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AVALIAÇÃO DA TOXICIDADE ORAL DO ÁCIDO ÚSNICO MICROENCAPSULADO

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Recife, fevereiro de 2008

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Ata da defesa de dissertação da Mestranda **Milena Sales Ferraz,** realizada em 29 de fevereiro de 2008, como requisito final para obtenção do título de Mestre em Bioquímica e Fisiologia da UFPE.

Às 09:20 horas, do dia vinte e nove de fevereiro de 2008, foi aberto, na Sala de Aulas do Mestrado – Depto. de Bioquímica, do Centro de Ciências Biológicas, da Universidade Federal de Pernambuco, o ato de defesa de dissertação da mestranda **Milena Sales Ferraz**, aluna do Curso de Mestrado em Bioquímica e Fisiologia/CCB/UFPE. Iniciando os trabalhos a Profa. Dra. Nereide Stela Santos Magalhães fez a apresentação da aluna, de sua orientadora, ela própria, sua co-orientadora Profa. Dra. Noemia Pereira da Silva Santos, bem como da Banca Examinadora composta pelos professores doutores: Nereide Stela Santos Magalhães, na qualidade de Presidente, Luiz Bezerra de Carvalho Júnior, Nicácio Henrique da Silva, ambos do Depto. de Bioquímica/UFPE e Eliana Martins Lima, da Faculdade de Farmácia da UFG. Após as apresentações, a Profa. Dra. Nereide Stela Santos Magalhães convidou a aluna para a apresentação de sua dissertação intitulada: "**Avaliação da Toxicidade Oral do Ácido Úsnico Microencapsulado**", e informou que de acordo com o Regimento Interno do Curso, a candidata dispõe de até 50 (cinqüenta) minutos para apresentação do trabalho e o tempo de argüição para cada examinador, juntamente com o tempo gasto pela aluna para responder às perguntas será de 30 (trinta) minutos. A aluna procedeu à explanação e comentários acerca do tema em **35 (trinta e cinco) minutos**. Após a apresentação da mestranda, a Sra. Presidente convidou a Banca Examinadora para ocupar seus lugares e passou a palavra ao primeiro examinador, Profa. Dra. Eliana Martins Lima que agradeceu o convite, fez alguns comentários e sugestões, e iniciou sua argüição. Ao final, a referida professora deu-se por satisfeita. Em seguida, a Sra. Presidente passou a palavra para o Prof. Dr. Luiz Bezerra de Carvalho Júnior, que agradeceu o convite, fez alguns comentários e sugestões, e iniciou sua argüição. Ao final, o referido professor deu-se por satisfeito. Logo após, a Sra. Presidente passou a palavra para o Prof. Dr. Nicácio Henrique da Silva, que agradeceu ao convite, fez alguns comentários e sugestões, iniciando sua argüição. Ao final, o referido professor deu-se por satisfeito. Em seguida, a Sra. Presidente, na qualidade de orientadora, usou da palavra para tecer alguns comentários a respeito do trabalho da aluna, agradecer à Banca Examinadora e parabenizar a candidata. Finalmente, a sessão foi suspensa para proceder ao julgamento pela Banca Examinadora, a qual se reuniu na Secretaria do Curso. Após alguns comentários, a Banca decidiu, por unanimidade, conceder a menção "**Aprovada com Distinção**". Nada mais havendo a tratar, lavrei a presente ata que vai assinada por mim, Secretário, e demais membros da Banca Examinadora. Recife, 29 de fevereiro de 2008.

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À Deus, pela luz com a qual ilumina os caminhos de minha vida e sigo aprendendo, crescendo e realizando meus sonhos com a certeza que Ele está zelando por mim.

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LISTA DE ABREVIATURAS

ALT – Alanina aminotransferase

AST – Aspartato aminotransferase

ATP – Adenosina trifosfato

CI_{50} – Concentração necessária para inibir 50% do crescimento celular

CIM – Concentração inibitória mínima

DNA – Ácido desoxirribonucléico

FDA – Food and Drug Administration

Hep-2 – Carcinoma epidermóide de laringe

ME-AU – Microesferas contendo ácido úsnico

NCI-H 292 – Carcinoma de pulmão humano

PEG – Polietilenoglicol

PGA – Polímero de ácido glicólico

PLA – Polímero de ácido lático

PLGA – Co-polímero de ácido lático e glicólico

PVA – Álcool polivinílico

RNA – Ácido ribonucléico

UA – Ácido úsnico

UA-MS – Ácido úsnico microencapsulado

RESUMO

O objetivo do presente estudo é avaliar a toxicidade subcrônica oral do ácido úsnico encapsulado em microesferas de copolímero de ácido láctico e glicólico em comparação com sua forma em suspensão. As microesferas contendo ácido úsnico foram preparadas utilizando a técnica de emulsão (A/O/A) seguida de evaporação do solvente e caracterizadas através do tamanho de partículas, carga superficial e taxa de encapsulação. No estudo de toxicidade, ratos machos Wistar receberam doses orais de 25 mg/Kg/dia de ácido úsnico encapsulado em microesferas (UA-MS) ou ácido úsnico (UA) em suspensão por 28 dias. O efeito tóxico causado pelos tratamentos foi avaliado através da eficiência de conversão alimentar, análise bioquímica e histopatológica e microscopia confocal de varredura a laser. As microesferas contendo UA apresentaram-se com tamanho de partículas de 4,92 ± 0,44 μm, carga de superfície de -25,7 ± 6,5 mV e eficiência de encapsulação de 99,0 ± 0,82 %. Uma diferença significativa foi observada na eficiência de conversão alimentar dos animais tratados com UA, mas nenhuma diferença foi observada no grupo tratado com UA-MS. Não houve diferença no peso dos órgãos em todos os grupos de animais. Um aumento significante de AST e ALT foi observado nos animais tratados com UA (44 ± 4.3 and 106.1 ± 10.4 U/L, respectivamente) quando comparado com o grupo não tratado (32.2 ± 3.9 and 82.5 ± 13.8 U/L, respectivemente). Contudo, alterações enzimáticas significantes não foram encontradas após o tratamento dos animais com UA-MS, desse modo confirma uma redução da hepatoxicidade do UA devido a sua microencapsulação. Mudanças não foram observadas nos níveis séricos de uréia e creatinina após o tratamento com UA ou UA-MS confirmando que o ácido úsnico não apresenta nefrotoxicidade. As análises histopatológicas do fígado demonstraram extensas áreas de degeneração vacuolar com áreas de necrose nos animais tratados com UA. Porém, os animais tratados com UA-MS não desenvolveram alterações hepáticas, sugerindo que a microencapsulação do ácido úsnico foi capaz de reduzir sua hepatotoxicidade. O UA foi detectado no fígado e rins dos animais tratados com ácido úsnico livre, através da fluorescência intrínseca do ácido úsnico, indicando a sua captura hepática e eliminação renal. Uma menor intensidade de fluorescência foi visualizada no fígado após tratamento com ácido úsnico microencapsulado, devido à liberação controlada pelas microesferas. Nenhuma fluorescência foi detectada no rim após tratamento dos animais com ácido úsnico encapsulado. Estes resultados sugerem que a microencapsulação do ácido úsnico pode reduzir sua hepatotoxicidade, desse modo permitindo o seu uso para aplicação terapêutica.

Palavras chaves: ácido úsnico, PLGA, microesferas, toxicidade oral, ratos Wistar.

ABSTRACT

The goal of the present study is to evaluate the subchronic oral toxicity of usnic acid encapsulated into poly(lactide-co-glicolide) microspheres in comparison with its suspension form. The microspheres containing usnic acid were prepared using the conventional double emulsion (w/o/w) solvent evaporation technique and characterized through diameter mean size of particles, surface charge potential and encapsulation efficiency. In toxicity study, Wistar male rats received oral doses of 25 mg/kg/day of usnic acid-loaded microspheres (UA-MS), or usnic acid (UA) in suspension for 28 days. The toxic effects caused by usnic acid were evaluated through feed conversion efficiency, biochemical and histopathological analyses, and confocal scanning laser microscopy. Microspheres containing UA presented a particle mean size of 4.92 ± 0.44 μm, surface charge of -25.7 ± 6.5 mV and drug entrapment efficiency was 99 ± 0.82 %. A significant statistical difference was observed in feed conversion efficiency of the animals treated with UA, but differences had not been observed in the group treated with UA-MS. No statistical differences in organ weights were found in all groups of animals. A significant increase in ALT and AST (44 ± 4.3 and 106.1 ± 10.4 U/L, respectively) was observed for the animals treated with UA when compared to the untreated group (32.2 ± 3.9 and 82.5 ± 13.8 U/L, respectively). Nevertheless, no significant enzyme alterations were found after the treatment of animals with UA-MS, thereby confirming a reduction of the hepatotoxicity of UA due to its microencapsulation. No changes were observed in the serum levels of urea and creatinine after treatment with UA or UA-MS, confirming that usnic acid not exhibited nefrotoxicity. Histopathological analysis demonstrated extensive areas of vacuolar degeneration with necrotic areas could be seen in the liver of animals treated with free usnic acid. However, animals treated with UA-MS not to developed hepatic alterations, suggesting that the microencapsulation of usnic acid was able reduce its toxicity. Usnic acid was detected in liver and kidney of the animals treated with UA, through its intrinsic fluorescence, indicating its hepatic uptake and renal elimination. Low intensity of fluorescence was visualized in liver after UA-MS treatment, due to controlled release of microspheres. No fluorescence was detected in kidney after treatment usnic acid-loaded microspheres. These findings suggest that the microencapsulation of usnic acid was able to reduce its hepatoxicity, thereby allowing its potential introduction for therapeutic proposes.

Keywords: Usnic acid, PLGA, microspheres, oral toxicity, Wistar rats

INTRODUÇÃO

1. ÁCIDO ÚSNICO

1.1. Propriedades físico-químicas do ácido úsnico

O ácido úsnico, [2,6-diacetil-7,9-dihidroxi-8-9b-dimetil-1,3(2H,9bH)-dibenzofurandiona; $C_{18}H_{16}O_7$] (figura 1), é um composto de origem natural resultante do metabolismo secundário de várias espécies de liquens, tais como *Cladonia* (Cladoniaceae), *Usnea* (Usneaceae), *Lecanora* (Lecanoraceae), *Ramalina* (Ramalinaceae) e *Parmelia* (Parmeliaceae) (INGÓLFSDÓTTIR, 2002). Caracteriza-se por ser uma substância de pigmentação amarelada e de baixa solubilidade em água (10 mg/100 mL a 25°C). Ocorre na natureza em duas formas enatioméricas (-) e (+), indicando uma projeção angular do grupamento metil localizado na posição 9b, podendo apresentar atividades biológicas e mecanismos de ação distintos (INGÓLFSDÓTTIR, 2002; COCCHIETTO et al, 2002).

Possui um ponto de fusão é em torno de 204ºC, peso molecular de 344,32 g/mol e uma DL_{50} em camundongos i.v. de 25mg/Kg (MERCK INDEX, 1989).

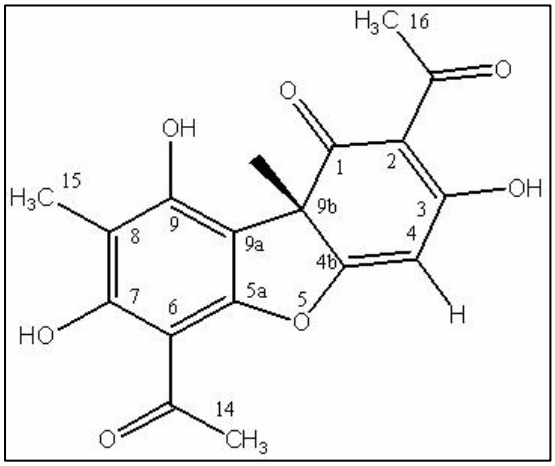

Figura 1. Estrutura química do ácido úsnico (INGÓLFSDÓTTIR, 2002).

1.2. Atividades biológicas do ácido úsnico

1.2.1. Atividade Antimicrobiana

De acordo com relatos disponíveis na literatura, o principal papel biológico do ácido úsnico na natureza é o de antibiótico. As primeiras avaliações sobre sua ação antibacteriana

datam da década de 50 e investigações nos últimos anos têm ampliado o conhecimento desse metabólito liquênico como antibiótico (COCCHIETTO et al., 2002).

A avaliação da atividade antimicobacteriana utilizando cinco compostos liquênicos diferentes revelou que a melhor ação foi exibida pelo ácido úsnico extraído da *Cladonia arbuscula*, a concentração inibitória mínima (CIM) de 32 μg/mL frente ao *Mycobacterium aurum* despertou interesse por mais investigações (INGÓLFSDÓTTIR et al., 1998).

Elo e colaboradores (2007) avaliaram a atividade antimicrobiana do ácido úsnico frente a sete cepas de microrganismos resistentes, tais como, enterococo vancomicina-resistente e estafilococo meticilina-resistente clinicamente isolados, foram utilizadas 12 concentrações do ácido único que variaram de 0,0195 a 40 mg/mL, o qual foi verificado uma alta atividade (CIM) frente a esses isolados, quando comparado com a ampicilina (droga de referência), mostrando o potencial valor clínico deste metabólito.

1.2.2. Atividade Antitumoral

A pesquisa desenvolvida por Ribeiro-Costa e colaboradores (2004) avaliou o efeito citotóxico do ácido úsnico livre e encapsulado em microesferas de PLGA contra carcinoma epidermóide de laringe (HEp-2) demonstrando que a concentração requerida para inibir 50% da proliferação celular (CI_{50}) foi de 12,6 e 14,4 μg/mL, respectivamente. Células de carcinoma de pulmão humano (NCI-H 292) também foram utilizadas na investigação da ação antiproliferativa do ácido úsnico livre e em nanocápsulas, os resultados desse trabalho exibiram uma atividade citotóxica considerável com CI_{50} de 10 e 13,8 μg/mL para o ácido úsnico livre e encapsulado, respectivamente (SANTOS et al., 2005).

A atividade antitumoral *in vivo* foi avaliada após administração intraperitoneal em camundongos Swiss de ácido úsnico livre e em microesferas foi avaliada contra Sarcoma- 180 revelando uma importante inibição do tumor de 63% para o ácido úsnico encapsulado e de 42% para o composto livre (RIBEIRO-COSTA et al., 2004).

Na pesquisa desenvolvida por Santos e colaboradores (2006) também foi estudada a atividade antitumoral *in vivo* do ácido úsnico em sua forma livre e nanoencapsulado frente ao Sarcoma-180. Neste estudo, obteve-se um aumento da inibição tumoral do ácido úsnico em nanocápsulas (68%) quando comparado com o metabólito livre (43%).

1.2.3. Atividade Antiprotozoária

Estudos da avaliação da atividade *in vitro* do ácido úsnico extraído da *Cladonia substellata* contra o protozoário *Trypanosoma cruzi* apontaram o potencial uso desse composto na doença de Chagas, uma vez que, este metabólito foi significativamente efetivo em várias concentrações (10 a 50 mg / mL) frente às formas epimastigotas, tripomastigotas e amastigotas causando danos às mitocôndrias e ao cinetoplasto (De CARVALHO et al., 2005).

1.2.4. Atividade Antiviral

Os efeitos citopáticos dos Vírus Herpes simples tipo I e Pólio tipo I foram inibidos *in vitro* quando células de rins de macaco infectadas foram tratadas com ácido úsnico nas concentrações de 1 a 5 μg por disco (PERRY et al., 1999).

Posteriormente, a inibição do Poliomavírus *in vitro* foi investigada usando células 3T6 (linhagem de fibroblastos de camundongo), demonstrando que o ácido úsnico inibiu severamente a replicação do DNA viral através de uma ação indireta de drástica inibição da transcrição do RNA. Salientando que esta ação aconteceu em concentrações não tóxicas (5 e 10 μg/mL) sendo observada considerável viabilidade das células 3T6 (aproximadamente 80%) durante o experimento (CAMPANELLA et al., 2002).

1.2.5. Atividade Antiinflamatória

Vijayakumar e colaboradores (2000) estudaram a ação antiinflamatória do (+)-ácido úsnico em ratos Wistar com edema de pata, os resultados revelaram uma significante redução do edema, utilizando uma dose de ácido úsnico de 100 mg/Kg de peso corpóreo, quando comparado com o ibuprofeno (na mesma dose), fármaco padrão de referência como antiinflamatório. No estudo de inflamação aguda, a redução do edema de pata foi de 0,82 mL (controle) para 0,55 e 0,47 mL (ácido úsnico e ibuprofeno, respectivamente). Através deste resultado, foi sugerido que o mecanismo de ação do ácido úsnico pode envolver uma inibição da síntese de prostaglandinas como outras drogas antiinflamatórias.

1.2.6. Gastroprotetora

Recentes estudos indicam que o ácido úsnico, isolado da *Usnea longissima*, está sendo aplicado no tratamento da úlcera gástrica em ratos. Induzida pela indometacina, lesões gástricas foram significativamente reduzidas por todas as doses utilizadas de ácido úsnico (25,

50, 100 e 200 mg/kg de peso corporal), quando comparado com o grupo tratado com a ranitidina (droga referência). Entretanto, a dose de 25mg/Kg de ácido úsnico apresentou maior inibição da lesão ulcerativa (92,9%). Esse efeito gastroprotetor do ácido úsnico pode ser atribuído ao seu efeito redutor contra o dano oxidativo e seu efeito inibitório na infiltração neutrofílica em estômago de ratos (ODABASOGLU et al., 2006).

1.3. Famacocinética do ácido úsnico

Em 1992, Krishna e Venkatarama estudaram a farmacocinética do ácido úsnico em coelhos após a administração oral e intravenosa de doses de 20 e 5 mg/Kg de peso corporal, respectivamente. Os resultados encontrados demonstraram um tempo de meia-vida de 11 horas para a via intravenosa e de 18 horas para a via oral e uma biodisponibilidade absoluta de 77,8 % após administração oral.

1.4. Mecanismo de ação e toxicologia do ácido úsnico

O mecanismo de ação do ácido úsnico não está completamente elucidado. Porém, Pramyothin e colaboradores (2004) constataram que o (+)-ácido úsnico age alterando a integridade da membrana celular, permitindo a liberação de enzimas hepatoespecíficas, principalmente transaminases, além de causar destruição da função mitocondrial exibindo uma perda do controle da respiração celular e na síntese de ATP. Esta investigação identificou que o ácido úsnico possui um efeito similar ao tetracloreto de carbono, que envolve geração de radical livre que resulta na injúria na membrana celular e mitocondrial, peroxidação dos lipídeos e morte celular.

Estudos posteriores enfatizaram a elevada hepatoxicidade do ácido úsnico, quando foi observado aproximadamente 100% de necrose após tratamento de cultura de hepatócitos de ratos com ácido úsnico (5 μM e 10 μM). Também foi visualizada redução considerável nos níveis de ATP. Além disso, constatar-se também inibição da respiração mitocondrial. Esses achados comprovam a inibição direta da função mitocondrial, levando ao aumento da produção de oxigênio reativo, uma forma de radical livre, pela cadeia transportadora de elétrons, levando à morte celular (HAN et al, 2004).

Nos Estados Unidos, esse dano hepatocelular foi confirmado quando indivíduos que consumiram LipoKinetix®, um suplemento dietético que contém usneato de sódio, apresentaram falência aguda do fígado (NEFF et al, 2004). Mais recentemente, dois pacientes

desenvolveram hepatotoxicidade severa após três meses consumindo três cápsulas por dia de UDP-1 (produto para perda de peso que contém 150 mg de ácido úsnico, 525 mg de carnitina e 1050 mg de piruvato de cálcio por cápsula). Um dos pacientes desenvolveu falência hepática fulminante e foi necessário o transplante do fígado. As análises histopatológicas revelaram infiltrados linfocitoplasmáticos e áreas de necrose no fígado dos pacientes usuários de UDP-1 (SANCHEZ et al, 2006).

Este efeito hepatotóxico do ácido úsnico tem sido amplamente investigado. Santos e colaboradores (2006) demonstraram, através de análises bioquímicas e histopatológicas, que há uma redução da toxicidade do ácido úsnico quando este é encapsulado em sistemas de liberação controlada de fármacos, tais como, nanocápsulas após administração em comundongos por via intraperitoneal.

Portanto, é necessário o desenvolvimento de sistemas de liberação controlada de medicamentos capazes de viabilizar a administração do ácido úsnico em uma formulação que melhore sua solubilidade, bem como, otimize a dose terapêutica diminuindo os efeitos tóxicos do mesmo pela diminuição da sua presença nos hepatócitos.

2. MICROPARTÍCULAS

Micropartículas são sistemas de liberação controlada de fármacos e podem ser definidas como pequenas partículas sólidas e esféricas com tamanho que varia de 1 a 1000 μm, e podem ser de dois tipos, as microcápsulas e as microesferas, as primeiras são sistemas reservatórios contendo a substância ativa revestida por polímeros, que constituem uma cápsula, por outro lado as microesferas são sistemas matriciais, onde o fármaco encontra-se uniformemente disperso e/ou dissolvido (SILVA et al, 2003).

As aplicações das técnicas de microencapsulação são muito variadas, desde de sua utilização para o mascaramento de sabores ou odores, até para a redução dos efeitos secundários provocados por alguns fármacos (SILVA et al, 2003). Por isso, uma grande variedade de compostos bioativos tem sido incorporados em microesferas, dentre esses podemos citar, drogas antineoplásicas e antituberlostáticas (TRUTER et al, 2001; HIROTA et al, 2007), agentes anestésicos (KOHANE et al, 2003), vírus (STURESSON et al, 1999), DNA (ZHOU et al, 2007), peptídeos e proteínas (SILVA et al, 2003).

Os sistemas de liberação, quando comparados a formas de dosagens convencionais, apresentam vantagens, tais como: liberação precisa em baixas doses, redução da concentração do fármaco em outros locais que não os órgãos e tecidos alvos minimizando os efeitos

colaterais indesejáveis e proteção dos principios ativos lábeis da degradação, até exercerem a sua função farmacológica. Além disso, são versáteis e podem ser administradas por diversas vias como: oral, parenteral, nasal e pulmonar (FREITAS et al, 2005 ; KUMAR, 2000). Porém, a via oral é a via preferencial devido à facilidade de administração, conveniência e aceitação.

O encapsulamento é uma alternativa válida, por constituírem sistemas nos quais protegem os fármacos da degradação pelo trato gastrointestinal, aumentam a permeabildade através do epitélio intestinal aumentando, assim, a biodisponibilidade oral dos mesmos (CHEN et al, 1998).

2.1. Absorção de micropartículas pelo trato gastrointestinal

Alguns mecanismos para a absorção de micropartículas no intestino após administração oral são descritos na lietratura. Contudo, a maioria dos estudos sugerem que são predominantemente capturadas no tecido linfático intestinal, ou seja, nas placas de Peyer's. Acredita-se que céluas M especializadas localizadas nas placas de Peyer são responsáveis pela internalização destas partículas (Figura 2) (CHEN et al, 1998 ; DELIE, 1998).

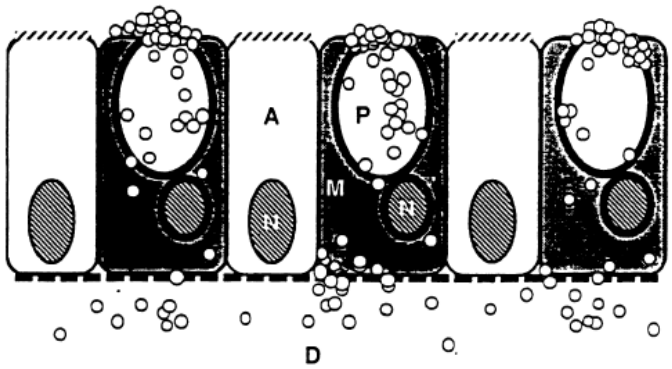

Figura 2. Diagrama esquemático de absorção de partículas pelas placas de Peyer (D): células M (M) alternadas com células colunares (A) contendo núcleo (N), partículas localizadas na superfície das células M e dentro de vacúolos (P) (CHEN et al, 1998).

A eficiência de absorção pelo trato gastrintestinal é afetada pelo tamanho da partícula administrada, isto é, partículas coloidais pequenas (submicrométricas) podem ser absorvidas e transportadas por uma via intracelular nos enterócitos, enquanto que partículas micrométricas são absorvidas exclusivamente pelas placas de Peyer's através das células M (CHEN, 1998).

Outro fator que influencia na absorção é o número de partículas presentes nas placas de Peyer's após administração oral que depende da hidrofobicidade relativa dos polímeros

usados para fabricação das micropartículas, ou seja, quanto mais hidrofóbica maior será a absorção (CHEN et al, 1998). Na tabela 1 estão exemplificados alguns exemplos de polímeros utilizados para a fabricação de microesferas e sua característica quanto a absorção pelas placas de Peyer's.

O copolímero de ácido lático e glicólico (PLGA) (figura 3) é um polímero muito utilizado na fabricação de sistemas de liberação como nanopartículas e micropartículas. Apresenta-se comercialmente com variações na proporção de seus dois monômeros (50/50, 75/25, 85/15, 90/10, 80/20, 70/30) motivo este que se torna peculiar às características fisico-químicas de cada apresentação como temperatura de transição, peso molecular, cristalinidade, morfologia e velocidade de biodegradação. O PLA é mais hidrofóbico, absorve menos água, e conseqüentemente sofre degradação mais lenta (PARK, 1995; HUSMANN et al, 2002).

Tabela 1. Absorção de microesferas de 1 a 10 μm preparadas com vários polímeros pelas placas de Peyer após administração oral (CHEN et al, 1998).

Polímeros	**Biodegradável**	**Absorção pelas placas de Peyer's**
Poli (estireno)	Não	Muito boa
Poli (metil metacrilato)	Não	Muito boa
Poli (hidroxibutirato)	Sim	Muito boa
Poli (D,L – lático)	Sim	Boa
Poli (L – lático)	Sim	Boa
Poli (D,L – lático-co-glicólico) 85:15	Sim	Boa
Poli (D,L – lático-co-glicólico) 50:50	Sim	Boa
Triacetato de celulose	Não	Nenhuma
Etilcelulose	Não	Nenhuma

A biodegradação do PLGA não envolve qualquer atividade enzimática, ele é hidrolisado em contato com os fluidos biológicos originando ácidos que são metabolizados no ciclo de Krebs em gás carbônico e água (TAMBER et al, 2005). Por isso, esse polímero foi aprovado pelo *Food and Drug Administration* (FDA) no uso de sistemas de liberação controlada (KUMAR et al., 2001).

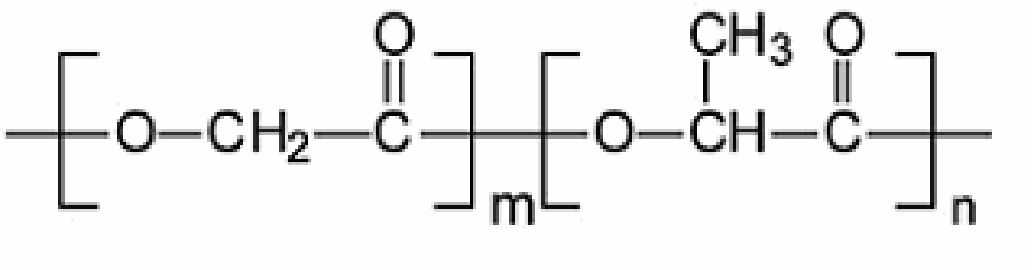

Figura 3. Estrutura molecular do poli ácido lático-co-glicólico (PLGA).

3. TOXICOLOGIA

A toxicidade de uma substância a um organismo vivo pode ser considerada como a capacidade de lhe causar injúria grave ou morte. Para que essa injúria ocorra é indispensável à interação do agente químico com o organismo. Essa interação é observada, experimentalmente, através da relação dose/resposta do organismo vivo. A partir desta relação dose/resposta pode-se calculadar estatisticamente a dose letal que pode causar a morte de 50% (DL_{50}) de uma dada população de organismos em condições experiementais adequadas. A DL_{50} é um índice empregado para classificar e comparar a toxicidade entre substâncias químicas (Tabela 2) (LIMA, 1995; OGA, 2003).

Tabela 2. Critérios para classificação de toxicidade de fármacos segundo a Comunidade Européia (OGA, 2003).

Categoria	**DL_{50} oral para ratos (mg/Kg peso corpóreo)**
Muito tóxico	Menor que 25
Tóxico	De 25 a 200
Nocivo	De 200 a 2.000

Além da determinação da DL_{50}, para se conhecerem os efeitos tóxicos de uma substância, a realização de testes toxicológicos devem ser procedidos. Estes testes (tabela 3), aplicados em animais de laboratório e sob condições previamente estabelecidas, permitem estabelecer os possíveis efeitos das substâncias em humanos expostos a elas (LOOMIS, 1989; OGA, 2003).

Tabela 3. Tipos de testes de toxicidade realizados em animais experimentais (OGA, 2003).

Tipos de testes	**Objetivo**	**Frequência de doses**	**Tempo de duração**
Aguda	Determinar a potênica do fármaco em casos de ingestão ou envenenamneto acidental ou avaliar os efeitos acumulativos	Única ou múltiplas	24 horas
Sub-crônica	Identificar e caracterizar os órgãos afetados após exposições repetidas.	Doses repetidas	21 a 90 dias
Crônica	Determinar efeito tóxico após exposição prolongada	Doses repetidas	6 meses a 2 anos

A finalidade dos testes toxicológicos é, portanto, fornecer dados que possam ser utilizados para a avaliação do risco do uso da substância para o homem, desde que sejam definidos a substância química, os indivíduos expostos e as condições de exposição (OGA, 2003).

OBJETIVOS

1. OBJETIVO GERAL

Determinar a toxicidade oral do ácido úsnico encapsulado em um sistema carreador na forma de microesferas de copolímero biodegradável de ácido láctico e glicólico (PLGA) em ratos Wistar, visando uma futura aplicação terapêutica.

2. OBJETIVOS ESPECÍFICOS

- Encapsular o ácido úsnico em microesferas de copolímero de ácido lático e glicólico (50/50), utilizando a técnica de emulsão múltipla A/O/A seguida de evaporação do solvente;
- Caracterizar físico-quimicamente as microesferas contendo ácido úsnico e determinar a eficiência de encapsulação;
- Avaliar a toxicidade oral do ácido úsnico em suspensão e microencapsulado em ratos da linhagem Wistar após tratamento com doses diárias de 25 mg/kg durante 28 dias;
- Analisar alterações das funções hepática e renal através da quantificação sorológica das enzimas transaminases e dos compostos nitrogenados uréia e creatinina;
- Avaliar as alterações morfológicas dos espécimes de tecidos obtidos dos animais tratados com ácido úsnico em suspensão e microencapsulado através da análise histopatológica;
- Avaliar qualitativamente a presença do ácido úsnico em tecidos utilizando técnica de microscopia confocal de varredura a lazer.

ARTIGO CIENTÍFICO

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Evaluation of the subchronic oral toxicity of usnic acid encapsulated into microspheres

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Abstract

The goal of the present study was to assess the subchronic oral toxicity of usnic acid encapsulated into poly(lactide-co-glicolide) microspheres in comparison with that of usnic acid suspension. Microspheres containing usnic acid were prepared and characterized by measuring the mean diameter and surface charge potential of particles, as well as the drug encapsulation efficiency. The toxic effects caused by the repeated 28-day oral administration of free and usnic acid-loaded microspheres in Wistar rats at doses of 25 $mg.kg^{-1}$ were evaluated through feed conversion efficiency, biochemical and histopathological analyses, and confocal scanning laser microscopy. Microspheres containing usnic acid presented a mean particle size of 4.92 ± 0.44 μm and a surface charge of -25.7 ± 6.5 mV. The drug encapsulation efficiency was 99 ± 0.82 % for 10 mg of usnic acid with 1:45 drug:polymer ratio (w/w). Statistically significant differences in the feed conversion efficiency and serum levels of transaminases were observed in animals treated with usnic acid suspension. The increase in alanine aminotransferase and aspartate aminotransferase serum levels after treatment with usnic acid suspension was 37.5 % and 30 %, respectively, suggesting that animals developed hepatic injury induced by usnic acid. Conversely, animals treated with microencapsulated usnic acid presented normal enzyme serum levels. In addition, normal blood urea nitrogen and creatinine were found for both treatments, confirming that usnic acid does not cause nephrotoxicity. Liver histology showed extensive and diffused degenerative vacuolization of hepatocytes and necrotic areas in the liver of animals treated with usnic acid suspension. However, such abnormalities were not found in the animals treated with usnic acid-loaded microspheres. Low fluorescence intensity was detected in the liver of animals treated with usnic acid- loaded microspheres, suggesting that the microencapsulation controlled the usnic acid release and protected the liver from its toxic effects. All these findings suggest that the usnic acid-loaded microspheres were able to reduce their hepatotoxicity, thereby allowing the introduction of usnic acid for therapeutic proposes.

Keywords: Usnic acid, PLGA-microspheres, oral toxicity, hepatotoxicity

1. Introduction

Usnic acid is a dibenzofuran derivative [2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bH)-dibenzofurandione; $C_{18}H_{16}O_7$] produced by a number of lichen species as a secondary metabolite. Many potential pharmacological uses of usnic acid have been reported, such as its antimicrobial and antiviral **(**Campanella et al., 2002**)**, antitrypanosomial (De Carvalho et al., 2004), antiinflammatory **(**Vijayakumar et al., 2000**)**, antiproliferative (Santos et al., 2005) and antitumour properties (Ribeiro-Costa et al., 2004; Santos et al., 2006).

Although usnic acid presents remarkable pharmacological properties (Cocchietto et al., 2002), its use in therapy is still limited by hepatotoxicity. In fact, toxic reactions, including ataxia leading to paralysis and death, have been reported in animals ingesting lichens containing usnic acid (Roach et al., 2006; Dailey et al., 2008).

Nowadays, despite the exixting research on the pharmacological use of usnic acid, data related to human toxicity are limited. The hepatotoxicity of usnic acid has been confirmed in patients that developed acute hepatitis or fulminating liver failure associated with the use of a dietary supplement (LipoKinetix®) that contains sodium usniate, the salt form of usnic acid. A case report of seven patients who presented symptoms characteristic of acute hepatitis, including fatigue, abdominal pain and jaundice has been published (Favreau et al., 2002). In addition, the results of the biochemical testing (aminotransferase serum levels) were consistent with acute hepatitis. However, none of the seven patients exhibited lactic acidosis, a typical failure of drug-induced liver injury due to mitochondrial dysfunction. Except for the patient who underwent liver transplantation, all patients recovered spontaneously after LipoKinetix® was suspended. Since then, several case reports have been published on hepatotoxicity in patients using diet supplements containing usnic acid (Durazo et al., 2004, Estes et al., 2003, Neef et al., 2004). More recently, Sanchez et al. (2006) reported that two patients developed severe liver failure after using LipoKinetix®, requiring emergency liver transplantation. Biopsy of liver patients showed acute hepatitis and extensive areas of necrosis, as well as leukocyte inflammatory infiltrates.

The mechanism of action of usnic acid has been extensively studied *in vitro* and seems to be related to the effect of usnic acid as uncoupling oxidative phosphorilation, as demonstrated by Abo-Khatwa et al. (1996) in murine liver mitochondria. Usnic acid caused inhibition of adenosine triphosphate synthesis and enhancement of Mg^{+2}-ATPase activity, which may contribute to its hepatic toxicity. Usnic acid exhibits characteristics similar to those of 2,4-dinitrophenol (DNP), a classical uncoupler of oxidative phosphorylation. As a result, a dose-dependent uncoupling effect was revealed with a slowdown of ATP synthesis, release

respiratory control and enhancement of Mg^{+2}-ATPase activity. The smallest usnic acid concentration required to produce full uncoupling of oxidative phosphorylation was 1 μM. It was postulated that usnic acid uncoupling effect is induced on the inner mitochondrial membrane by modifying its lipophilic features and protonophoric activities (Abo-Khatwa et al., 1996).

Usnic acid induced necrosis of cultured mouse hepatocytes through oxidative stress and disruption of the normal metabolic process of cells (Han et al., 2004). In point of fact, Pramyothin et al. (2004) found that (+)-usnic acid is able to damage the plasmatic cellular membrane of hepatocytes, allowing the release of cellular transaminases. It was found that usnic acid affects the integrity of mitochondria, thereby altering their function and the ATP synthesis. Moreover, that investigation showed that usnic acid presents a similar effect to carbon tetrachloride, which involves free radical formation that induces injury to the mitochondrial cell membrane, peroxidation of lipids and eventually cell death. Further, it was suggested that usnic acid might be metabolized by cytochrome P450 (CYP2E1). More recently, it was confirmed in cultured human hepatocytes that the oxidative metabolism of usnic acid is produced mainly by cytochrome P450 (CYP)1A2 to form three monohydrohylated metabolites (Foti et al., 2008). Furthermore, usnic acid was shown to be a relatively weak inhibitor of CYP2D6 and a potent inhibitor of CYP2C19.

In order to diminish or circumvent the hepatoxicity of usnic acid, the nano and microencapulation of this drug were proposed (Ribeiro-Costa et al., 2004; Santos et al., 2006). In this way, usnic acid was encapsulated into poly(lactide-co-glycolide) nanocapsules and its hepatotoxicity was evaluated after subcutaneous administration in mice for 15 days. Histopatological analysis revealed extensive necrotic areas on the liver tissue after treatment with free usnic acid, which was markedly reduced in the animals treated with usnic acid encapsulated into PLGA-nanocapsules (Santos et al., 2006). These findings clearly suggest that encapsulation may be a way of enabling usnic acid to be used in cancer therapy.

Therefore, the aim of this research was to evaluate the subchronic toxicity of usnic acid after oral administration of drug-loaded microspheres in comparison with the free drug using Wistar rats as an animal model. The hepatic and renal toxicities of usnic acid were investigated by means of biochemical analyses of serum levels of transaminases, blood urea nitrogen and creatinine. Histopatological and confocal scanning laser microscopy analyses were also performed.

2. Materials and methods

Poly (D,L-lactide-co-glycolide) acid (PLGA) 50:50 (0.57 dl/g) was purchased from Birmingham Polymers (Alabama, USA). The stabilizers poly vinyl alcohol (PVA, MW 30, 000-70,000) and polyethylene Glycol (PEG, MW 4,000), the cryoprotectant trehalose, the anesthetic Urethane® and usnic acid were obtained from Sigma-Aldrich (St. Louis, USA). Solvents and other chemicals were supplied by Merck (Darmstadt, Germany).

2.1. Preparation of usnic acid-loaded microspheres

The PLGA-microspheres containing usnic acid were prepared through the conventional double emulsion (w/o/w) solvent evaporation technique, as previously reported (Ribeiro-Costa et al., 2004). Briefly, PLGA (450 mg) and usnic acid (10 mg) were dissolved in methylene chloride (12 mL) and homogenized with 5 mL PEG 4000 (400 mg) solution, using an ultra-turrax® homogenizer (T25, Ika, Germany) at 8,000 rpm for 60 seconds in an ice bath, resulting in a simple emulsion (w/o). The resulting emulsion was then added to a continuous phase consisting of 50 mL PVA solution (0.5% w/v) and emulsified at 8,000 rpm for 30 seconds, resulting in a double emulsion (w/o/w). This emulsion was then stirred with a four-blade propeller (Mechanic stirrer RE 162/P, IKA, Germany) at 25 ºC ± 1 ºC at 400 rpm for four hours so as to allow the evaporation of solvent and microspheres formation. The microspheres were subsequently collected by centrifugation (Kubota KN-70 centrifuge, Japan) at 3,000 rpm for five minutes and washed three times with 25 mL of deionized water. Finally, usnic acid-loaded microspheres were dispersed with 1 % trehalose solution (w/v), which was frozen overnight at - 80 ºC before lyophilisation (EZ-DRY, FTS System, New York, USA) for 16 hours. The PLGA-microspheres containing usnic acid were appropriately stored at 25 ºC ± 1 ºC in a vacuum desiccator.

2.2. Characterization of usnic acid-loaded microspheres

The particle size and surface charge (zeta potential) of usnic acid-loaded microspheres were measured using a Zetasizer® (Nano-ZS90, Malvern, United Kingdom). Results are presented as the average of at least three measurements of different diluted samples of the same batch.

The usnic acid content in microspheres was evaluated using UV-VIS spectroscopy (Ultrospec® 3000 pro, Amersham Biosciences, Sweden) at a wavelength of 280 nm. Samples of lyophilized usnic acid-loaded microspheres (11.5 mg) were diluted with 25 mL of a mixture of methylene chloride and methanol (3:2) under ultrasound agitation for 10 min. An aliquot of this solution was then diluted to a theoretical concentration of 6 $\mu g.mL^{-1}$. The standard curve of usnic acid was prepared in methanol at concentrations ranging from 2 to 14 $\mu g.mL^{-1}$. The assays were performed in triplicate.

2.3. Subchronic oral toxicity of usnic acid-loaded microspheres

A subchronic oral toxicity study through repeated 28-day doses of usnic acid-loaded microspheres was carried out in Wistar male rats, according to the experimental protocol (OECD, 1995) approved by the Animal Ethics Committee of the Federal University of Pernambuco. Animals weighing from 120 to 200 g were randomly distributed in three groups of 10 animals each. The two treated groups received daily oral doses of 25 mg/kg of usnic acid-loaded microspheres (UA-MS) after redispersion in saline solution at 25 °C or usnic acid in suspension (sodium bicarbonate solution at pH to 8.5) for 28 days. The control group received oral saline solution. The animals rested under suitable laboratory conditions of temperature (25 °C ± 2 °C) and lighting (12:12 light: dark cycles) and were given free access to water and standard food. They were daily observed for their general clinical behaviour and their body weights were recorded on the first day of the experiment and subsequently at 7, 14, 21 and 28 days. Food consumption was measured over successive periods of 7 days by weighing the feeders. The feed conversion efficiency percentage was calculated as weekly body weight gain (g) per weekly food consumption (g). On day 29 of the treatment, the animals were anaesthetized with Urethane® and sacrificed by cardiac puncture, blood being collected in suitable glass tubes. Serum samples were obtained after blood centrifugation at 3,500 rpm (KUBOTA centrifuge, Tokyo, Japan) for 20 min and subsequently subjected to standard biochemical analyses. Organs (liver, kidney, spleen, heart, lung and intestine) were excised, weighed and submitted to conventional treatment for histopathological examination by light microscopy. In addition, the possible presence of usnic acid in different tissues was assessed using confocal scanning laser microscopy, exploiting the intrinsic fluorescence of usnic acid.

2.4. Biochemical analyses

Routine clinical chemistry analyses were conducted on all animals at the end of the treatment period. The animals´ liver function was assessed by evaluating the serum levels of alanine aminotransferase (ALT), and aspartate aminotransferase (AST). The serum levels of the blood urea nitrogen (BUN) and creatinine (CRE) were likewise evaluated as markers of kidney function. Analyses were performed by spectroscopy (Ultrospec® 3000 Pro, Amersham Biosciences, Sweden) using standard biochemical analysis kits (Katal Biotecnologica, Brazil).

2.5. Histopathological analysis

Samples of liver, kidney, spleen, heart, lung and intestine were removed immediately after the animal´s death and fixed in a 10 % buffered formaline solution for at least 24 h. All tissue samples were embedded in paraffin wax, sectioned (3–5 μm thickness) and stained with hematoxylin and eosin using a standard protocol. Histological examination was performed by light microscopy (Olympus BH-2 microsope, Japan) and the presence of any cell abnormalities and infiltrations was observed.

2.6. Confocal scanning laser microscopy

Fragments of tissues were embedded in an optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, USA) and stored at -80 ^{o}C overnight for subsequent use. Sections of tissues were performed on cryostat (8 μm) and mounted in glass microscopic slides using glycerol/PBS (1:3). Digital images were obtained with a confocal scanning laser microscope (LEICA LSM, Heidelberg, Germany), by exploiting the intrinsic fluorescence of usnic acid.

2.7. Statistics

Data were expressed as mean ± SD (standard deviation, n=10/group). Statistical differences between mean data of the biochemical analysis, organ weights of the animals and feed conversion efficiency of the animal groups receiving different treatments were calculated by one-way analysis of variance (ANOVA). As usual, the level of significance was set at $p < 0.05$.

3. Results

3.1. Characterization of usnic acid-loaded microspheres

PLGA-microspheres containing acid usnic presented a particle mean diameter of 4.92 ± 0.44 μm. Measurement of the zeta potential, which was – 25.7 ± 6.5 mV, assessed the surface charge of usnic acid-loaded microspheres. The drug encapsulation efficiency was 99 ± 0.82 % for an initial amount of 10 mg of usnic acid and 1:45 drug:polymer ratio.

3.2. Subchronic oral toxicity of usnic acid-loaded microspheres

All the animals presented normal clinical behaviour and no death was recorded during the treatment. A significant difference (p<0.05) was observed between the average feed consumption efficiency of the animals treated with free usnic acid (22 ± 3 %) and the control animal group (30 ± 1.3 %). Conversely, no statistically significant difference (p<0.05) was found between the group of animals treated with usnic acid-loaded microspheres and the untreated group (Table 1). I should be emphasized that the animals did not exhibit any weight loss throughout the treatment.

Table 1. Body weight, feed consumed (in parenthesis) and feed conversion efficiency of Wistar rats treated with usnic acid suspension (UA) and usnic acid-loaded microspheres (UA-MS) with a daily dose of 25 mg/kg for 28 days. The control group received saline solution.

Days of analysis	Animal treatments		
	Control	UA	UA-MS
1	123.3 ± 10.0	182.5 ± 10.3	146.5 ± 26.6
7	160.0 ± 20.2	215.0 ± 18.4	180.0 ± 24.9
	(34.6 ± 3.3)	(37.7 ± 13.0)	(35.7 ± 6.6)
14	206.7 ± 27.3	254.0 ± 18.8	229.5 ± 21.4
	(43.2 ± 7.9)	(45.14 ± 2.6)	(37.14 ± 7.9)
21	252.2 ± 32.3	287.0 ± 20.0	269.0 ± 21.8
	(43.57 ± 3.4)	(46.0 ± 2.12)	(45.4 ± 4.0)
28	285.0 ± 31.8	311.0 ± 19.7	294.5 ± 25.9
	(32.68 ± 3.1)	(36.9 ± 3.1)	(39.6 ± 3.4)
FCE (%)	30 ± 1.3	22 ± 3*	27 ± 8

The data were represented as mean body weight (g/rat ± SD), mean consumed feed (g/rat/day ± SD) and average feed conversion efficiency (FCE) defined as 100 × [(g gained weight)/(g consumed feed) ± SD] (n = 10).

[*] $p<0.05$ *vs* Control Group.

The absolute and relative weights of the animals´ organs at the end of the treatment are presented in Table 2. The results show no statistically significant differences ($p<0.05$) between the organ weights of treated and untreated animals for both absolute and relative values.

Table 2. Absolute and relative organ weights of Wistar rats treated with usnic acid suspension (UA) and usnic acid-loaded microspheres (UA-MS) with a daily dose of 25 mg/kg for 28 days.

Organs	Animal treatments and organ weights					
	Control		UA		UA-MS	
	Absolute (g)	Relative (%)	Absolute (g)	Relative (%)	Absolute (g)	Relative (%)
Liver	9.95 ± 1.75	3.54 ± 0.78	10.6 ± 2.03	3.43 ± 0.76	10.2 ± 1.96	3.5 ± 0.76
Spleen	0.81 ± 0.23	0.29 ± 0.04	0.88 ± 0.19	0.28 ± 0.05	0.95 ± 0.14	0.32 ± 0.05
Kidneys	2.05 ± 0.20	0.72 ± 0.06	2.14 ± 0.20	0.70 ± 0.07	2.14 ± 0.24	0.71 ± 0.08
Heart	1.05 ± 0.13	0.37 ± 0.03	1.12 ± 0.15	0.36 ± 0.03	1.09 ± 0.1	0.36 ± 0.04
Lung	1.36 ± 0.15	0.48 ± 0.06	1.50 ± 0.20	0.48 ± 0.07	1.30 ± 0.11	0.44 ± 0.05

Data are presented as mean ± SD (n = 10). Relative organ weight values were calculated in relation to the mean body weight of animals at the end of the treatment (Table 1).

3.3. Biochemical analyses

Statistically significant changes in the biochemistry of animals treated with oral repeated doses of usnic acid (25 mg/kg) for 28 days were found (Fig. 1, Table 3). A significant increase ($p<0.05$) in serum levels of ALT and AST (44.0 ± 4.3 and 106.1 ± 10.4 U/L, respectively) was noted for the animals treated with usnic acid as compared with the control group (32.2 ± 3.9 and 82.5 ± 13.8 U/L, respectively). Nevertheless, no significant alterations in serum enzyme levels were found after treatment of the animals with usnic acid-loaded microspheres (ALT = 34.3 ± 4.0 and AST =89.8 ± 8.9) in comparison with the untreated animals.

The serum levels of BUN and CRE of the control group were 50.9 ± 7.0 mg/dL and 0.707 ± 0.13 mg/dL, respectively. Furthermore, no significant changes in the serum levels of BUN and CRE after treatment of the animals with usnic acid suspension (54.5 ± 9.8 e 0.747 ± 0.05, respectively) or usnic acid-loaded microspheres (48.5 ± 7.4 e 0.727 ± 0.8, respectively) were found (Fig. 1, Table 3).

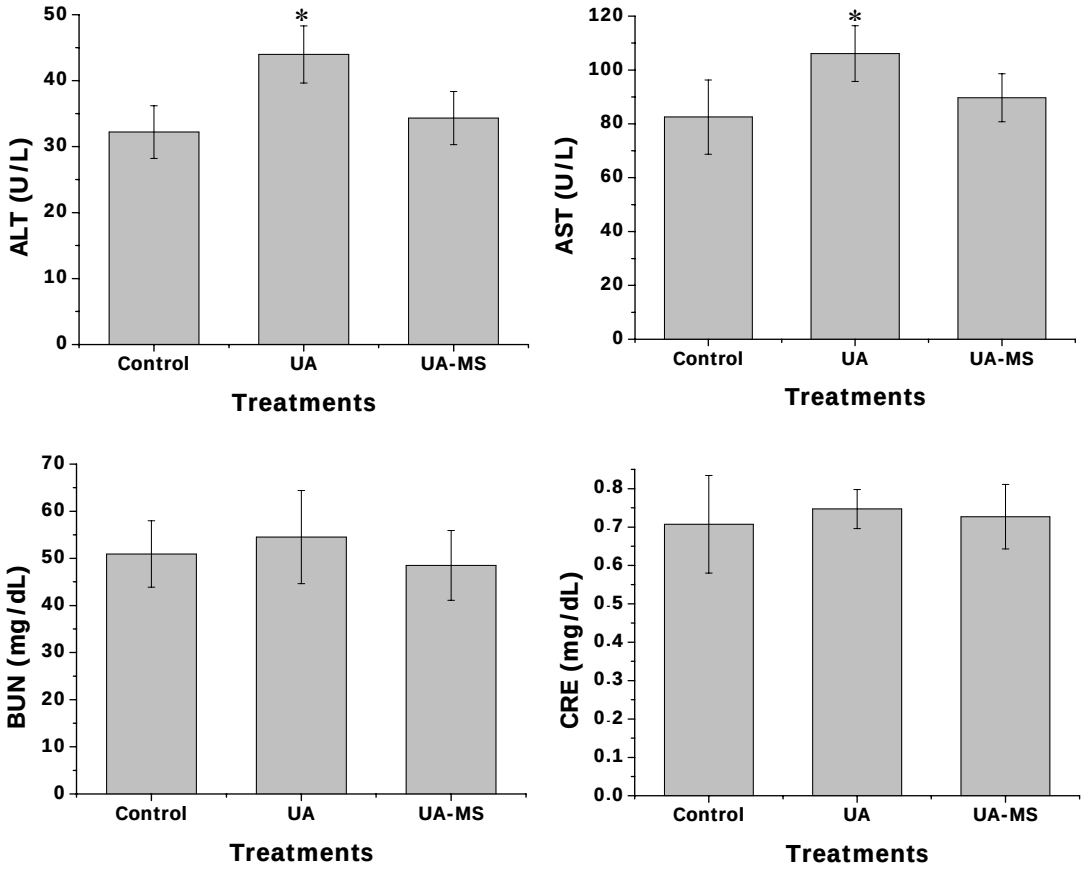

Fig. 1. Serum biochemical findings in Wistar rats treated with usnic acid suspension (UA) and usnic acid-loaded microspheres (UA-MS) at a daily oral dose of 25 mg/kg for 28 days. The data are presented as the mean ± SD (n = 10). * p<0.05 *vs* Control group.

Table 3. Statistical variance analysis (ANOVA) of serum levels of AST, ALT, BUN and CRE after treatment with usnic acid (UA) and usnic acid-loaded microspheres (UA-MS).

Treatments	Biochemical analysis	F-statistic	$F_{critical}$ (df)	Probe value p=	Null-hypothesis H_0
Control × UA	AST	18.4222	F_{18}^{1} =4.4139	0.0004	rejected
	ALT	40.2039	F_{18}^{1} =4.4139	5.6 x 10^{-6}	rejected
Control × UA-MS	AST	1.9111	F_{18}^{1} =4.4139	0.1837	cannot be rejected
	ALT	1.4392	F_{18}^{1} =4.4139	0.2458	cannot be rejected
Control×UA×UA-MS	CRE	0.4675	F_{27}^{2} =3.3541	0.6315	cannot be rejected
	BUN	1.3686	F_{27}^{2} =3.3541	0.2715	cannot be rejected

3.4. Histopathological analysis

Severe morphological abnormalities were observed in the liver of rats treated with usnic acid suspension compared with that of untreated animals (Fig. 2A). Extensive and diffuse areas of degenerative vacuolization of hepatocytes and necrotic areas can be seen in the liver of the animals treated with usnic acid suspension. Degenerated hepatocytes with characteristic pycknotic nuclei were visualized in region surrounding the central vein. Degenerative ballooned hepatocytes with multiple cytoplasmic vacuolization and a disrupted arrangement of the anatomizing plates of hepatocytes were visualized, revealing the damaged cytoarchitecture of the liver. It should be pointed out that, although the endothelium of the central vein was damaged, the bile ducts were well preserved (Fig. 2A, inset). In contrast, the liver of the animals treated with usnic acid-loaded microspheres was well preserved and no injury was visualized in the central vein region (Fig. 2B). No morphological abnormalities were found in kidneys, spleen, heart, lung or intestine of the animals treated with usnic acid or usnic acid-loaded microspheres (data not shown).

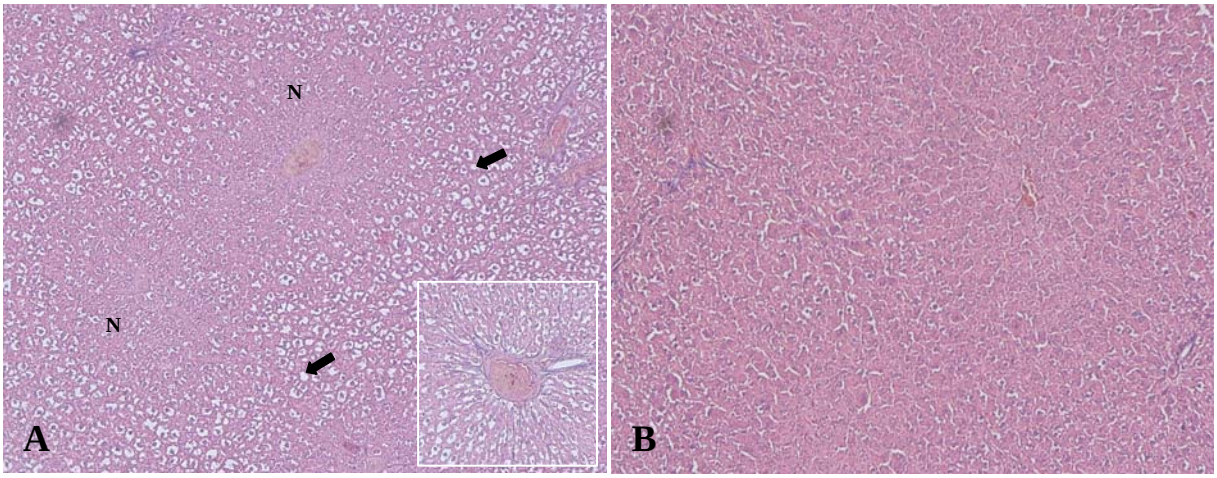

Fig. 2. Histopatological analysis of the liver of Wistar rats treated with usnic acid suspension (A) and usnic acid-loaded microspheres (B) at a daily oral dose of 25 mg/kg for 28 days. The inset in A shows a bile duct. N denotes diffuse areas of necrosis in the liver and arrows indicate cytoplasmic vacuolization of hepatocytes. Magnification 100 ×.

3.5. Confocal scanning laser microscopy

Figure 3 shows cryostat sections of a rat liver illustrating the distribution of fluorescence in hepatocytes after the 28-day oral administration of usnic acid suspension (Fig.

3A) and usnic acid-loaded microspheres (Fig. 3B). The liver of the animals treated with usnic acid suspension presented a higher intensity of fluorescence when compared with those treated with usnic acid-loaded microspheres.

Fluorescence was detected in the kidneys of the animals after treatment with usnic acid suspension, as shown in Fig 3C. However, no fluorescence was detected in the kidneys of animals treated with usnic acid-loaded microspheres. Spleen, lung and heart presented intrinsic fluorescence, and as a result, the presence of usnic acid in these tissues was not evaluated.

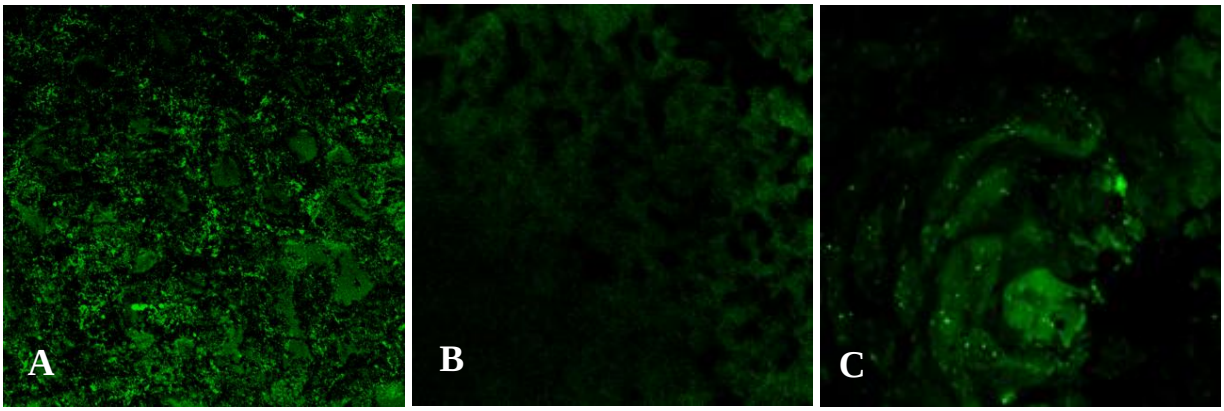

Fig. 3. Confocal scanning laser microscopic images of liver (A, B) and kidney (C) of Wistar rats after oral treatment with usnic acid suspension (A, C) or usnic acid-loaded microspheres (B) at a daily dose of 25 mg/kg for 28 days. Original magnification of 400 ×.

4. Discussion

Usnic acid provides an example of a drug causing hepatotoxicity by mitochondrial injury. The development of both fundamental and clinical research on the mechanisms of drug-induced liver injury is warranted. In fact, drug-induced hepatic injury is a major clinical issue, which also has a considerable economic and social impact on the pharmaceutical industry because represents the most frequent cause of post-marketing withdrawal of new drugs.

No systematic subchronic or chronic study of toxicity of usnic acid has been performed in rodents. Although several case histories of animals and human toxicity and related cytotoxicity studies have been reported in the literature, more comprehensive *in vivo* and *in vitro* toxicity evaluations of usnic acid need to be undertaken to elucidate the mechanism of its hepatotoxicity more thoroughly.

In the present study, the protective effect of the microencapsulation of usnic acid into PLGA-microspheres on liver injury caused by usnic acid was evaluated in a subchronic oral toxicity protocol using repeated 28-day doses (25 mg/kg) of usnic acid suspension and usnic acid-loaded microspheres administered to Wistar rats. Within this framework, PLGA-microspheres containing usnic acid were produced and characterized as spherical and negatively-charged surface particles, which were finely redispersed in water prior to being administered to the animals.

No alterations in clinical behaviour or death of animals were observed during the treatment. A reduction in feed conversion efficiency was determined for the animals treated with usnic acid suspension in comparison with the untreated animals. Nevertheless, no weight loss was found in either untreated or treated animals. It is likely that the reduction in feed conversion efficiency is related to the hepatic injury induced by usnic acid treatment.

The liver injury induced by usnic acid was assessed at a biochemical level by measuring the serum levels of ALT and AST as markers for hepatotoxicity. The serum levels of transaminases are often considered as the gold standard clinical chemical markers of liver function (Josef et al., 2008). It is well known that an increase in these enzyme levels in blood may represent conditions that alter the permeability of cell membranes to such a extent that ALT and AST leak into the blood, and this increase is almost proportional to the number of affected hepatocytes (Hall, 1992). In the present study, the nominal increase in ALT and AST serum levels after treatment with free usnic acid were 37.5 % and 30 %, respectively. These high serum levels of transaminases in the animals treated with usnic acid suspension suggest that the animals developed liver injury induced by the usnic acid. Conversely, the animals treated with microencapsulated usnic acid exhibited normal enzyme serum levels, confirming a decrease in the hepatotoxicity of usnic acid resulting from its encapsulation into PLGA-microspheres.

Our enzymatic results are at odds with ones previously reported (Pramyothin et al., 2004) for an acute toxicity evaluation in rats after treatment with usnic acid (dissolved in 0.1 ml DMSO) at doses of 50 mg/kg and 200 mg/kg for 5 days. No statistically significant alterations were found for serum levels of ALT (72.1 ± 2.7 and 78.7 ± 6.5 U/L) and AST (90.7 ± 15.7 and 101.7 ±14.4 U/L) for the treatment with usnic acid at a total dose of 250 mg and 1g, respectively. The discrepancy between our data and those previously reported can be accounted for the duration of the treatment, as the effect of usnic acid on the serum levels of transaminases does not seem to be dose-dependent. A higher total dose of usnic acid was administered to animals (1 g) for five days (Pramyothin et al., 2004) against 700 mg

administered for 28 days in the present study. Since the release of ALT and AST only occurs after damage to the hepatocyte membranes, the duration of exposure to the drug is a key factor in the serum levels of transaminases.

Measuring the serum levels of the burn urea nitrogen and creatinine assessed the effect of the treatment with usnic acid and usnic acid-loaded microspheres on the renal function of animals. Normal BUN and creatinine were found after both treatments, suggesting that usnic acid does not cause nephrotoxicity, as the renal function of the animals was preserved. As recently pointed out, usnic acid is eliminated by renal excretion as glucuronide conjugates. Usnic acid undergoes glucuronidation effected by uridine diphosphate-glucuronyltransferase UGT1A1 and UGT1A3, forming two regio-isomeric glucuronide conjugates (Foti et al., 2008).

Histopatological analyses revealed a diffuse degenerative vacuolization of hepatocytes and widespread necrotic areas in the liver of the animals treated with usnic acid suspension, indicating serious damage to the cytoarchitecture of the liver. The endothelium of the central vein was severely damaged, but no abnormalities were observed in the bile ducts. It can thus be inferred that usnic acid does not induce liver injury by cholestasis, since the bile ducts remained preserved after the subchronic oral treatment. However, these results should be carefully borne in mind and further investigations carried out, given that large intrahepatic bile ducts express CYP2E1 and may be more susceptible to damage by drugs metabolized by this enzyme (Le Sage et al., 1999) as in the case of usnic acid.

The histopatological results presented here are in agreement with those previously reported (Santos et al., 2006) for a subchronic toxicity study of free usnic acid and usnic acid-loaded nanocapsules after treatment of mice with 15 mg/kg/day intraperitoneal doses for 15 days. In that investigation, necrotic areas were observed in the liver of animals treated with usnic acid suspension, but this abnormality was remarkably reduced in animals treated with usnic acid encapsulated into PLGA-nanocapsules.

Usnic acid can restrain the function of the mitochondrial respiratory chain, thereby reducing the oxidative phosphorilation and depleting intracellular ATP levels. Indeed, the inhibition of enzymes in the normal respiratory chain can also produce excessive reactive oxidative species (ROS), instigating further cellular injury. ROS is assumed to play a key role in several forms of hepatic injury, including that associated with iron and copper overload (Bissel et al., 2001).

The findings of Pramyothin et al. (2004) suggest that usnic acid is metabolized in rat hepatopcytes by CYP2E1, as aniline hydroxylase activity increased (32.8 ± 1.7 and 46.5 ±

2.3) with increased doses of usnic acid (1 mM and 10 mM, respectively). In that study, isolated rat liver mitochondria were used as a model to evaluate the direct effect of usnic acid on mitochondrial function. It was found that usnic acid (4.5 μM) caused maximal stimulation of respiration (five-fold for gluatamate plus malate and three-fold for succinate as substrate) and produced a five-fold increase in ATPase activity.

Carbon tetrachloride (CCl_4) is the conventional model for free radical-induced liver injury and its main effects are high serum levels of ALT and AST, severe damage to hepatocytes, necrosis and tissue degeneration (Avasarala et al., 2006; Wu et al., 2007). Pramyothin et al. (2004) showed that usnic acid presents a mechanism of action similar to that of CCl_4. Based on all these findings, our study to a certain extent confirmed the similarity between the effects of usnic acid and CCl_4 on liver injury.

The presence of usnic acid in the liver and kidneys of the animals treated with usnic acid-loaded microspheres in comparison with usnic acid suspension was detected using confocal scanning laser microscopy, exploiting the intrinsic fluorescence of usnic acid. Confocal microscopic images showed less intensity of fluorescence in the liver of the animals treated with usnic acid-loaded microspheres, suggesting that microencapsulation controlled the usnic acid release and protected the liver from its toxic effects. This event was probably due to the decrease in drug exposure in the liver. The fluorescence detected in the kidneys of the animals treated with usnic acid suspension confirmed the renal filtration and excretion of usnic acid, which presents a clearance of 12.2 ± 3.0 ml/h/kg after intravenous administration of 5 mg/kg and a mean absolute bioavailability following oral administration of 77.8 % (Krishna and Ventakaranana, 1992).

In the present study, it was revealed for the first time that the oral administration of usnic acid encapsulated into PLGA-microspheres has a protective effect against the acute liver damage induced by usnic acid. This hepatoprotection could be explained by the controlled release of usnic acid from the microspheres, and its subsequent sustained metabolization by hepatocytes.

In conclusion, the current investigation provided convincing data that usnic acid-loaded microspheres might be considered as a therapeutic dosage form for the oral administration of usnic acid. The subchronic oral toxicity assay showed that a decrease in the hepatotoxicity of usnic acid was achieved as a result of its encapsulation into PLGA-microspheres.

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CONCLUSÕES

- Microesferas de PLGA contendo ácido úsnico foram obtidas e apresentaram forma esférica com distribuição homogênea de tamanho de partículas; com carga de superfície negativa, que confere estabilidade às partículas em suspensão, e eficiência de encapsulação de praticamente 100 % de ácido úsnico para uma relação de 1:45 fármaco:polímero;

- Na avaliação da toxicidade oral do ácido úsnico, uma diferença estatisticamente significativa foi observada na eficiência de conversão alimentar dos animais tratados com ácido úsnico com relação ao controle. No entanto, nenhuma diferença foi observada no grupo tratado com ácido úsnico microencapsulado;

- Não houve diferença estatisticamente significativa no peso dos animais durante o tratamento e dos órgãos dos animais após o tratamento com ácido úsnico livre e microencapsulado com relação ao controle;

- Um aumento significante das transaminases (AST e ALT) foi observado nos animais tratados com ácido úsnico quando comparado com o grupo não tratado. Contudo, alterações enzimáticas significantes não foram encontradas após o tratamento dos animais com ácido úsnico microencapsulado, confirmando uma redução da hepatoxicidade do ácido úsnico devido a sua microencapsulação;

- Mudanças não foram observadas nos níveis séricos de uréia e creatinina após o tratamento com ácido úsnico em suspensão ou microencapsulado, confirmando que o ácido úsnico não apresenta nefrotoxicidade;

- As análises histopatológicas do fígado demonstraram extensas áreas de degeneração vacuolar com áreas de necrose nos animais tratados com ácido úsnico. Porém, os animais tratados com ácido úsnico microencapsulado não desenvolveram alterações hepáticas,

sugerindo que a microencapsulação do ácido úsnico foi capaz de reduzir sua hepatotoxicidade;

- O ácido úsnico foi detectado no fígado e rins dos animais tratados com ácido úsnico livre, através da fluorescência intrínseca do ácido úsnico, indicando a sua captura hepática, filtração e eliminação renal. Uma menor intensidade de fluorescência foi visualizada no fígado após tratamento com ácido úsnico microencapsulado, devido à liberação controlada pelas microesferas. Nenhuma fluorescência foi detectada no rim após tratamento dos animais com ácido úsnico encapsulado.

- Estes resultados sugerem que a microencapsulação do ácido úsncio pode reduzir sua hepatotoxicidade, desse modo permitindo o seu uso para aplicação terapêutica.

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