeutic scheme, the animals received IndOH or IndOH-NC (1 mg·kg^{-1}, i.p.), 60 min after the injection of carrageenan. The control groups received the vehicle solutions: calcium carbonate 3% (control group) or unloaded nanocapsules (NC group) (1 mL·kg^{-1}, i.p.), at the same schedules of administration.

Complete Freund's adjuvant (CFA)-induced rat paw oedema – sub-chronic protocol

The protocol used was similar to that described by Stein *et al*. (1988), with minor modifications. Briefly, isoflurane-anaesthetized animals received an i.d. injection in one hindpaw (right paw) of CFA (1 mg·mL^{-1}; 100 μL; heat-killed and dried *Mycobacterium tuberculosis*, each millilitre of vehicle containing 0.85 mL paraffin oil plus 0.15 mL mannide monooleate), which was suspended in a 1:1 oil/saline emulsion (in a total volume of 200 μL per paw). As a control, the contralateral paw (left paw) received 200 μL of saline. In this model, the animals were treated with IndOH or IndOH-NC (1 mg·kg^{-1}, i.p.), 2 h post-CFA injection, and once a day for 3 days. The control groups received the corresponding vehicle solutions at the same intervals of time. The oedema was measured by using a plethysmometer (Ugo Basile) at several time points following CFA injection (2, 4, 6, 8, 24, 48 and 72 h), and it is expressed in mL as the difference between the right and left paws.

CFA-elicited oedema – arthritis model

The adjuvant-induced arthritis model employed in the present study was similar to that described by Lorton *et al*. (2000), with some modifications. For this purpose, the oedema was induced by CFA injection, as indicated above, and it was assessed daily in a plethysmometer, between days 14 and 21 post-CFA administration. Animals were treated with IndOH or IndOH-NC (1 mg·kg^{-1}, i.p.), twice a day, for 8 days, starting at the 14th day of CFA injection, until the 21st day. Control groups received the respective vehicle solutions.

Determination of cytokine levels in serum

In the arthritis group, the animals were killed on the 21st day by isoflurane inhalation, and blood samples were collected by cardiac puncture. The blood samples were centrifuged at 1300 *g* at 4°C for 10 min. The supernatant was rapidly frozen and stored at –70°C for later measurement of tumour necrosis factor α (TNF-α), interleukin (IL)-6 and IL-10 levels using specific enzyme-linked immunosorbent assay (ELISA) kits, according to the recommendations of the supplier (R&D Systems).

Evaluation of gastrointestinal damage

The occurrence of gastrointestinal lesions was evaluated in the arthritis group, at the end of experiments (21 days after arthritis induction by CFA). For this purpose, rats were killed, and the intestine (duodenum, jejunum and ileum) was slit open opposite the attached mesenteric tissue. The organs were washed with saline and the mucosal surfaces were macroscopically examined according to an arbitrary scale previously reported (Guterres *et al*., 2001). Accordingly, the number and the gravity of erosions were scored on a scale of five grades: grade 0, no lesion; grade 0.5, hemorrhagic point; grade 1, ulcer length <2 mm; grade 2, ulcer length >2 mm; grade 3, lesion with perforation and haemorrhage. Experimental data were obtained by multiplying the score by the number of lesions. The mean scores for each group were calculated and expressed as lesion indexes.

Statistical analysis

The results are presented as the mean ± SEM of 5–8 animals. The statistical significance between groups was assessed by means of one-way analysis of variance (ANOVA) followed by Tukey's test. *P*-values less than 0.05 ($P < 0.05$) were considered significant. The percentages of inhibition between groups IndOH and IndOH-NC were determined in percentage, on the basis of the area under the curve. The statistical significance between groups was assessed by means of unpaired Student's *t*-test. *P*-values less than 0.05 were considered significant.

Results

Physico-chemical characterization of nanocapsule formulations

The nanocapsule formulations were prepared by interfacial deposition of polymer without the need of any subsequent step of purification. IndOH-NC and unloaded nanocapsules presented a macroscopic homogeneous aspect, such as white bluish opalescent liquids. After preparation, the average particle sizes were 240 nm (IndOH-NC) and 226 nm (unloaded nanocapsules). The suspensions showed monomodal size distributions and polydispersity indexes lower than 0.19, indicating narrow size distributions. The pH values were 5.95 (IndOH-NC) and 6.05 (unloaded nanocapsules). The zeta potential values were –6.9 and –7.3 mV respectively. The indomethacin content was 0.991 ± 0.012 mg·mL^{-1} and the encapsulation efficiency was close to 100%.

Carrageenan-induced paw oedema – acute protocol

We firstly examined the effects of IndOH or IndOH-NC treatment, on the oedema induced by carrageenan. The results demonstrated that prophylactic administration of IndOH or IndOH-NC (1 mg·kg^{-1}, i.p., 30 min before carrageenan) markedly inhibited the oedema induced by carrageenan, when compared with the respective control groups, with inhibition of 61 ± 4% and 63 ± 3% respectively (Figure 1). In addition, the oedema elicited by i.d. injection of carrageenan into the rat paw was significantly reduced by the therapeutic administration of IndOH or IndOH-NC (1 mg·kg^{-1}, i.p.) administered 60 min after carrageenan, with inhibition of 31 ± 11% and 44 ± 7% respectively (Figure 2). Comparison of the inhibition observed for IndOH and IndOH-NC did not reveal any significant difference in the effect of the tested formulations

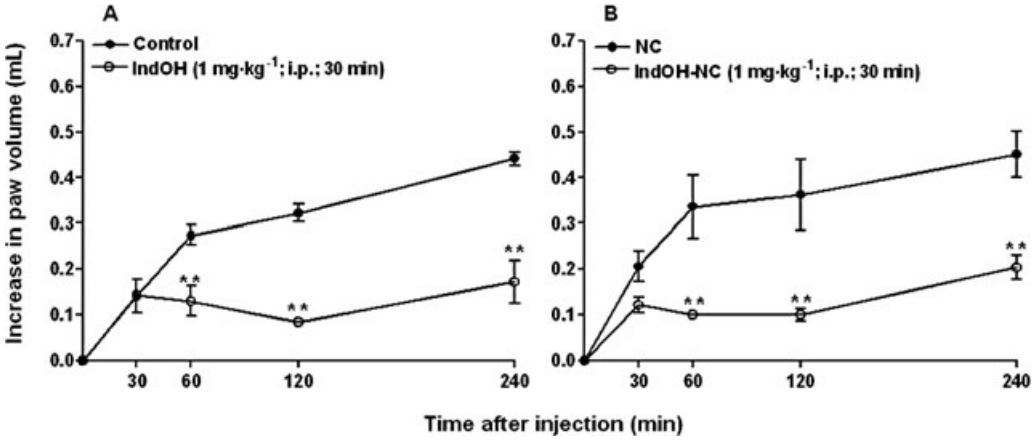

Figure 1 Effect of indomethacin-loaded nanocapsules (IndOH-NC, 1 mg·kg^{-1}, i.p., 30 min before), on rat paw oedema induced by carrageenan (300 μg·paw^{-1}, acute model – prophylactic treatment). (A) Indomethacin in solution (IndOH) and calcium carbonate 3% (Control); (B) Indomethacin-loaded nanocapsules (IndOH-NC) and unloaded nanocapsules (NC). Each point represents the mean of 6–8 animals and vertical lines show the SEM. Asterisks denote the significance levels in comparison to respective control values: $^{**}P < 0.01$.

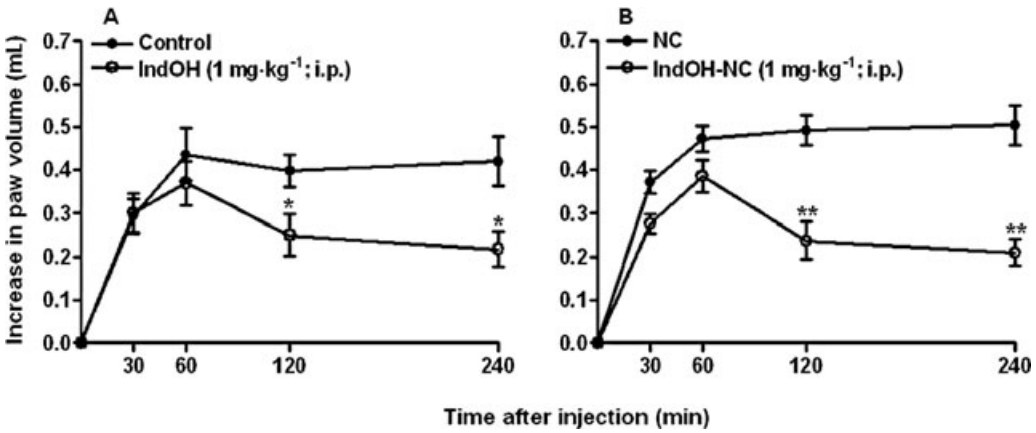

Figure 2 Effect of indomethacin-loaded nanocapsules (IndOH-NC, 1 mg·kg^{-1}, i.p., after 60 min), on rat paw oedema induced by carrageenan (300 mg paw^{-1}, acute model – therapeutic treatment). (A) Indomethacin in solution (IndOH) and calcium carbonate 3% (Control); (B) Indomethacin-loaded nanocapsules (IndOH-NC) and unloaded nanocapsules (NC). Each point represents the mean of 6–8 animals and vertical lines show the SEM. Asterisks denote the significance levels in comparison to respective control values: $^{*}P < 0.05$, $^{**}P < 0.01$.

of indomethacin, in either the prophylactic or therapeutic schedules of treatment ($P > 0.05$) (Figures 1 and 2).

CFA-induced rat paw oedema – sub-chronic protocol

In this experimental set, we compared the effects of IndOH and IndOH-NC in a short period of evaluation following CFA application (until 3 days). The results depicted in Figure 3 show that administration of IndOH or IndOH-NC (1 mg·kg^{-1}, i.p., 2 h after induction of paw oedema, and once a day, for 3 days) significantly reduced the oedema induced by CFA injection, according to assessment in the sub-chronic protocol. The calculated inhibition was 21 ± 2% (IndOH) and 33 ± 4% (IndOH-NC) and these values were significantly different ($P < 0.05$; Figure 3).

CFA-elicited oedema – arthritis model

It is well known that CFA injection into the rat paw evokes a marked and time-related local oedema, which is observed as early as 2 h after and persists for up to 28 days, and presenting signs of systemic alterations. Hence, assessment of CFA-induced oedema in the later phases (after 14 days) is widely adopted as an arthritis model (Lorton *et al.*, 2000). In this study, animals received either IndOH or IndOH-NC (1 mg·kg^{-1}, i.p., twice a day, for 8 days), between the 14th and the 21st day after CFA injection. Both indomethacin formulations were able to significantly reduce the long-term oedema caused by CFA. In these experiments, IndOH-NC exhibited a greater inhibition (35 ± 2%), than IndOH (14 ± 2%) ($P < 0.01$) (Figure 4).

Determination of cytokine levels in serum

The serum of animals in the arthritis group was collected at 21 days after CFA injection, and it was used for determining effects of the different indomethacin formulations on the systemic alterations of cytokine levels. The results demonstrate that the treatment with IndOH-NC (1 mg·kg^{-1}, i.p., twice a day, for 8 days), between the 14th and the 21st day

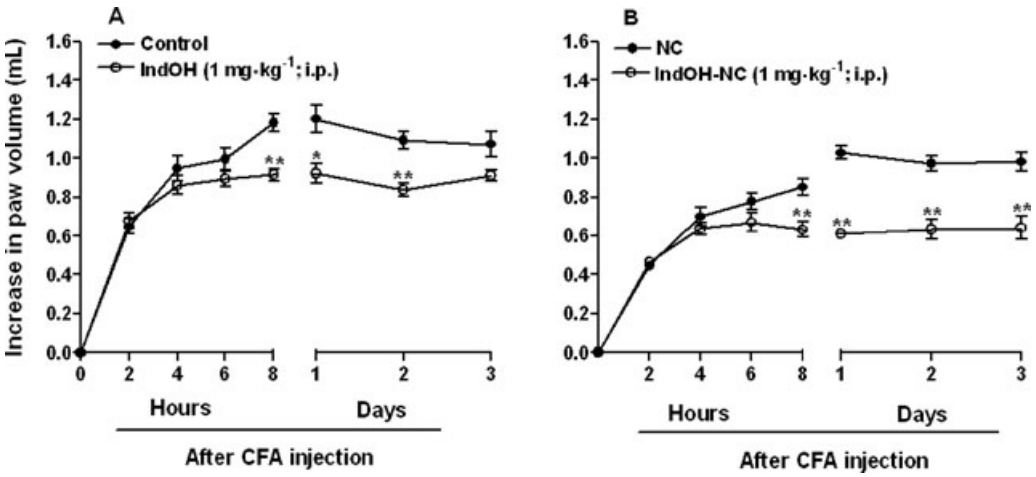

Figure 3 Effect of indomethacin-loaded nanocapsules (IndOH-NC, 1 mg·kg^{-1}, i.p. daily, 2 h after induction of paw oedema by CFA, for 3 days), on rat paw oedema induced by CFA (sub-chronic model). (A) Indomethacin in solution (IndOH) and calcium carbonate 3% (control); (B) Indomethacin-loaded nanocapsules (IndOH-NC) and unloaded nanocapsules (NC). Each point represents the mean of 6–8 animals and vertical lines show the SEM. Asterisks denote the significance levels in comparison to respective control values. *$P < 0.05$, **$P < 0.01$.

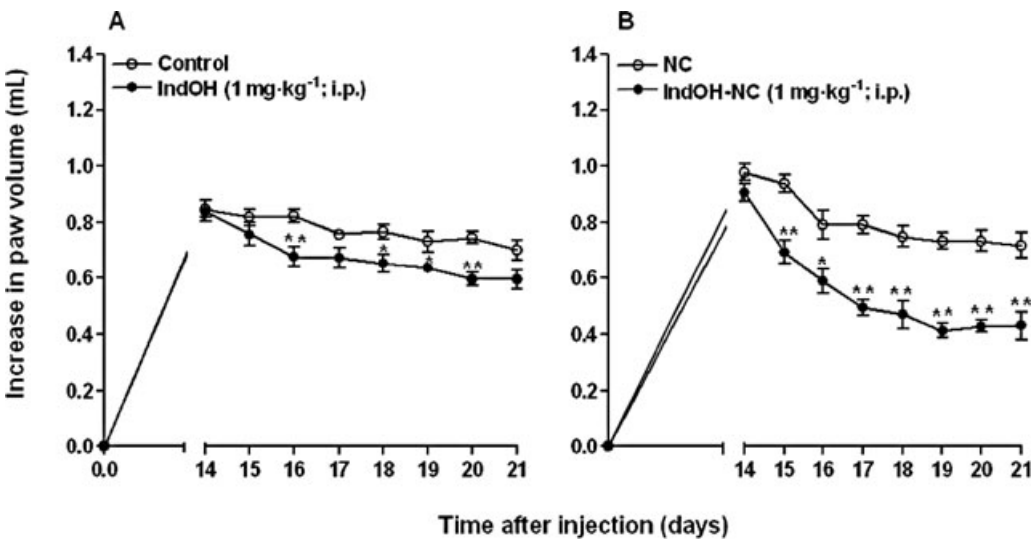

Figure 4 Effect of indomethacin-loaded nanocapsules (IndOH-NC, 1 mg·kg^{-1}, i.p., 14 days after induction of paw oedema, twice a day, for 8 days), on rat paw oedema induced by CFA (arthritis model). (A) Indomethacin in solution (IndOH) and calcium carbonate 3% (control); (B) Indomethacin-loaded nanocapsules (IndOH-NC) and unloaded nanocapsules (NC). Each point represents the mean of 6–8 animals and vertical lines show the SEM. Asterisks denote the significance levels in comparison to respective control values. *$P < 0.05$, **$P < 0.01$.

after CFA injection, produced a striking inhibition in the production of the pro-inflammatory cytokines TNF-α and IL-6 in serum of CFA-injected rats, by 83 ± 8% and 84 ± 11% respectively (Figure 5A and B). Furthermore, the same treatment with IndOH-NC induced a marked increase of the anti-inflammatory cytokine IL-10 by 196 ± 55% (Figure 5C). Conversely, the administration of IndOH (at the same schedule of administration) failed to significantly alter the systemic production of all analysed cytokines (Figure 5).

Evaluation of gastrointestinal damage

This series of experiments was designed to evaluate the gastrointestinal toxicity of IndOH-NC in relation to IndOH, after the long-term administration of both formulations. For the first time, the efficacy and the toxicity were determined using the same groups treated with nanoencapsulated NSAIDs. For this purpose, the intestines in the arthritis group of rats (killed at 21 days) were analysed and the indices of damage were determined separately for duodenum, jejunum and ileum. As shown in the Figure 6, the lesion indices in the animals treated with IndOH-NC were significantly reduced when compared with the IndOH group, by 58 ± 16%, 72 ± 6% and 69 ± 2%, for duodenum, jejunum and ileum respectively. When the total lesion index was calculated (i.e. the total score for the three intestinal regions) the reduction was 68 ± 5% in the IndOH-NC group, compared with the IndOH-treated rats (Figure 6). The animals treated with NC presented a low but significant increase (10 ± 2%) in the total lesion indexes when compared with the control group (Figure 6).

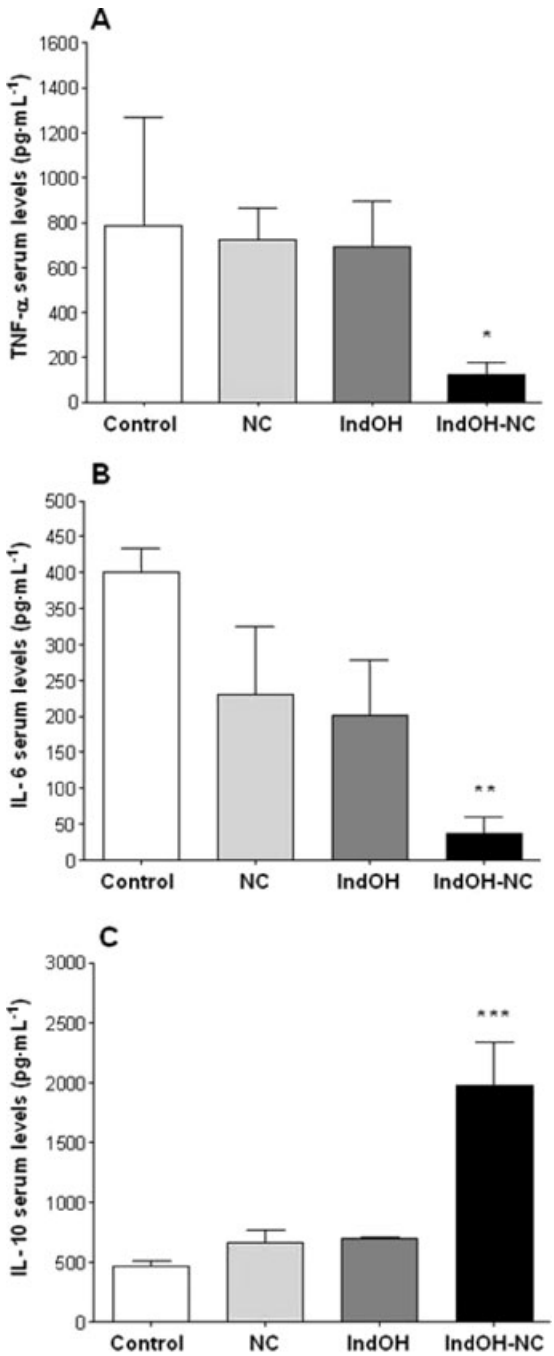

Figure 5 Effect of indomethacin-loaded nanocapsules (IndOH-NC) (1 mg·kg^{-1} IndOH-NC i.p., 14 days after induction of paw oedema, twice a day, for 8 days) on (A) TNF-α and (B) IL-6 and IL-10 levels in serum of animals in the arthritis model. Each point represents the mean of 5–8 animals and vertical lines show the SEM. Significantly different from the control, unloaded nanocapsules (NC) and indomethacin in solution (IndOH) groups for *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Discussion

The present study was conducted to investigate the potential actions of IndOH-NC in experimental models of inflammation in rats. To this end, three classical models of infmallation *in vivo* were employed to evaluate the short and long-term effects of IndOH-NC, in comparison with IndOH: carrageenan-induced acute oedema, CFA-induced sub-chronic inflammation and CFA-induced arthritis. We have also attempted to compare the gastrointestinal toxicity found in rats chronically treated with either IndOH-NC or IndOH.

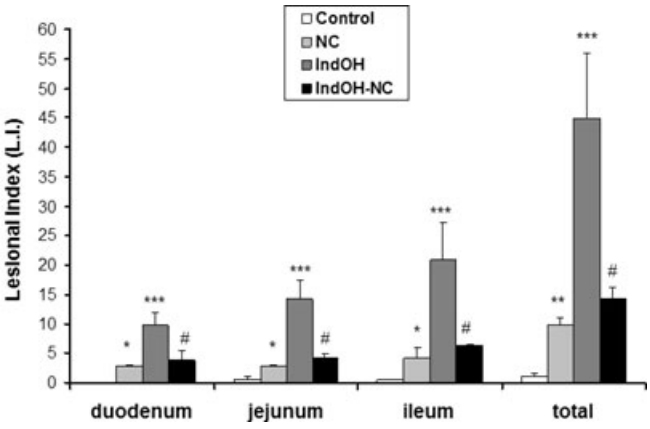

Figure 6 Effect of indomethacin-loaded nanocapsules (IndOH-NC) (1 mg·kg^{-1} IndOH-NC i.p. every 12 h, 14 days after induction of paw oedema, for 8 days) on intestine lesional index (LI) of animals in the arthritis group. Each point represents the mean of 6–8 animals and vertical lines show the SEM. *Significantly different from the control group ($P < 0.05$). **Significantly different from the control group ($P < 0.01$). ***Significantly different from the control and unloaded nanocapsules (NC) groups ($P < 0.001$). #Significantly different from the indomethacin in solution group (IndOH) ($P < 0.001$).

The injection of carrageenan into the rat hindpaw represents a model commonly employed to study acute inflammation and pain. The application of carrageenan causes a rapid formation of oedema, allied to an exacerbated sensitivity to thermal and mechanical stimuli (Rocha *et al.*, 2006). In this regard, carrageenan-induced rat paw oedema is widely used to characterize the mechanisms of action of new anti-inflammatory drugs or formulations, including NSAIDs (Velo *et al.*, 1973; Kawamura *et al.*, 2000; Quintão *et al.*, 2005). We assessed the effects of IndOH-NC in comparison to IndOH, when both formulations were dosed by two distinct schedules of administration, before (prophylactic) or after (therapeutic), the i.d. injection of carrageenan. Our results indicate that IndOH-NC displays an anti-inflammatory efficacy, which is similar to that observed for IndOH, according to assessment in both regimens of treatment. However, no significant difference was observed between the anti-inflammatory efficacy for IndOH-NC and IndOH in the carrageenan acute model of inflammation.

Considering the kinetic properties of polymeric nanocapsules, we decided to investigate whether IndOH-NC might exhibit increased efficacy in long-term models of inflammation. First, we have assessed its effects in the sub-chronic model of inflammation induced by CFA, in which the oedema was measured until 3 days after the application of the inflammatory agent. This experimental set revealed that IndOH-NC presented a significantly higher efficacy in comparison to IndOH. This encouraging result prompted us to test the anti-inflammatory efficacy of IndOH-NC in an experimental model of clinical relevance: CFA-induced arthritis. Repeated treatment with IndOH-NC produced a marked inhibition of

CFA-induced long-term oedema formation (between 14 and 21 days), which was significantly greater than that obtained with IndOH. One plausible explanation for these effects is that, the nanoencapsulation improves drug efficacy and drug bioavailability (Couvreur *et al*., 2002; Schaffazick *et al*., 2003) by providing a more sustained drug release to the inflamed site, according to evaluation in the CFA-arthritis model. It is important to note that the dose of indomethacin used in the present study (1 mg·kg^{-1}) is sub-therapeutic, showing that anti-inflammatory actions of IndOH-NC were noticeably enhanced when compared with the same dose of indomethacin in solution.

It is well known that lowered pH is one of the hallmarks of rheumatoid arthritis (Andersson *et al*., 1999; Levick, 1990). This pH decline may lead a delay in the indomethacin release from the nanocapsules, enhancing its anti-inflammatory effect. Furthermore, plasma protein binding is known to limit indomethacin cellular uptake, by reducing the free fraction of the drug in the circulation (Parepally *et al*., 2006). In this regard, some characteristics of nanoparticulated systems such as the carrier size, the polymer type, as well as their surface features, might induce steric stabilization of nanoparticles, thus inhibiting protein binding and increasing blood circulation time (Brigger *et al*., 2002; Brioschi *et al*., 2007). In this context, a recent publication (Zhang *et al*., 2007) revealed that indomethacin concentrations in plasma were prolonged in the group treated subcutaneously with indomethacin-loaded micelles, compared with indomethacin in aqueous solution. Furthermore, the nanoparticles can accumulate in inflamed tissues due to the greater microvascular permeability in those sites. Additionally in our study, the polymeric nanocapsules were prepared with polysorbate 80, a hydrophilic coating able to delay the protein plasma binding, increasing the particle blood circulation time. Accordingly, all of these factors might well have contributed to the increased efficacy of IndOH-NC observed in the present study.

As reported in the literature, CFA injection can elicit the release of a series of inflammatory mediators, including cytokines. Cytokine production is an important event related to the onset and/or maintenance of inflammatory diseases, such as asthma, arthritis, sepsis and inflammatory bowel disease, among others (Laufer, 2003; Meyer, 2003; Stokkers and Hommes, 2004; Ulloa and Tracey *et al*., 2005; Woodfolk, 2006). Herein, we sought to determine whether the anti-inflammatory effects of IndOH-NC in the CFA-arthritis model, were associated with changes in cytokine generation. Interestingly, our data demonstrate that treatment with IndOH-NC was able to produce a significant decrease of the pro-inflammatory cytokines TNF-α and IL-6, in the serum of arthritic rats. More relevantly, the administration of IndOH-NC also induced a marked increase in the serum levels of the anti-inflammatory cytokine IL-10. Conversely, no significant effect on cytokine production was observed when rats were treated with indomethacin in solution. Thus, on the basis of this series of results, it is possible to infer that increased efficacy of IndOH-NC in comparison to free IndOH, is likely to be related to its ability to alter cytokine production in the inflammatory scenario.

Treatment with NSAIDs has been associated with development of adverse and severe gastrointestinal effects (Asako *et al*., 1992; Tries *et al*., 2002). Accordingly, another important aspect assessed in our study was the gastrointestinal toxicity of IndOH-NC, when dosed in a chronic schedule of administration. Present data clearly demonstrated that animals treated with IndOH-NC showed a significant reduction of intestinal lesion indices, when compared with the animals that received indomethacin in solution. This allows us to suggest that IndOH-NC formulation displayed a lower level of adverse effects than that after IndOH, presenting a desirable, increased gastrointestinal tolerance. Surprisingly, the animals treated with NC also exhibited a significant increase in the intestinal lesion indices when compared with the control group. As previously reported by our group, acute treatment with the NC formulation did not present a significant gastrointestinal toxicity (Guterres *et al*., 2001; Schaffazick *et al*., 2003). It is important to note that in the present study the animals were treated chronically (1 mg·kg^{-1} i.p. every 12 h for 8 days). Therefore, the low gastrointestinal toxicity caused by NC formulations was probably due to this prolonged period of treatment. A relevant point to be discussed is that cytokines might exhibit an important role in mucosal defence (Robinson *et al*., 2008). Therefore, the reduction of TNFα and IL-6 production, associated with the elevation of IL-10 levels might well contribute to the reduced gastrointestinal toxicity observed in the IndOH-NC-treated group.

In summary, the data reported herein clearly demonstrate that polymeric nanocapsules are able to successfully carry indomethacin into the inflammatory sites. Of note, in long-term models of inflammation following CFA injection, IndOH-NC presented an increased anti-inflammatory efficacy, allied to an improved gastrointestinal safety. Thus, the present findings allow us to suggest that IndOH-NC might constitute a relevant and apparently gastrointestinal safe therapeutic alternative for the treatment of chronic inflammatory diseases, such as rheumatoid arthritis with NSAIDs.

Acknowledgements

This study was supported by the Brazilian agencies: CNPq/Brazil (Edital Universal), FINEP, Rede Nanocosméticos/CNPq-MCT. A.B. and E.J. were recipients of Brazilian Capes and CNPq fellowships. A.C.C.V.Z. was recipient of a Brazilian BPA-PUCRS fellowship. The authors thank Juliano Soares for his excellent technical assistance.

Conflict of interest

None.

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DISCUSSÃO

Nos últimos anos, materiais biodegradáveis nanoestruturados para aplicações nas diversas áreas biomédicas, principalmente como carreadores de fármacos, biossensores e biomarcadores tem sido foco de intenso estudo (Emerich et al., 2006; Emerich et al., 2007). Na área farmacêutica os principais objetivos com carreadores de fármacos concentram-se em aumentar o controle da liberação, a especificidade e a seletividade ao local de ação do fármaco, bem como a diminuição da dose e de seus efeitos colaterais (Couvreur & Vauthier, 2006). O uso de carreadores de fármacos está direcionado principalmente para o desenvolvimento de novas terapias contra o câncer, principalmente naqueles onde o tratamento ainda é muito limitado, como é o caso dos tumores do SNC.

Os gliomas destacam-se entre os tumores como sendo os mais refratários frente aos tratamentos, mesmo com o contínuo avanço da radioterapia, quimioterapia e técnicas cirúrgicas (Ohgaki & Kleihues, 2009). Dessa forma, novas estratégias terapêuticas se fazem necessárias.

Estudos recentes realizados pelo nosso grupo de pesquisa demostraram que a indometacina exerce um efeito antiproliferativo em linhagens celulares de gliomas através de uma parada na progressão do ciclo celular (Bernardi et al., 2006). Além disso, demostrou-se que este efeito antiproliferativo da indometacina é mediado, pelo menos em parte, pelo aumento no catabolismo de purinas extracelulares (Bernardi et al., 2007). Considerando a possibilidade de um potencial uso clínico da indometacina na terapia dos gliomas, o principal aspecto limitante dessa terapia seria a dificuldade para atingir as células tumorais devido às limitações impostas pela BHE, uma vez que, após administrado, mais de 90% deste fármaco liga-se a

proteínas plasmáticas reduzindo a fração livre do fármaco na circulação (Parepally et al., 2006).

Nesse contexto, aliado às inúmeras vantagens que a nanobiotecnologia pode oferecer, a nossa hipótese é que nanocápsulas poliméricas seriam capazes de vetorizar a indometacina às células tumorais, sendo, portanto essa formulação mais eficaz em reduzir o crescimento de gliomas quando comparado com o fármaco não vetorizado.

Para validar nossa hipótese, primeiramente comparamos o efeito citotóxico da indometacina em nanocápsulas com o efeito da indometacina em solução em linhagens celulares de gliomas. As linhagens de gliomas, apesar de apresentarem algumas limitações, têm sido extensivamente utilizadas como modelos para estudos de gliomas *in vitro* com as mais diversas abordagens. Essas células são consideradas modelos adequados de estudo uma vez que apresentam diversas características biológicas dos tumores *in vivo*, como alto poder proliferativo, invasivo e expressão de proteínas específicas, sendo representativas de GBM (Grobben et al*.,* 2002).

Nossos resultados mostram que a indometacina, quando nanoencapsulada, foi pelo menos 2 vezes mais citotóxica, sendo mais eficaz em diminuir a proliferação e viabilidade celular de linhagens de gliomas quando comparada com o mesmo fármaco em solução (Capítulo 1, Figuras 1A e 2A, Tabela 1). Para melhor compreender esse efeito do tratamento com indometacina em nanocápsulas, utilizamos, nas mesmas condições experimentais, nanocápsulas contendo éster etílico de indometacina. O éster etílico de indometacina tem sido muito utilizado na literatura como uma ferramenta para elucidar o mecanismo de liberação de fármacos das nanocápsulas (Jäger et al., 2007; Cruz et al., 2006; Pohlmann et al.,

2004). Isso se deve principalmente às diferenças na sua cinética de liberação (Cruz et al., 2006). Enquanto a indometacina encontra-se majoritariamente adsorvida na matriz polimérica, sendo liberada em poucos minutos, o éster etílico encontra-se predominantemente no núcleo da nanocápsula, precisando de 24h para ser totalmente liberado para o meio externo. Essas diferenças na cinética de liberação explicam os resultados obtidos com as linhagens de gliomas, de modo que o tratamento com o éster etílico de indometacina em nanocápsulas foi menos eficaz do que o tratamento com éster etílico em solução (Capítulo 1, Figuras 1B e 2B, Tabela 1). Um curto período de exposição das células (1 e 3h) às formulações confirmam esses resultados, sugerindo que as formulações não exercem uma resposta temporal, sendo necessário apenas o período de 1h para as nanocápsulas serem internalizadas pelas células e assim desencadear os efeitos observados após 48 h (Capítulo 1, Figura 3). Ainda considerando as diferenças na cinética de liberação, nós decidimos investigar se a indometacina e o éster etílico de indometacina poderiam exercer um efeito sinérgico nas células de gliomas. Para tanto, utilizamos nanocápsulas que possuiam simultaneamente indometacina e éster etílico de indometacina. Quando as células foram tratadas com esta formulação, a redução do número de células foi sinergisticamente maior quando comparado com as formulações que continham apenas indometacina ou éster etílico de indometacina na mesma concentração (Capítulo 1, Figura 4). O efeito sinérgico observado poderia ser explicado pelo fato da indometacina ser liberada inicialmente causando um efeito antiproliferativo e, a liberação prolongada e sustentada do éster etílico de indometacina, que é hidrolisado formando indometacina, ser capaz de sustentar esse efeito antiproliferativo.

Considerando a inespecificidade da maioria dos agentes antitumorais e consequentemente os efeitos adversos que são decorrentes da quimioterapia, nós avaliamos se o efeito citotóxico das formulações aqui estudadas seria seletivo para as células tumorais. Para tanto, utilizou-se culturas organotípicas de hipocampo de ratos como modelo de tecido não-tumoral. O modelo de cultura organotípica foi desenvolvido em 1981 por Gähwiler e modificado por Stoppini e colaboradores em 1991. Esse modelo é uma ferramenta adequada para o estudo de novos compostos farmacológicos uma vez que se aproxima das condições observadas *in vivo*, mantendo as características fisiológicas, a arquitetura celular e as conexões intercelulares (Stoppini et al., 1991; Xiang et al., 2000; Holopainen, 2005). Nossos resultados mostraram que a exposição das culturas organotípicas às formulações de nanocápsulas contendo indometacina ou éster etílico de indometacina não provocou dano celular ao tecido não-tumoral, enquanto que nas mesmas condições experimentais essas formulações causaram significativa morte celular das células de glioma em cultura (Capítulo 1, Figuras 5 e 6). É importante ressaltar que a concentração em que a cultura organotípica foi exposta (50 μM) é 10 vezes maior que a concentração que foi citotóxica (5 μM) para as linhagens de gliomas, sugerindo uma citotoxicidade seletiva dessa formulação para as células tumorais (Capítulo 1, Figuras 5 e 6).

Realizados esses experimentos iniciais com modelos *in vitro*, seguimos o nosso estudo avaliando o efeito do tratamento sistêmico com indometacina em nanocápsulas em modelo *in vivo* de gliomas implantados em cérebro de ratos. O modelo de implante de células C6 de glioma em cérebro de ratos foi primeiramente proposto por Takano e colaboradores (2001) e é adequado para estudos que investigam a biologia desses tumores, bem como o estudo de novas abordagens

terapêuticas no tratamento dos gliomas. A literatura relata que as células C6 de glioma implantadas em cérebro de ratos *Wistar* e *Sprague-Dawley* expressam vários fatores de crescimento como FGF, EGF, PDGF e VEGF e seus receptores, apresentando significativamente a capacidade proliferativa e o poder de invasão (Chicoine & Silbergeld, 1995; Takano et al., 2001; Auguste et al., 2001). Em estudo prévio realizado em nosso laboratório, validou-se esse modelo para o estudo de drogas com potencial ação quimioterápica, uma vez que ratos tratados com temozolomida, fármaco de escolha para o tratamento de gliomas, apresentaram um aumento significativo da sobrevida (Morrone et al., 2006).

No presente estudo, utilizando modelo *in vivo* de gliomas, as células foram implantadas no *striatum* dos animais e após 10 dias do implante do tumor, a indometacina em nanocápsulas foi administrada intraperitonealmente durante 10 dias consecutivos. Através da análise histopatológica das lâminas de H&E foi possível observar que os animais tratados com concentrações sub-terapêuticas de indometacina em nanocápsulas apresentaram uma significativa redução no volume do tumor (Capítulo 2, Figura 1). Outro aspecto importante é que os tumores apresentaram características histopatológicas que são semelhantes àquelas encontradas nos GBMs de pacientes, como crescimento nos espaços intraventricular e intraparenquimal, além do elevado índice proliferativo, características de pseudopaliçada periférica, proliferação vascular, edema, necrose coagulativa, dentre outros (Capítulo 2, Tabela 2 e Figura 2). É importante ressaltar que o tratamento com indometacina em nanocápsulas, além de reduzir o tamanho do tumor, foi capaz de reduzir as características histopatológicas de malignidade dos tumores como as características de pseudopaliçada periférica, índice mitótico, necrose, hemorragia intratumoral e proliferação vascular (Capítulo 2, Tabela 2 e

Figura 2). Esses resultados foram observados apenas quando os animais foram tratados com indometacina em nanocápsulas, sendo que o tratamento com indometacina em solução não causou alterações significativas em relação aos animais controles (Capítulo 2, Figura 2, Tabela 2). Além disso, um dado importante foi que apenas 50% dos animais tratados com indometacina em nanocápsulas apresentaram no tecido cerebral uma massa tumoral possível de ser analisada e quantificada pela coloração de H&E (Capítulo 2, Tabela 2). Os outros 50% dos animais apresentaram um tecido cerebral que continha células tumorais isoladas com características semelhantes a tumores residuais. Para confirmar a hipótese de que os tumores teriam involuído com o tratamento de indometacina em nanocápsulas e que estas células seriam células tumorais, realizou-se uma análise de imunohistoquímica com a nestina, uma proteína de citoesqueleto, característica de células tronco-neurais e GBMs (Dahlstrand et al., 1992; Wiese et al., 2004; Schiffer et al., 2006). Embora seja expressa apenas em uma subpopulação de células de GBM, a nestina é considerada um marcador específico para este tipo de tumor, estando relacionada com o potencial tumorigênico (Singh et al., 2004; Mao et al., 2007; Ma et al., 2008). Além disso, Stronjinik e colaboradores relatam que a expressão de nestina está diretamente relacionada com o aumento do grau de malignidade dos gliomas, podendo ser relacionada com a sobrevida dos pacientes (Stronjinik et al., 2001). Além disso, as células nestina positivas estariam localizadas principalmente na periferia da massa tumoral, estando relacionada com a capacidade de invadir o tecido normal adjacente (Stronjinik et al., 2007; Maderna et al., 2007). Nossos resultados mostraram que as células que apresentaram características de tumor residual apresentaram imunodetecção positiva para nestina que, embora menos intensa que nos tumores dos animais controles, confirmaram a

nossa hipótese de que seriam células de GBM que involuíram com o tratamento com indometacina em nanocápsulas (Capítulo 2, Figura 3).

Como já citado na introdução desta tese, dentre as várias características envolvidas na agressividade dos gliomas, pode-se destacar a proliferação descontrolada e a angiogênese que esses tumores apresentam (Konopka & Boni, 2003). Muitas estratégias terapêuticas têm sido desenvolvidas na tentativa de inibir a angiogênese em gliomas como o bloqueio na produção de fatores de crescimento, a neutralização de fatores de crescimentos circulantes, a inibição da ativação de receptores tirosina-cinases e consequente inibição das vias de sinalização que levam à proliferação celular (Lakka et al., 2008; Wong et al., 2009). Através da técnica de imunohistoquímica para Ki67 e VEGF, marcadores de proliferação e angiogênese, respectivamente, nossos resultados demonstraram que a indometacina em nanocápsulas exerce efeito antiproliferativo e anti-angiogênico nos gliomas enquanto a indometacina em solução não apresentou efeitos significativos (Capítulo 2, Figura 4, Tabela 3). A redução na marcação com Ki67 nos tumores dos animais tratados com indometacina em nanocápsulas confirmam os resultados apresentados no presente trabalho onde se observou uma redução significativa no índice mitótico através da quantificação por H&E (Capítulo 2, Tabela 1). Os efeitos farmacológicos antiproliferativo e anti-angiogênico são fundamentais na terapia anti-glioma, uma vez que as propriedades invasivas desses tumores são dependentes da presença de novos vasos e da capacidade proliferativa das células (Kim & Lee, 2009). A diminuição na neovascularização poderia representar um suprimento vascular insuficiente que, dessa forma, poderia ser um fator limitante para o crescimento tumoral. De acordo com dados da literatura, a angiogênese é determinada pelo balanço entre fatores pró- e anti-angiogênicos que são secretados

principalmente pelas próprias células tumorais e células endoteliais, dentre outras (Fukumura et al., 1998 ; Tandle et al., 2004 ; Kim & Lee, 2009). Em tumores sólidos incluindo os gliomas, a expressão de VEGF tem sido predominantemente maior em regiões adjacentes às regiões de necrose (Shweiki et al., 1992; Ferrara, 2004). Esses dados juntamente com outros trabalhos da literatura indicam que em regiões da massa tumoral onde há concentrações reduzidas de oxigênio, há a indução do fator-1 induzível por hipóxia que ativa a transcrição de genes que elevam à expressão de VEGF (Sheweiki et al., 1992; Maxwell et al., 1997, Vredenburgh et al., 2007). Assim, pode-se dizer que a angiogênese e a proliferação celular estão intimamente relacionadas, uma vez que VEGF é capaz de suprimir a expressão de proteínas pró-apoptóticas como *Bax* e, ao mesmo tempo, ativar a sinalização de vias de proliferação e sobrevivência celular como MAPK e PI3K/AKT (Wheeler-Jones et al., 1997 ; Gerber et al., 1998a; Gerber at al., 1998b). Dessa forma, a redução do volume do tumor e das características de malignidade observadas no presente estudo pode ser explicada pelos efeitos antiproliferativo e anti-angiogênico desencadeados pela indometacina em nanocápsulas. Além disso, é importante ressaltar que COX-2 e citocinas têm sido descritas como fatores que podem regular a expressão de VEGF em diferentes tumores (Williams et al., 1999; Kang et al., 2007). Considerando que os gliomas superexpressam COX-2 (Joki et al., 2000; Matsuo et al., 2001; Prayson et al., 2002) e que a indometacina é um potente inibidor dessa enzima, essa inibição poderia explicar, pelo menos em parte, o efeito anti-angiogênico aqui observado.

Considerando os importantes resultados observados até o momento com o tratamento dos animais com indometacina em nanocápsulas, nós investigamos a biodisponibilidade da indometacina no tecido cerebral. Através da análise por HPLC,

a indometacina foi quantificada no hemisfério ipsilateral e também no hemisfério contralateral ao implante do tumor. Nossos resultados mostraram que as nanocápsulas utilizadas foram capazes de vetorizar a indometacina para o tecido cerebral e que concentrações maiores do fármaco foram detectadas no hemisfério cerebral onde o tumor foi implantado (Capítulo 2, Figura 5). Além disso, é importante ressaltar que os animais tratados com indometacina em solução não apresentaram concentrações detectáveis do fármaco em ambos os hemisférios cerebrais (Capítulo 2, Figura 5). Com base nesses resultados, sugerimos que a indometacina em solução não foi eficaz em reduzir o tamanho do tumor por não ter conseguido atingir o tecido cerebral e consequentemente o tecido tumoral, provavelmente pelo fato de não ter sido capaz de atravesar a BHE. Os mecanismos pelos quais nanopartículas poliméricas atravessam a BHE ainda não são completamente elucidados. Entretanto, considerando as hipóteses sugeridas pela literatura e os resultados aqui obtidos, podemos sugerir alguns mecanismos. Considerando que o polisorbato 80 está presente na composição das nanocápsulas utilizadas no presente estudo, e que este é capaz de promover a adsorção de proteínas plasmáticas como a apolipoproteína E às nanocápsulas, nós sugerimos que as nanopartículas com apolipoproteína E adsorvida poderiam ser transportadas pelas células endoteliais da BHE através de endocitose receptor-mediada. Entretanto, não pode ser descartada a possibilidade de adesão das nanocápsulas nas células endoteliais da BHE e subsequente trancitose, a modulação das *tight junction* ou a inibição da glicoproteína-P (Calvo et al., 2001; Kreuter et al., 2003; Vauthier et al., 2003). O fato de a indometacina estar presente em maior concentração no hemisfério cerebral onde o tumor foi implantado pode ser explicado por vários motivos. Primeiramente, deve-se ressaltar que os fatores pró-angiogênicos secretados pelas células do

glioma, bem como o processo inflamatório que acompanha o crescimento tumoral, contribuem para alterações de permeabilidade microvascular, alterado a permeabilidade da BHE e facilitando dessa forma o direcionamento das nanopartículas para o tecido tumoral. Além disso, as diferenças na vasculatura e permeabilidade microvascular do tumor e do tecido saudável adjacente poderiam explicar o acúmulo das nanocápsulas no tecido tumoral, uma vez que devido ao tamanho reduzido, nanopartículas são capazes de penetrar em pequenos capilares. É importante ressaltar que o compartimento intersticial dos tumores sólidos é predominantemente constituído de colágeno, hialuronato e proteoglicanos, que formam um gel hidrofílico (Jain, 1987). Essas características poderiam explicar o acúmulo das nanocápsulas no tecido tumoral resultando no denominado “efeito de aumento de retenção e permeabilidade”. Além disso, considerando que as nanocápsulas utilizadas no presente estudo são constituídas de polímeros biodegradáveis, uma liberação sustentada do fármaco no sítio de ação poderia ter contribuído para os efeitos observados. A soma de todos esses fatores provavelmente contribuiu para a vetorização cerebral e consequentemente efeito antitumoral observado.

Uma vez que os agentes quimioterápicos utilizados na clínica são, na maioria das vezes, inespecíficos causando, portanto efeitos adversos aos tecidos normais, nós investigamos os potenciais efeitos adversos do tratamento com indometacina em nanocápsulas. Nossos resultados mostraram que o tratamento com indometacina em nanocápsulas não causou mortalidade nos animais e ainda impediu a perda de peso em decorrência do tumor, observada nos animais controles (Capítulo 2, Figura 6). A necropsia dos animais ao término dos tratamentos não demonstrou alterações macroscópicas nos órgãos examinados (fígado, estômago,

rim, coração e pulmão). Além disso, a análise microscópica desses órgãos através da coloração de H&E indicou ausência de toxicidade. A ausência de toxicidade hepática foi confirmada pela dosagem das enzimas alanina aminotrasferase, aspartato aminotransferase, fosfatase alcalina e gama-glutamil transferase. Assim, pode-se afirmar que os efeitos da indometacina em nanocápsulas, pelo menos na concentração utilizada no presente estudo e nestas condições experimentais, exerceram citotoxicidade seletiva para as células tumorais.

Um resultado bastante promissor do presente trabalho foi que o tratamento com indometacina em nanocápsulas foi capaz de aumentar a sobrevida dos animais (Capítulo 2, Figura 7). O aumento da sobrevida é um objetivo bastante almejado no tratamento de pacientes com gliomas, uma vez que ainda não existe cura para essa doença. Apesar dos grande avanços em neuroradiologia, neurocirurgia, radioterapia e quimioterapia, a sobrevida média dos pacientes com gliomas pouco se alterou nos últimos 30 anos (Bondy et al., 2008). Dessa forma, este resultado pode ser considerado bastante promissor.

Assim, o conjunto de resultados obtidos nestes dois primeiros capítulos desta tese nos mostram que a indometacina foi vetorizada ao tecido cerebral pelas nanocápsulas, sendo detectada majoritariamente no tecido tumoral, exercendo portanto um efeito citotóxico seletivo. Os resultados observados são bastante promissores, considerando as limitações das atuais terapias farmacológicas para gliomas. Além disso, estamos aliando as inúmeras vantagens que as nanopartículas oferecem quando se busca uma vetorização de fármacos à minimização dos efeitos adversos de um fármaco que já possui dados farmacocinéticos bastante conhecidos por ser amplamente utilizado na terapêutica com outras finalidades.

Como já mencionado na Introdução desta tese, o tratamento de doenças neurodegenerativas é outro grande desafio devido principalmente às limitações impostas BHE. Considerando os resultados obtidos no Capítulo 2, onde as nanocápsulas poliméricas foram eficazes em vetorizar a indometacina ao tecido cerebral e que AINEs têm sido descritos pela literatura como promissores no tratamento de várias desordens neurodegenerativas (Mingheti et al., 2007 ; Canlelario-Jalil & Fiebich, 2008; Ahmad et al., 2009), nós investigamos o potencial efeito neuroprotetor da indometacina em nanocápsulas em culturas organotípicas de hipocampo de ratos submetidas à privação de oxigênio e glicose. Como descrito anteriormente, Capítulo 1, a cultura organotípica é um modelo adequado para o estudo de novos compostos farmacológicos devido à manutenção da citoarquitetura do tecido e pela possibilidade de ser mantida em cultivo por longos períodos preservando as características de maturação observadas *in vivo* (Stoppini et al., 1991; Xiang et al., 2000; Tavares et al., 2001; Holopainen, 2005). Além disso, estudos de morte celular (Horn et al., 2005; Cavaliere et al., 2006; Frozza et al., 2008) e inflamação (Skibo et al., 2000; Hailer et al., 2005; Strassburger et al., 2008) são extensamente realizados neste modelo, o que justifica a sua utilização no presente trabalho.

No Capítulo 3, inicialmente avaliamos se o tratamento com indometacina em nanocápsulas durante um período de 60 min de privação de oxigênio e glicose (POG), o qual mimetiza um insulto isquêmico, bem como durante as 24h subsequentes (período de re-oxigenação), seria capaz de prevenir o dano celular induzido. Nossos resultados mostraram que o tratamento com indometacina em nanocápsulas exerceu um significativo efeito neuroprotetor frente à injúria desencadeada pela POG (Capítulo 3, Figura 1). Por outro lado, quando as culturas

foram tratadas nas mesmas condições experimentais com a indometacina em solução, este efeito neuroprotetor não foi observado. Uma plausível explicação para estes resultados é que as nanocápsulas estariam proporcionando um aumento na distribuição da indometacina pelo tecido, sendo que esta liberação pelas nanocápsulas proporcionaria maiores concentrações intracelulares do fármaco. Além disso, considerando que o processo inflamatório está presente no insulto isquêmico, este poderia estar contribuindo para a distribuição da indometacina através das alterações de permeabilidade entre as células.

Na sequência, investigamos os mecanismos moleculares pelos quais a indometacina em nanocápsulas poderia estar exercendo esse efeito neuroprotetor. Inicialmente analisamos vias de sinalização celular que têm sido descritas como fundamentais para a sobrevivência celular. As vias da ERK1/2 e JNK são ativadas de forma sustentada após um insulto isquêmico exercendo assim um papel fundamental no dano neuronal tardio (Sugino et al., 2000; Nozaki et al., 2001; Ferrer et al., 2003). A ativação dessas enzimas leva à transcrição de fatores e citocinas pró-inflamatórias envolvidas na morte celular (Xia et al., 1995). Dessa forma, manipulações farmacológicas que consigam reduzir essa ativação são consideradas promissoras para o tratamento da isquemia cerebral (Mehta et al., 2007). Nossos resultados mostraram que o tratamento com indometacina em nanocápsulas foi capaz de prevenir significativamente a fosforilação e consequente ativação de ERK1/2 e JNK, levando os níveis de fosforilação dessas enzimas a níveis comparáveis com os controles (Capítulo 3, Figuras 2 e 3).

A morte celular observada nas culturas submetidas à POG é acompanhada pelo processo inflamatório que contribui para o dano celular observado. Após um insulto isquêmico, os astrócitos são ativados induzindo a um aumento na expressão

da proteína GFAP, o que caracteriza o processo denominado astrogliose reativa (Pekny & Nilsson, 2005). Além disso, juntamente com a microglia ativada, os astrócitos secretam moléculas que são capazes de induzir excitotoxicidade, estresse oxidativo e inflamação. Dentre estas moléculas pode-se citar o glutamato, citocinas como TNF-α, IL-1β e IL-6, prostaglandinas e o radical superóxido (Block et al., 2007). Contribuindo para a resposta inflamatória no tecido isquêmico, a proteína iNOS é superexpressa por astrócitos e microglia em situações de estresse celular possuindo papel fundamental na neurotoxicidade. A superexpressão na iNOS contribui para a síntese de NO, que conjugado ao ânion superóxido forma peroxinitrito, composto altamente reativo também envolvido com a neurotoxicidade (Xie et al., 2002). Além disso, o NO por si só é capaz de induzir a morte celular de neurônios, principalmente por sua capacidade de inibição da cadeia respiratória e pelo aumento da liberação de glutamato e consequente excitotoxicidade por ele desencadeada (Bal-Price & Brown, 2001). Já foi demonstrado também que a iNOS pode ter a sua expressão aumentada em resposta à secreção de citocinas como TNF-α, IL-1β e IL-6 (Minghetti & Levi, 1998; Combs et al., 2001). Além disso, sabe-se que o NO é capaz de aumentar a expressão de COX-2, propagando assim a reação inflamatória (Paoletti et al., 1998). Os resultados do presente trabalho mostraram que o tratamento com indometacina em nanocápsulas foi capaz de reduzir a ativação glial como observado pelo significativo decréscimo no imunoconteúdo das proteínas iNOS e GFAP (Capítulo 3, Figuras 4 e 5). Além disso, o tratamento com indometacina em nanocápsulas reduziu a ativação microglial, como pode ser observado pela diminuição da reatividade da Isolectina B_4 (Capítulo 3, Figura 6). Assim, nós propomos que o efeito neuroprotetor da indometacina em nanocápsulas poderia ser consequência da prevenção da ativação glial e microglial.

Considerando esses resultados observados sobre parâmetros inflamatórios, nós seguimos nossa investigação avaliando o envolvimento das citocinas no efeito neuroprotetor desencadeado pela indometacina em nanocápsulas. Como observado, os resultados do tratamento com essa formulação causou um significativo decréscimo nos níveis secretados das citocinas IL-6, IL-1β e TNF-α (Capítulo 3, Figuras 7A, 7B e 7C). Essa redução poderia contribuir, pelo menos em parte, com o efeito neuroprotetor aqui observado. Citocinas pró-inflamatórias são descritas por desempenhar um papel importante na isquemia cerebral (Han and Yenari et al., 2003). Estudos clínicos demonstram que o aumento de IL-6 no soro de pacientes que sofreram isquemia cerebral tem sido relacionado com o agravamento neurológico destes pacientes (Vila et al., 2001). Em modelos experimentais de isquemia, a expressão aumentada de IL-1β por neurônios e astrócitos, está relacionada com a indução da liberação de ácido araquidônico e estimulação de iNOS (Huang et al., 2006). O TNF-α é uma das principais citocinas liberadas pela microglia ativada, pelos astrócitos reativos e pelos neurônios, sendo considerado o principal mediador da neuroinflamação (Sriram & O'Callaghan, 2007). Em concentrações aumentadas, é um importante estimulador de neurotoxicidade principalmente por alterar a permeabilidade da BHE, facilitando assim a infiltração de células do sistema imunológico (Sriram & O'Callaghan, 2007). Além disso, o TNF-α é capaz de inibir a recaptação de glutamato pela glia, potencializando assim o efeito citotóxico do glutamato (Zou & Crews, 2005). Já foi demonstrado também que níveis aumentados de TNF-α são capazes de aumentar os níveis da COX-2, responsável pela formação de prostanóides e propagação da resposta inflamatória, culminando também com a neurotoxicidade e morte neuronal (Eligini et al., 2005). Assim, considerando que a indometacina é um potente inibidor de COX-2, essa inibição

poderia explicar, pelo menos em parte, o efeito neuroprotetor aqui observado. É importante ressaltar, que as citocinas secretadas pelas células que sofreram um insulto isquêmico talvez não sejam diretamente responsáveis pela neurotoxicidade observada, entretanto são capazes de induzir direta ou indiretamente a ativação glial e microglial, de modo que se torna um ciclo de retroalimentação positiva culminando em excitotoxicidade. Considerando que citocinas pró-inflamatórias, proteínas como ERK1/2 e JNK, além da COX-2 podem ser superexpressas no período de reoxigenação (Wang et al., 2007), este momento é descrito como crucial para uma intervenção farmacológica com o intuito de prevenir a morte neuronal tardia, uma característica da lesão isquêmica (Mehta et al., 2007).

Outro resultado relevante, é que o tratamento com indometacina em nanocápsulas foi capaz de aumentar os níveis da proteína anti-inflamatória IL-10 (Capítulo 3, Figura 7D). O aumento desta citocina está relacionado com efeitos neurológicos benéficos em pacientes que sofreram um insulto isquêmico (Vila et al., 2003; Wang et al., 2007). Além disso, a IL-10 pode atuar inibindo as ações de IL-1β e TNF-α, suprimindo a ativação e/ou expressão de seus receptores específicos (Wang et al., 2007).

Com base nos resultados apresentados neste terceiro Capítulo, podemos afirmar que indometacina em nanocápsulas foi eficaz em reduzir o dano celular ocasionado pela POG devido à ação simultânea de vários mecanismos: 1) prevenção da ativação das vias ERK1/2 e JNK; 2) prevenção da ativação glial e microglial; 3) redução nos níveis de citocinas pró-inflamatórias como TNF-α, IL-1β e IL-6; 4) aumento nos níveis da citocina anti-inflamatória IL-10.

Embora mais estudos utilizando modelo *in vivo* de isquemia cerebral sejam necessários para confirmar a nossa hipótese, a indometacina em nanocápsulas

pode ser considerada um bom candidato para a intervenção famacológica em insultos isquêmicos, uma vez que ela pode ser vetorizada ao tecido cerebral, como mostrado no Capítulo 2, além de atuar bloqueando a neuroinflamação envolvida na excitotoxicidade desencadeada por um evento isquêmico.

Considerando os efeitos anti-inflamatórios da indometacina em nanocápsulas observados no SNC (Capítulo 3), o Capítulo 4 teve por objetivo comparar a eficácia anti-inflamatória de concentrações sub-terapêuticas da indometacina em nanocápsulas e da indometacina em solução administradas intraperitonealmente em modelos de inflamação periférica. Para tanto, utilizou-se os seguintes modelos de edema de pata em ratos: modelo de inflamação aguda induzido por carragenina, modelo de inflamação subcrônica induzida por CFA e modelo de inflamação crônica induzido por CFA (modelo de artrite). A artrite é uma doença inflamatória crônica que compromete a qualidade de vida dos pacientes por ela acometidos devido às suas severas manifestações clínicas. Além disso, considerando que o uso crônico da indometacina, em doenças como a artrite, está frequentemente associado a distúrbios gastrointestinais, investigamos se esses efeitos colaterias poderiam ser minimizados quando a indometacina fosse administrada na formulação de nanocápsulas poliméricas.

Como mencionado na Introdução, a injeção intraplantar de carragenina ou CFA são considerados modelos adequados para o estudo de inflamação e dor, sendo amplamente utilizados para a caracterização de novos fármacos ou de compostos com potencial atividade anti-inflamatória (Valo et al., 1973; Kawamura et al., 2001; Quintão et al., 2008). Assim, inicialmente nós comparamos o efeito da indometacina em nanocápsulas com o efeito da indometacina em solução em modelo agudo de inflamação utilizando dois protocolos de tratamento, um profilático

e outro terapêutico. No protocolo profilático as formulações foram administradas intraperitonealmente uma única vez antes da indução do edema, enquanto que no protocolo terapêutico as formulações foram administradas da mesma maneira, porém após a indução do edema. Em ambos os protocolos, o tratamento com indometacina em nanocápsulas reduziu o edema induzido pela carragenina, de maneira similar àquele desencadeado pela indometacina em solução (Capítulo 4, Figuras 1 e 2). O fato do tratamento com indometacina em nanocápsulas não ter sido mais eficaz em reduzir o edema poderia ser explicado pelo fato de que estes protocolos de tratamento não foram suficientes para as nanocápsulas proporcionarem uma maior biodisponibilidade da indometacina no tecido inflamado. Quando utilizamos o modelo subcrônico de inflamação, onde o edema foi avaliado 3 dias após a aplicação do CFA e as formulações foram administradas durante 3 dias consecutivos, a indometacina em nanocápsulas mostrou-se mais eficaz em reduzir o edema quando comparada ao efeito da indometacina em solução (Capítulo 4, Figura 3). Da mesma forma, quando utilizamos o modelo de artrite, modelo crônico de inflamação onde as formulações foram administradas durante 8 dias consecutivos, a eficácia anti-inflamatória da indometacina em nanocápsulas foi ainda mais significativa (Capítulo 4, Figura 4). Os resultados observados poderiam ser explicados pelo fato da administração repetida de indometacina em nanocápsulas ser capaz de propiciar uma eficiente vetorização do fármaco para o local de ação aumentando assim a sua biodisponibilidade. De acordo com o que é descrito pela literatura, as alterações de permeabilidade microvascular do processo inflamatório poderiam facilitar dessa forma o direcionamento das nanocápsulas para o tecido inflamado propiciando assim o seu acúmulo (Couvreur e Vauthier, 2006). Além disso, considerando que no processo inflamatório da artrite reumatóide existe uma

diminuição do pH (Andersson et al., 1999; Levick et al., 1990) associado a alterações na drenagem linfática, essas alterações poderiam explicar a maior eficácia observada pelo tratamento com indometacina em nanocápsulas, uma vez que nesta condição de pH diminuído a indometacina é liberada mais lentamente das nanocápsulas, havendo assim a possibilidade de um efeito sustentado.

Considerando que as citocinas possuem papel fundamental na resposta inflamatória presente nos modelos de artrite (Laufer, 2003; Meyer, 2003), nós avaliamos se a eficácia anti-inflamatória da indometacina em nanocápsulas observada no modelo de artrite utilizado no presente estudo poderia ser mediada por alterações na produção das citocinas IL-6, TNF-α e IL-10. As dosagens no soro dos animais tratados com indometacina em nanocápulas demonstraram uma significativa redução nos níveis das citocinas pró-inflamatórias TNF-α e IL-6 (Capítulo 4, Figuras 5A e 5B). Outro resultado importante observado neste estudo foi o significativo aumento nos níveis da citocina anti-inflamatória IL-10 após o tratamento com indometacina em nanocápsulas (Capítulo 4, Figura 5C). No grupo de animais tratados com indometacina em solução, nenhuma alteração significativa nos níveis de citocinas foi observada quando comparada ao grupo controle (Capítulo 4, Figura 5). Assim, a maior eficácia anti-inflamatória observada pelo tratamento com indometacina em nanocápsulas pode ser explicado, pelo menos em parte, pela sua maior biodisponibilidade e consequente habilidade em alterar a produção de citocinas no cenário inflamatório, especialmente o TNF-α. Esta capacidade do tratamento com indometacina em nanocápsulas alterar os níveis séricos de TNF-α é extremamente relevante uma vez que os pacientes acometidos pela artrite, além de utilizarem AINEs, frequentemente fazem uso de terapia anti-TNF-α para minimizar a sintomatologia da doença.

A tentativa de minimizar os efeitos gastrointestinais adversos causados pela indometacina tem sido um objetivo constante de estudos utilizando carreadores de fármacos (Ammoury et al., 1991; Fawaz et al., 1993). Dessa forma, considerando as limitações do emprego terapêutico da indometacina na clínica, um resultado bastante relevante deste Capítulo 4 é que o tratamento crônico com indometacina em nanocápsulas foi capaz de diminuir de forma significativa a toxicidade gastrointestinal quando comparado com o tratamento de indometacina em solução (Capítulo 4, Figura 6). A vetorização da indometacina pelas nanocápsulas foi capaz de prevenir a toxicidade gastrointestinal causada pela indometacina, provavelmente pelo fato das nanocápsulas impedirem o contato direto do fármaco com a mucosa intestinal. Este resultado está de acordo com estudos prévios de nosso grupo de pesquisa que demonstram que nanopartículas são capazes de prevenir os efeitos ulcerativos de AINEs como o diclofenaco (Guterres et al., 1995; Guterres et al., 2000; Guterres et al., 2001). É importante considerar que a produção de citocinas pode exibir um importante papel protetor da mucosa gastrointestinal (Robinson et al., 2008). Dessa forma, a redução nos níveis de TNF-α e IL-6, associado com o aumento dos níveis de IL-10 poderia contribuir para a redução da toxicidade gastrointestinal observada nos animais tratados com indometacina em nanocápsulas. Com base nos resultados apresentados neste Capítulo 4, podemos concluir que as nanocápsulas foram eficazes em vetorizar a indometacina aos sítios de inflamação, apresentando uma eficácia terapêutica de modo que esta formulação poderia ser considerada um candidato promissor para o tratamento de doenças inflamatórias crônicas como a artrite reumatóide.

CONCLUSÕES

Gerais:

Os resultados do presente estudo apresentam novas possibilidades de aplicação terapêutica para a indometacina, fármaco que possui dados farmacocinéticos já bem estabelecidos pelo fato de ser amplamente utilizado na terapêutica com finalidade anti-inflamatória.

Específicas:

- ✓ A indometacina em nanocápsulas foi mais eficaz em reduzir a viabilidade e a proliferação celular em linhagens de gliomas e esse efeito citotóxico foi seletivo para as células tumorais como evidenciado pela ausência de toxicidade sobre culturas organotípicas de hipocampo de ratos. (Capítulo 1).

- ✓ As nanocápsulas foram capazes de vetorizar a indometacina ao tecido cerebral, sendo que o tratamento sistêmico com essa formulação foi eficaz em reduzir o volume do tumor e suas características histopatológicas de malignidade, devido aos efeitos antiproliferativo e anti-angiogênico observados. (Capítulo 2).

- ✓ O tratamento com indometacina em nanocápsulas exerceu efeito neuroprotetor em culturas organotípicas de hipocampo de ratos submetidas à privação de oxigênio e glicose. Esse efeito foi mediado pelo diminuição da

neuroinflamação envolvida na excitotoxicidade do insulto isquêmico. (Capítulo 3).

- ✓ O tratamento com indometacina em nanocápsulas apresentou maior eficácia anti-inflamatória quando comparada ao tratamento com indometacina em solução nos modelos de inflamação subcrônica e crônica (modelo de artrite). A vetorização da indometacina pelas nanocápsulas foi capaz de prevenir a toxicidade gastrointestinal observada pelo tratamento com indometacina em solução (Capítulo 4).

PERSPECTIVAS

Como continuação desse trabalho, pretende-se trabalhar com os seguintes objetivos:

- ✓ Utilizando nanopartículas fluorescentes, determinar através de microscopia confocal, se estas são internalizadas pelas células de glioma e em quais compartimentos celulares encontram-se localizadas;
- ✓ Determinar, através da imunodetecção para CD133, se o tratamento com indometacina em nanocápsulas é capaz de atuar sobre a subpopulação de células-tronco neurais presentes no gliomas;
- ✓ Investigar os possíveis mecanismos de transdução de sinal envolvidos no efeito antiproliferativo e anti-angiogênico da indometacina em nanocápsulas nos gliomas;
- ✓ Estudar o efeito do tratamento de outros AINEs em nanocápsulas sobre o crescimento de gliomas implantados em cérebro de ratos;
- ✓ Estudar o efeito neuroprotetor da indometacina em nanocápsulas em um modelo *in vivo* de isquemia cerebral e investigar os possíveis mecanismos de ação através da análise dos mesmos parâmetros de neuroinflamação que foram estudados nas culturas organotípicas;
- ✓ Estudar a eficácia anti-inflamatória do tratamento com outros AINEs em nanocápsulas em modelos de inflamação aguda e crônica em ratos.
- ✓ Avançar investigando o potencial uso clínico da indometacina em nanocápsulas no tratamento de gliomas e de artrite.

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ANEXO

OUTROS ARTIGOS CIENTÍFICOS REALIZADOS EM CO-AUTORIA DURANTE O PERÍODO DO DOUTORADO:

1. BRAGANHOL E, TAMAJUSUKU ASK, **BERNARDI A**, WINK MR, BATTASTINI A MO. Ecto-5'-nucleotidase/CD73 inhibition by quercetin in human U128MG glioma cell line. *Biochimica et Biophysica Acta, General Subjects*, 1770: 1352 – 1359, 2007.

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10. HOPPE JB, FROZZA RL, HORN AP, COMIRAN R, **BERNARDI A**, CAMPOS MM, BATTASTINI AMO, SALBEGO C. β-amyloid neurotoxicity in organotypic hippocampal slice culture is attenuated by melatonin: involvement of GSK-3β, *tau* and neuroinflammation. Manuscrito submetido à revista *Journal of Pineal Research.*

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