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ESTUDO TOXICOLÓGICO COMPARATIVO REFERENTE AO HALOPERIDOL NA SUA FORMA LIVRE E NANOENCAPSULADA

DISSERTAÇÃO DE MESTRADO

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Santa Maria, RS, Brasil
2015

ESTUDO TOXICOLÓGICO COMPARATIVO REFERENTE AO HALOPERIDOL NA SUA FORMA LIVRE E NANOENCAPSULADA

Katiane Roversi

Dissertação apresentada ao Programa de Pós-Graduação em Farmacologia da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Farmacologia**

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ESTUDO TOXICOLÓGICO COMPARATIVO REFERENTE AO HALOPERIDOL NA SUA FORMA LIVRE E NANOENCAPSULADA

elaborada por
Katiane Roversi

como requisito parcial para obtenção do grau de
Mestre em Farmacologia

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Santa Maria, 14 de agosto de 2015.

Dedico esta dissertação ao meu querido pai Adecir,

e minhas irmãs Karine, Karoline e Francini.

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"Que os vossos esforços desafiem as impossibilidades,
lembrai-vos de que as grandes coisas do homem
foram conquistadas do que parecia impossível."

Charles Chaplin

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Farmacologia
Universidade Federal de Santa Maria

ESTUDO TOXICOLÓGICO COMPARATIVO REFERENTE AO HALOPERIDOL NA SUA FORMA LIVRE E NANOENCAPSULADA

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Data e Local de Defesa: Santa Maria, 14 de agosto de 2015

A associação de haloperidol (HP) a nanocápsulas poliméricas proporciona uma significante melhora na eficácia terapêutica, prolonga o tempo de ação e reduz efeitos adversos motores em ratos. No entanto, tendo em vista a toxicidade do HP sobre órgãos como fígado e rim e o pouco conhecimento sobre a toxicidade das nanocápsulas poliméricas, o objetivo deste estudo foi avaliar os efeitos da formulação contendo haloperidol nanoencapsulado sobre parâmetros bioquímicos e marcadores de estresse oxidativo (EO) nestes mesmos tecidos, além do dano no DNA no sangue. Para este estudo, 28 ratos foram separados em quatro grupos experimentais (n=7) e tratados com solução aquosa contendo 5% polissorbato 80 (v/v) (grupo C), suspensão de haloperidol livre (grupo FH), suspensão de nanocápsulas branca (grupo B-Nc) e suspensão de haloperidol nanoencapsulado (grupo H-Nc). Todas as suspensões foram administradas aos animais (0,5 mg/kg-ip) uma vez por dia, durante 28 dias. Os resultados mostraram que o tratamento subcrônico com FH causou danos no fígado, evidenciado pelo aumento nos níveis de peroxidação lipídica e diminuição das defesas antioxidantes como vitamina C e superóxido dismutase, diminuição na integridade celular e aumento nos níveis plasmáticos das enzimas AST e ALT. O FH também causou danos no rim, mas em menor extensão, e causou danos ao DNA sanguíneo. Por outro lado, ratos tratados com H-Nc não apresentaram estas alterações. A partir deste estudo comparativo foi possível evidenciar que o haloperidol nanoencapsulado (H-Nc) não causou toxicidade subcrônica hepática e renal aos animais, preservando estes tecidos dos danos oxidativos e da perda da integridade celular, os quais foram observados nos animais tratados com o fármaco livre.

Palavras-chave: integridade celular, haloperidol, nanopartículas poliméricas, nanotoxicologia, estresse oxidativo

ABSTRACT

Dissertation of Master´s Degree
Post-Graduate Program in Pharmacology
Federal University of Santa Maria, RS

COMPARATIVE TOXICOLOGICAL STUDY RELATED TO HALOPERIDOL IN ITS FREE FORM AND NANOENCAPSULATED

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Date and Place of defense: August 14th, 2015, Santa Maria.

The association of haloperidol (HP) with polymeric nanocapsules causes a significant improve in therapeutic efficacy prolongs the drug time of action and reduces motor side effects in rats. However, in view of the HP toxicity on organs such as liver and kidney and besides due the lack of knowledge about the toxicity of polymeric nanocapsules, the objective of this study was to evaluate the effects of formulation containing haloperidol-loaded lipid-core nanocapsules on biochemical parameters and oxidative stress (OS) markers in the same tissues, besides DNA damage in blood. For this study, 28 rats were divided in four groups (n = 7) and treated with aqueous solution containing 5% polysorbate 80 (v/v) (C group), free haloperidol suspension (FH group), blank nanocapsules suspension (B-Nc group) and haloperidol-loaded lipid-core nanocapsules suspension (H-Nc group). All suspensions were administered in the animals (0,5 mg/kg-ip) once a day, for 28 days. The results showed that a subchronic treatment with FH increased oxidative damage evidenced by the elevation in lipid peroxidation levels and diminution in antioxidant defenses like vitamin C and superoxide dismutase enzyme, decreased cell integrity and increased plasma levels of AST and ALT enzymes. FH also caused damage to kidney, but to a lesser extent, and caused damage to blood DNA. On the other hand, rats treated with H-Nc did not present these alterations. Through this comparative study was possible evidence that H-Nc did not show subchronic toxicity in liver and kidney of animals, preserving these tissues from oxidative damage and loss of cell integrity, which were observed in animals treated with free drug.

Keywords: cellular integrity, haloperidol, polimeric nanocapsules, nanotoxicology, oxidative stress.

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ARTIGO CIENTÍFICO

LISTA DE ABREVIATURAS

DA - dopamina
EO - estresse oxidativo
HP - haloperidol
PCL - poli (ε-caprolactona)

SUMÁRIO

APRESENTAÇÃO

Esta dissertação está estruturada em seções dispostas em: Introdução, Objetivos, Artigo Científico, Conclusões, Perspectivas e Referências.

Os itens materiais e métodos, resultados, discussão dos resultados e referências encontram-se inseridos no artigo contido na seção **ARTIGO CIENTÍFICO,** representando a íntegra deste estudo. As **REFERÊNCIAS** referem-se somente às citações que aparecem no item **INTRODUÇÃO** desta dissertação.

1 INTRODUÇÃO

1.1 Psicoses e Esquizofrenia

O termo psicose descreve os distúrbios psiquiátricos graves, geralmente de origem desconhecida ou idiopática, nos quais são encontrados, além dos distúrbios do comportamento, a incapacidade de pensar coerentemente e de compreender a realidade (SILVA, 2002). A esquizofrenia é um transtorno mental crônico e debilitante que impede de forma significativa o funcionamento social e profissional normal (MUESER; McGURK, 2004; Van OS; KAPUR, 2009). A doença é caracterizada, fundamentalmente, pela presença de sintomas positivos, caracterizados por delírios e alucinações, e sintomas negativos, como a perda de motivação e oscilação emocional (TANDON; NASRALLAH; KESHAVAN, 2009; ANDREASEN; BLACK, 2009).

Embora muitos fatores têm sido associados com a esquizofrenia, incluindo fatores genéticos, influências ambientais no início da vida, processos neurobiológicos, psicológicos e sociológicos (AKDENIZ; TOST; MEYER-LINDENBERG, 2014; SCHMITT et al., 2014; BERGEN et al., 2014), o mecanismo subjacente a esta doença é desconhecido, sendo que várias hipóteses bioquímicas já foram desenvolvidas na tentativa de explicar a gênese da doença. Entre todas as hipóteses, a mais aceita é a da hiperfunção do sistema dopaminérgico na via mesolímbica encefálica (HOWES; KAPUR, 2009). No entanto, sabe-se que além do sistema dopaminérgico, outros sistemas de neurotransmissores centrais também desempenham algum papel, sendo provável seu envolvimento simultâneo (LIEBERMAN; MAILMAN; DUNCAM, 1998).

A hipótese dopaminérgica da esquizofrenia descreve que os sintomas positivos da doença são resultado do aumento da liberação de dopamina (DA) subcortical, a qual aumenta a ativação dos receptores dopaminérgicos do tipo D_2 da via dopaminérgica mesolímbica (LARUELLE et al., 2005; SHEN; LIAO; TSENG, 2012). Com relação aos sintomas negativos e cognitivos, a hipótese dopaminérgica os atribui a uma redução da ativação do receptor D_1 no córtex pré-frontal (SHEN; LIAO; TSENG, 2012) e diminuição da atividade do núcleo caudado (ABI-DARGHAM, 2004). Em resumo, acredita-se atualmente que uma hiperatividade dopaminérgica subcortical

(mesolímbica) e um déficit na atividade dopaminérgica no córtex pré-frontal estariam envolvidos na esquizofrenia (HOWES; KAPUR, 2009; WALTER et al., 2009; POGARELL et al., 2012).

Algumas observações confirmam a hipótese dopaminérgica: primeiramente, que a eficácia dos neurolépticos está correlacionada com a sua ligação aos receptores D_2, antagonizando os efeitos da DA (SEEMAN, 1975; 1976; CREESE; BURT; SNYDER, 1976; MIYAMOTO et al., 2005) e que agonistas diretos e indiretos de DA, como a anfetamina e apomorfina, respectivamente são capazes de induzir pseudopsicose em animais (LYON, 1991; BYRNES; HAMMER, 2000; CURRAN et al., 2004). Uma evidência adicional para essa hipótese resulta de estudos de imagem em humanos que demonstram aumento região-específico da transmissão dopaminérgica em indivíduos esquizofrênicos (KEGELES et al., 2010)

1.2 Fármacos Antipsicóticos

Os antipsicóticos estão entre os psicotrópicos mais prescritos na clínica, constituindo uma farmacoterapia amplamente empregada no controle dos sintomas psicóticos e em distúrbios do comportamento associados à demência e drogas de adição (RAJA, 1995; ARRUDA; MORAIS; PARTATA, 2012)**.** Eles têm sido a base do tratamento para as pessoas com diagnóstico de esquizofrenia ou psicose desde que foram introduzidas na década de 1950 e são recomendados para a psicose aguda, e rotineiramente são prescritos para tratamento a longo prazo para a melhora dos sintomas em curso e a prevenção de recaídas (LLORENTE; URRUTIA, 2006; MONCRIEFF, 2015). É importante ressaltar que tais medicamentos tratam apenas os sintomas e não apresentam perspectivas de cura da doença mental (RANG et al., 2004). Além disso, a farmacoterapia sozinha com antipsicóticos produz apenas uma melhora limitada nos sintomas negativos, função cognitiva, convívio social e qualidade de vida, sendo que poucos pacientes retornam totalmente ao seu estado pré-mórbido (KERN et al., 2009; PATTERSON; LEEUWENKAMP, 2008).

Os fármacos antipsicóticos são subdivididos em típicos e atípicos, conforme sua respectiva capacidade em desenvolver efeitos adversos extrapiramidais ou não, como também sua potencial eficácia sobre os sintomas negativos da esquizofrenia (BLIN, 1999). Os antipsicóticos

típicos incluem clorpromazina, flufenazina e haloperidol, os quais atuam de modo principal bloqueando receptores dopaminérgicos D_2 (CREESE; BURT; SNYDER, 1976). São conhecidos por causarem os chamados "efeitos extrapiramidais", pelo fato de antagonizarem a via dopaminérgica extrapiramidal, envolvida no controle motor, causando alterações como acatisia, bradicinesia e discinesia tardia (LLORENTE; URRUTIA, 2006). Também levam ao desenvolvimento de alterações endócrinas, como hiperprolactinemia, a qual provoca galactorreia, ginecomastia e alterações da libido, devido ao bloqueio da via dopaminérgica túbero-infundibular, que controla a secreção de prolactina (BAPTISTA, 1999).

Os antipsicóticos atípicos, incluindo a risperidona, clozapina e a olanzapina, bloqueiam receptores serotoninérgicos e dopaminérgicos (CORDIOLI, 2005; GOLAN et al., 2009). Estes apresentam a vantagem de produzirem efeitos extrapiramidais de modo menos acentuado e de serem mais efetivos contra os sintomas negativos da esquizofrenia (KANE et al., 1988). Porém, seu uso ainda é muito restrito, em função de seu alto custo e também por causarem outros efeitos adversos como ganho de peso corporal, desenvolvimento de diabetes *melittus*, além de discrasias sanguíneas, o que pode ser potencialmente fatal, necessitando de monitoramento intensivo (HENDERSON, 2002).

1.3 Haloperidol

O haloperidol, 4-[4-(4-clorofenil)-4-hidroxi-1-piperidinil]-1-(4-fluorofenil)-1-butanona (Figura 1), é o protótipo da classe das butirofenonas, sendo que foi descoberto em 1958 pelo belga Paul Janssen (JANSSEN, 1998). A sua descoberta como fármaco antipsicótico marcou um acontecimento decisivo na história desses fármacos, tornando-se o medicamento para controle das psicoses mais vendido no mundo (DENIKER, 1998). Ele pertence à classe dos antipsicóticos clássicos ou típicos, e se destaca por sua potência, especificidade, longa duração de ação (NIEMEGEERS, 1983; FROTA, 2001), além de ampla disponibilidade e baixo custo de aquisição (EMSLEY et al., 1999; PONTO et al., 2010). Além disso, o haloperidol destaca-se por ser muito eficaz no controle dos sintomas positivos da esquizofrenia, (TIRONE; PARENTI; GROPPETTI, 1985), transtornos afetivos, delírios, alucinações, confusão mental e psicoses

agudas e crônicas que apresentem agitações psicomotoras (MENEGATTI et al., 2004; GRANGER; ALBU, 2005).

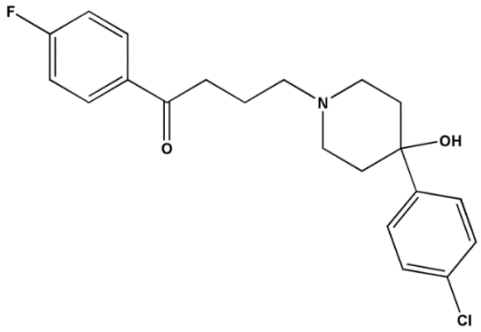

Figura 1 - Estrutura química do haloperidol
Fonte: adaptado de Moor et al. (1992)

A ação farmacológica do haloperidol envolve o bloqueio dos receptores dopaminérgicos D_2 na via mesolímbica (CREESE; BURT; SNYDER, 1976; MIYAMOTO et al., 2002; ANANTH; PARAMESWARAN; HARA, 2004). Além disso, é um potente antagonista dos receptores σ e apresenta fraca atividade antagonista em receptores muscarínicos, histaminérgicos H_1, α-adrenérgicos e serotoninérgicos (DOLLERY, 1991; POTTER; HOLLISTER, 20).

Após administração do haloperidol por via intramuscular o pico das concentrações plasmáticas ocorre dentro de 15 a 60 minutos e a biodisponibilidade é de aproximadamente 75% (JAVAID, 1994). Já por via oral, as concentrações plasmáticas máximas ocorrem geralmente dentro de 3-6 horas (KUNKA; PEREL, 1989) e a biodisponibilidade cai para 65% (HOLLEY et al., 1983). A meia vida do fármaco é de cerca de 20 horas, podendo esta ser muito variável (intervalo 10-36 horas) (FORSMAN; OHMAN, 1976; HOLLEY et al., 1983). Após dose única oral o fármaco leva em torno de 28 dias para atingir eliminação completa (DOLLERY, 1991), sendo esta predominantemente urinária (BERESFORD; WARD, 1987). Dentre os metabólitos formados, destaca-se o haloperidol piridinium (HPP^+), reconhecido por sua hepatotoxicidade (SILVEIRA, 2007) e neurotoxicidade (BLOOMQUIST et al., 1993, 1994; PARIKH; KHAN; MAHADIK, 2003).

1.3.1 Efeitos adversos

Os efeitos indesejáveis relatados para o haloperidol são os efeitos motores extrapiramidais, como distonia aguda, rigidez muscular, tremores e acatisia, além de agitação, cefaleias, náuseas, retenção urinária, hipotensão, depressão, comprometimento da função sexual, sensibilidade à luz solar, hiperprolactinemia e arritmias ventriculares (MIYAMOTO et al., 2002). Além disso, é considerado o agente causal mais frequente da síndrome neuroléptica maligna (ALLEN et al., 1998).

O haloperidol também é conhecido por causar efeitos adversos locais em alguns órgãos específicos, como rim (UYANIK et al, 2009) e fígado (HALICI et al, 2009; DINCSOY; SAELINGER, 1982). GAERTNER et al (2001) e ZIMMERMAN (1999) mostraram uma elevação das transaminases hepáticas, as quais podem indicar dano hepático, em pacientes tratados com o medicamento. Outros estudos demonstraram um aumento da alanina aminotransferase em ratos tratados com haloperidol, bem como uma diminuição nas defesas antioxidantes no fígado desses animais (DALLA CORTE et al., de 2008), aumentando portanto, o dano oxidativo nesse órgão (BEASLEY et al., 2011). Além disso, já foi demonstrado que o haloperidol é capaz de estimular as vias apoptóticas no fígado de ratos (GEBRESELASSIE; BOWEN, 2004; WEI et al., 2006; HANAGAMA et al., 2008).

1.4 Nanotecnologia

A nanotecnologia pode ser vista como a criação de materiais funcionais, dispositivos e sistemas através do controle da matéria na escala nanométrica, implicando em sistemas que apresentem novos fenômenos e propriedades, que são dependentes do tamanho (AZEVEDO, 2002). O uso da nanotecnologia vem revolucionado o campo da medicina, permitindo que nanopartículas sejam utilizadas para diagnóstico, monitoramento, tratamento de doenças e como ferramenta biomédica em pesquisas (MEDINA et al., 2007; PRIMO et al., 2008; RODRIGUES et al., 2009; FALQUEIRO et al., 2011). Uma área que vem merecendo destaque é a utilização de

nanopartículas para liberação seletiva de fármacos, o que promove um aumento da eficácia e diminuição da toxicidade do medicamento (BARRATT, 2000; KREUTER, 2001; BECK et al., 2005).

As nanopartículas poliméricas são sistemas carreadores de fármacos que apresentam diâmetro inferior a 1 µm. Dentre estas incluem-se as nanocápsulas, que são constituídas por um invólucro polimérico disposto ao redor de um núcleo oleoso (QUINTANA-GUERRERO et al., 1998; SCHAFFAZICK et al., 2003). Essas nanopartículas têm recebido uma atenção especial como sistemas de liberação devido a sua estabilidade e maior facilidade na modificação de sua superfície (HERRERO-VANRELL et al., 2005). Quanto a liberação de fármacos, esses sistemas fornecem inúmeras vantagens quando comparados à liberação convencional, por serem capazes de alterar parâmetros farmacocinéticos, podendo melhorar a biodistribuição, prolongar a meia-vida dos fármacos, aumentar a biodisponibilidade e possibilitar uma liberação homogênea, controlada e sustentada. Assim, os efeitos adversos sistêmicos podem ser reduzidos, bem como a dose e a frequência de administração do fármaco, fazendo com que aumente a aderência ao tratamento por parte do paciente (GUTERRES; ALVES; POHLMANN, 2007; DE MARTIMPREY et al., 2009).

Dentre os polímeros utilizados, destaca-se a poli(ε-caprolactona) (PCL), cuja propriedade de biodegradabilidade foi identificada pela primeira vez em 1973 (MURTHY, 1997). Devido à natureza biocompatível e biodegradável, a PCL tem sido amplamente estudada em formulações que visam um controle na liberação de fármacos. Sua elevada permeabilidade a uma gama de medicamentos permite a distribuição uniforme dos fármacos na matriz polimérica e por ser um polímero semi-cristalino apresenta uma degradação mais lenta o que facilita a liberação sustentada do fármaco (GUTERRES; ALVES; POHLMANN, 2007), por até mesmo vários meses (HAKKARAINEN; ALBERTSSON, 2002; SINHA et al., 2004).

Inúmeras substâncias, com as mais variadas atividades terapêuticas, mostraram-se capazes de serem associadas a sistemas nanocarreadores. Como exemplo: quimioterápicos (CALLEWAERT et al., 2013; KUO; LIANG, 2011; WOHLFART et al., 2011), antiinflamatórios (BERNARDI; FROZZA; HORN, 2010; NAJAFABADI; ABDOUS; FAGHIHI, 2014), antibióticos (PANDEY; KHULLER, 2006; XU et al., 2009; WANG et al., 2010) antifúngicos (CHEN et al., 2011; XU et al., 2011), antipsicóticos (DIMER et al., 2014;

GOVENDER et al., 2015; PARIHK; BOMMANA; SQUILLANTE, 2010), ansiolíticos (PRIPREM et al., 2008; TORABI et al., 2013), entre outros.

Na literatura encontra-se estudos que mostram o preparo de nanocápsulas contendo fármacos antipsicóticos (MUTHU; AGRAWAL; SINGH, 2014), como a risperidona (SINGH; MUTHU, 2007; MUTHU; SINGH, 2008; MUTHU et al, 2009; SILVA et al., 2011), clozapina (VENKATESWARLU; MANJUNATH, 2004; MANJUNATH; VENKATESWARLU, 2005; MORAES et al., 2015), sulpirida, (PARIHK et al., 2010); olanzapina (VIVEK et al., 2007; SEJU; KUMAR; SAWANT, 2011; DIMER et al., 2014), tioridazina (LAI et al., 2006), clorpromazina (GOVENDER et al., 2015) e o próprio haloperidol (BUDHIAN; SIEGEL; WINEY, 2005; 2007; 2008). Caracterização físico-química destas nanoestruturas mostraram uma modificação no perfil de liberação *in vitro* da risperidona (MUTHU; SINGH, 2008; MUTHU et al., 2009), tioridazina (LAI et al., 2006), olanzapina (VIVEK et al., 2007; SEJU; KUMAR; SAWANT, 2011) e haloperidol (BUDHIAN SIEGEL; WINEY, 2008; PIAZZA et al., 2014), possibilitando uma liberação mais sustentada e controlada.

Estudos de eficácia terapêutica e efeitos colaterais foram realizados com nanocápsulas de risperidona, e como resultado verificou-se um aumento da eficácia terapêutica, quando comparado com a forma livre do fármaco (MUTHU; SINGH, 2008; MUTHU et al., 2009). Com relação ao haloperidol nanoencapsulado, estudos em ratos demonstraram a vantagem desta formulação sobre a livre, visto que foi observado uma melhora na eficácia terapêutica e um prolongamento no tempo de ação do fármaco no organismo (BENVEGNÚ et al., 2011), além de uma redução nos efeitos adversos motores (BENVEGNÚ et al., 2011; BENVEGNÚ et al, 2012), minimizando danos oxidativos nas regiões cerebrais envolvidas no controle do movimento (BENVEGNU et al., 2012).

1.4.1 Nanotoxicologia

A nanotecnologia tem sido promovida como uma tecnologia revolucionária no desenvolvimento de dispositivos médicos, diagnósticos, encapsulamento e liberação de fármacos (LINKOV; SATTERSTROM; COREY, 2008). No entanto, a crescente produção e utilização de

nanomateriais, especificamente na forma de nanopartículas, para diversas aplicações biomédicas têm levantado sérias preocupações sobre sua segurança para a saúde humana (OBERDÖRSTER, 2010), tornando necessário abordagens diversificadas de investigação, a fim de avaliar os riscos em potencial (NEL et al., 2006).

Devido ao pequeno tamanho, a razão de área superficial para massa de nanopartículas é elevada, e uma grande fração de elétrons reativos fica exposta à superfície. Isto tem sido proposto para aumentar a toxicidade. Neste contexto, Oberdörster e colaboradores definiram nanotoxicologia como a ciência da engenharia de nanodispositivos e nanoestruturas que lida com seus efeitos sobre organismos vivos (OBERDÖRSTER et al., 2005). Propõe-se também que a nanotoxicologia irá desenvolver e implementar protocolos específicos de nano, a fim de investigar e ganhar o conhecimento necessário para determinar a toxicidade potencial da multiplicidade de diferentes nanopartículas (CLIFT et al., 2011).

Estudos indicam que o tamanho pequeno, a extensa superfície de contato e a capacidade de gerar espécies reativas de oxigênio estão relacionadas à capacidade de determinadas nanopartículas induzirem injúria tecidual, inflamação, fibrose, citotoxicidade e outras alterações (OBERDÖRSTER et al., 2005; NEL et al., 2006). No entanto, os estudos que demonstraram esses efeitos tóxicos referem-se a nanopartículas inorgânicas (XIONG et al., 2011; ZHU et al., 2009), e a formulações contendo nanotubos de carbono (SHVEDOVA et al., 2003; LAM et al., 2003;.KIPEN; RADOMSKI et al, 2005;.CHEN et al, 2006; DONALDSON et al., 2006; HU et al., 2011), sendo que até o momento pouco se sabe sobre a possível toxicidade e segurança de nanocápsulas poliméricas com PCL (HU et al., 2011; BULCÃO et al., 2013).

Alguns autores testaram nanomateriais (HUANG et al., 2010; FANG et al., 2009) e nanoesferas (KIM et al., 2003) contendo PCL, e nenhum mostrou toxicidade *in vivo*. Quanto as nanocápsulas poliméricas contendo PCL, apenas um grupo de pesquisadores realizou estudos de toxicidade e relatou que estas não apresentam toxicidade (BULCÃO et al., 2013, 2014).

A literatura aponta resultados terapêuticos promissores com o haloperidol na forma de nanocápsulas poliméricas, as quais promoveram um aumento do tempo de ação e redução dos efeitos adversos, quando comparado ao fármaco livre (BENVEGNU et al., 2011, 2012). No entanto, pouco se sabe sobre a toxicidade dessas formulações contendo o polímero PCL, utilizadas na liberação de fármacos; sendo que até o presente momento não foram encontrados relatos de estudos que avaliassem os possíveis efeitos tóxicos desta nanocápsula de haloperidol

sobre rim, fígado e sangue. Sendo assim, esse estudo terá como objetivo avaliar a possível toxicidade de nanocápsulas poliméricas de haloperidol, em relação ao fármaco na sua forma livre, em fígado, rim e sangue de ratos.

2 OBJETIVOS

2.1 Objetivo Geral

Realizar uma avaliação comparativa entre o haloperidol nanoencapsulado e o fármaco livre, considerando a toxicidade a diferentes tecidos de ratos.

2.2 Objetivos Específicos

-Avaliar marcadores de dano oxidativo e defesas antioxidantes em fígado e rim de ratos tratados subcronicamente com haloperidol livre e nanoencapulado;

-Avaliar a integridade celular em fígado e rim de ratos tratados subcronicamente com haloperidol livre e nanoencapulado;

-Avaliar indicadores de dano hepático e renal no plasma de ratos tratados subcronicamente com haloperidol livre e nanoencapulado;

- Avaliar indicadores de dano no DNA em sangue total de ratos tratados subcronicamente com haloperidol livre e nanoencapsulado.

3 ARTIGO CIENTÍFICO

Os resultados inseridos nesta dissertação apresentam-se sob a forma de artigo científico, o qual se encontra aqui estruturado. Os itens materiais e métodos, resultados, discussão dos resultados e referências, encontram-se no próprio artigo, o qual está disposto na mesma forma em que foi publicado.

HALOPERIDOL-LOADED LIPID-CORE POLYMERIC NANOCAPSULES REDUCE DNA DAMAGE IN BLOOD AND OXIDATIVE STRESS IN LIVER AND KIDNEYS OF RATS

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RESEARCH PAPER

Haloperidol-loaded lipid-core polymeric nanocapsules reduce DNA damage in blood and oxidative stress in liver and kidneys of rats

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Abstract Haloperidol (HP) nanoencapsulation improves therapeutic efficacy, prolongs the drug action time, and reduces its motor side effects. However, in a view of HP toxicity in organs like liver and kidneys in addition to the lack of knowledge regarding the toxicity of polymeric nanocapsules, our aim was to verify the influence of HP-nanoformulation on toxicity and oxidative stress markers in the liver and kidneys of rats, also observing the damage caused in the blood. For such, 28 adult male Wistar rats were designated in four experimental groups ($n = 7$) and treated with vehicle (C group), free haloperidol suspension (FH group), blank nanocapsules suspension (B-Nc group), and haloperidol-loaded lipid-core nanocapsules suspension (H-Nc group). The nanocapsules formulation presented the size of approximately 250 nm. All suspensions were administered to the animals (0.5 mg/kg/day-i.p.) for a period of 28 days. Our

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results showed that FH caused damage in the liver, evidenced by increased lipid peroxidation, plasma levels of aspartate aminotransferase, and alanine aminotransferase, as well as decreased cellular integrity and vitamin C levels. In kidneys, FH treatment caused damage to a lesser extent, observed by decreased activity of δ-aminolevulinate dehydratase (ALA-D) and levels of VIT C. In addition, FH treatment was also related to a higher DNA damage index in blood. On the other hand, animals treated with H-Nc and B-Nc did not show damage in liver, kidneys, and DNA. Our study indicates that the nanoencapsulation of haloperidol was able to prevent the subchronic toxicity commonly observed in liver, kidneys, and DNA, thus reflecting a pharmacological superiority in relation to free drug.

Keywords Cellular integrity · Haloperidol · Polymeric nanocapsules · Nanotoxicology · Oxidative stress · Nanomedicine

Introduction

Haloperidol (HP) is a typical neuroleptic drug widely used for the treatment of psychosis and other mental diseases due to its high therapeutic potential and low cost (Ponto et al. 2010). The basic pharmacological action of HP includes blockade of dopamine (DA) receptors, especially the sub-type D2 (Creese et al. 1976; Ananth et al. 2004). When chronically used, HP is related to extrapyramidal side effects, including from parkinsonism, akathisia, and muscular dystonia to a disabling movement condition known as tardive dyskinesia (Dayalu and Chou 2008; Andreassen and Jorgensen 2000). Besides these extrapyramidal symptoms, HP treatment has been also related to additional adverse effects in specific target organs such as liver (Halici et al. 2009) and kidneys, to a lesser extent (Uyanik et al. 2009). While an increased level of transaminases in HP-treated patients has been indicative of hepatic damage (Gaertner et al. 2001; Zimmerman 1999), experimental studies showed increased levels of alanine aminotransferase levels (ALT) and oxidative damage in the liver of HP-treated rats (Dalla Corte et al. 2008; Beasley et al. 2011). Additional studies also indicated apoptosis development in the liver of rats exposed to this antipsychotic drug (Wei et al. 2006; Gebreselassie and Bowen 2004; Hanagama et al. 2008).

In recent years, nanotechnology has transformed the field of medicinal products, allowing the use of nanoparticles for diagnosis, monitoring and treatment of diseases, and acting also as a promising tool in biomedical research (Medina et al. 2007; Primo et al. 2008; Rodrigues et al. 2009; Falqueiro et al. 2011). In this sense, polymeric nanoparticles have attracted considerable attention as potential drug carriers due to their innovative property of enhancing therapeutic efficacy while minimizing side effects of a variety of drugs associated with these systems (Benvegnú et al. 2011, 2012; Callewaert et al. 2013; Ourique et al. 2011; Bernardi et al. 2009; Ianiski et al. 2012; Gao et al. 2010; Mora-Huertas et al. 2010). However, the growing manufacturing and application of nanoparticles have caused serious concerns regarding their safety for human health (Oberdörster 2010), once little is known about their toxic potential (Bulcão et al. 2013; Hu et al. 2011).

Considering that the use of antipsychotics is strongly associated with the development of adverse effects, the literature has shown the benefits of nanoencapsulation of some of these drugs, such as (i) risperidone (Kumar et al. 2009; Singh et al. 1988; Muthu and Singh 2009; Silva et al. 2011); (ii) clozapine (Venkateswarlu and Manjunath 2004; Manjunath and Venkateswarlu 2005); (iii) sulpiride (Parihk et al. 2010); (iv) olanzapine (Dimer et al. 2014; Seju et al. 2011); (v) thioridazine (Lai et al. 2006); and (vi) haloperidol (Budhian et al. 2005, 2007, 2008; Benvegnú et al. 2011, 2012). In addition, physicochemical characterization of these nanostructures showed changes in the in vitro release profile of risperidone (Muthu and Singh 2008, 2009), thioridazine (Lai et al. 2006), olanzapine (Vivek et al. 2007; Seju et al. 2011), and haloperidol (Budhian et al. 2008), enabling a more sustained and controlled release.

Studies on the efficacy and adverse effects have been performed with risperidone and olanzapine nanocapsules, and the results showed an increase in therapeutic efficacy compared to the free form of the

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drug (Muthu and Singh 2008; Muthu and Singh 2009; Dimer et al. 2014). Regarding HP nanocapsules, experimental studies have shown advantages of this formulation over free drug, once the nanoencapsulated drug showed higher therapeutic efficacy and prolonged time of action (Benvegnú et al. 2011) as well as reduced extrapyramidal side effects (Benvegnú et al. 2011, 2012). Such beneficial effects were associated to lower oxidative damage in brain areas involved in the motor control (Benvegnú et al. 2012).

Considering the clinical and experimental evidences regarding the toxicity of HP in organs such as liver and kidneys, and the lack of knowledge regarding the toxicity of polymeric nanocapsules, our aim was to assess the effects of a formulation of HP-loaded lipid-core nanocapsules on biochemical parameters and oxidative stress (OS) markers in liver and kidneys of rats.

Experimental procedures

Chemicals

Haloperidol was obtained from Galena (Campinas-SP, Brazil). Fish oil capsules (Achè-Guarulhos-SP, Brazil), containing 1 g oil/capsule with 120 mg of docosahexaenoic acid (DHA) and 180 mg of eicosapentaenoic acid (EPA) were used. Poly (ε-caprolactone) and sorbitan monostearate (Span 60®) were obtained from Sigma (Tatuapé-SP, Brazil). Polysorbate 80 was acquired from Delaware (Porto Alegre, RS, Brazil). All other chemicals and solvents used were from pharmaceutical laboratories or high-performance liquid chromatography (HPLC) grade, and used as received.

Animals

Adult male Wistar rats (200 ± 50 g) were kept in Plexiglas cages with free access to food and water in a room with controlled temperature (22–23 °C), and a 12-h light/dark cycle with lights on at 7:00 am. The experimental protocol of this study was approved by the Animal Ethics Committee of Universidade Federal de Santa Maria (CIETEA-22/2010), affiliated to CONCEA, and adhered to the ''Principles of Laboratory Animal Care'' and international rules of ethics in research.

Preparation of nanocapsules and haloperidol-free solution

Nanocapsules (H-Nc) were prepared by interfacial deposition of preformed polymer, as described by Fessi et al. (1989). Briefly, the organic solution consisted of fish oil (0.8 mL), poly(ε-caprolactone) (250 mg), haloperidol (12.6 mg), sorbitan monostearate (192 mg), and acetone (67 mL), poured into an aqueous solution (134 mL) containing polysorbate 80 (192 mg) under magnetic stirring. Acetone was removed, and the suspension was concentrated by evaporation to a final volume of 25 mL (0.50 mg/mL of haloperidol). Blank nanocapsules (B-Nc) were prepared using the same protocol described above, without the drug. Haloperidol-free suspension (0.50 mg/mL) was prepared in an aqueous solution using 5 % (w/v) polysorbate 80.

Physicochemical characterization of nanocapsules

Particle sizes, polydispersity index, and zeta potential ($n = 3$) were measured by photon correlation spectroscopy (Zetasizer Nanoseries ZEN 3600, Malvern Instruments, UK). Drug content (mg mL^{-1}) was determined ($n = 3$) according to a previously described and validated protocol (Benvegnú et al. 2011). Encapsulation efficiency was determined by the ultrafiltration–centrifugation technique. Afterward, the concentration of free drug was determined in the clear supernatant following this technique (Ultrafree-MC® 10,000 MW, Millipore, Bedford, USA). Encapsulation efficiency (%) was calculated by the difference between total and free drug concentrations determined in the nanocapsule suspension (drug content) and in the ultrafiltrate, respectively, using the same protocol (Benvegnú et al. 2011). The morphological analysis was conducted by transmission electron microscopy (TEM; Jeol, JEM 1200 Exll, Centro de Microscopia Eletrônica, UFRGS) operating at 80 kV. For this analysis, the diluted suspension was deposited in Formvar–Carbon support film on specimen grid (electron microscopy sciences) and negatively stained with 2 % (w/v) uranyl acetate solution.

In vivo experiment

Twenty-eight rats were randomly divided into four groups ($n = 7$) and treated with aqueous solution

containing 5 % polysorbate 80 (v/v) (C group), free haloperidol suspension, prepared in water with 5 % polysorbate 80 (v/v) (FH group), blank nanocapsules suspension (B-Nc group), and haloperidol-loaded lipid-core nanocapsules suspension (H-Nc group). All suspensions were administered to the animals (0.5 mg/kg-ip) once a day, for 28 days.

On the 29th day of the experiment, all animals were anesthetized (thiopental, 50 mg/kg- ip) and euthanized by exsanguination (blood was collected by cardiac puncture in heparinized tubes). An aliquot of the collected whole blood was separated for comet assay, and the remaining was centrifuged at 1300 g (15 min) to obtain plasma and used to determine the biochemical markers of hepatic and renal damage. Liver and kidneys were immediately removed; one section of these tissues was homogenized with 0.1 M Tris–HCl (1:10), pH 7.4, centrifuged at 3640 g (15 min, at 4 °C); and the supernatants were used to quantify the OS markers. The other sections of liver and kidneys were sliced (0.4 mm) in a McIlwain chopper, and used for cell integrity assay.

Oxidative stress parameters

Malondialdehyde assay

Malondialdehyde (MDA) levels were quantified using the HPLC technique according to Grotto et al. (2007). A volume of 75 µL of homogenate was added to 25 µL of standard or water and 25 µL of NaOH 3 N, and incubated at 60 °C for 30 min in a shaking water bath system. After this procedure, 125 µL of H_3PO_4 6 % and 125 µL of TBA 0.8 % were added, and the mixture was heated at 90 °C for 45 min. The mixture was subsequently cooled, 50 µL of 10 % sodium dodecyl sulfate (SDS) was added, and the extraction with 300 µL of *n*-butanol was carried out by vortex mixing for 1 min, and centrifuged at 3000×*g* for 10 min. 20 µL of the butanol layer was injected into HPLC with a visible detector, using a reverse-phase column and eluted as previously described. The separation of the MDA–(TBA) 2 adduct was performed using a 150 × 9 × 4 mm^3 silica-based reversed-phase C18 column (Eurospher-100) with a particle size of 5 µm. The mobile phase was a mixture of 2.5 mmol/L KH_2PO_4 pH 7.0 and methanol (50:50 v/v). The sample run lasted 8 min, with a flow rate of 0.6 mL/min isocratically maintained throughout. The column was kept at 40 °C, and the absorbance of the eluent was monitored at 532 nm.

δ-Aminolevulinate dehydratase (ALA-D) activity assay

ALA-D activity was assayed according to the method of Sassa (1982) with minor modifications, by measuring the rate of porphobilinogen formation for 1 h at 37 °C in the absence of the reducing agent dithiothreitol. The enzyme reaction was initiated after 10 min of pre-incubation by the addition of ALA to a final concentration of 4 mmol/L in PBS pH 6.8, and incubation was carried out for 1 h at 37 °C. The reaction product was measured at 555 nm, and the activity of ALA-D was expressed as nmol PBG/mg protein/h.

Antioxidant defenses

Catalase (CAT) activity was spectrophotometrically quantified in tissues by the method of Aebi (1995), which includes monitoring the disappearance of H_2O_2 in the presence of cell homogenate (pH 7.0 at 25 °C) at 240 nm. The enzymatic activity was expressed in µmol H_2O_2/min/g tissue.

Superoxide dismutase (SOD) was spectrophotometrically assayed as described by Misra and Fridovich (1972). Briefly, epinephrine rapidly auto-oxidizes at pH 10.2 producing adrenochrome, a pink-colored product that can be detected at 480 nm. The addition of samples containing SOD inhibits the auto-oxidation of epinephrine. The inhibition rate was monitored during 120 s at intervals of 15 s. The amount of enzyme required to produce 50 % inhibition at 40 °C was defined as one unit of enzyme activity. The enzymatic activity was expressed in U/g tissue.

Vitamin C (VITC) levels in liver and kidneys were estimated as described by Galley et al. (1996) with some modifications (Jacques-Silva et al. 2001). Tissue homogenate was precipitated in one volume of cold 5 % trichloroacetic acid solution followed by centrifugation. An aliquot of the supernatants was mixed with 2,4-dinitrophenylhydrazine (4.5 mg/mL) and 13,3 % trichloroacetic acid, and incubated for 3 h at 37° C. After the addition of 65 % sulfuric acid, an orange red color was produced at 520 nm. A standard curve using ascorbic acid was used to calculate the content of VITC and expressed as µg VITC/g tissue.

Cellular integrity by MTT assay

The cellular integrity of liver and kidney slices was quantified by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to a dark violet formazan product by mitochondrial dehydrogenases (Mosmann 1983). MTT reduction assays were performed in plates containing 500 µL of phosphate-buffered saline, and the reaction was started by adding MTT to a final concentration of 0.1 mg/mL. After 1 h of incubation at 37 °C, the medium was removed and the slices dissolved in dimethylsulfoxide. MTT reduction was spectrophotometrically measured by the difference in absorbance between 570 and 630 nm. After the determination of protein content (Lowry et al. 1951) in the slices, MTT value was corrected by protein content and expressed as percentage of control.

Biochemical analysis

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, urea, and albumin levels were determined in the plasma of animals with Bioclin® commercial kit.

Comet assay

The alkaline comet assay followed the method described by Singh et al. (1988) and was performed according to general comet assay guidelines. The whole blood of animals was suspended in agarose and spread onto a glass microscope slide pre-coated with agarose (preparation was made in duplicate). The following step was the removal of the coverslips, followed by the immersion of slides in a previously prepared lysis solution (NaCl 2.5 M, EDTA 100 mM, Tris 10 mM, distilled water, DMSO 10 %, and Triton X-100 1 %). After lysis, electrophoresis was carried out, when cells encapsulated in gel on a wafer were exposed to an electric current, causing them to migrate out of the core segments of DNA free of resulting breaks. After the slides were soaked in buffer for 20 min in the electrophoresis apparatus, power was turned on and the run started at 25 V and 300 mA for 20 min. Then neutralization was carried out, and the slides were transferred to a coppling with neutralization solution (Tris 0.4 M, distilled water) in the dark for 15 min. The nearly dry slides were put into a coppling with absolute ethanol for 5 min in order to promote the fixation. After complete drying, they were stained with 30 µL ethidium bromide (20 µL/mL) and immediately analyzed. Comet observations were made at ×400 magnification using a fluorescence microscope (Olympus) equipped with an excitation filter of 510–550 nm connected to a camera. Comet Score™ software was obtained from the public domain (http://www.tritekcorp.com/products_cometscore.php), and DNA damage was quantified through the measurement of DNA percentage in the tail (% DNA). Positive reference control consisted of whole blood mixed with methyl methanesulfonate at final concentration 8×10^{-5} M. This mixture was incubated at 37 °C for 2 h.

Statistical analysis

Homogeneity of variances was analyzed with Levene's test. All assessments were analyzed with two-way ANOVA followed by Duncan's multiple range test, when appropriate. Data were analyzed using Statistica software (11.0) and expressed as mean ± SEM. Significance was considered when $p < 0.05$.

Results

Physicochemical characteristics of polymeric nanocapsules are shown in Table 1 and Fig. 1

All formulations appeared macroscopically homogeneous, and their aspect was similar to a milky bluish opalescent fluid (Tyndall effect). Both blank and haloperidol nanocapsule suspensions presented particles within the submicrometric range (between 200 and 300 nm), low polydispersity (≤0.25), negative zeta potentials, drug content near the theoretical value (0.50 mg mL^{-1}) for the suspension, and excellent encapsulation efficiency (95.01 %) (Table 1). TEM analysis showed uniform and round particles, and similar size to that found by photon correlation spectroscopy technique (approximately 200 nm) (Fig. 1).

Table 1 Physicochemical characteristics of blank and haloperidol-loaded nanocapsules (B-Nc and H-Nc)

Nanocapsules	Particle size (nm)	PDI	Zeta Potential (mv)	Drug content (mg mL^{-1})	Encapsulation efficiency (%)
B-Nc	251 ± 8	0.21 ± 0.2	−11 ± 0.6	–	–
H-Nc	250 ± 7	0.21 ± 0.0	−12 ± 0.9	0.5 ± 0.0	95.01 ± 0.4

Data are expressed as mean ± SD: it represent the variation between the different batches

B-Nc blank nanocapsules, *H-Nc* haloperidol-loaded lipid-core nanocapsules

PDI polydispersity index

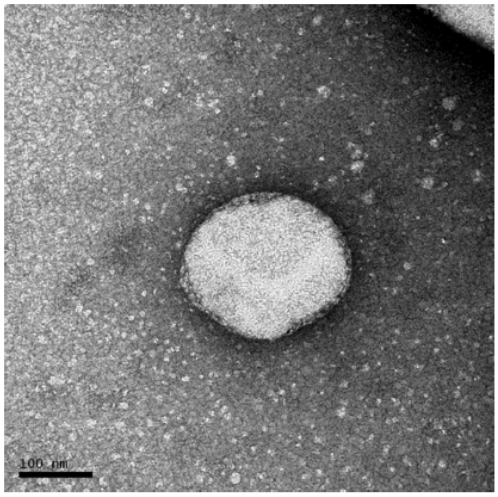

Fig. 1 Transmission electron microscopy (TEM) images of haloperidol nanocapsule. *Bar* 100 nm (200,000×)

Estimation of malondialdehyde (MDA) levels, δ-Aminolevulinate dehydratase (ALA-D) activity, and cellular integrity (MTT assay) in liver of rats treated with haloperidol formulations are shown in Fig. 2

Two-way ANOVA of MDA levels, ALA-D activity, and cellular integrity revealed a significant main effect of the treatment in liver [$F = (3, 24) = 4.21$; $p < 0.05$, 2.80; $p < 0.05$ and 17.61; $p < 0.001$, respectively].

Post hoc test revealed that FH group showed increased levels of MDA in liver (Fig. 2a) and decreased cellular integrity (Fig. 2c) in relation to both C and H-Nc groups, ALA-D activity of FH was comparable to control, but lower in comparison to H-Nc group (Fig. 2b).

Estimation of Malondialdehyde (MDA) levels, δ-Aminolevulinate dehydratase (ALA-D) activity, and cellular integrity in kidneys of animals treated with haloperidol formulations are shown in Fig. 3

Two-way ANOVA of ALA-D activity in kidneys revealed a significant main effect of treatment [$F = (3, 24) = 6.12$; $p < 0.05$].

While no differences in MDA levels (Fig. 3a) and cellular integrity (Fig. 3c) were observed in kidneys, post hoc test showed that FH treatment decreased ALA-D activity in this organ when compared to both C and H-Nc group (Fig. 3b).

Estimation of antioxidant defenses in liver of animals treated with haloperidol formulations are shown in Fig. 4

Two-way ANOVA of VITC, SOD, and CAT revealed a significant main effect of the treatment in liver [$F = (3, 24) = 20.06$; $p < 0.001$, 4.21; $p < 0.05$ and 3.02; $p < 0.05$, respectively]. Post hoc test showed that FH-treated group showed lower levels of VIT C (Fig. 4a) and increased SOD activity (Fig. 4c) when compared to both C and H-Nc groups, while CAT activity (Fig. 4b) in the latter was lower than FH.

Estimation of antioxidant defenses in kidneys of animals treated with haloperidol formulations are shown in Fig. 5

Two-way ANOVA of VITC [$F = (3, 24) = 5.63$; $p < 0.05$] evidenced a significant main effect of the treatment in kidneys. Post hoc test indicated that FH group showed decreased VIT C levels (Fig. 5a) in relation to both C and H-Nc groups, while SOD levels (Fig. 5c) and CAT activity (Fig. 5b) showed no differences among the different experimental groups.

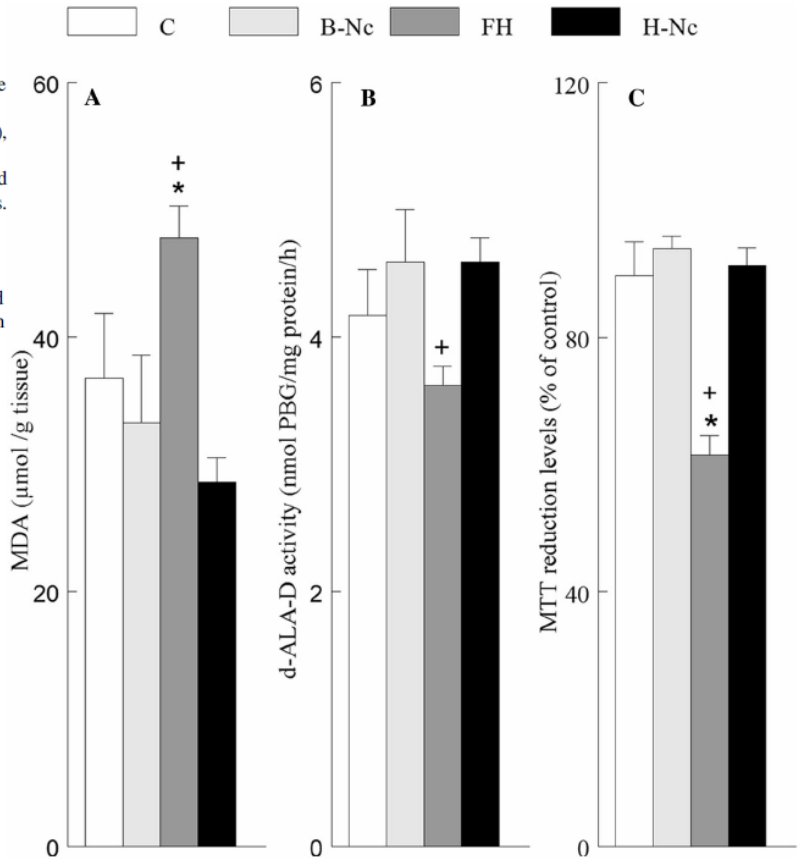

Fig. 2 Influence of daily administration of free (FH) or nanoencapsulated (H-Nc) haloperidol (0.5 mg/kg/mL-ip once a day, for 28 days) on malondialdehyde (MDA) levels (**a**), *d*-aminolevulinate dehydratase activity (*d*-ALA-D activity) (**b**), and cellular integrity (**c**) in liver of rats. Data are expressed as mean ± SEM. ($n = 7$). *Plus symbol* indicates significant difference from haloperidol-loaded lipid-core nanocapsules suspension (H-Nc) group. *Asterisk* indicates significant difference from control (C) group. ($p < 0.05$ for all comparisons)

Estimation of DNA damage index (DI) in whole blood of animals treated with haloperidol formulations are shown in Fig. 6

Two-way ANOVA of DNA-DI revealed a significant main effect of the treatment [$p = (3, 24) = 18.87$; $p < 0.001$] in blood. Post hoc test showed that FH treatment was related to an increased DNA damage index (DI) in whole blood when compared to the other three experimental groups (C, H-Nc, and B-Nc) (Fig. 6f).

Estimation of biochemical parameters in plasma of animals treated with haloperidol formulations are shown in Table 2

Two-way ANOVA of AST and ALT revealed a significant main effect of the treatment [$p = (3, 24) = 5.20$; $p < 0.05$ and 3.94; $p < 0.05$, respectively].

Post hoc test showed that FH treatment was able to increase AST and ALT levels in relation to both control and H-Nc groups. In addition, the different treatments exerted no changes in albumin, creatinine, and urea levels, which were comparable to one another.

Discussion

An increased clinical use of atypical antipsychotics has been observed in the last two decades, especially due to their improved extrapyramidal tolerability compared to the typical agents such as haloperidol (Csernansky et al. 2002; Möller et al. 2008). However, recent data from routine clinical practice point out the development of adverse reactions for atypical antipsychotics, which have not been reported by clinical trials (Casey 2004; Newcomer 2005; Trifiro 2010). In this

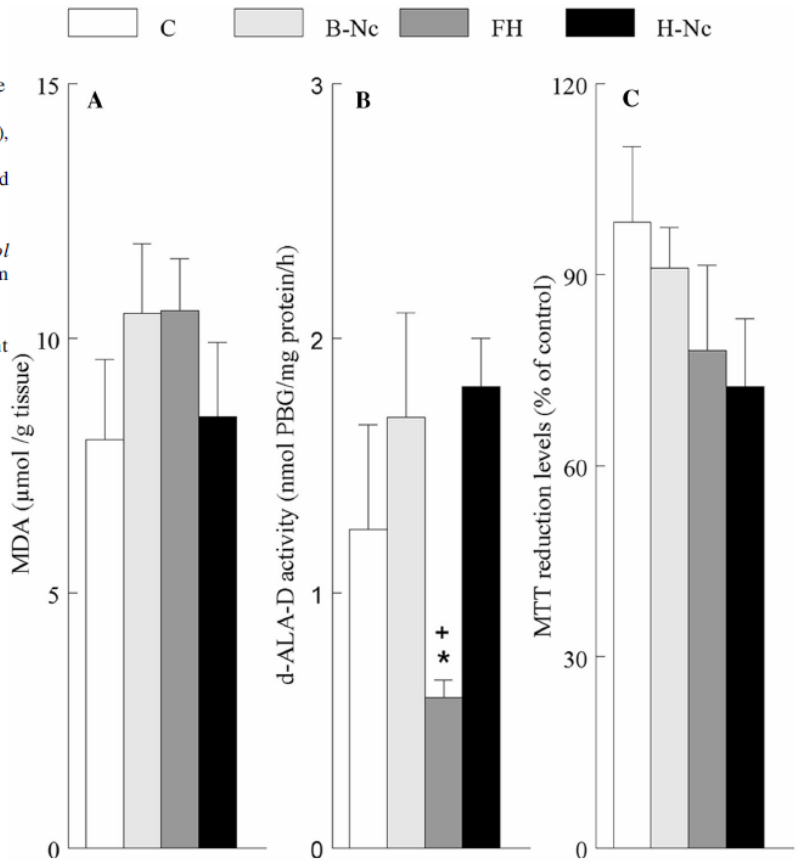

Fig. 3 Effects of daily administration of free (FH) or nanoencapsulated (H-Nc) haloperidol (0.5 mg/kg/mL-ip once a day, for 28 days) on malondialdehyde (MDA) levels (**a**), *d*-aminolevulinate dehydratase activity (*d*-ALA-D activity) (**b**), and cellular integrity (**c**) in kidneys of rats. Data are expressed as mean ± SEM ($n = 7$). *Plus symbol* indicates significant difference from haloperidol-loaded lipid-core nanocapsules suspension (H-Nc) group. *Asterisk* indicates significant difference from control (C) group. ($p < 0.05$ for all comparisons)

sense, haloperidol (HP) is a typical antipsychotic drug that remains widely prescribed (Bolcato et al. 2014) due to its high potency and low cost (Ponto et al. 2010). In fact, despite wide clinical application, HP treatment remains causing intense side effects, what requires additional studies that allow delaying or minimizing its damage to vital tissues, especially liver and kidneys, as well as blood, which are the central aim of the current study. Thus, studies regarding alternative methods that target the minimization of HP-induced adverse effects constitute a promising approach to the psychiatric clinic, directing to improve the quality of life of psychiatric patients.

Following this idea, a recent experimental study carried out by our group reported that HP-loaded lipid-core nanocapsules were related to an increased and prolonged activity in an animal model of amphetamine-induced pseudo-psychosis (Benvegnú et al. 2011) when compared to free HP. In addition, this innovative formulation was also related to decreased motor side effects and lower oxidative damage in extrapyramidal brain areas, in relation to free drug (Benvegnú et al. 2012). In fact, in the cited study, HP was encapsulated in the concentration of 0.25 mg/mL in nanocapsules containing fish oil, proving the therapeutic potential of this nanosystem (Benvegnú et al. 2012). Based on this, the current study was performed with a similar fish oil nanoformulation in which haloperidol content was duplicated (0.50 mg/mL) in order to increase the drug dose. Indeed, physicochemical properties obtained for this more recent formulation were similar to the first, which contained smaller amounts of drug. In addition, the increase in drug content did not affect the system, displaying suitable nanotechnological characteristics such as particle size, zeta potential, polydispersity index, encapsulation efficiency, and morphology, as well as drug delivery

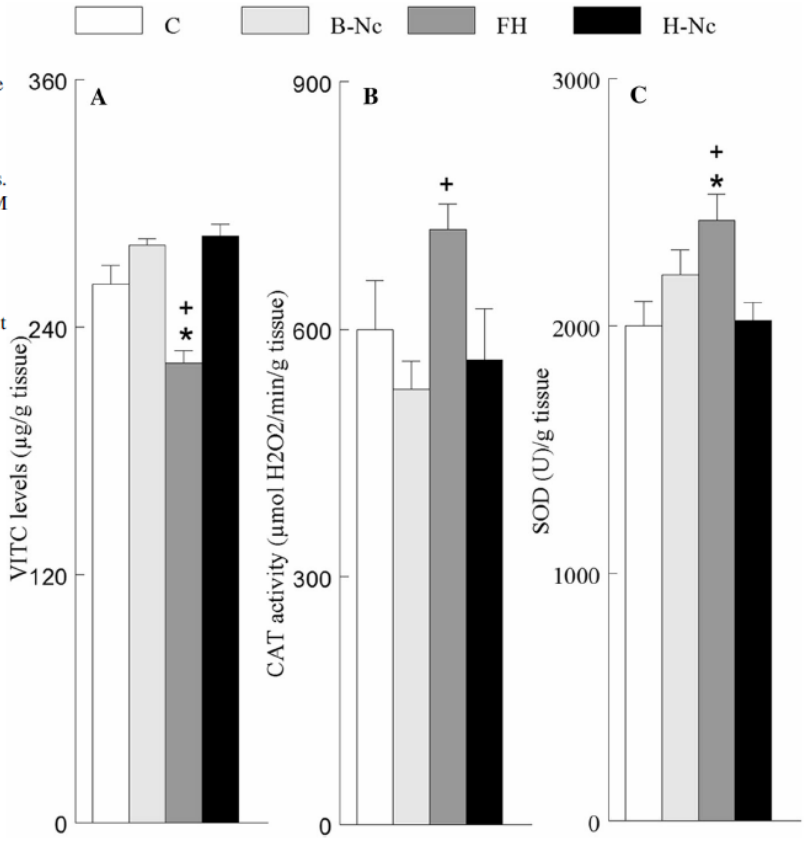

Fig. 4 Effects of daily administration of free (FH) or nanoencapsulated (H-Nc) haloperidol (0.5 mg/kg/mL-ip once a day, for 28 days) on vitamin C (VITC) levels (**a**), catalase (CAT) activity (**b**), and superoxide dismutase (SOD) (**c**) in liver of rats. Data are expressed as mean ± SEM ($n = 7$). *Plus symbol* indicates significant difference from haloperidol-loaded lipid-core nanocapsules suspension (H-Nc) group. *Asterisk* indicates significant difference from control (C) group. ($p < 0.05$ for all comparisons)

systems, proving the drug content to be adequate. Thus, this new nanoformulation is viable, showing ideal size, homogeneity, and stability of the particles in suspension and excellent drug encapsulation, as already reported (Benvegnú et al. 2011, 2012).

Besides neurotoxicity in the extrapyramidal system, chronic use of HP is frequently related to hepatic damage. Experimental studies have shown the hepatotoxicity of HP (Halici et al. 2009; Gaertner et al. 2001) by the increase in oxidative damage biomarkers, reduced antioxidant defenses, and disruption of cell integrity (Beasley et al. 2011; Dalla Corte et al. 2008). Our current findings confirmed this HP-induced hepatotoxicity, which was evidenced by (i) increased hepatic lipid peroxidation (LP): in fact, this increase indicates generation of reactive species (RS), which is able to stimulate the autocatalytic process of lipid peroxidation, increasing the formation of products such as MDA (Benzi 1993), thereby compromising cell integrity and function; (ii) decreased cellular viability (by MTT assay): MTT assay has been widely used to measure toxicity of different substances (Schiff et al. 1985), because it penetrates the cells by endocytosis, being reduced by NADH reductase and other enzymes to formazan, and reflecting the reductive potential of the cytoplasm and the cell viability (Liu and Schubert 1997); and (iii) increased plasmatic AST and ALT levels, which are indicative of hepatic damage: ALT is a cytoplasmic enzyme released into circulation in response to damage in the structural integrity of liver cells (Sallie et al. 1991), and it can also affect cell organelles such as the mitochondria, releasing compartmented enzymes such as AST.

In addition to liver damage, rats treated with free HP also presented decreased hepatic levels of VITC, which is an important water-soluble reducing agent (Padh 2005). On the other hand, our findings also showed that HP treatment was able to increase the hepatic SOD levels, whose antioxidant action is required to remove superoxide radical (Fridovich

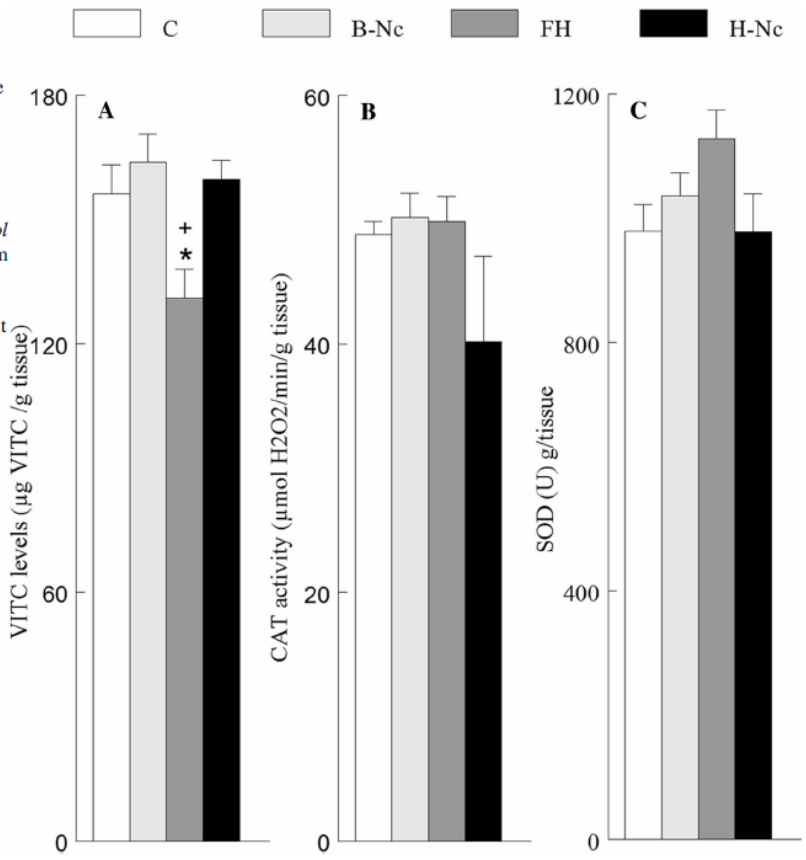

Fig. 5 Effects of daily administration of free (FH) or nanoencapsulated (H-Nc) haloperidol (0.5 mg/kg/mL-ip once a day, for 28 days) on vitamin C (VITC) levels (**a**), catalase (CAT) activity (**b**), and superoxide dismutase (SOD) (**c**) in kidneys of rats. Data are expressed as mean ± SEM ($n = 7$). *Plus symbol* indicates significant difference from haloperidol-loaded lipid-core nanocapsules suspension (H-Nc) group. *Asterisk* indicates significant difference from control (C) group. ($p < 0.05$ for all comparisons)

1986; Yu 1994). These changes observed in the liver of haloperidol-treated animals can indicate an overproduction of haloperidol pyridinium (HPP^+), which is produced following HP administration. In this sense, this drug is metabolized by hepatic cytochrome P450 system, which generates HPP^+, a potent inhibitor of mitochondrial respiration (Rollema et al. 1994; Usuki et al. 1996; Wright et al. 1998). Additionally, HPP^+ is able to increase RS generation, whose accumulation may cause oxidative damage (Burkhardt et al. 1993; Prince et al. 1997; Maurer and Moller 1997), especially in the liver (Silveira 2007). Indeed, HP is able to contribute to OS generation of an additional source: the increased turnover of catecholamines, favoring their metabolism as well as RS accumulation (Sagara, 1998; Yokoyama and Okano 1996). Taken together, these mechanisms are able to explain both oxidative damage and lower cell integrity in the liver, as observed in the haloperidol-treated animals of the current study.

Besides hepatotoxicity, haloperidol treatment has been also related to nephrotoxicity (Uyanik et al. 2009; Sawas and Gilbert 1985), whose literature data are yet controversial (Gulaboglu et al. 2006; Dalla Corte et al. 2008). Therefore, it is important to assess whether free HP is harmful to kidneys, or its nanoencapsulation may exert protective effects on these vital organs. Our current findings showed that free HP did not cause renal oxidative damage, as observed by the comparable levels of lipid peroxidation in kidneys. Furthermore, renal cellular integrity was also preserved, as observed by the MTT assay. In addition, free haloperidol caused no changes in plasma levels of creatinine, urea, and albumin, indicating that the renal function integrity was maintained. On the other hand, it was able to reduce vitamin C levels and ALA-D

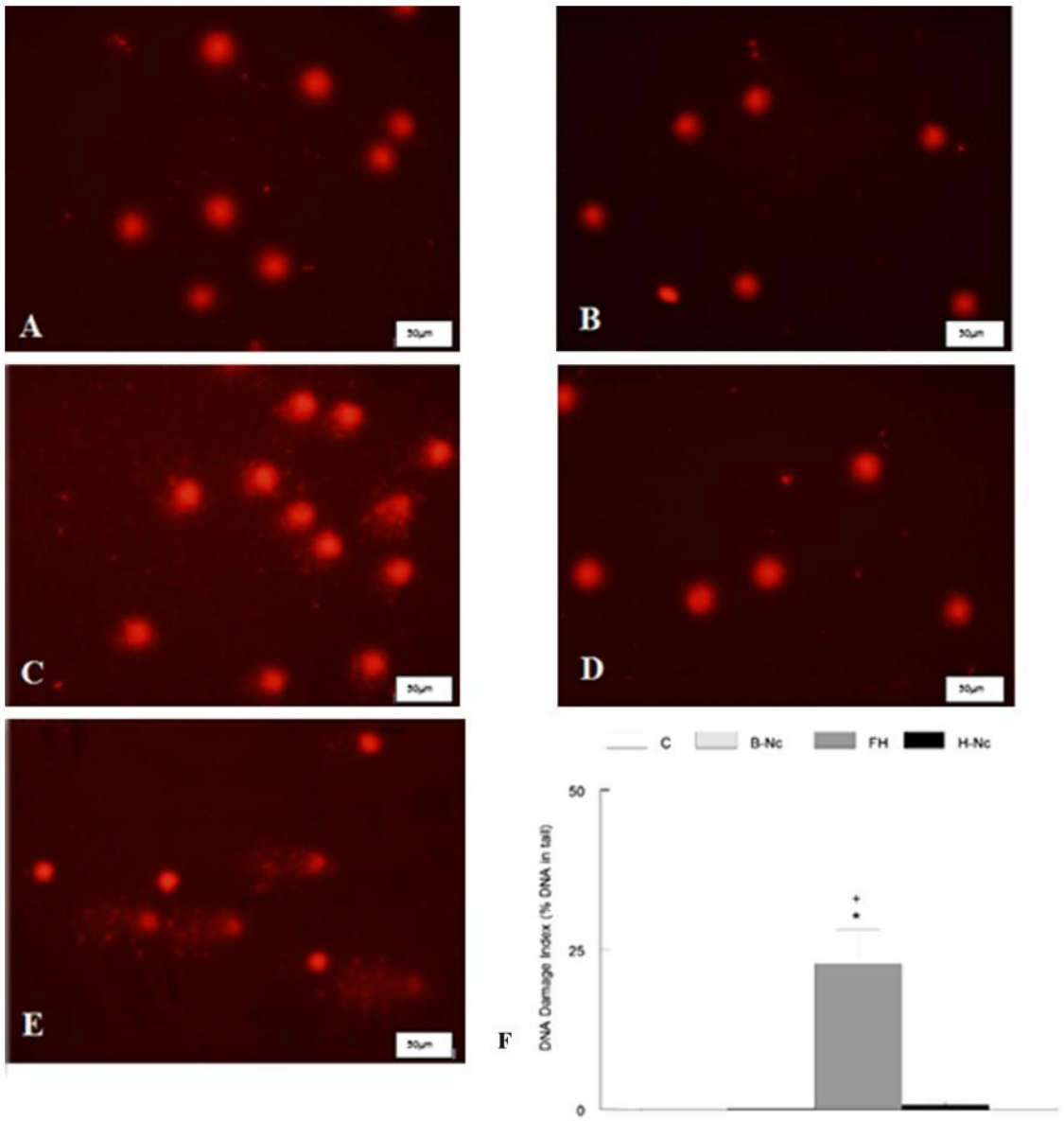

Fig. 6 Effects of daily administration of free or (FH) nanoencapsulated (H-Nc) haloperidol (0.5 mg/kg/mL-ip once a day, for 28 days) on DNA damage index in whole blood of rats. Control group (**a**), blank nanocapsules (B-Nc) (**b**), free haloperidol (**c**), nanoencapsulated haloperidol (**d**), positive control (**e**), and percentage of DNA damage index (**f**) in each group where data are expressed as mean ± SEM ($n = 7$). *Plus symbol* indicates significant difference from haloperidol-loaded lipid-core nanocapsules suspension (H-Nc) group. *Asterisk* indicates significant difference from control (C) group. ($p < 0.05$ for all comparisons)

activity in kidneys, indicating some pro-oxidant event (Gonçalves et al. 2009a, b, c). Based on this, we suggest that haloperidol exerts mild damage to kidneys, since biomarkers of oxidative damage together with antioxidant defenses such as SOD and CAT were not modified by this drug. A hypothesis that supports the minor extension of renal damage of haloperidol is that it is more extensively metabolized

Table 2 Effect of free haloperidol or haloperidol-loaded nanocapsules on biochemical parameters in plasma of rats

Parameter	C	B-Nc	FH	H-Nc
AST	158.50 ± 10.04	165.0 ± 0.40	206.00 ± 15.76*,+	149.43 ± 8.55
ALT	52.40 ± 6.03	63.5 ± 2.87	68.83 ± 4.19*,+	49.2 ± 5.12
Albumin	3.00 ± 0.07	2.95 ± 0.13	3.08 ± 0.05	2.95 ± 0.04
Creatinine	0.34 ± 0.02	0.31 ± 0.06	0.48 ± 0.07	0.40 ± 0.01
Urea	44.20 ± 2.17	43.75 ± 2.65	46.71 ± 1.96	43.57 ± 2.56

Data are expressed as mean ± SEM ($n = 7$)

C control, *B-Nc* blank nanocapsules, *FH* free haloperidol, *H-Nc* haloperidol-loaded lipid-core nanocapsules

Units: *AST* U/L plasm, *ALT* U/L plasm, *Albumin* g/dL plasm, *Creatinine* mg/dL plasm, *Urea* mg/dL plasm

+ Indicates significant difference from H-Nc group

* Indicates significant difference from control (C) group. ($p < 0.05$ for all comparisons)

in the liver, once only about 1 % of the administered drug is eliminated through this via (Fukunishi et al. 2002).

Furthermore, regarding free haloperidol nephrotoxicity, its genotoxic role has been little investigated, and the findings are conflicting (Asanami and Shimono 2009; Loveday et al. 1989; Van Cauteren et al. 1987; Balbi et al. 1980). Our current outcomes showed that free haloperidol was able to cause damage to DNA, which was assessed in whole blood. The development of oxidative damage may be a possible reason for this toxic effect of haloperidol, once haloperidol and HPP^+ metabolite are precursors of RS and OS, thus affecting the cellular integrity and consequently the genetic material of blood cells. This analysis was performed in blood because it is a tissue vulnerable to damage due to the drug residence time in the circulation (Andem et al. 2013). Also, due to the fact that nanocapsules remain more time in the circulation surface (Bender et al. 2012; Venturini et al. 2011), the analysis of these new formulations on DNA damage in blood cells deserves special attention.

In the current study, our findings showed that rats treated with haloperidol-loaded lipid-core nanocapsules (H-Nc) did not present hepatotoxic, renal, and DNA damage. It indicated that the nanoencapsulation was able to minimize the HP-induced oxidative damage. In fact, the nanocapsules system enables a controlled and sustained delivery of the drug, thus preventing its bioaccumulation in vital organs (Beck et al. 2005, 2006, 2007; Fontana et al. 2009; Marchiori et al. 2010) such as liver and kidneys. In addition, the presence of polysorbate 80 on the surface of the nanocapsules allows the system to remain longer in the bloodstream, avoiding the macrophages phagocytosis (Bender et al. 2012; Soppimath et al. 2001; Brigger et al. 2002; Venturini et al. 2011). In fact, this process may delay the biotransformation of HP, preventing the subsequent accumulation of toxic metabolites that may cause hepatic and renal oxidative damage. Our findings are in accordance with other studies that were performed with nanocapsules containing different drugs, thus indicating that the modifications in drug delivery are able to prevent hepatic (Santos et al. 2006; Gao et al. 2011) and renal (Avgoustakis et al. 2002; Boulikas 2004) damage related to the administration of free form of the drug. Regarding DNA damage, HP-loaded lipid-core nanocapsules were able to prevent this event, indicating that this nanoparticle system was able to protect blood cells from damage related to HP. Again, it is possible to observe that drug delivery through nanosystems allows a reduction of HP side effects, possibly due to a controlled drug release. These results are in agreement with a recent study by Grillo et al. (2012), which showed changes in distribution of substances, avoiding damage to DNA.

Besides the lower toxicity of haloperidol nanocapsules in different vital tissues, as already discussed, the lower toxicity of blank nanocapsules in liver, kidneys, and blood is of particular importance for the current study. Our findings are in agreement with other in vivo studies that showed safety of nanomaterials containing polymers (Huang et al. 2010; Fang et al. 2009; Kim et al. 2003). In fact, it is worth pointing out that literature regarding toxicity or safety of nanocapsules that contain Poly(e-caprolactone) (PCL) (Hu et al. 2011; Bulcão et al. 2013) is still poor, as toxicity studies about polymeric nanocapsules containing PCL

have so far been scantily performed (Bulcão et al. 2013, 2014). This indicates the innovative contribution of the current study regarding the low toxicity of this compound.

In summary, our study shows that HP-loaded lipid-core nanocapsules did not show toxicity to vital organs such as liver and kidneys, and caused no damage to blood DNA, differently from the observed with the free form of the drug. While the literature is poor in studies about the general toxicity of HP, especially in vital tissues such as liver, kidneys, and blood, our study shows that its nanoencapsulation was able to minimize these toxic events. Additional studies involving molecular targets are needed to confirm and support the absence of toxicity of this innovative formulation.

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Conflict of interest Authors report no conflicts of interest.

Compliance with Ethical Standards The experimental protocol of this study was approved by the Animal Ethics Committee of Universidade Federal de Santa Maria (CIETEA-22/2010), affiliated to CONCEA, and adhered to the ''Principles of Laboratory Animal Care'' and international rules of ethics in research.

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4 CONCLUSÕES FINAIS

Através dos dados apresentados neste estudo, pode-se concluir que após tratamento subcrônico, o haloperidol livre foi capaz de causar toxicidade no fígado, evidenciado através do aumento dos níveis de peroxidação lipídica, diminuição da integridade celular e maiores níveis plasmáticos das enzimas AST e ALT, cujo aumento confirma a disfunção hepática causada pelo fármaco na sua forma livre. Adicionalmente, o haloperidol livre causou danos ao rim dos animais, porém em menor extensão, e também provocou danos ao DNA sanguíneo dos animais. Em contrapartida, o tratamento com haloperidol nanoencapsulado não causou toxicidade hepática ou renal para os animais, demonstrado pela prevenção do desenvolvimento de EO, manutenção da integridade celular e dos níveis das enzimas AST e ALT. Esta mesma formulação também não causou danos ao DNA sanguíneo. Portanto, além de não provocar toxicidade nestes órgãos, a nova formulação foi capaz de prevenir as alterações causadas pelo fármaco livre. No entanto, estudos adicionais são necessários para confirmar a ausência de toxicidade do haloperidol nanoencapsulado a outros tecidos. A continuidade do estudo também deverá incluir a busca de mecanismos farmacocinéticos e/ou farmacodinâmicos, os quais podem estar envolvidos na prevenção dos danos aqui mostrados. Nossa expectativa é que o presente estudo, juntamente com pesquisas futuras, possam fortalecer as bases da terapêutica antipsicótica em favor desta nanoformulação inovadora.

5 PERSPECTIVAS

Com base nos resultados obtidos no presente trabalho, faz-se necessário os seguintes estudos:

- Avaliação toxicológica do haloperidol nanoencapsulado após seu uso em tratamento crônico.
- Quantificação das formulações de haloperidol em nível plasmático, em outros tecidos, além de diferentes áreas cerebrais.
- Efeito do tratamento com diferentes formulações de haloperidol sobre parâmetros de memória e cognição de ratos.

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