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EFEITO DO TRATAMENTO COM NANOCÁPSULAS POLIMÉRICAS CONTENDO CLOZAPINA SOBRE PARÂMETROS DE ESTRESSE OXIDATIVO EM RATOS WISTAR

Dissertação de Mestrado

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Dissertação apresentada ao Programa de Pós-Graduação *Stricto sensu* em Bioquímica da Fundação Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Bioquímica.

Orientadora: Prof.ª Dr.ª Vanusa Manfredini

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“Suba o primeiro degrau com fé.
Não é necessário que você veja toda a escada.
Apenas dê o primeiro passo.”

Martin Luther King

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Bioquímica
Fundação Universidade Federal do Pampa

EFEITO DO TRATAMENTO COM NANOSSISTEMAS CONTENDO CLOZAPINA SOBRE PARÂMETROS DE ESTRESSE OXIDATIVO EM RATOS WISTAR

Autora: Angélica Aparecida da Costa Güllich
Orientadora: Vanusa Manfredini
Local e Data da Defesa: Uruguaiana, 22 de Janeiro de 2014.

A clozapina é um antipsicótico de segunda geração, da família da dibenzodiazepina sendo o tratamento de primeira escolha na esquizofrenia refratária, devido ao seu efeito marcante sobre os sintomas positivos e negativos da doença. No entanto, seus efeitos adversos graves limitam o uso na terapia clínica, como agranulocitose, cardiotoxicidade e hepatotoxicidade. Em virtude dessas complicações torna-se necessário uma forma farmacêutica capaz de vetorizar o fármaco para seu local de ação, reduzindo efeitos indesejáveis e minimizando o estresse oxidativo, fazendo dos nanossistemas uma ferramenta promissora para este fim. O objetivo desse trabalho foi verificar o efeito do tratamento com nanossistemas contendo clozapina sobre parâmetros de estresse oxidativo em ratos Wistar. O estudo foi constituído de oito grupos de ratos machos Wistar (n = 6), os animais receberam os seguintes tratamentos: solução salina (NaCl 0,9% 1,0 mL/Kg i.p.), clozapina livre (25 mg/Kg i.p.), nanocápsulas brancas sem revestimento (1,0 mL/Kg i.p.), nanocápsulas contendo clozapina sem revestimento (25 mg/Kg i.p.), nanocápsulas brancas revestidas com quitosana ou polietilenoglicol (1,0 mL/Kg i.p.), nanocápsulas contendo clozapina revestidas com quitosana ou polietilenoglicol (25 mg/Kg i.p.). Os animais receberam as formulações uma vez ao dia durante sete dias consecutivos, sendo eutanasiados no oitavo dia. Foram coletados sangue total e os órgãos coração, fígado e rim para análises posteriores. A terapia com nanossistemas contendo clozapina foi eficaz em manter os níveis hematológicos dentro da normalidade. A quantificação dos marcadores enzimáticos para funções cardíaca, hepática e renal demonstrou níveis séricos significativamente diminuídos ($p < 0,05$) quando comparado ao grupo clozapina livre, ficando mais evidente a melhora clínica no grupo nanocápsulas contendo clozapina revestidas com quitosana nos marcadores cardíacos e hepáticos. A análise histopatológica dos órgãos mostrou que os diferentes nanossistemas contendo clozapina foram capazes de reduzir danos teciduais. A atividade das enzimas antioxidantes catalase, superóxido dismutase e glutationa peroxidase apresentou-se significativamente elevada nos grupos com diferentes nanossistemas, assim como a atividade da glutationa reduzida, sendo do grupo nanocápsulas contendo clozapina revestidas com quitosana os melhores resultados. Quanto ao dano oxidativo em lipídios de membrana, proteínas plasmáticas e no material genético, a clozapina livre induziu o dano enquanto que os diferentes nanossistemas contendo clozapina foram capazes que reduzi-lo, sendo mais evidente no grupo nanocápsulas contendo clozapina revestidas com polietilenoglicol. Logo, os achados demonstram que diferentes revestimentos podem atuar de maneira diversificada e específica para cada órgão ou tecido pelo qual possuem maior afinidade. A nanoencapsulação da clozapina é uma ferramenta terapêutica promissora, capaz de atenuar os efeitos nocivos do fármaco, minimizando o estresse oxidativo, tornando-a um fármaco mais seguro aos pacientes.

Palavras-chave: esquizofrenia, clozapina, nanossistemas, vias de estresse oxidativo.

ABSTRACT

Dissertation of Master's Degree
Program of Post-Graduation in Biochemistry
Federal University of Pampa

EFFECT OF TREATMENT WITH NANOSYSTEMS CONTAINING CLOZAPINE ON OXIDATIVE STRESS PARAMETERS IN RATS WISTAR

Author: Angélica Aparecida da Costa Güllich
Advisor: Vanusa Manfredini
Date and Place of Defense: Uruguaiana, January 22, 2014

Clozapine is a second-generation antipsychotic, of family dibenzodiazepine being treatment of first choice in refractory schizophrenia, owing outstanding effect about positive and negative symptoms disease. However, severe adverse effects limit their use in clinical therapy, such as agranulocytosis, hepatotoxicity and cardiotoxicity. In view these complications became necessary a pharmaceutical form able to vectorize the drug its site of action, reducing side effects and minimizing oxidative stress, making the nanosystems a promising tool for this purpose. The aim this study was to investigate the effect of treatment with nanosystems containing clozapine on oxidative stress parameters in Wistar rats. The study consisted of eight groups of male Wistar rats (n = 6) animals received the following treatments: saline solution (NaCl 0.9% 1.0 mL/Kg i.p.), free clozapine (25 mg/Kg i.p.), blank uncoated nanocápsulas (1.0 mL/Kg i.p.), clozapine-loaded uncoated nanocapsules (25 mg/Kg i.p.), blank chitosan-coated or polyethyleneglycol-coated nanocápsulas (1.0 mL/Kg i.p.), clozapine-loaded chitosan-coated or polyethyleneglycol-coated nanocapsules (25 mg/Kg i.p.). The animals received the formulation once a day for seven consecutive days and euthanized in the eighth day. It was collected global blood and organs heart, liver and kidney for further analysis. The nanosystems containing clozapine therapy was effective in maintaining blood levels within the normal range. Quantification of biochemical markers for cardiac, hepatic and renal function showed serum levels significantly decreased ($p < 0.05$) when compared free clozapine group, becoming more evident clinical improvement in the clozapine-loaded chitosan-coated nanocapsules group in the markers cardiac and hepatic. The histopathological analysis of the organs showed that the different nanosystems containing clozapine were able to reduce tissue damage. The activity of antioxidant enzymes catalase, superoxide dismutase and glutathione peroxidase were significantly higher in the groups with different nanosystems, such as the activity of reduced glutathione, being the clozapine-loaded chitosan-coated nanocapsules group the best results. How to oxidative damage in membrane lipids, plasma proteins and genetic material, the free clozapine induced damage while different nanosystems containing clozapine were able to reduce, being more evident in the clozapine-loaded polyethyleneglycol-coated nanocapsules. Thus, our findings suggest that different coatings can act in diverse and specific way for each organ or tissue through which possess higher affinity. The nanoencapsulation of clozapine is a promising therapeutic tool, able of mitigating the harmful effects of drug, minimizing oxidative stress, making a safer drug to patients.

Keywords: schizophrenia, clozapine, nanosystems, oxidative stress pathways.

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

APG	-	Antipsicóticos de Primeira Geração
ASG	-	Antipsicóticos de Segunda Geração
BIOTECH	-	Laboratório de Biotecnologia da Reprodução
CAPES	-	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CAT	-	Catalase
CZP	-	Clozapina
DNA	-	Ácido Desoxirribonucléico
ER	-	Esquizofrenia Refratária
ERN	-	Espécies Reativas de Nitrogênio
ERO	-	Espécies Reativas de Oxigênio
GESTOX	-	Grupo de Estudos em Estresse Oxidativo
GPx	-	Glutationa Peroxidase
GSH	-	Glutationa Reduzida
H_2O_2	-	Peróxido de Hidrogênio
HOCl	-	Ácido Hipocloroso
N_2O	-	Óxido Nitroso
NC	-	Nanocápsulas
$NO^{\bullet}$	-	Radical Óxido Nítrico
NP	-	Nanopartículas
OMS	-	Organização Mundial de Saúde
O_2	-	Oxigênio Singleto
$O_2^{\bullet -}$	-	Radical Ânion Superóxido
$OH^{\bullet}$	-	Radical Hidroxila
$ONOO^-$	-	Ânion Peroxinitrito
PEG	-	Polietilenoglicol
PPGBIOQ	-	Programa de Pós-Graduação em Bioquímica
QTS	-	Quitosana
RL	-	Radical Livre
$RO^{\bullet}$	-	Radical Alcoxila
$ROO^{\bullet}$	-	Radical Peroxila

SNC	-	Sistema Nervoso Central
SOD	-	Superóxido Dismutase
TBARS	-	Substâncias Reativas ao Ácido Tiobarbitúrico

SUMÁRIO

APRESENTAÇÃO

A presente dissertação foi dividida em três partes principais. Na **parte I** encontram-se a **INTRODUÇÃO**, **REVISÃO BIBLIOGRÁFICA** e **OBJETIVOS**. Os resultados que fazem parte desta dissertação estão apresentados sob a forma de manuscritos, nos itens **MANUSCRITO I**, **MANUSCRITO II** e **MANUSCRITO III**, os quais se encontram na **parte II** deste trabalho. As seções materiais e métodos, resultados, discussão e referências, encontram-se nos próprios manuscritos e representam a íntegra deste estudo. O item **CONCLUSÃO** encontra-se na **parte III** desta dissertação, apresentando interpretações e comentários gerais sobre os resultados mostrados nos manuscritos deste trabalho. No item **PERSPECTIVAS**, estão expostos os possíveis estudos para dar continuidade a este trabalho. O item **REFERÊNCIAS** refere-se somente às citações que aparecem nos itens introdução e revisão bibliográfica desta dissertação.

PARTE I

1 INTRODUÇÃO

A esquizofrenia é um transtorno psicótico, ou grupo de transtornos, ocorrendo normalmente na fase mais tardia da adolescência ou no início da idade adulta. Geralmente, há prejuízo das funções ocupacionais caracterizado por afastamento social, perda de interesse e/ou capacidade de agir (HÄFNER & VAN DER HEIDEN, 2003; FALKAI et al., 2006).

O termo esquizofrenia refratária (ER) ou esquizofrenia resistente ao tratamento refere-se à persistência de sintomas positivos moderados a graves, com o paciente fazendo uso de medicação. Alguns autores levam em consideração sintomas negativos e cognitivos, bem como a incapacidade do indivíduo retornar ao seu melhor nível de funcionamento (PEUSKENS, 1999; MELTZER & KOSTACOGLU, 2001; ELKIS & MELTZER, 2007).

A clozapina (CZP) é o antipsicótico de escolha, em indivíduos com ER, uma vez que há evidências da maior eficácia clínica em relação aos demais antipsicóticos (ELKIS & MELTZER, 2007; MCILWAIN et al., 2011). Antipsicótico de segunda geração derivado dibenzodiazepínico, suas principais indicações incluem o tratamento da esquizofrenia não responsiva a fármacos antipsicóticas convencionais e de pacientes com intolerância a outros antipsicóticos (ABIDI & BHASKARA, 2003; MCILWAIN et al., 2011).

A clozapina sofre extenso metabolismo de primeira passagem, e a metabolização do fármaco ocorre no fígado, predominantemente, originando dois metabólitos, a N-desmetilclozapina e a clozapina-N óxido, esses são reativos e parecem danificar as células, como as do fígado e medula óssea (BUUR-RASMUSSEN & BROSEN, 1999).

A limitação clínica na utilização da CZP se deve a gravidade dos seus efeitos adversos. Entre as complicações mais graves que este agente antipsicótico pode causar estão a granulocitopenia e agranulocitose (ALVIR, 1993; GASZNER, MAKKOS & KOSZA, 2002; DUTT et al., 2010; RAMÍREZ et al., 2011; MANU et al. , 2012; PONS et al., 2012). As cardiomiopatias, em que a mortalidade cardiovascular é uma das principais causas de óbito em pacientes com esquizofrenia, sendo que, o risco é duas vezes maior do que a população em geral (LEITÃO-AZEVEDO et al., 2007; RAMÍREZ et al., 2011; RONALDSON et al., 2011; MANU et al. , 2012), e, outro efeito adverso é a hepatotoxicidade relacionada aos

metabólitos da CZP, podendo induzir alteração das transaminases e causar insuficiência hepática (HUMMER et al., 1997; NEVES et al., 2006).

Estudos demonstram que o estresse oxidativo está envolvido na progressão de inúmeras doenças, dentre as quais estão às doenças neurológicas e a esquizofrenia (DIETRICH-MUSZALSKA et al., 2009, 2012; DIETRICH-MUSZALKA & OLAS, 2009).

Estudos realizados em ratos tratados com haloperidol e/ou CZP demonstraram que estes fármacos foram capazes de induzir dano oxidativo em diversas estruturas no sistema nervoso central (SNC) (POLYDORO et al., 2004; AGOSTINHO et al., 2007). Achados similares foram relatados em um estudo que avaliou o dano oxidativo no cérebro de ratos induzido pela administração crônica de haloperidol e/ou CZP e/ou olanzapina, em que a CZP induziu dano oxidativo, embora menor que o haloperidol, e, não foi verificado dano oxidativo induzido por olanzapina (REINKE et al., 2004). Outros trabalhos mostram que pacientes esquizofrênicos possuem elevados níveis de homocisteína, grupamentos carbonil e isoprostanos em plasma decorrentes do estresse oxidativo (DIETRICH-MUSZALKA et al., 2009, 2012; DIETRICH-MUSZALKA & OLAS, 2009).

Portanto, em virtude dessas complicações graves associadas ao uso da CZP torna-se necessário a utilização de uma forma farmacêutica capaz de vetorizar esse fármaco para seu local de ação, no caso o SNC, reduzindo seus efeitos periféricos indesejáveis, fazendo dos sistemas nanoparticulados uma ferramenta promissora. As nanopartículas (NP) poliméricas, como as nanocápsulas (NC), vem sendo utilizadas como carreadores para diversos fármacos com diferentes objetivos terapêuticos, como a vetorização dos mesmos para a biofase com consequente redução de efeitos adversos (HANS & LOWMAN, 2002).

Dentre as matérias-primas que podem ser utilizadas na preparação das NP estão a quitosana (QTS) e o polietilenoglicol (PEG). A QTS polímero de origem natural, com características de bioadesividade e a biocompatibilidade, possui cargas positivas que podem facilitar a interação com componentes de carga negativa das membranas celulares, auxiliando na permeação do fármaco. O PEG, polímero comumente utilizado possui como finalidade a modificação da superfície, é hidrofílico, não-iônico e biocompatível (HANS & LOWMAN, 2002).

Aspectos relacionados à segurança dos sistemas nanoparticulados ainda devem ser estudados, como o dano oxidativo. Até o presente, existem poucos estudos publicados na literatura consultada que mostrem o envolvimento do estresse oxidativo e a CZP tanto em modelo animal quanto em humanos, bem como estudos nanotoxicológicos que contemplem nanossistemas contendo CZP e o dano oxidativo celular e tecidual.

2 REVISÃO BIBLIOGRÁFICA

2.1 Esquizofrenia

A esquizofrenia é uma forma grave de doença mental, sendo considerada um transtorno psicótico maior, ou grupo de transtornos, que apresenta diferentes sintomas de forma heterogênea (CROW, 1985; HÄFNER & VAN DER HEIDEN, 2003; WHO, 2013). Geralmente, aparece na fase mais tardia da adolescência ou no início da idade adulta, por ser uma doença de etiologia complexa acomete diferentes indivíduos, além de possuir uma alta variabilidade nos mesmos indivíduos no decorrer do tempo (CROW, 1985; MARENCO & WEINBERGER, 2000; FALKAI et al., 2006).

Normalmente, há prejuízo das funções ocupacionais caracterizada por afastamento social, perda de interesse ou capacidade de agir (HÄFNER & VAN DER HEIDEN, 2003). A psicopatologia da doença se caracteriza por fenômenos positivos, negativos e cognitivos (CROW, 1985). Os sintomas positivos incluem aqueles que o indivíduo não deveria apresentar, mas os apresentam como delírios, alucinações, sintomas catatônicos, agitação e desconfiança. Já os sintomas negativos se referem aqueles que o indivíduo deveria apresentar, mas não os apresentam como a redução das expressões emocionais, diminuição da produtividade do pensamento e da fala, retraimento social e monotonia afetiva. Além disso, o indivíduo apresenta sintomas desorganizados incluindo desorganização do pensamento e do comportamento associada ao comprometimento da atenção (FALKAI et al., 2006).

Estudos vem sendo realizados com o intuito de determinar o papel de variáveis como fatores genéticos, bioquímicos e alterações na morfologia cerebral no desenvolvimento da esquizofrenia. Segundo a hipótese do neurodesenvolvimento, a patologia seria o resultado de um distúrbio do desenvolvimento cerebral durante os períodos pré e perinatal. Porém, a natureza precisa dessa alteração cerebral, bem como de sua patogênese, ainda não foi totalmente definida (MARENCO & WEINBERGER, 2000).

Sua prevalência é de aproximadamente 1 caso a cada 100 pessoas, bastante comum na população, mas essa estimativa pode variar de acordo com a metodologia utilizada nos diferentes estudos (HÄFNER & VAN DER HEIDEN, 2003; FALKAI et al., 2006). Segundo dados da Organização Mundial de Saúde (OMS), cerca de 7 a cada 1000 indivíduos da população adulta são acometidos pela doença, principalmente na faixa etária de 15 a 35

anos. São cerca de 24 milhões de pessoas no mundo diagnosticadas com esquizofrenia (WHO, 2013).

Não há cura para a esquizofrenia, porém a doença possui tratamento, sendo este mais eficaz se iniciado nos estágios iniciais da doença. Mais de 50% dos casos de esquizofrenia não recebem cuidados e tratamento adequados, sendo que desses, 90% dos indivíduos acometidos pela doença estão em países em desenvolvimento (WHO, 2013). O tratamento farmacológico deve ser introduzido com rigor e cautela. Recomenda-se iniciar a terapêutica antipsicótica em doses baixas, seguido de ajuste gradual da dose de acordo com a melhora e bem estar de cada paciente (FALKAI et al., 2006).

Os antipsicóticos de primeira geração (APG), também chamados de antipsicóticos convencionais ou típicos, tem sua dose terapêutica e sua tendência de causar efeitos colaterais extrapiramidais intimamente relacionadas a sua afinidade por receptores de dopamina, particularmente aos receptores D2. A eficácia dos APG na redução de sintomas positivos e na prevenção de recidivas é considerada inquestionável, contudo, as limitações da intervenção com APG decorrem do alcance limitado desse tipo de tratamento sobre os sintomas negativos da doença, bem como sobre a disfunção cognitiva, fatos esses que contribuem para a baixa qualidade de vida e déficits funcionais dos pacientes (FALKAI et al., 2006).

Antipsicóticos de segunda geração (ASG), ou antipsicóticos atípicos, tem farmacocinética similar à dos APG. Os ASG são rápida e predominantemente absorvidos após administração oral, e frequentemente passam por extenso metabolismo de primeira passagem hepático (BURNS, 2001). São altamente lipofílicos, altamente ligados a proteínas, e tendem a se acumular no cérebro e em outros tecidos (FALKAI et al., 2006).

Os APG estão associados ao alto risco de efeitos colaterais extrapiramidais e risco moderado de sedação. Enquanto que, os ASG tem maior eficiência e menor incidência de efeitos adversos, dados demonstrados em diferentes estudos evidenciam a superioridade da classe em relação aos demais antipsicóticos (FACTOR, 2002; ABIDI & BHASKARA, 2003; FALKAI et al., 2006).

Além do mais, APG acarretam efeitos adversos importantes principalmente aqueles relacionados ao movimento, como sintomas extrapiramidais e disforia, o que acaba por comprometer a tolerabilidade e aderência dos pacientes ao tratamento (MÖLLER, 2000a; 2000b).

Tais fatos estimularam o desenvolvimento dos ASG, cuja maior vantagem é a baixa propensão a efeitos extrapiramidais, principalmente a discinesia tardia. Além disso, os ASG demonstraram maior eficácia no tratamento dos sintomas negativos, dos distúrbios cognitivos

e dos sintomas depressivos da doença, tem seu perfil clínico descrito como o espectro mais amplo de eficácia clínica (MÖLLER, 2000a; 2000b). Existem alguns riscos relacionados aos ASG, como distúrbios de utilização da glicose, metabolismo de lipídios e ganho de peso, também descritos e relacionados a alguns dos APG, porém, podem ser ainda mais pronunciados em alguns ASG (FALKAI et al., 2006).

Como primeira escolha para a terapêutica clínica farmacológica da esquizofrenia, devem ser utilizados, preferencialmente, os ASG devido sua reconhecida superioridade em relação aos demais medicamentos. Alternativamente, recomenda-se o uso de APG no limite inferior da faixa terapêutica. Essa recomendação é baseada principalmente na melhor tolerabilidade e no risco reduzido de discinesia tardia dos ASG (FALKAI et al., 2006).

A seleção de uma medicação antipsicótica é guiada pela experiência prévia do paciente, considerando sua resposta terapêutica e a relação da dose efetiva com os efeitos colaterais relatados pelo indivíduo. Para reduzir o risco de sintomas extrapiramidais, recomenda-se o emprego de doses tão baixas quanto possível, especialmente quando se optar pelo uso de APG (FALKAI et al., 2006).

Estudos demonstram que, dentre os ASG, é a CZP que possui maior eficácia e menores efeitos adversos, inclusive aqueles relacionados aos sintomas extrapiramidais, sendo considerada superior aos demais agentes neurolépticos (FALKAI et al., 2006; MCILWAIN et al., 2011).

2.1.1 Esquizofrenia refratária

A esquizofrenia é uma enfermidade crônica, onde mais de 80% dos pacientes exibem algum tipo de disfunção, seja ele, social ou ocupacional, portanto, torna-se difícil estabelecer uma linha divisória entre a esquizofrenia responsiva e a refratária ao tratamento (MELTZER, 1990).

Há uma distinção entre a cronicidade e a refratariedade. Existem diversas e diferentes doenças crônicas, como diabetes ou hipertensão, que apesar de sua cronicidade respondem ao tratamento, onde os pacientes tem suas doenças estabilizadas pelo uso contínuo de medicação específica (ELKIS & MELTZER, 2007).

O termo ER, ou esquizofrenia resistente ao tratamento, é por vezes incorretamente aplicado a pacientes que se mantêm sintomáticos por não aderirem ao tratamento (ELKIS &

MELTZER, 2007). A aderência ao tratamento deve ser verificada, se necessário, pela determinação das concentrações séricas dos medicamentos (FALKAI et al., 2006).

A ER é normalmente caracterizada pela persistência de sintomas positivos moderados a graves (PEUSKENS, 1999; FALKAI et al., 2006). Porém, mesmo que os sintomas positivos entrem em remissão com o tratamento, outros sintomas residuais frequentemente persistem (FALKAI et al., 2006).

Não há um consenso a respeito do conceito de ER. Alguns pesquisadores mencionam que devem ser levados em consideração ainda, sintomas negativos e cognitivos, como a gravidade da disfunção cognitiva, comportamentos bizarros, sintomas afetivos, déficits funcionais e sociais, a má qualidade de vida, bem como a incapacidade do paciente em retornar ao seu melhor nível (MELTZER & KOSTACOGLU, 2001; FALKAI et al., 2006).

Estudos apontam que cerca de 10% a 30% dos esquizofrênicos não respondem ou tem pouca resposta a um tratamento neuroléptico convencional, e até 30% dos pacientes tem respostas parciais ao tratamento, ou seja, obtém melhora na psicopatologia, mas continuam a ter alucinações e delírios leves a graves (BRENNER et al., 1990; LOUZÃ NETO, 1995; ESSOCK et al., 1996; HENNA, 1999). É extremamente necessário estabelecer se a resposta terapêutica foi insuficiente em relação ao tratamento adotado (FALKAI et al., 2006).

Diretrizes para o tratamento da esquizofrenia da American Psychiatric Association e algoritmos do Texas Medication Algorithm Project estabelecem que após o fracasso de dois ou três tratamentos com antipsicóticos atípicos, o paciente deve ser considerado como portador de ER (LEHMAN et al. 2004; MILLER et al. 2004).

Outros autores mencionam que deve-se levar em consideração o fracasso do emprego alternativo de pelo menos dois antipsicóticos, onde um deve ser um agente atípico, após a medicação ser administrada nas doses recomendadas por pelo menos 6 até 8 semanas (FALKAI et al., 2006). Caso o quadro seja confirmado significa que o paciente é candidato ao tratamento com CZP, droga aprovada para o tratamento da ER, sendo assim, o tratamento de primeira escolha para essa enfermidade (LOUZÃ NETO, 1995; HENNA, 1999; LEHMAN et al. 2004; MILLER et al. 2004).

Estudos demonstram de forma consistente que a CZP possui maior eficácia e menores efeitos adversos, sendo considerada superior aos agentes neurolépticos convencionais na terapêutica da ER (FALKAI et al., 2006).

2.2 Radicais livres, estresse oxidativo e defesas antioxidantes

Radical livre (RL) é o termo utilizado para caracterizar espécies atômicas ou moleculares que contenham um ou mais elétrons desemparelhados na sua camada de valência, o que as torna espécies altamente reativas que agem como eletrófilos capazes de reagir com qualquer composto que esteja próximo, assumindo o papel de agentes oxidantes (HALLIWELL, 1991; GILLHAN, PAPACHRISTODOULOU & THOMAS, 1997; HALLIWELL & GUTTERIDGE, 2007).

No entanto, o termo RL não serve para designar todos os agentes reativos, pois alguns não possuem elétrons desemparelhados na sua camada mais externa, como o peróxido de hidrogênio (H_2O_2). Mesmo não sendo um RL, esse composto químico pode gerar dano celular, particularmente por ser capaz de reagir com o radical ânion superóxido ($O_2^{\bullet-}$), mediado por íons de ferro ou cobre, formando o radical hidroxila ($OH^{\bullet}$). Este, por sua vez, é altamente reativo (HALLIWELL, 1991).

Dentre os oxidantes mais importantes envolvidos em processos patológicos estão às espécies reativas de oxigênio (ERO) e as espécies reativas de nitrogênio (ERN). As principais ERO distribuem-se em dois grupos: radicalares e não radicalares. Fazem parte do grupo das radicalares: $O_2^{\bullet-}$, $OH^{\bullet}$, radical peroxila ($ROO^{\bullet}$) e radical alcoxila ($RO^{\bullet}$). Já o grupo das não radicalares é composto por: oxigênio singleto (O_2), H_2O_2 e ácido hipocloroso (HOCl). As ERN incluem-se o radical óxido nítrico ($NO^{\bullet}$), óxido nitroso (N_2O) e o ânion peroxinitrito ($ONOO^-$), dentre outros compostos (GILLHAM, PAPACHRISTODOULOU & THOMAS, 1997).

As espécies reativas podem ser formadas no organismo de diversos modos. Nos organismos aeróbios geralmente ocorre com a redução de uma molécula de O_2 a $O_2^{\bullet-}$, uma reação de óxido-redução. Os radicais podem ser formados por processos de oxidação provenientes do metabolismo aeróbico, portanto são produzidos naturalmente ou por uma disfunção biológica (HALLIWELL, 1991; BARREIROS & DAVID, 2006). Durante a fosforilação oxidativa também podem ser formados, mecanismo este usado pelas células para produzir energia. Podem ainda ser produzidos durante a oxidação de ácidos graxos, reações do citocromo P450 e de células fagocíticas. Algumas enzimas também são capazes de gerar ERO, sob condições normais ou patológicas. Além de fontes exógenas como tabaco, radiações, luz ultravioleta, solventes e alguns fármacos (BIESALSKI, 2002; JUNQUEIRA & RAMOS, 2005).

Em condições fisiológicas normais, as ERO desempenham um papel importante nos seres vivos. Dentre as suas funções no organismo a regulação da resposta imune, participando do processo de defesa contra infecções, sinalização intracelular, induzindo a apoptose sendo capaz de eliminar bactérias e partículas em um processo de fagocitose (HALLIWELL, 1991; BIESALSKI, 2002; HALLIWELL & GUTTERIDGE, 2007; RAY, HUANG & TSUJI, 2012).

O campo de RL e antioxidantes, ou biologia redox, é fundamental para a vida aeróbica. Constantemente espécies reativas modulam suas atividades sintetizando antioxidantes. Este equilíbrio permite que algumas espécies reativas executem funções úteis como minimizar os danos oxidativos (HALLIWELL, 2011).

No entanto, quando ocorre um aumento das ERO ou ERN e/ou uma diminuição da capacidade antioxidante, ou seja, um desequilíbrio entre os sistemas, as espécies reativas são capazes de lesar componentes celulares direta ou indiretamente, modificando sua estrutura e/ou função e gerando o estresse oxidativo. As espécies reativas são capazes de danificar biomoléculas como lipídeos de membrana, proteínas e também o ácido desoxirribonucléico (DNA) (SCHAFER & BUETTNER, 2001; MONAGHAN, METCALFE & TORRES, 2009).

Estudos sugerem diversas e abundantes evidências, demonstrando o envolvimento do estresse oxidativo na progressão e patogênese de inúmeras doenças que afetam praticamente todos os sistemas do corpo humano, como anemia falciforme (MANFREDINI et al., 2008), Diabete Mellitus tipo 2 (MANFREDINI et al., 2010), erros inatos do metabolismo (RIBAS et al., 2010), doenças neurológicas e também a esquizofrenia (DIETRICH-MUSZALSKA et al., 2009, 2012; DIETRICH-MUSZALKA & OLAS, 2009; HALLIWELL, 2012a; 2012b).

Tendo em vista o papel do estresse oxidativo em diferentes patologias, é importante a ação dos antioxidantes na manutenção da saúde, bem como na prevenção e tratamento de doenças (NIKI, 2010). A produção contínua de RL durante os processos metabólicos ocasionou o desenvolvimento de mecanismos de defesa antioxidante para limitar os níveis intracelulares de ERO e ERN, bem como para impedir a indução danos oxidativos (SIES, 1993).

Para proteger o organismo do ataque dessas espécies reativas, existe uma série de sistemas de defesa antioxidante, como o sistema enzimático que é a primeira via de defesa do organismo, onde enzimas específicas inativam algumas das ERO. Desta forma, o sistema antioxidante enzimático diminui as ERO e, consequentemente o dano às estruturas biológicas, fazem parte deste grupo as enzimas antioxidantes catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GPx) (GUTTERIDGE & HALLIWELL, 2000; BELLÓ, 2002; HALLIWELL & GUTTERIDGE, 2007).

Outro sistema de defesa é o sistema não enzimático o qual é representado por compostos antioxidantes que podem ter origem endógena ou serem obtidas através da alimentação. Estes são subdivididos em compostos antioxidantes hidrofílicos como glutationa reduzida (GSH), vitamina C e polifenóis, e em compostos antioxidantes lipofílicos como a vitamina E e carotenoides (GUTTERIDGE & HALLIWELL, 2000).

Os agentes antioxidantes são substâncias capazes de inibir e/ou reduzir a oxidação, mesmo quando presentes em baixas concentrações em relação a seu substrato oxidável, com consequente redução dos danos causados pelos RL nas células. Desta forma, esses agentes tem a função de proteção das células contra os efeitos deletérios dos RL, dos processos ou reações que levam à oxidação de macromoléculas ou estruturas celulares. Podendo prolongar a fase de iniciação ou então inibir a fase de propagação, mas não podem prevenir completamente a oxidação (BIANCHI & ANTUNES, 1999; GUTTERIDGE & HALLIWELL, 2000; BARREIROS & DAVID, 2006).

2.2.1 Esquizofrenia X estresse oxidativo

Os RL induzem o estresse oxidativo e danos em todos os tipos de moléculas biológicas e podem estar envolvidos na patologia da esquizofrenia (HALLIWELL, 2006; DIETRICH-MUSZALKA & OLAS, 2009; HALLIWELL, 2012a; 2012b). O cérebro e o SNC são propensos ao estresse oxidativo, pois estão insuficientemente equipados com sistemas de defesa antioxidante para prevenir o dano oxidativo imposto pelas doenças neurodegenerativas (HALLIWELL, 2006).

Em pacientes esquizofrênicos ocorre a desregulação do metabolismo de ERO e ERN, tal processo pode ser verificado através das análises das atividades anormais das enzimas antioxidantes, além de outros biomarcadores de estresse oxidativo como a peroxidação lipídica em plasma, glóbulos vermelhos, plaquetas e/ou líquido cefalorraquidiano (REDDY & YAO, 1996; YAO et al., 1998; DIETRICH-MUSZALKA, OLAS & RABE-JABLONSKA, 2005). Tais achados tem sido associados com discinesia tardia, sintomas negativos e sinais neurológicos (LI et al., 2006).

Estudos sugerem que o excesso de formação de ERO pode desempenhar um papel crucial na etiologia da esquizofrenia. A disfunção da membrana celular causada pela peroxidação lipídica pode ser secundária a uma patologia mediada por RL e pode contribuir

para a sintomatologia e complicações do tratamento. Estudos realizados por meio da avaliação da atividade da enzima antioxidante SOD em plaquetas evidenciaram a indução de estresse oxidativo em pacientes esquizofrênicos, sugerindo que a supressão da atividade da SOD em pacientes com esquizofrenia está associada com maior geração de ERO e da peroxidação lipídica **(**DIETRICH-MUSZALKA, OLAS & RABE-JABLONSKA, 2005).

Relatos na literatura mencionam que o nível de isoprostanos, outro indicador de estresse oxidativo, em pacientes com esquizofrenia em fase aguda de psicose é extremamente elevado, sendo que o aumento da produção de isoprostanos reflete o estresse oxidativo, bem como o dano oxidativo de lipídios (DIETRICH-MUSZALKA & OLAS, 2009).

A modificação de proteínas desempenha um papel essencial na patogênese da doença, além do mais, foram relatadas também alterações nos níveis da GSH, cisteína e cisteinilglicina, as quais são compostos *scavenger* fisiológicos de RL, os quais encontraram-se níveis diminuídos, ao passo que os níveis de homocisteína encontrados foram significativamente elevados no plasma de pacientes esquizofrênicos em fase aguda de psicose (DIETRICH-MUSZALKA et al., 2009; 2012).

Alguns dos neurolépticos podem alterar parâmetros de estresse oxidativo e função cognitiva do indivíduo, potencializando ainda mais os sintomas da doença. Além de induzir efeitos citotóxicos, foi demonstrado que ambos neurolépticos típicos e atípicos, podem alterar a função cognitiva em humanos e modelos animais (CLEGHORN et al., 1990). A atividade antioxidante diminuída é considerada como uma das causas da discinesia tardia em pacientes esquizofrênicos em curso com tratamento prolongado com neurolépticos. APG, como o haloperidol, são capazes de potencializar o estresse oxidativo, enquanto que os ASG, como a CZP, produzem menor dano oxidativo (AGOSTINHO et al., 2007).

Estudos realizados em ratos tratados com haloperidol e/ou CZP demonstraram que estes fármacos foram capazes de induzir dano oxidativo em diversas estruturas no SNC, verificado através de análises de peroxidação lipídica pelo nível de substâncias reativas ao ácido tiobarbitúrico (TBARS), carbonilação de proteínas, e atividade das enzimas antioxidantes CAT e SOD (POLYDORO et al., 2004; AGOSTINHO et al., 2007).

Achados similares foram relatados em estudo que avaliou o dano oxidativo, através do TBARS e da carbonilação de proteínas, no cérebro de ratos induzido pela administração crônica de haloperidol, CZP e/ou olanzapina. Onde, a CZP induziu dano oxidativo, embora menor que o haloperidol, não foi verificado dano oxidativo induzido por olanzapina. Sendo assim, o dano e efeitos adversos provocados pela CZP tem sido associado ao estresse oxidativo, tanto por formação de RL quanto pela inibição de defesas celulares enzimáticas e

não-enzimáticas (REINKE et al., 2004). Também foi demonstrado que a clozapina induz estresse oxidativo em neutrófilos, que pode desencadear agranulocitose (FEHSEL, 2005).

2.3 Clozapina

A CZP (Figura 1) é considerada o protótipo dos fármacos antipsicóticos atípicos, sendo o advento do fármaco o marco para uma nova classe de antipsicóticos, denominados de atípicos ou ASG, um derivado dibenzodiazepínico (FACTOR, 2002; FLEISCHHACKER, 2002; ABIDI & BHASKARA, 2003; GASZNER & MAKKOS, 2004).

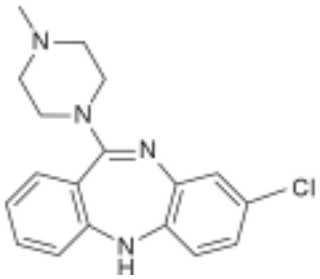

FIGURA 1 – Estrutura química da clozapina
Fonte: Adaptado de Sanders-Bush & Hazelwood, 2012, p.359

É o mais eficaz tratamento para a ER, devido ao seu perfil terapêutico distinto com menores efeitos secundários, sendo que suas principais indicações incluem o tratamento de pacientes que tem pouca ou nenhuma resposta terapêutica a outros antipsicóticos, ou seja, esquizofrenia não responsiva a fármacos antipsicóticos convencionais e de pacientes com intolerância a outros antipsicóticos (FACTOR, 2002; ABIDI & BHASKARA, 2003; IQBAL, 2005; TANDON, CARPENTER & DAVIS, 2007; MCILWAIN et al., 2011; KANE, 2012).

Os ASG, incluindo a CZP, induzem consideravelmente menor efeito extrapiramidal, numa faixa de dose terapêutica efetiva quando comparados aos APG. Sendo que a CZP foi o primeiro fármaco a apresentar efeitos extrapiramidais insignificantes (FLEISCHHACKER, 2002; NEWCOMER, 2005; DASKALAKIS & GEORGE, 2009; MCILWAIN et al., 2011).

Trata-se de um agente antipsicótico efetivo que não induz catalepsia e possui a capacidade de melhorar a discinesia tardia, além de apresentar um perfil terapêutico melhorado em relação aos APG, sendo eficaz sobre os sintomas positivos e negativos da ER, e tendo sua superioridade eficácia terapêutica reconhecida (GASZNER & MAKKOS, 2004; NEWCOMER, 2005; DASKALAKIS & GEORGE, 2009; MCILWAIN et al., 2011).

Relatos na literatura demonstram que a CZP é um fármaco que possui alto potencial oxidante, embora produza menor dano oxidativo quando comparada a outros fármacos (POLYDORO et al., 2004; REINKE et al., 2004; AGOSTINHO et al., 2007). A CZP é metabolizada no fígado, originando, predominantemente, dois metabólitos a N-desmetilclozapina e a clozapina-N óxido, os quais são reativos e podem danificar as células e tecidos (BUUR-RASMUSSEN & BROSEN, 1999).

No entanto, são os efeitos adversos da CZP, devido a sua baixa biodisponibilidade, que tem limitado seu uso clínico, além de possuir um prejudicial efeito sobre o perfil metabólico dos pacientes. A CZP é um fármaco lipofílico, por isso é rapidamente absorvida por via oral (JANN, 1991; MELTZER, 2004; NEWCOMER, 2005).

Das inúmeras complicações ocasionadas pela CZP, a mais devastadora, que este agente pode causar é a granulocitopenia, podendo chegar à sua forma mais grave, a agranulocitose (ALVIR, 1993; GASZNER, MAKKOS & KOSZA, 2002; DUTT et al., 2010; RAMÍREZ et al., 2011; MANU et al. , 2012; PONS et al., 2012; NUNES et al., 2013).

Esse efeito colateral é o problema mais significativo na utilização clínica da clozapina, pois a agranulocitose gera uma condição de risco de morte ao paciente, no qual o número de células brancas do sangue é fortemente reduzido, e a resistência a infecções pode ser severamente diminuída (BERGEMANN et al., 2007).

Em particular, a neutropenia e agranulocitose, são o foco de preocupação durante o tratamento com este antipsicótico, com uma incidência de agranulocitose de cerca de 1% e de neutropenia de cerca de 3% (ATKIN et al. 1996; LAMBERTENGHI, 2000; WULFF, 2007; MANU et al. , 2012; PONS et al., 2012).

O tratamento com CZP exige um estreito acompanhamento clínico e triagem hematológica programada obrigatória, tendo em vista que a grande maioria dos efeitos hematológicos induzidos pela CZP ocorre principalmente durante os primeiros três meses de terapia, com o maior risco nas primeiras 6 a 18 semanas de tratamento (ATKIN et al. 1996; GASZNER, MAKKOS & KOSZA, 2002).

É importante destacar entre os riscos associados ao fármaco estão as cardiomiopatias, que em casos mais extremos pode levar a morte súbita do indivíduo. A morte em decorrência de alguma disfunção cardiovascular é uma das principais causas de óbito em pacientes com esquizofrenia, sendo que o risco é duas vezes maior do que na população em geral (LEITÃO-AZEVEDO et al., 2007; RAMÍREZ et al., 2011; RONALDSON et al., 2011). Em graus variáveis, todos os antipsicóticos podem causar efeitos colaterais cardíacos. Estudos constaram alterações metabólicas nos portadores de esquizofrenia usuários de ASG com

prevalência de aproximadamente 80% de dislipidemia e 40% de glicemia alterada (LEITÃO-AZEVEDO et al., 2006; 2007).

A miocardite induzida por CZP está entre as patologias cardiovasculares que mais acometem indivíduos com ER (RAMÍREZ et al., 2011; RONALDSON et al., 2011; MANU et al., 2012). Relatos indicam que o uso da CZP está associado ao risco de miocardite em 1:500 a 1:10.000 dos pacientes tratados (WARNER et al., 2000; LA GRENADE, GRAHAM & TRONTELL, 2001). Se o diagnóstico for provável, a CZP deve ser suspensa imediatamente, e o paciente encaminhado urgentemente para um especialista (MARDER et al., 2004).

Outro efeito adverso é a hepatotoxicidade relacionada aos metabólitos da CZP, cerca de 40% dos pacientes apresentam alguma alteração nas transaminases, podendo causar insuficiência hepática em 0,06% dos pacientes (HUMMER et al., 1997; NEVES et al., 2006).

Tais riscos exigem um monitoramento laboratorial adequado, onde o acompanhamento clínico é pré-requisito, como medida de segurança, para o uso deste fármaco. O uso do medicamento deve ser interrompido imediatamente sempre que houver suspeita de complicações do quadro clínico (GASZNER, MAKKOS & KOSZA, 2002).

Em virtude das complicações graves associadas ao uso de CZP torna-se necessária a utilização de uma forma farmacêutica capaz de vetorizar esse fármaco para seu local de ação, o SNC, reduzindo seus efeitos adversos. Uma forma de alcançar esse objetivo seria por meio dos sistemas nanoparticulados, os quais são capazes não apenas de conferir proteção ao fármaco contra a degradação para que menos metabólitos reativos sejam gerados, bem como também na redução da toxicidade e ocorrência de efeitos adversos. Sendo considerada uma ferramenta útil para esse fim, as NC podem favorecer o aumento do uso clínico da CZP podendo beneficiar e melhorar a qualidade de vida de diversos pacientes (SOPPIMATH et al., 2001; HANS & LOWMAN, 2002; BERNARDI et al., 2009).

2.4 Sistemas carreadores de fármacos

Dentre os sistemas carreadores de fármacos, os sistemas nanoparticulados, compostos que apresentam diâmetro entre 10 e 1000 nm, tem despertado atenção devido às suas vantagens como vetores, destacando-se os lipossomas e as partículas poliméricas (COUVREUR, FATAL & ANDREMONT, 1991; SOPPIMATH et al., 2001).

As NP poliméricas tem atraído interesse como sistemas de entrega de fármacos, podendo ser utilizadas no transporte e liberação de fármacos de maneira controlada e efetiva em locais específicos, especialmente no SNC (RIEUX et al., 2006). A metodologia utilizada no preparo das NP poliméricas define o tipo da formulação, podendo obter-se NC ou nanoesferas, as quais diferem uma da outra de acordo com sua composição e organização estrutural (Figura 2) (COUVREUR, FATAL & ANDREMONT, 1991; SCHAFFAZICK et al., 2003).

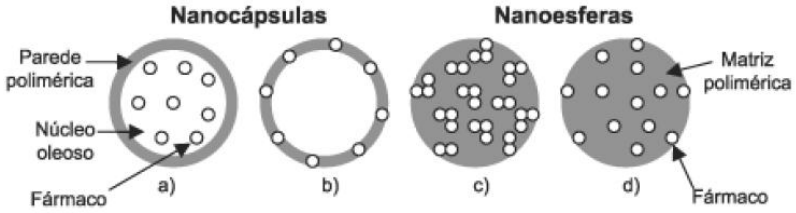

FIGURA 2 – Representação esquemática de nanocápsulas e nanoesferas poliméricas. a) fármaco dissolvido no núcleo oleoso das nanocápsulas; b) fármaco adsorvido à parede polimérica das nanocápsulas; c) fármaco retido na matriz polimérica das nanoesferas; d) fármaco adsorvido ou disperso molecularmente na matriz polimérica das nanoesferas.
Fonte: Schaffazick et al., 2003, p.726

As NC são constituídas por um invólucro polimérico disposto ao redor de um núcleo oleoso, podendo o fármaco estar dissolvido neste núcleo e/ou adsorvido à parede polimérica. Já as nanoesferas, não apresentam óleo em sua composição, são formadas por uma matriz polimérica, onde o fármaco pode ficar retido ou adsorvido (COUVREUR, FATAL & ANDREMONT, 1991; ALLÉMANN, GURNY & DOELKER, 1993).

A viabilidade da administração de fármacos, de ação no cérebro, utilizando NP poliméricas pode abrir novas perspectivas para o tratamento da esquizofrenia, principalmente pela possibilidade de atividade biológica em doses baixas. Estudo realizado com NP carregadas com o antipsicótico risperidona demonstraram que os nanossistemas foram capazes de prolongar o efeito do fármaco e de reduzir os efeitos extrapiramidais (MUTHU et al. 2009).

Estudos envolvendo NP de clozapina tem apresentado resultados promissores como boa biocompatibilidade, grande área de superfície, boa propriedade de dispersão e maior biodisponibilidade do fármaco lipofílico (VENKATESWARLU & MANJUNATH, 2004; MANJUNATH & VENKATESWARLU, 2005; MASHHADIZADEH & AFSHAR, 2013).

Diversos autores descrevem a enorme utilidade de sistemas nanoparticulados na terapêutica clínica de diversos fármacos, porém estudos relacionados à segurança desses

sistemas ainda são limitados e ainda devem ser estudados como o dano oxidativo. Algumas das propriedades desses sistemas que provaram benefícios terapêuticos, porém podem levar a acúmulos celulares e toxicidade em longo prazo (LANDSIEDEL et al., 2009; SINGH et al., 2009).

2.4.1 Nanocápsulas

As nanocápsulas estão sendo utilizadas como carreadores para diversos fármacos com diferentes objetivos terapêuticos visando à vetorização dos mesmos para a biofase, com consequente redução dos seus efeitos adversos (HANS & LOWMAN, 2002; CHAKRAVARTHI et al., 2010).

Estudos realizados com NC carregadas com haloperidol demonstraram que a nanoencapsulação em sistemas poliméricos é uma ferramenta terapêutica promissora. Os dados demonstraram a viabilidade da preparação das NC para melhorar a eficácia terapêutica do haloperidol. O efeito do antipsicótico nanoencapsulado foi mantido durante um maior tempo e os distúrbios motores extrapiramidais foram reduzidos quando comparado com a administração do fármaco livre, bem como induziu uma melhoria dos marcadores de estresse oxidativo (BENVEGNÚ et al. 2011; BENVEGNÚ et al. 2012).

As matérias-primas utilizadas nas formulações das NC são de extrema importância, pois influenciam no desempenho dos nanossistemas, devem ser considerados aspectos relacionados à toxicidade, via de administração, biocompatibilidade e biodegradabilidade dos polímeros.

2.4.2 Quitosana

Dentre as matérias-primas que podem ser utilizadas na preparação de NP está a QTS um polímero de origem natural (HANS & LOWMAN, 2002). A QTS é um polissacarídeo, unido por ligações β-1,4 entre os açúcares da sua cadeia, com alto grau de N-acetilação. Apresenta como vantagem, propriedades biológicas dentre elas biocompatibilidade, biodegradabilidade e bioadesividade. Possui a capacidade de formação de gel o que a elevada

capacidade de adsorção e a biodegradabilidade (FELT, BURI & GURNY, 1998; DASH et al., 2011).

Apresenta cargas positivas, facilitando a interação com componentes de carga negativa das membranas celulares, auxiliando a permeação do fármaco (HANS & LOWMAN, 2002). Outra propriedade muito importante é a sua capacidade de se ligar a moléculas aniônicas, tais como fatores de crescimento, glicanos e DNA (DASH et al., 2011).

Tem sido documentado que NP de QTS têm eficiente encapsulação e liberação controlada de fármacos. A QTS tem mostrado melhorar a velocidade de dissolução de drogas fracamente solúveis, e, portanto, pode ser explorada para a melhoria da biodisponibilidade de fármacos. Vários agentes terapêuticos, tais como quimioterápicos, anti-inflamatórios, antibióticos, anti-trombótico, esteroides, proteínas, aminoácidos e diuréticos tem sido incorporados em sistemas à base de QTS (DASH et al., 2011). Estudo com NP de CZP revestidos com QTS e polissorbato 80 apresentaram melhoria da hidrofilidade de superfície, facilitando a permeação do fármaco (ISHAK et al., 2013).

2.4.3 Polietilenoglicol

O PEG é o polímero mais comumente utilizado com a finalidade de modificação da superfície, hidrofílico, não-iônico e biocompatível (HANS & LOWMAN, 2002).

Sistemas nanoparticulados de longa circulação possuem a sua superfície modificada com PEG, pois esse polímero é capaz de evitar o reconhecimento pelos anticorpos aumentando o tempo na circulação sistêmica. São capazes de diminuir a captação de fármacos por órgãos e tecidos pertencentes ao sistema retículo endotelial, como a medula óssea e o fígado, potencialmente relacionados com a toxicidade da clozapina (MOSQUEIRA et al., 2001; HANS & LOWMAN, 2002).

Autores demonstraram que este revestimento reduz a captação hepática de NP, simultaneamente, aumentando sua permanência na circulação (GREF et al.,1994; BAZILE et al., 1995; CALVO et al., 2001). A engenharia de polímeros de superfície reduz a agregação de NP e a fagocitose devido à blindagem eficaz do revestimento PEG, dificultando o reconhecimento pelo sistema retículo endotelial (ISHAK et al., 2013). Devido à conformação das cadeias de PEG a adsorção de proteínas a superfície das NP é diminuída, com consequente redução de processos fagocitários (SOPPIMATH et al., 2001).

3 OBJETIVOS

3.1 Objetivo geral

Verificar o efeito do tratamento com nanossistemas contendo clozapina sobre parâmetros de estresse oxidativo em ratos Wistar.

3.2 Objetivos específicos

- Realizar a análise hematológica e contagem de plaquetas após o tratamento com os nanossistemas contendo clozapina;
- Quantificar os marcadores bioquímicos para funções cardíaca (CK, CK-MB e homocisteína), hepática (TGO e TGP) e renal (ureia e creatinina);
- Realizar a análise histopatológica dos órgãos: coração, fígado e rim;
- Determinar o dano oxidativo lipídico em plasma;
- Determinar o dano oxidativo em proteínas plasmáticas;
- Determinar o dano oxidativo no material genético (DNA) em sangue total;
- Determinar a atividade das enzimas antioxidantes CAT, SOD e GPx em eritrócitos;
- Determinar os níveis de GSH em eritrócitos;
- Determinar o dano oxidativo em biomoléculas (lipídios, proteínas e DNA) no homogenato do cérebro de ratos Wistar após o tratamento com os nanossistemas contendo clozapina.

PARTE II

MANUSCRITO I

Clozapine linked to nanosystems improves hematological, biochemical and histopathological parameters in Wistar rats

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Clozapine linked to nanosystems improves hematological, biochemical and histopathological parameters in Wistar rats

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Abstract

Clozapine is an atypical antipsychotic used effectively in refractory schizophrenia . However , it has some serious side effects, such as agranulocytosis, cardiomyopathy and sudden death in some cases, limiting their use. The nanosystems has attracted the attention as pharmaceutical form able to vectorize the drug directly to target tissue minimizing undesirable effects. This study aimed to evaluate hematological, biochemical and histopathological profile of different nanosystems containing clozapine inWistar rats. The study consisted of eight groups of male Wistar rats (n = 6), animals received the following treatments: saline solution (SAL) (NaCl 0.9% 1.0 mL/Kg i.p.), free clozapine (CZP) (25 mg/Kg i.p.), blank uncoated nanocápsulas (BNC) (1.0 mL/Kg i.p.), clozapine-loaded uncoated nanocapsules (CNC) (25 mg/Kg i.p.), blank chitosan-coated nanocapsules (BCSN) (1.0 mL/Kg i.p.), clozapine-loaded chitosan-coated nanocapsules (CCSN) (25 mg/Kg i.p.), blank polyethyleneglycol-coated nanocapsules (BPEGN) (1.0 mL/Kg i.p.), clozapine-loaded polyethyleneglycol-coated nanocapsules (CPEGN) (25 mg/Kg i.p.). The animals received the formulation once a day for seven consecutive days, being euthanized in the eighth day. It was global blood for hematologic and biochemical analyzes and organs heart, liver and kidney for histopathological analysis. The hematologic analysis revealed a significant reduction ($p < 0.05$) of parameters evaluated induced by CZP, while the groups treated with nanosystems equate SAL group. However, in CCN group became evident a significant increase in global count of leukocytes, featuring an inflammatory process. The evaluation of markers cardiac, hepatic and renal function revealed a significant increase of parameters evaluated induced by CZP, groups treated with nanosystems showed significant improvement these markers. Histopathological evaluation revealed that CZP group is able induce tissue damage, nanosystems a less damage was observed. The findings show that different coatings can act in diverse and specific way. These results indicate that the drug when linked to different nanosystems is able to mitigate the harmful effects of drug.

Keywords: refractory schizophrenia, clozapine, nanosystems, hematological parameters, biochemical parameters, histopathology.

1 Introduction

Clozapine is an effective atypical antipsychotic used, particularly, in the treatment of patients with refractory schizophrenia to other neuroleptics. Clozapine is therapeutically effective, in both positive and negative symptoms. Unlike other neuroleptics, no produces significant extrapyramidal side effects (JANN, 1991; JANN et al., 1993; SILVA, et al., 2001; LOUS et al., 2003; ELKIS & MELTZER, 2007; MCILWAIN et al., 2011).

As most drugs administered orally, clozapine is absorbed into systemic circulation by porta system and undergoes firs-pass metabolism, thus having low oral availability (SWARTZ, 2001). Metabolized in liver, yields two metabolites, N-desmethylclozapine and clozapine-N-oxide, which are reactive and can cause cellular damage (BUUR-RASMUSSEN & BROSEN, 1999).

Reports in literature show that typicals antipsychotics potentiate oxidative stress, and, although atypicals antipsychotics produce less damage, still have high potential oxidant, such as clozapine (AGOSTINHO et al., 2007). Some studies also reported that chronic exposure to clozapine resulted in significant changes in the activity of antioxidant enzymes, and produce oxidative damage in brain rats (POLYDORO et al., 2004; REINKE et al., 2004).

Clozapine has limited clinical use owing their potential adverse effects, which are associated, mainly, their reactive metabolites. Among the most serious complications are granulocytopenia and agranulocytosis. Being that most part hematological complications clozapine-induced occurs, mainly, during the first three months of therapy, making it necessary accompaniment of patients with frequent monitoring hematologic (BECHELLI & CAETANO, 1992; GASZNER, MAKKOS & KOSZA, 2002; DUTT et al., 2010; RAMÍREZ et al., 2011; MANU et al. , 2012). Another risk associated with clozapine are already established cardiotoxic effects, such as tachycardia, arrhythmia, hypertension, cardiomyopathies, as well as myocarditis may lead to sudden death if not diagnosed quickly (MERRILL; WILLIAM & GOFF, 2005; MERRILL et al., 2006; WEHMEIER; HEISER & REMSCHMIDT, 2005; RAMÍREZ et al., 2011; MANU et al. , 2012). Different hypotheses are considered, however is proposed that myocarditis can be caused by hypersensitivity reactions reflected in histopathological finding presence of cellular infiltrate characterized by eosinophils (MERRILL; WILLIAM & GOFF, 2005). Cardiovascular mortality is a major cause of death in patients with schizophrenia (MERRILL et al., 2006; RAMÍREZ et al., 2011). Hepatotoxicity also is a risk associated with the drug, studies have reported alterations

in transaminases levels, besides can cause liver failure (HUMMER et al., 1997; MACFARLANE et al.,1997).

The pharmacotherapeutic profile of this drug makes essential research to increase its spectrum of use, therefore, to improve their therapeutic application of nanoparticulate systems can be an alternative to circumvent the limitations this drug (JANN et al., 1993). Advances in nanotechnology over the past three decades have had significant impact on clinical diagnosis and therapy (SALATA, 2004). Among the various drug delivery systems nanoparticles, polymeric nanoparticles have received great attention owing stability (VAUTHIER et al., 2003). In addition, small size combined with the use of suitable polymers can offer numerous benefits to nanocoated drugs, some which include vectorization of active drug to target tissue and/or cell, improved oral bioavailability, controlled release in target tissue, solubilization for intravascular delivery and protection against enzymatic degradation, especially to stomach acids (HAIXIONG et al., 2002).

Among the raw materials which can be used in the preparation of nanoparticles is chitosan (CS) wtih features as bioadhesiveness and biocompatibility, and facilitate interaction with components of cellular membranes owing their positive charge which help permeation of drug. Another raw material is polyethylene (PEG) polymer most commonly used for the purpose of modifying the surface, hydrophilic, biocompatible and non-ionic (HANS & LOWMAN, 2002).

However, the aim this study was to evaluate the hematological, biochemical and histopathological profile of different nanosystems containing clozapine in Wistar rats.

2 Material e methods

2.1 Materials and reagents

All chemicals were of analytical grade. All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Kits for analysis of hematological profile and biochemical markers of cardiac, hepatic and renal function (Labtest, Lagoa Santa - Minas Gerais, Brazil).

2.2 Experimental animals

This study was approved by the Ethics Committee on Animal Use (CEUA), Federal University of Pampa (UNIPAMPA) under Protocol nº. 034/2012, which is affiliated to the Brazilian College of Animal Experimentation (COBEA). Since the experiments were conducted in accordance with the ethical and technical principles of animal experimentation established by the National Council for the Control of Animal Experimentation (CONCEA) and Law nº. 11.794 of 08 October 2008 which establishes procedures for the scientific use of animals (BRASIL, 2008; 2013).

Were used 48 adult Wistar rats, weighing about 250g, coming from the Bioterio of Federal University of Santa Maria. The animals remained in the Bioterio of Federal University of Pampa, Campus Uruguaiana, under standard environmental conditions, maintained in cabinets with dark/light cycle of 12 hours. They were fed *ad libitum* diet, this being appropriate in quantity and quality to maintain their health, with free access to drink water, *ad libitum*.

The animals were divided into eight experimental groups consisting of 6 animals each. The groups were treated as follows: saline solution NaCl 0.9% 1.0 mL/Kg (SAL), free clozapina 25 mg/Kg (CZP), blank uncoated nanocapsules 1.0 mL/Kg (BNC), clozapine-loaded uncoated nanocapsules 25 mg/Kg (CNC), blank chitosan-coated nanocapsules 1.0 mL/Kg (BCSN), clozapine-loaded chitosan-coated nanocapsules 25 mg/Kg (CCSN), blank polyethyleneglycol-coated nanocapsules 1.0 mL/Kg (BPEGN), clozapina-loaded polyethyleneglycol-coated nanocapsules 25 mg/Kg (CPEGN). The animals received the formulation once a day for seven consecutive days and euthanized in the eighth day.

The dose of administration of suspensions containing clozapine used was 25 mg/Kg in a volume of 1.0 mL/Kg and route of administration was intraperitoneal (CANADIAN COUNCIL ANIMAL CARE, 1993; POLYDORO et al., 2004; REINKE et al., 2004). The animals receive the formulations once a day, always at the same time, during seven consecutive days. In the eighth day, the rats were euthanized, the blood was collected and processed for evaluation of hematological profile and markers cardiac, hepatic and renal function. The organs were also collected: heart, liver and kidney for histopathological evaluation.

2.3 Preparation of the suspensions of nanocapsules

The nanocapsules (NC) were prepared using the interfacial precipitation of the preformed polymer method. The organic phase was constituted with poli(ε-caprolactone), TCM, Lipoid S45® and CZP dissolved in acetone kept under heating and stirring. This phase was poured in aqueous phase with polysorbate 80. After the formation of suspension of NC, the acetone and part of the water are evaporated (1.5 mg/mL of CZP). To obtain the formulation covered with PEG, this was added to the aqueous phase of the suspension. For the covering with CS, aqueous acid solution of polysaccharide was added to the NC solution and kept under constant stirring for a period an hour. Unloaded NC were prepared (BNC).

2.4 Physico-chemical characterization of nanocapsules

The formulations were characterized by the diameter, specific surface area (SPAN) (Mastersizer, Malvern), Zeta potential (Zetasizer, Malvern), pH, drug content and encapsulation efficiency (EE) (HPLC-PDA) (BIENIEK et al., 2011).

2.5 Hematological parameters

Blood samples were collected in tubes containing EDTA (Vacutainer - Becton, Dickinson and Company - New Jersey - USA), and analyzed immediately after collection. The hemograms, complete blood count (CBC) were performed in an automatic counter Cell-Dyn 3200 Hematology Analyzer (Abbott Diagnostics, Santa Clara, CA, USA). The evaluation included an analysis of several hematological parameters such as total count of red blood cells (RBC), hemoglobin (Hb), absolute erythrocyte indices the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), RDW, hematocrit (Ht), total white cell count (WBC), differential leukocyte count, and platelet count and mean platelet volume (MPV). Blood smears were prepared and stained by Kit Panoptic fast (Renylab Chemicals & Pharmaceuticals, Barbacena - Minas Gerais, Brazil).

2.6 Markers function

Blood samples were collected in tubes containing gel without anticoagulant (Vacutainer - Becton, Dickinson and Company - New Jersey - USA), the samples remained at

room temperature for 30 min to coagulate. Serum was collected after centrifugation at 1500 rpm for 10 min and analyzed immediately.

2.6.1 Markers cardiac function

2.6.1.1 CK and CK-MB analysis

Were evaluated markers of cardiac function creatine kinase (CK) and isoenzyme creatine kinase (CK-MB) cardiac muscle. The CK and CK-MB analysis were performed using A25 Biosystems automated analyzer (SA Biosystems, Barcelona, Spain). All assays were performed in triplicate.

2.6.1.2 Determination of homocysteine levels

The measurement of plasma homocysteine was performed by high efficiency liquid chromatography coupled to mass spectrometry (LC-MS/MS). The equipment used was a Waters liquid chromatograph Model 2690 and a mass spectrometer Micromass Four. The mobile phase used contained acetronitrila/formic acid and the flow rate was used 0.7 mL/min. Briefly, plasma was mixed with 20μL of internal standard homocystine-d8 (2 nmols) in an eppendorf tube. Then were added 20μL of reducing solution (dithiothreitol 500 mM) , leaving to stand for 15 minutes at room temperature. Two hundred microliters of desproteinizante solution (1 mL of formic acid and 0.5 mL of trifluoroacetic acid in 1L of acetonitrile) were added and the sample was centrifuged at 3000 rpm for 2 minutes at room temperature. One hundred microliters of the supernatant was transferred to a vial for injection and finally, 1μL of the sample was injected into LC-MS/MS using an autosampler maintained at 4°C for the detection and quantification of plasma homocysteine . The peak transition of homocysteine and homocysteine-d8 occur respectively at m/z 136 to m/z 90 and m/z 140 to m/z 94. A calibration curve was prepared in parallel from a solution of D,L- homocysteine 200μM, obtaining the following concentrations : 2.5, 5.0, 10.0 , 25.0, 50.0 and 100.0 μM, to allow measurement of homocysteine in the sample (NELSON et al. 2003). All assays were performed in triplicate.

2.6.2 Markers hepatic function: GOT and GPT analysis

Markers of liver function Glutamic Oxaloacetic Transaminase (GOT) and Glutamic Pyruvic Transaminase (GPT) were evaluated. Analyses were performed using automated analyzer A25 Biosystems (Biosystems SA, Barcelona, Spain). All assays were performed in triplicate.

2.6.3 Markers renal function: urea and creatinine analysis

Markers urea and creatinine renal function were evaluated. Analyses were performed using automated analyzer A25 Biosystems (Biosystems SA, Barcelona, Spain). All assays were performed in triplicate.

2.7 Histopathological analysis

After seven days of treatment, the animals were euthanized and the thoracic abdominal cavity was opened. The heart, liver and kidney were excised each animal. The organs were rapidly dissected and tissue sections (5 mm) fixed by immersion at room temperature in 10% formalin solution. For the histological examinations, paraffin embedded tissue sections of aorta were stained with hematoxylin-eosin (H&E). The tissue samples were for examined and photographed under a light microscope for observation of structural abnormality.

2.8 Statistical analysis

Data were expressed as mean ± standard deviation (SD). Comparisons between groups were performed using a two-way analysis of variance (ANOVA), followed by post hoc of Bonferroni for multiple comparison tests. Results were considered statistically significant when $p<0.05$. The statistical analysis was performed using the software GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

3 Results

The formulations containing CZP showed a pH higher than the formulations without drug. BNC and CNC group showed a similar diameter of 139 ± 1 and 137 ± 2 nm

respectively. BPEGN and CPEGN group showed different values, 140 ± 1 and 142 ± 1 nm, respectively, the same was observed to BNCS and CCSN group, 135 ± 2 and 141 ± 1, respectively. All formulation showed SPAN values inferior to 1.5. The zeta potential was negative for all formulation, except those covered with CS. CNC and CPEGN group presented drug content near 100% and the EE was above 95%, but around 70% for CCSN (Table 1).

TABLE 1

Values (mean ± SD) of particle mean diameter (D[4,3], nm), SPAM, zeta potential (mV) and pH of clozapine nanoformulations (n = 3 batches)

Formulation	D[4,3] Nm	SPAN ± DP	Zeta potential (mV)	pH ± DP
BNC	139±1	1.2 ± 0.2	-33.2 ± 0.5	5.5 ± 0.01
CNC	137 ± 2	1.3 ± 0.1	-20.7 ±1.6 [a]	7.1 ± 0.01 [a]
BCSN	135 ± 2	1,1 ± 0.1	7.2 ±1.5	4.3 ± 0.005
CCSN	141 ± 1[b]	1.4 ± 0.001	29 ± 0.8[b]	4.5 ± 0.005 [b]
BPEGN	140 ± 1	1.3 ± 0.1	-22 ± 0.6	5.2 ± 0.005
CPEGN	142 ± 1	1,2 ± 0.1	-10.6 ± 1.3[c]	6.9 ± 0.005 [c]

[a] Difference between BNC x CNC ($p < 0.05$)
[b] Difference between BCSN x CCSN ($p < 0.05$)
[c] Difference between BPEGN x CPEGN ($p < 0.05$)

The results of several hematological parameters evaluated are shown in figures following (Figure 1, Figure 2 and Figure 3).

The findings demonstrated that CZP group had significantly decreased values ($p < 0.05$) compared to SAL group for RBC, as well as Hb and Ht indicates the cytotoxic potential of drug (Figure 1A, 1B and 1C, respectively). The groups treated with nanossystems got better and significant results for RBC and Hb compared to CZP group. The nanocapsules coated with CS and PEG were statistically different of uncoated nanocapsules, obtaining better results for RBC. In Hb parameter coating with PEG got better performance. However, the groups that received nanocapsules showed a significant decrease in Ht. Figure 1D shows the MCV values for all treated groups were significantly different compared to SAL group, but was only BPEGN was statistically different of CZP group. Figure 1E shows values for MCH, as BNC as CPEGN and nanocapsules coated with CS were significantly different of SAL group. All treated groups nanosystems were statistically different of CZP group. Figure 1F shows values for MCHC as BNC as BCSN and BPEGN differed significantly of SAL and CZP group, already CPEGN group differ only CZP group. Finally, in Figure 1G are values for

RDW where BCSN group was statistically diferent of SAL and CZP group, and BPEGN group different only CZP group.

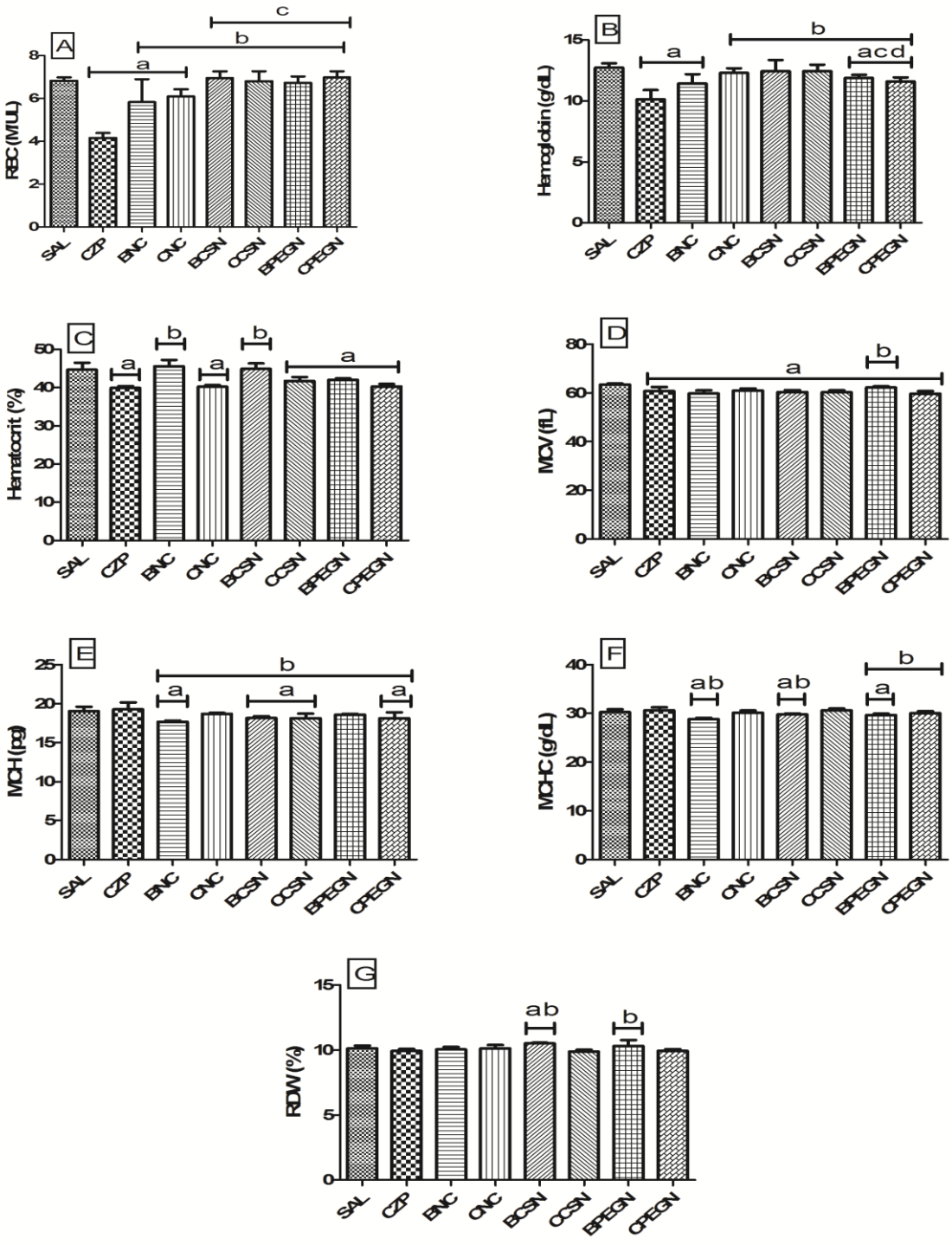

FIGURE 1 - Hematological parameters of red blood cells in Wistar rats exposed to different nanosystems treatments. In A: values for RBC; B: values for Hb; C: values for Ht; D: values for MCV; E: values for MCH; F: values for MCHC; G: values for RDW. SAL: saline solution; CZP: clozapine free; BNC: blank uncoated nanocapsules; CNC: clozapine-loaded uncoated nanocapsules; BCSN: blank chitosan-coated nanocapsules; CCSN: clozapine-loaded chitosan-coated nanocapsules; BPEGN: blank polyethyleneglycol-coated nanocapsules; CPEGN: clozapina-loaded polyethyleneglycol-coated nanocapsules. [a] Significantly different SAL ($p < 0.05$); [b] Significantly different CZP ($p < 0.05$); [c] Significantly different BNC and CNC ($p < 0.05$); [d] Significantly different BCSN and CCSN ($p < 0.05$).

The values for WBC and differential leukocyte counts are shown in Figure 2. CZP group had parameters significantly decreased ($p < 0.05$) for WBC, as well as neutrophilic, monocytic and lymphocytic series, however a significant increase was observed for eosinophilic serie (Figure 2A, Figure 2B, Figure 2C and Figure 2D, respectively). All groups treated with nanosystems got better and significant results when compared with CZP group for WBC, the nanocapsules coated CS and PEG were statistically different of uncoated nanocapsules getting better results, coat with CS was the best performance (Figure 2A). All groups treated with nanosystems were significantly different when compared to CZP group for differential count of neutrophils, the nanocapsules coated with PEG had a significant increased of neutrophilic serie and significantly different of the others and nanocapsules (Figure 2B). All treated groups, except BPEGN group, had significantly decreased values lymphocytic serie when compared with SAL group. Except CCSN group, all groups treated with nanosystems had better results than CZP group to lymphocytic serie (Figure 2C). The group that received CS-coated nanocapsules showed an increase in monocytic and basophilic series significantly higher than SAL and CZP groups, as well as differed significantly of the other nanocapsules (Figure 2D and Figure 2F). All groups were treated with nanosystems were significantly different when compared with SAL and CZP groups in eosinophilic series, CPEGN group showed an increase in eosinophilic series significantly higher compared to the other groups (Figure 2E).

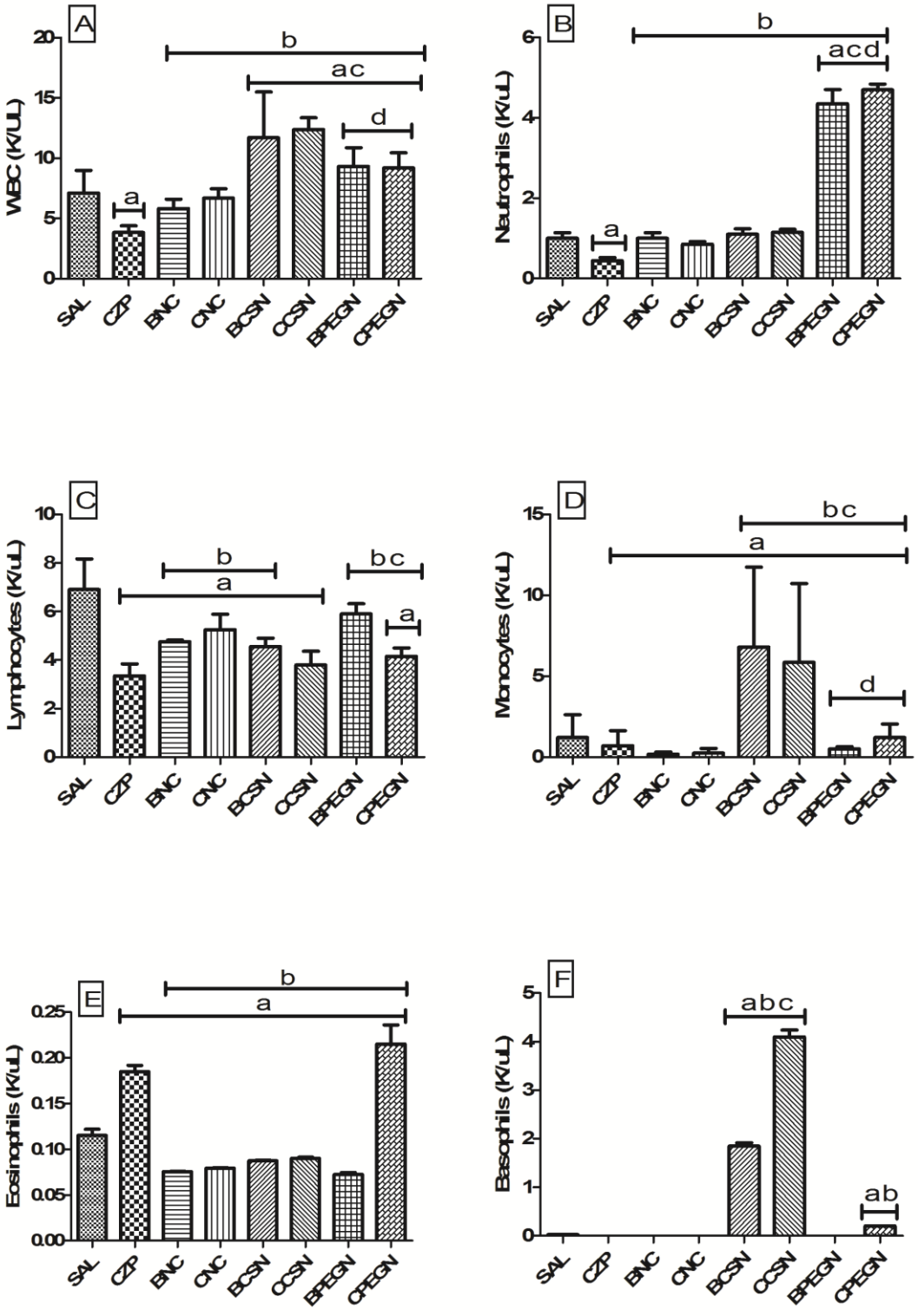

FIGURE 2 - Hematological parameters of white blood cells in Wistar rats exposed to different nanosystems treatments. In A: values for WBC; B: values for Neutrophils; C: values for Lymphocytes; D: values for Monocytes; E: values for Eosinophils; F: values for Basophils. SAL: saline solution; CZP: clozapine free; BNC: blank uncoated nanocapsules; CNC: clozapine-loaded uncoated nanocapsules; BCSN: blank chitosan-coated nanocapsules; CCSN: clozapine-loaded chitosan-coated nanocapsules; BPEGN: blank polyethyleneglycol-coated nanocapsules; CPEGN: clozapina-loaded polyethyleneglycol-coated nanocapsules. [a] Significantly different SAL ($p < 0.05$); [b] Significantly different CZP ($p < 0.05$); [c] Significantly different BNC and CNC ($p < 0.05$); [d] Significantly different BCSN and CCSN ($p < 0.05$).

The global parameters MPV and platelet count are shown in Figure 3. All treatment groups were significantly different when compared to SAL group. The uncoated nanocápsulas, coated with CS and BPEGN groups were significantly different when compared to CZP group (Figure 3A). BNC and CCSN group were significantly different SAL and CZP groups. BCSN group showed differences only CZP group. Nanocapsules coated with CS were different of the other nanosystems (Figure 3B).

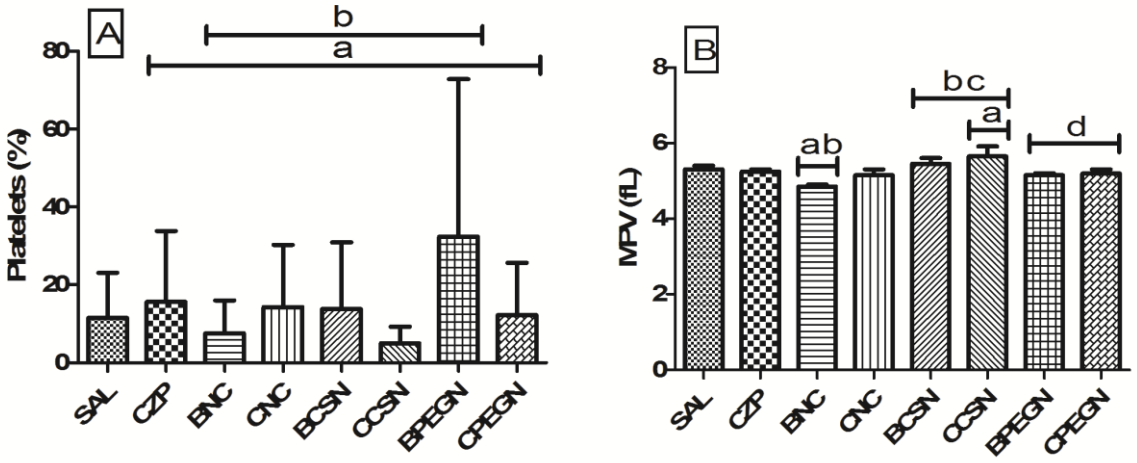

FIGURE 3 - Hematological parameters of plaquets in Wistar rats exposed to different nanosystems treatments. In A: values for Plaquets; B: values for MPV. SAL: saline solution; CZP: clozapine free; BNC: blank uncoated nanocapsules; CNC: clozapine-loaded uncoated nanocapsules; BCSN: blank chitosan-coated nanocapsules; CCSN: clozapine-loaded chitosan-coated nanocapsules; BPEGN: blank polyethyleneglycol-coated nanocapsules; CPEGN: clozapina-loaded polyethyleneglycol-coated nanocapsules. [a] Significantly different SAL ($p < 0.05$); [b] Significantly different CZP ($p < 0.05$); [c] Significantly different BNC and CNC ($p < 0.05$); [d] Significantly different BCSN and CCSN ($p < 0.05$).

The results for CK, CK-MB and homocysteine analysis are shown in Figure 4. The result for CK showed that there was a significant increase ($p < 0.05$) in all treated groups when compared with SAL group, however all groups treated with nanosystems showed a significant improvement when compared CZP group, being nanocapsules with CS-coated the best performance (Figure 4A). CK-MB there was a significant increase induced by CZP group, the groups treated with different nanosystems showed a significant improvement in the CK-MB, and groups treated with nanocapsules uncoated and CS-coated the best results (Figure 4B). The results for homocysteine there was a significant increase induced by CZP group, however when linked to the nanosystems there was a significant decrease in these levels, again the group treated with CS-coated nanocapsules had better results (Figure 4C).

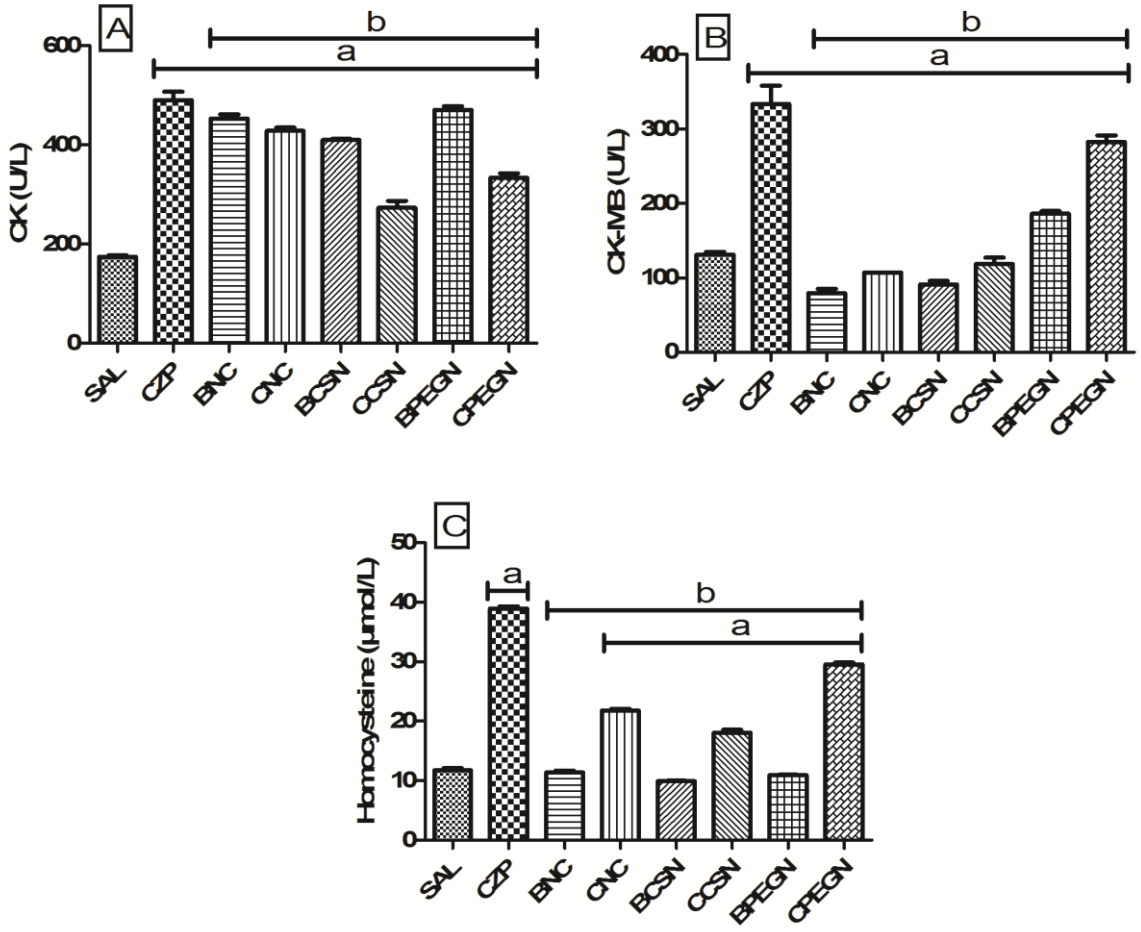

FIGURE 4 – Markers cardiac function in Wistar rats exposed to different nanosystems treatments. In A: values for CK; B: values for CK-MB; C: values for Homocysteine. SAL: saline solution; CZP: clozapine free; BNC: blank uncoated nanocapsules; CNC: clozapine-loaded uncoated nanocapsules; BCSN: blank chitosan-coated nanocapsules; CCSN: clozapine-loaded chitosan-coated nanocapsules; BPEGN: blank polyethyleneglycol-coated nanocapsules; CPEGN: clozapina-loaded polyethyleneglycol-coated nanocapsules. [a] Significantly different SAL ($p < 0.05$); [b] Significantly different CZP ($p < 0.05$).

The results for GOT and GPT are showed in Figure 5. The result for GOT demonstrate that there was a significant increase ($p < 0.05$) of the parameters evaluated induced by CZP group, as well as for GPT (Figure 5A, Figure 5B, respectively). All groups treated with nanossystems showed a significant improvement in markers liver function in relation to CZP group. The group treated with CS-coated nanocapsules obtained better results.

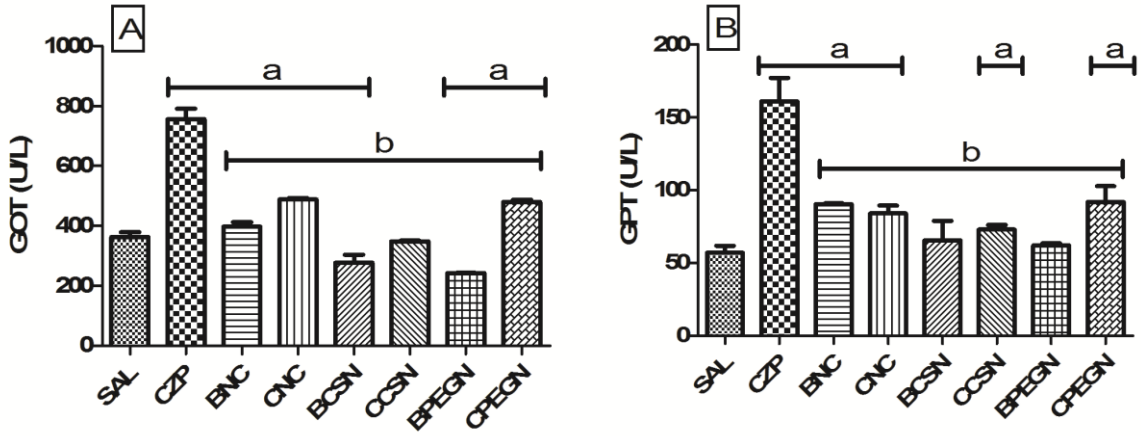

FIGURE 5 – Markers hepatic function in Wistar rats exposed to different nanosystems treatments. In A: values for GOT; B: values for GPT. SAL: saline solution; CZP: clozapine free; BNC: blank uncoated nanocapsules; CNC: clozapine-loaded uncoated nanocapsules; BCSN: blank chitosan-coated nanocapsules; CCSN: clozapine-loaded chitosan-coated nanocapsules; BPEGN: blank polyethyleneglycol-coated nanocapsules; CPEGN: clozapina-loaded polyethyleneglycol-coated nanocapsules. [a] Significantly different SAL ($p < 0.05$); [b] Significantly different CZP ($p < 0.05$).

The results for the analysis of urea and creatinine are showed in Figure 6. The results of treated groups to urea is present similar to SAL and CZP group suggesting that nanoparticles systems are not nephrotoxic, with the exception of the groups treated with nano-coated with CS (Figure 6A). For creatinine results were significantly decreased compared to SAL group, as well as groups receiving uncoated nanocápsulas, coated with PEG and CCNS were significantly decreased compared CZP group (Figure 6B).

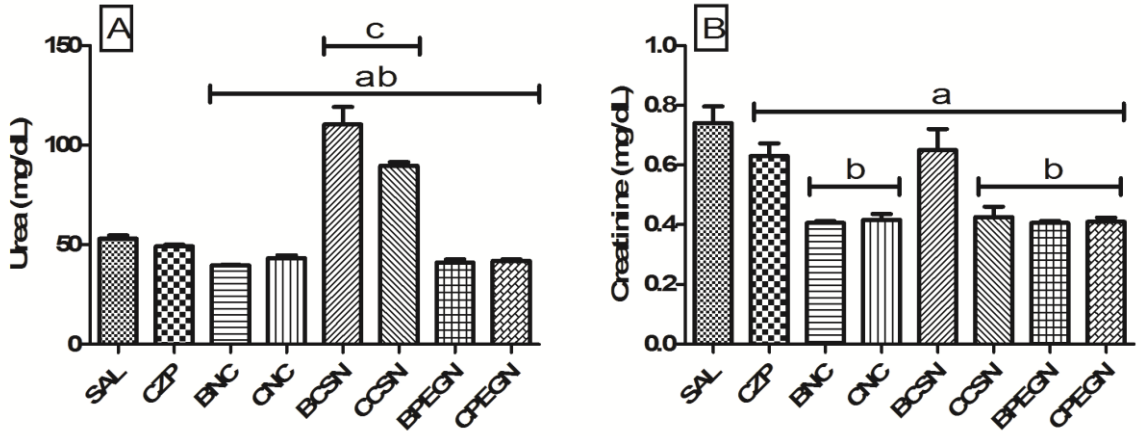

FIGURE 6 – Markers renal function in Wistar rats exposed to different nanosystems treatments. In A: values for Urea; B: values for Creatinine. SAL: saline solution; CZP: clozapine free; BNC: blank uncoated nanocapsules; CNC: clozapine-loaded uncoated nanocapsules; BCSN: blank chitosan-coated nanocapsules; CCSN: clozapine-loaded chitosan-coated nanocapsules; BPEGN: blank polyethyleneglycol-coated nanocapsules; CPEGN: clozapina-loaded polyethyleneglycol-coated nanocapsules. [a] Significantly different SAL ($p < 0.05$); [b] Significantly different CZP ($p < 0.05$).

In Figure 7 are showed images of histopathological findings of heart, the histological evaluation revealed that the group receiving CZP had significant changes in organ, as heart congestion and relaxation of cells cardiac.

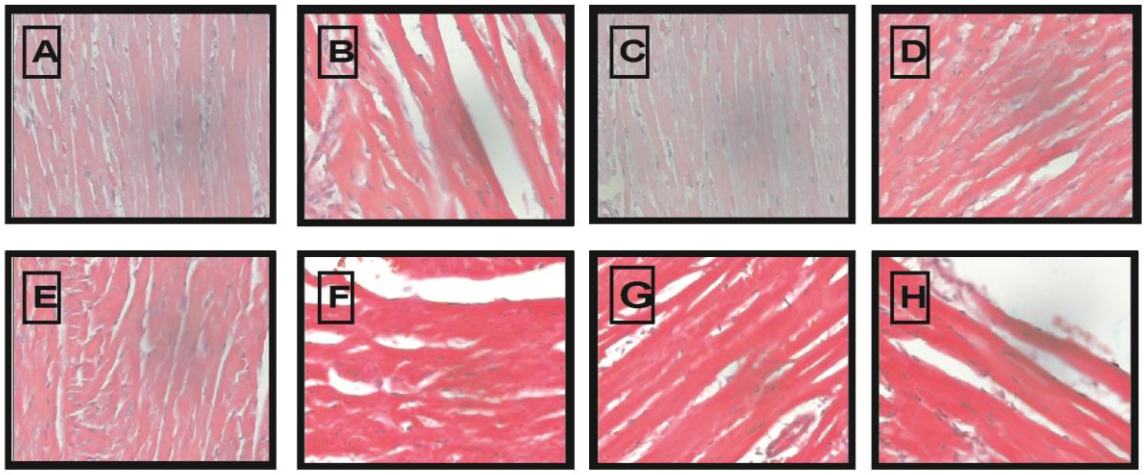

FIGURE 7 – Histopathological analysis of heart in Wistar rats exposed to different nanosystems treatments. In A: SAL group; B: CZP group; C: BNC group; D: CNC group; E: BCSN group; F: CCSN group; G: BPEGN group; H: CPEGN group. SAL: saline solution; CZP: clozapine free; BNC: blank uncoated nanocapsules; CNC: clozapine-loaded uncoated nanocapsules; BCSN: blank chitosan-coated nanocapsules; CCSN: clozapine-loaded chitosan-coated nanocapsules; BPEGN: blank polyethyleneglycol-coated nanocapsules; CPEGN: clozapina-loaded polyethyleneglycol-coated nanocapsules.

In Figure 8 are showed images of histopathological findings of the liver. The histological evaluation revealed that the group receiving CZP had significant changes in organ, as liver necrosis.

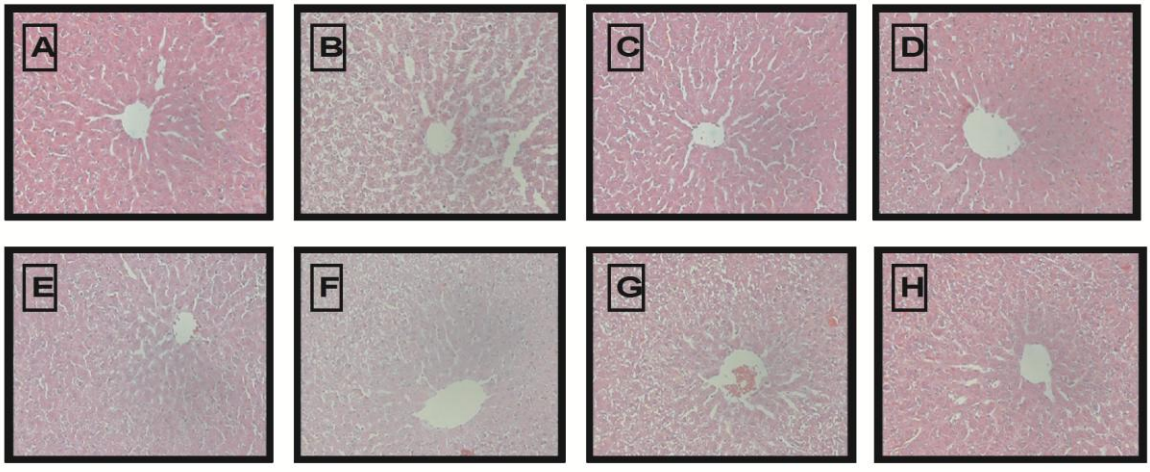

FIGURE 8 – Histopathological analysis of liver in Wistar rats exposed to different nanosystems treatments. In A: SAL group; B: CZP group; C: BNC group; D: CNC group; E: BCSN group; F: CCSN group; G: BPEGN group; H: CPEGN group. SAL: saline solution; CZP: clozapine free; BNC: blank uncoated nanocapsules; CNC: clozapine-loaded uncoated nanocapsules; BCSN: blank chitosan-coated nanocapsules; CCSN: clozapine-loaded chitosan-coated nanocapsules; BPEGN: blank polyethyleneglycol-coated nanocapsules; CPEGN: clozapina-loaded polyethyleneglycol-coated nanocapsules.

In Figure 9 are showed images of the histopathologic findings of kidney. The histological revealed that the group which had received CZP significant changes in organ, as an increased cell volume, tubular degeneration, increasing the scapular area, necrosis and glomerular death.

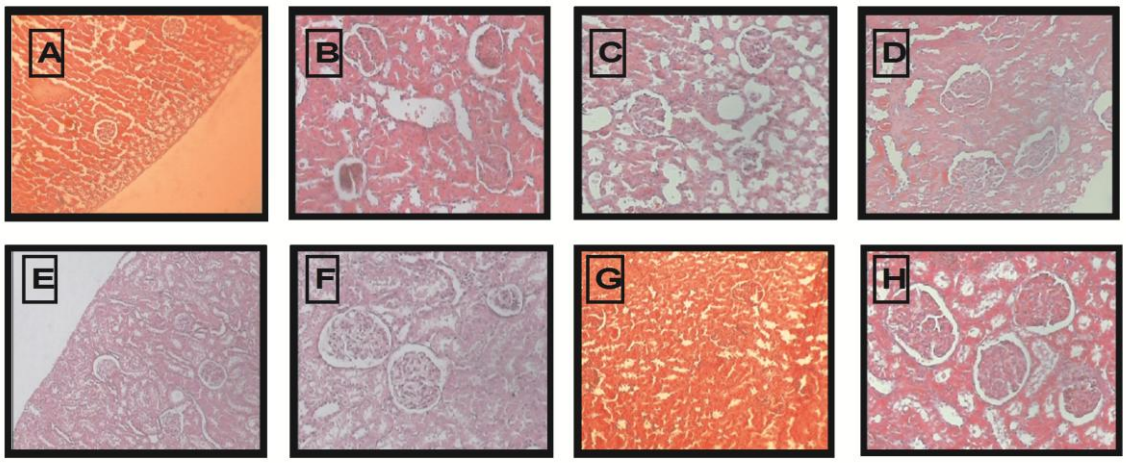

FIGURE 9 – Histopathological analysis of kidney in Wistar rats exposed to different nanosystems treatments. In A: SAL group; B: CZP group; C: BNC group; D: CNC group; E: BCSN group; F: CCSN group; G: BPEGN group; H: CPEGN group. SAL: saline solution; CZP: clozapine free; BNC: blank uncoated nanocapsules; CNC: clozapine-loaded uncoated nanocapsules; BCSN: blank chitosan-coated nanocapsules; CCSN: clozapine-loaded chitosan-coated nanocapsules; BPEGN: blank polyethyleneglycol-coated nanocapsules; CPEGN: clozapina-loaded polyethyleneglycol-coated nanocapsules.

However, our findings suggest that when clozapine is linked to different nanosystems, the treated groups have less tissue damage in organs heart, liver and kidney.

4 Discussion

The results obtained for hematological parameters demonstrated that clozapina is a drug able to generate damage in cellular level corroborating findings in the literature (BECHELLI & CAETANO, 1992; BUUR-RASMUSSEN & BROSEN, 1999; GASZNER, MAKKOS & KOSZA, 2002). Decreased levels of hemoglobin may feature anemia confirmed by severe decrease in RBC and associated with decreased hematocrit levels.

Furthermore, CZP group had severe leukopenia indicating that hematopoiesis is suppressed, which contributes to severe neutropenia, lymphopenia and monocytopenia found CZP group. Reports in the literature mention neutropenia and agranulocytosis as risk associated with the use of CZP, but the mechanisms that lead to changes white blood cells are not fully understood, making a focus of concern during treatment with this antipsychotic

(ATKIN et al., 1996; LAMBERTENGHI, 2000; WULFF, 2007; DUTT et al., 2010; PONS et al., 2012).

However, it was observed that the group treated with CS-coated nanocapsules had a severe leukocytosis compared SAL group suggest an inflammatory process, characterized by lymphopenia suggesting a depression of the immune system, confirmed by monocyte acting as second line of defense organism, in addition basophilia.

This condition can be caused owing CS, although is a natural and biodegradable polymer is not absorbed in the intestine (NASCIMENTO et al , 2001; . HANS & LOWMAN, 2002; SILVA et al , 2006). According studies among mammals, humans are rare example where specific chitinases are ausent. Thus, it is not possible hydrolyze the chitosan extensively, had only partial hydrolysis by bacteria of normal flora and non-specific hydrolases. This feature has to be interesting characteristic because, polymer is cationic, it is soluble in the stomach (pH range 1-3), however, precipitates in the intestine (pH 6.5). This physical- chemical characteristic is the key point in the mechanism of chitosan, because it interferes with the emulsification of lipids in the stomach and reaching the intestine, owing precipitation, the complex is not absorbed (SILVA et al., 2006).

Neutropenia, granulocytopenia or extreme, agranulocytosis, can result in decreased production or use of accelerated granulocytes. However, the most common cause neutropenia is induced by drugs and toxic agents, such as clozapina, so the data is consistent with other work that demonstrated that granulocytopenia one of the most devastating side effects caused by clozapine occurring immediately the first weeks of treatment (GASZNER; MAKKOS & KOSZA, 2002). Despite the variety of proposed hypotheses, the exact mechanism of clozapine -induced granulocytopenia and agranulocytosis is unknown, although some studies suggest that there is a direct myelotoxic effect and antibodies against myeloid cells and mature granulocytes (CLAAS, 1989). A potential mechanism involves the metabolic activation of clozapine by free radicals generated by peroxidase, or genetic factors may be involved in the etiology of agranulocytosis. Other adverse reactions hemopoietic as thrombocytopenia may occur rarely (GASZNER; MAKKOS & KOSZA, 2002).

It has been hypothesized that activation of metabolites of CZP may cause neutropenia and/or agranulocytosis, causing cell death by oxidative stress-induced apoptosis and, finally, directly targeting stromal cells of bone marrow (PIRMOHAMED & PARK, 1997; HUSAIN et al., 2006; PEREIRA & DEAN, 2006; FLANAGAN & DUNK, 2008).

The evaluation of complete blood count and platelet count for the groups treated with nanosystems revealed that polymerics nanocapsules can leave hematopoietic parameters to normalized levels.

The evaluation of markers cardiac function revealed that there was a significant increase ($p < 0.05$) of the evaluated parameters induced by CZP. These results corroborate the literature where most of the effects of the drug occurs in the first weeks of treatment (MERRILL; WILLIAM & GOFF, 2005; MERRILL et al., 2006; WEHMEIER; HEISER & REMSCHMIDT, 2005; RAMÍREZ et al., 2011). The groups treated with different nanosystems containing clozapine showed a significant improvement in markers cardiac function, and the group treated nanosystems containing clozapine CS-coated had better results. These results demonstrate that the drug when linked to nanosystems is able to mitigate the harmful effects of drug making safer.

Studies suggest that homocysteine levels is significantly elevated in the plasma of patients with schizophrenia, clozapine having an oxidant potential can act in the formation of reactive species, which in excess can further contribute to the increased homocysteine levels as well as aspects the specific schizophrenic symptoms and complications of their treatment (DIETRICH-MUSZALKA, A. et al., 2012). The data show that clozapine induced a significant increase in the levels of homocysteine and when bound to nanoparticles had decreased their level indicating the effectiveness of nanosystems.

The markers of renal function GOT and GPT levels were significantly increased in the group receiving CZP, which was expected because CZP is a drug which undergoes extensive first-pass metabolism and is metabolized almost exclusively in liver (HUMMER et al., 1997; MACFARLAN et al.,1997; CHANG et al., 2009). The groups treated with different nanosystems containing clozapine showed a significant improvement in markers liver function in relation to CZP group. Again the group treated with CS-coated nanocapsules obtained better results.

Marker renal urea the results of groups are similar SAL group suggesting that the nanoparticle systems were not nephrotoxic. The CS-coated nanocapsules showed a significant increase in the levels of urea. For the results of creatinine were significantly reduced compared SAL group suggesting that nanoparticle systems are not nephrotoxic because of decreased creatinine values have no clinical significance. Although our findings were not found impairment in renal function induced by CZP, reports in the literature show that the drug can cause urinary incontinence can be potentially able reduce bladder tone of the internal sphincter (HANES et al., 2013).

The findings show that different coatings can act in diverse and specific way each organ or tissue through which possess higher affinity.

Histological evaluation revealed that our findings corroborate the literature indicating that clozapina is a drug able of inducing tissue damage, but our histological evaluation showed that when clozapine is linked to different nanosystems is able to mitigate the harmful effects and reduce the damage to tissue level (KELLY et al., 2009; RAMIREZ et al., 2011).

5 Conclusion

Our findings demonstrated that clozapine when nanoencapsuladed showed better and significant results, suggesting that the nanosystems are able to mitigate the harmful effects of drug. Different coatings used in the formulations can act in diverse and specific way for each organ or tissue which has highest affinity. However, it was concluded that the nanosystems have a safe toxicological profile which combined with its effectiveness indicate a potential to establish clinical benefits as an effective therapy against refractory schizophrenia. Nanomedicine can be an alternative to the administration of clozapine and its Nanoencapsulation in polymeric systems is a promising therapeutic tool to be further elucidated.

Conflicts of interest statement

All authors report no conflict of interest.

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MANUSCRITO II

Clozapine linked to nanosystems improves oxidative stress parameters in Wistar rats

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Clozapine linked to nanosystems improves oxidative stress parameters in Wistar rats

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Abstract

Free radicals are oxidants agents highly reactive that in excess can react with lipids of membranes, proteins and genetic material being associated with various diseases such as schizophrenia, where occurs deregulation of metabolism these reactive species. Antipsychotics can potential oxidative stress, such as clozapine. Used as treatment of choice in refractory schizophrenia has clinical therapy is limited owing their side effects associated with their reactive metabolites. The feasibility of drug delivery for action in the brain using polymeric nanocapsules can open new perspectives for the treatment of schizophrenia, particularly to possibility minimize of oxidative damage, thus reducing the adverse effects of the drug. This study aimed to evaluate oxidative damage of biomolecules lipids, proteins and DNA and antioxidant defenses in Wistar rats after treatment with nanosystems containing clozapine. The study consisted of eight groups of male Wistar rats (n = 6), animals received the following treatments: saline solution (SAL) (NaCl 0.9% 1.0 mL/Kg i.p.), free clozapine (CZP) (25 mg/Kg i.p.), blank uncoated nanocápsulas (BNC) (1.0 mL/Kg i.p.), clozapine-loaded uncoated nanocapsules (CNC) (25 mg/Kg i.p.), blank chitosan-coated nanocapsules (BCSN) (1.0 mL/Kg i.p.), clozapine-loaded chitosan-coated nanocapsules (CCSN) (25 mg/Kg i.p.), blank polyethyleneglycol-coated nanocapsules (BPEGN) (1.0 mL/Kg i.p.), clozapine-loaded polyethyleneglycol-coated nanocapsules (CPEGN) (25 mg/Kg i.p.). The animals received the formulation once a day for seven consecutive days and euthanized in the eighth day. After euthanasia global blood was collected and immediately processed for further analysis. Lipid peroxidation analyses showed a significant increase ($p < 0.05$) induced by CZP and CS indicating lipid membrane damage, CNC and CPEGN groups had a reduction in lipid peroxidation levels indicating the protective character of nanosystems, better performance was obtained for CNC group. The carbonylation of proteins was induced by CZP, while the nanosystems-treated groups showed a significant improvement in these levels suggesting protective character nanosystems to protein damage, PEG coating showed better results. CZP group was able to induce oxidative genetic damage, while nanocapsules conferred a protective character causing less damage to DNA, PEG again better performance. The frequency of micronucleus showed damage induced by CZP and CNC group, other groups had a significant reduction in damage. A significant improvement of the antioxidant enzymes SOD, CAT and GPx, the best performance was CS-coated, similar results were obtained for GSH levels. The findings show that different coatings may confer a protective character, minimizing lipid, protein and genetic damage caused by the drug. These results indicate that the drug nanoencapsuladed is a useful alternative to mitigate the harmful effects of CZP.

Keywords: refractory schizophrenia, clozapine, nanosystems, oxidative stress, lipid peroxidation, protein carbonylation, genetic damage, antioxidant defenses.

1 Introduction

Free radicals are oxidants agents highly reactive that in excess can react with membrane lipids, proteins and genetic material being associated with various diseases such as schizophrenia, carcinogenesis, neurological dysfunction, pulmonary, autoimmune diabetes and vascular (TOURÉ & XUEMING, 2010). In schizophrenic patients occurs deregulation of metabolism of reactive oxygen species and nitrogen (REDDY & YAO, 1996; YAO et al., 1998; DIETRICH-MUSZALKA, OLAS & RABE-JABLONSKA, 2005).

Antipsychotics may potential oxidative stress, studies have showed that clozapine has oxidant potential. Chronic exposure to clozapine resulted in significant reduction in the activity of antioxidant enzymes, and produce oxidative damage in different structures of rat brain (POLYDORO et al., 2004; REINKE et al., 2004; AGOSTINHO et al., 2007).

Clozapine is the treatment of choice in refractory schizophrenia owing to be effective in both positive and negative symptoms. However, the clinical therapy is limited owing their side effects associated with their reactive metabolites (JANN, 1991; JANN et al., 1993; SILVA, et al., 2001; LOUS et al., 2003; ELKIS & MELTZER, 2007; MCILWAIN et al., 2011). Studies have reported that activation of metabolites can CZP cell death by oxidative stress (PIRMOHAMED & PARK, 1997; HUSAIN et al., 2006; PEREIRA & DEAN, 2006; FLANAGAN & DUNK, 2008).

In this context, one of the hypotheses involving oxidative stress and CZP is the potential mechanism involving metabolic activation of CZP by free radicals generated by peroxidase, or genetic factors (GASZNER, MAKKOS & KOSZA, 2002).

The feasibility of administering drugs, with action in the brain, using polymeric nanocapsules can open new perspectives for the treatment of schizophrenia, especially the possibility of biological activity at low doses (MUTHU et al. 2009). Studies describe the enormous usefulness of nanoparticle therapeutic systems, but studies of security these systems are still limited and should be further studied as oxidative damage. Some of the properties of these systems that proven therapeutic benefits can lead to cellular accumulation and toxicity in long time (LANDSIEDEL et al., 2009; SINGH et al., 2009).

However, the aim this study was to evaluate oxidative damage of biomolecules lipids, proteins and DNA and antioxidant defenses in Wistar rats after treatment with nanosystems containing clozapine.

2 Material e methods

2.1 Materials and reagents

All chemicals were of analytical grade. All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 Experimental animals

This study was approved by the Ethics Committee on Animal Use (CEUA), Federal University of Pampa (UNIPAMPA) under Protocol nº. 034/2012, which is affiliated to the Brazilian College of Animal Experimentation (COBEA). Since the experiments were conducted in accordance with the ethical and technical principles of animal experimentation established by the National Council for the Control of Animal Experimentation (CONCEA) and Law nº. 11.794 of 08 October 2008 which establishes procedures for the scientific use of animals (BRASIL, 2008; 2013).

Were used 48 adult Wistar rats, weighing about 250g, coming from the Bioterio of Federal University of Santa Maria. The animals remained in the Bioterio of Federal University of Pampa, Campus Uruguaiana, under standard environmental conditions, maintained in cabinets with dark/light cycle of 12 hours. They were fed *ad libitum* diet, this being appropriate in quantity and quality to maintain their health, with free access to drink water, *ad libitum*.

The animals were divided into eight experimental groups consisting of 6 animals each. The groups were treated as follows: saline solution NaCl 0.9% 1.0 mL/Kg (SAL), free clozapina 25 mg/Kg (CZP), blank uncoated nanocapsules 1.0 mL/Kg (BNC), clozapine-loaded uncoated nanocapsules 25 mg/Kg (CNC), blank chitosan-coated nanocapsules 1.0 mL/Kg (BCSN), clozapine-loaded chitosan-coated nanocapsules 25 mg/Kg (CCSN), blank polyethyleneglycol-coated nanocapsules 1.0 mL/Kg (BPEGN), clozapina-loaded polyethyleneglycol-coated nanocapsules 25 mg/Kg (CPEGN). The animals received the formulation once a day for seven consecutive days and euthanized in the eighth day.

The dose of administration of the solutions containing clozapine used was 25 mg/Kg in a volume of 1.0 mL/Kg and route of administration was intraperitoneal (CANADIAN COUNCIL ANIMAL CARE, 1993; POLYDORO et al., 2004; REINKE et al., 2004). The animals receive the formulations once a day, always at the same time, during seven

consecutive days. In the eighth day, the rats were euthanized, after euthanasia the global blood and immediately processed for further analysis.

2.3 Preparation of the suspensions of nanocapsules

The nanocapsules (NC) were prepared using the interfacial precipitation of the preformed polymer method. The organic phase was constituted with poli(ε-caprolactone), TCM, Lipoid S45® and CZP dissolved in acetone kept under heating and stirring. This phase was poured in aqueous phase with polysorbate 80. After the formation of suspension of NC, the acetone and part of the water are evaporated (1.5 mg/mL of CZP). To obtain the formulation covered with PEG, this was added to the aqueous phase of the suspension. For the covering with CS, aqueous acid solution of polysaccharide was added to the NC solution and kept under constant stirring for a period an hour. Unloaded NC were prepared (BNC).

2.4 Physico-chemical characterization of nanocapsules

The formulations were characterized by the diameter, specific surface area (SPAN) (Mastersizer, Malvern), Zeta potential (Zetasizer, Malvern), pH, drug content and encapsulation efficiency (EE) (HPLC-PDA) (BIENIEK et al., 2011).

2.5 Oxidative parameters

2.5.1 Lipid peroxidation

As an index of production of reactive species were measured by the spectrophotometric method the formation of thiobarbituric acid reactive substance (TBARS) during an acid-heating reaction, which is widely adopted as a sensitive method for measuring lipid peroxidation (OHKAWA et al., 1979). The results are expressed as equivalents of malondialdehyde (MDA) (nmolMDA/mL). All assays were performed in triplicate.

2.5.2 Carbonylation proteins

Oxidative damage of proteins was assessed by the spectrophotometric method for the determination of carbonyl groups based on the reaction with DNPH (LEVINE et al., 1990). All assays were performed in triplicate.

2.5.3 Comet assay

The assessment of DNA damage index was performed by comet assay (SINGH et al. 1988).

2.5.4 Test micronucleus

The frequency of micronuclei was evaluated in leukocytes. Global blood was collected and a sample was placed on the surface of the blade and made a smear, the blood was spread over the surface of the blade. After 24 hours, the slides were fixed in 96% ethanol for 30 min. The slides were stained with Panoptic dye and washed in water and put to dry. After drying the cells analyzed were considered as micronuclei the particles in relation to the main core: not exceed 1/3 of their size, are clearly separated with discernible edges and with the same color and refringence core (SCHMID, 1975).

2.6 Antioxidant Defenses

2.6.1 Catalase

The evaluation of catalase (CAT) was performed according to the method described by Aebi , 1984. All assays were performed in triplicate.

2.6.2 Superoxide dismutase

Measurement of superoxide dismutase (SOD) activity in erythrocytes was determined using the RANSOD ® Kit (Randox Laboratories, UK). This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4 nitrophenol)-5-phenyltetrazole chloride (INT) to form a red compound formazan. The activity

of superoxide dismutase was measured by the degree of inhibition of this reaction to 505 nm. All assays were performed in triplicate.

2.6.3 Glutathione Peroxidase

Measurement of glutathione peroxidase (GPx) activity in erythrocytes was determined using the Ransel ® Kit (Randox Laboratories, UK), according to Paglia & Valentine, 1967. All assays were performed in triplicate.

2.6.4 Total Glutathione

The quantification of the total glutathione (GSH) levels in RBCs was taken at 412 nm, observing the appearance of a yellow color oxidation product of 5,5'-bisditio-2-nitrobenzoic acid (DTNB). The standard containing 1 mM GSSG and white were measured separately (AKERBOOM & SIES, 1981). All assays were performed in triplicate.

2.6 Statistical analysis

Data were expressed as mean ± standard deviation (SD). Comparisons between groups were performed using a two-way analysis of variance (ANOVA), followed by post hoc of Bonferroni for multiple comparison tests. Results were considered statistically significant when $p<0.05$. The statistical analysis was performed using the software GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

3 Results

The formulations containing CZP showed a pH higher than the formulations without drug. BNC and CNC group showed a similar diameter of 139 ± 1 and 137 ± 2 nm respectively. BPEGN and CPEGN group showed different values, 140 ± 1 and 142 ± 1 nm, respectively, the same was observed to BNCS and CCSN group, 135 ± 2 and 141 ± 1, respectively. All formulation showed SPAN values inferior to 1.5. The zeta potential was negative for all formulation, except those covered with CS. CNC and CPEGN group presented drug content near 100% and the EE was above 95%, but around 70% for CCSN (Table 1).

TABLE 1

Values (mean ± SD) of particle mean diameter (D[4,3], nm), SPAM, zeta potential (mV) and pH of clozapine nanoformulations (n = 3 batches)

Formulation	D[4,3] nm	SPAN ± DP	Zeta potential (mV)	pH ± DP
BNC	139±1	1.2 ± 0.2	-33.2 ± 0.5	5.5 ± 0.01
CNC	137 ± 2	1.3 ± 0.1	-20.7 ±1.6 [a]	7.1 ± 0.01 [a]
BCSN	135 ± 2	1,1 ± 0.1	7.2 ±1.5	4.3 ± 0.005
CCSN	141 ± 1[b]	1.4 ± 0.001	29 ± 0.8[b]	4.5 ± 0.005 [b]
BPEGN	140 ± 1	1.3 ± 0.1	-22 ± 0.6	5.2 ± 0.005
CPEGN	142 ± 1	1,2 ± 0.1	-10.6 ± 1.3[c]	6.9 ± 0.005 [c]

[a] Difference between BNC x CNC ($p < 0.05$)
[b] Difference between BCSN x CCSN ($p < 0.05$)
[c] Difference between BPEGN x CPEGN ($p < 0.05$)

In Figure 1 are showed the values obtained for oxidative stress parameters. Lipid peroxidation analyses showed a significant increase ($p < 0.05$) induced by CZP and CS indicating lipid membrane damage, however, CNC and CPEGN groups had a reduction in the lipid peroxidation levels indicating protective character of nanosystems (Figure 1A). Among the nanocapsules CNC group had better performance in analysis showing the protective potential of the nanocapsules. The carbonylation of proteins induced by CZP, again the CS coating induced more damage when compared other groups of nanosystems, the other treated groups showed a significant improvement these levels suggesting protective character nanosystems to protein damage, again CNC and PEG showed the best results (Figure 1B). CZP group was able induce genetic oxidative damage, while nanocapsules showed a protective character causing less DNA damage (Figure 1C), PEG coating had better performance. The frequency of micronucleus showed damage induced by CZP and CNC groups, the other groups had a significant reduction in damage (Figure 1 D).

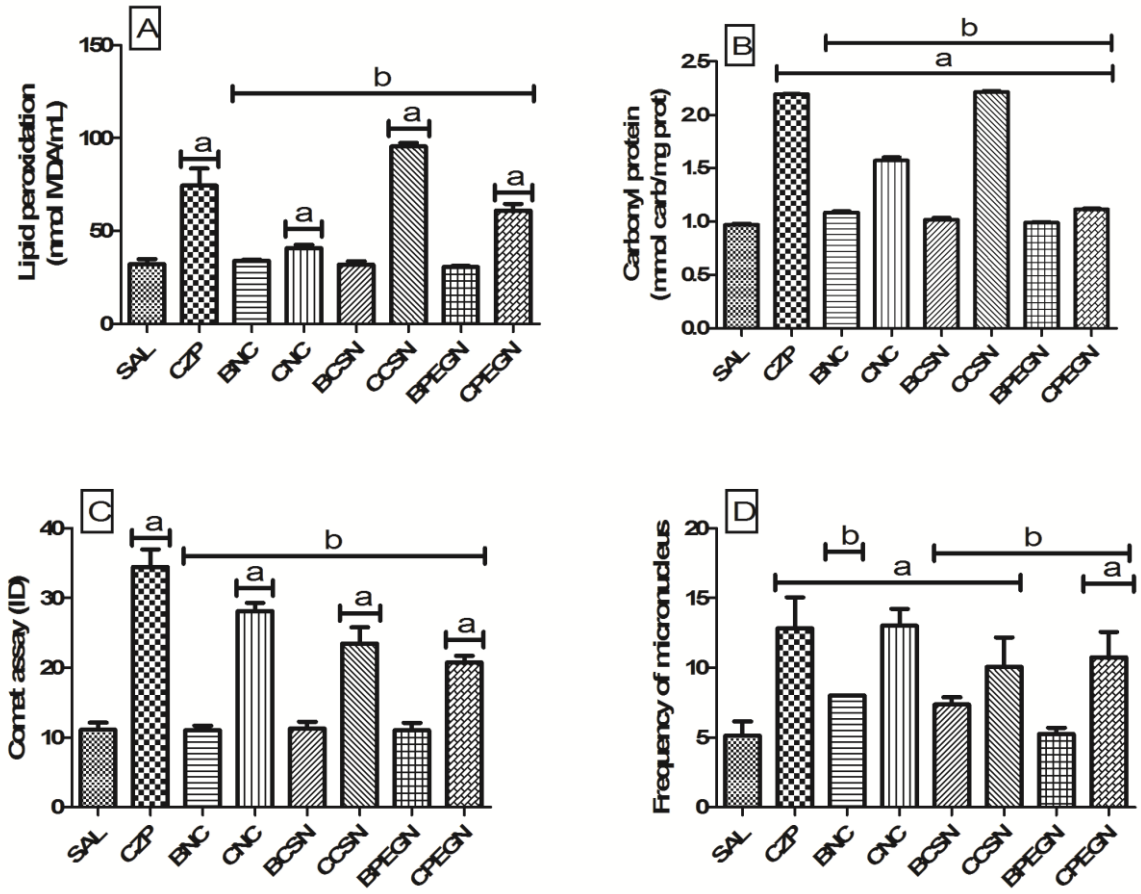

FIGURE 1 - Oxidative damage markers in Wistar rats exposed to different nanosystems treatments. In A: Lipid peroxidation levels; B: Carbonyl protein contents; C: Comet assay; D: Micronucleus test. SAL: saline solution; CZP: clozapine free; BNC: blank uncoated nanocapsules; CNC: clozapine-loaded uncoated nanocapsules; BCSN: blank chitosan-coated nanocapsules; CCSN: clozapine-loaded chitosan-coated nanocapsules; BPEGN: blank polyethyleneglycol-coated nanocapsules; CPEGN: clozapina-loaded polyethyleneglycol-coated nanocapsules. [a] Significantly different SAL ($p < 0.05$); [b] Significantly different CZP ($p < 0.05$).

Figure 2 are showed the results for antioxidant defenses. There was a reduction in the antioxidant defenses induced by CZP confirming the oxidant potential of drug. Evidenced a significant improvement of antioxidant enzymes SOD, CAT and GPx, the best performance by coating CS, similar results were obtained for GSH levels (Figure 2A, 2B, 2C, 2D, respectively).

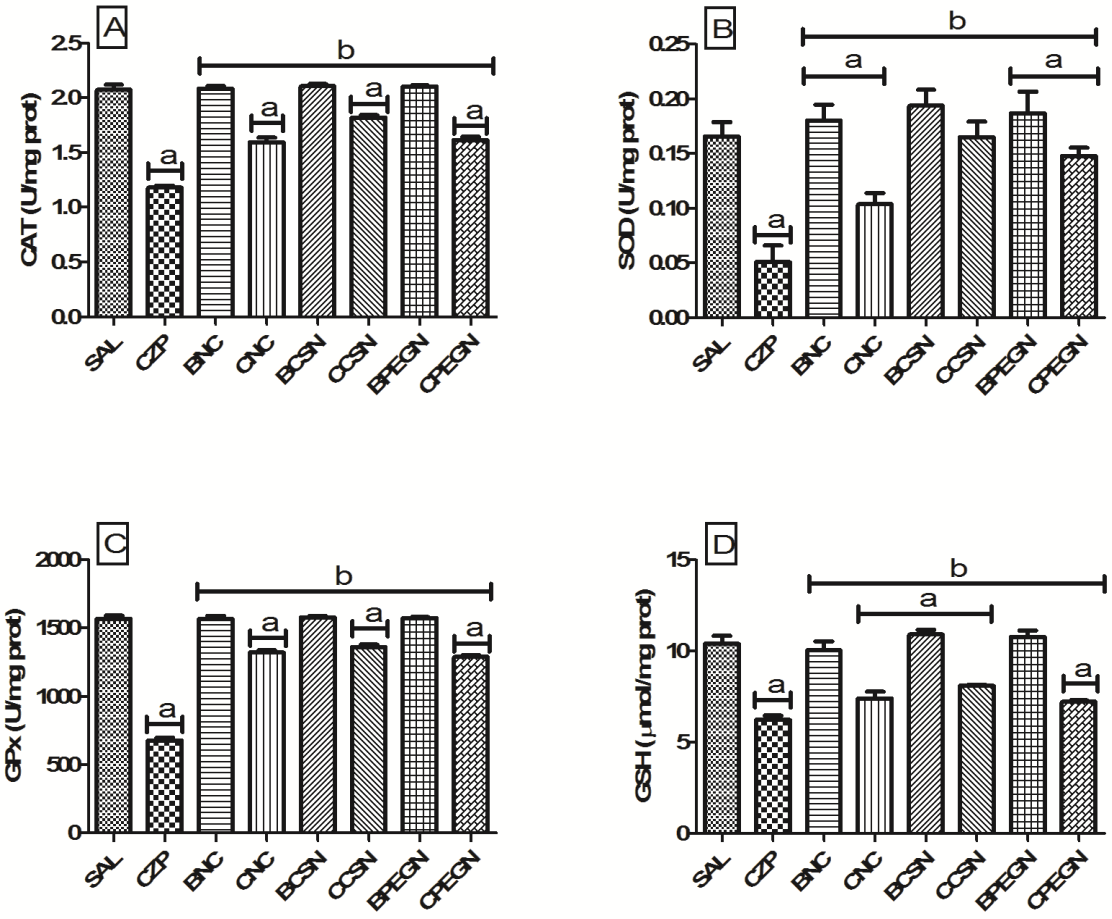

FIGURE 2 - Antioxidant defenses parameters in Wistar rats exposed to different nanosystems treatments. In A: Catalase levels; B: Superoxide dismutase levels; C: Gluthatione peroxidase levels; D: Total gluthatione levels. SAL: saline solution; CZP: clozapine free; BNC: blank uncoated nanocapsules; CNC: clozapine-loaded uncoated nanocapsules; BCSN: blank chitosan-coated nanocapsules; CCSN: clozapine-loaded chitosan-coated nanocapsules; BPEGN: blank polyethyleneglycol-coated nanocapsules; CPEGN: clozapina-loaded polyethyleneglycol-coated nanocapsules. [a] Significantly different SAL ($p < 0.05$); [b] Significantly different CZP ($p < 0.05$).

4 Discussion

The findings showed that different coatings can act protecting cells of lipid, protein and genetic damage. Our findings demonstrate that CZP linked to nanosystems is able to mitigate the effects in oxidative stress parameters and increase antioxidant defenses, thus are able to minimize the cytotoxic potential of CZP.

This occurs probably owing vectoring of drug through of the nanocapsules, making with the drug no undergo extensive hepatic metabolism and thus generating less toxic metabolites. Similar findings were obtained with haloperidol nanoencapsuladed demonstrated

beneficial effects of nanosystems in motor system and oxidative damage in brain regions (BENVEGNÚ et al., 2012).

Lipids oxidative damage is a complex process involving the interaction of reactive oxygen species with polyunsaturated fatty acids, components of cell membranes. This process results in structural disorganization and loss of selectivity of the membranes, which can lead to cell death (GUTTERIDGE & HALLIWELL, 2000). Both CZP as CS group induced lipid peroxidation indicating potential oxidative damage, our findings corroborate the literature, studies in rats treated with haloperidol and/or CZP demonstrated that these drugs were able to induce lipid damage in various structures in the central nervous system (POLYDORO et al., 2004; REINKE et al., 2004; AGOSTINHO et al., 2007).

Proteins are immediate to oxidative modification caused by reactive oxygen species, altering their structure, causing loss of function and protein fragmentation targets. The carbonylation of proteins was induced by CZP and CS generating protein damage, similar findings were reported in a study that evaluated the oxidative damage in rat brain induced by chronic administration of haloperidol (POLYDORO et al., 2004). Other studies showed that schizophrenic patients have elevated levels of carbonyl groups and isoprostanes in plasma owing oxidative stress (DIETRICH-MUSZALKA et al., 2009, 2012; DIETRICH-MUSZALKA & OLAS, 2009).

The CZP was able to induce genetic oxidative damage, although the nanocapsules conferring protective character are still able to generate DNA damage. This may stem from changes in DNA by direct interaction when the nanocapsules cross cell membranes obtaining direct access to the core, or indirect damages owing oxidative damage and inflammatory responses. These materials may remain accumulated in the cells, which can trigger severe responses such as mutagenesis and carcinogenesis (LANDSIEDEL et al., 2009).

The results showed a reduction of damage lipid of membranes, protein and genetic in groups with PEG and uncoated nanocapsules in comparison with CS coatings, leading to a neuroprotective action related to the type of coating.

Many studies have showed that drug-loaded nanoparticles are an efficient tool for drug delivery, enhancing the therapeutic effect and reduce adverse side effects (BECK et al., 2005; 2006; WU et al., 2008; BERNARDI et al., 2009; FONTANA et al., 2011). Moreover, these nanosystems are able to promote the permeation of drugs across the blood brain barrier, as has been demonstrated by several authors, suggesting its use as an alternative to drug delivery in the brain (BERNARDI et al., 2009; 2010; WANG et al., 2009; XIN-HUA et al., 2011).

Our findings demonstrate the beneficial effects of nanocapsules about antioxidant defense corroborating the literature that chronic exposure to clozapine resulted in significant reduction of antioxidant enzymes SOD and CAT (POLYDORO et al., 2004). The enzyme system is the primary pathway of antioxidant defense, being mainly represented by antioxidant enzymes GPx, CAT and SOD (GUTTERIDGE e HALLIWELL, 2000; BELLÓ, 2002). Thus, the enzymatic and non-enzymatic antioxidant system reduces reactive oxygen species and consequently the damage to biological structures (BELLÓ, 2002).

5 Conclusion

Our findings showed oxidative damage to membrane of lipids, proteins and genetic material induced by clozapine as well as the reduction of antioxidant defenses. When the drug was administered through nanosystems the oxidative damage has been reduced. Regarding the type of coating, uncoated and PEG-coated nanocapsules can realize a more efficient protective activity compared CS nanocapsules. Thus, it is concluded that the nanosystems is a potential alternative for the clinical treatment of clozapine.

Conflicts of interest statement

All authors report no conflict of interest.

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MANUSCRITO III

Clozapine linked to nanosystems reduces oxidative damage to biomolecules lipids, proteins and DNA in brain of rats Wistar

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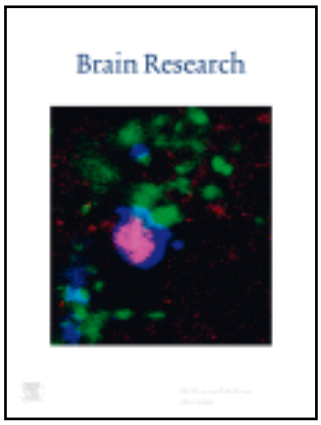
Em fase de preparação para submissão para Brain Research



Clozapine linked to nanosystems reduces oxidative damage to biomolecules lipids, proteins and DNA in brain of rats Wistar

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Abstract

Second-generation antipsychotic, clozapine, is used in the treatment of refractory schizophrenia. The reactive species, in excess, can have a crucial role in the etiology of this disease. Clozapine, like other neuroleptics, can change oxidative stress parameters potentiating disease symptoms. The clinical limitation of clozapine owing their serious adverse effects, the nanocapsules have attracted attention as carriers of several drugs with different therapeutic goals, such as vectorization of the same for target tissue with consequent reduction of side effects. This study aimed to evaluate oxidative damage of biomolecules lipids, proteins and DNA in the brain of Wistar rats after treatment with nanosystems containing clozapine. The study consisted of eight groups of male Wistar rats (n = 6), animals received the following treatments: saline solution (SAL) (NaCl 0.9% 1.0 mL/Kg i.p.), free clozapine (CZP) (25 mg/Kg i.p.), blank uncoated nanocápsulas (BNC) (1.0 mL/Kg i.p.), clozapine-loaded uncoated nanocapsules (CNC) (25 mg/Kg i.p.), blank chitosan-coated nanocapsules (BCSN) (1.0 mL/Kg i.p.), clozapine-loaded chitosan-coated nanocapsules (CCSN) (25 mg/Kg i.p.), blank polyethyleneglycol-coated nanocapsules (BPEGN) (1.0 mL/Kg i.p.), clozapine-loaded polyethyleneglycol-coated nanocapsules (CPEGN) (25 mg/Kg i.p.). The animals received the formulation once a day for seven consecutive days and euthanized in the eighth day. After euthanasia, the brain was collected each animal and immediately the organ homogenate was processed for further analysis. The evaluation of lipid peroxidation showed a significant increase ($p < 0.05$) induced by CZP and other groups treated with the drug indicating lipid membrane damage, CNC and CPEGN groups obtained a reduction of lipid peroxidation, indicating the protective character of nanosystems. The carbonylation of proteins was induced by CZP, while the nanosystems-treated groups showed a significant improvement these levels suggesting protective character nanosystems protein damage. The CZP was able to induce oxidative genetic damage, while the nanocapsules conferred a protective character causing less damage to DNA. The findings show that different coatings can act protecting target tissues and/or cell decreasing lipid, protein and genetic damage. These results indicate that the drug when linked to different nanocapsules is able to mitigate the harmful effects of the drug.

Keywords: refractory schizophreni, clozapine, nanosystems, oxidative stress, lipid peroxidation, protein carbonylation, genetic damage.

1 Introduction

Clozapine is an effective atypical antipsychotic used, particularly, in the treatment of patients with refractory schizophrenia to other neuroleptics. Clozapine is therapeutically effective, in both positive and negative symptoms. Unlike other neuroleptics, no produces significant extrapyramidal side effects (JANN, 1991; JANN et al., 1993; SILVA, et al., 2001; LOUS et al., 2003; ELKIS & MELTZER, 2007; MCILWAIN et al., 2011).

The clozapine has limited clinical use owing potential adverse effects, which are mainly associated to reactive metabolites. The metabolism of drug generates two reactive metabolites, the N-desmethylclozapine and clozapine N-oxide, which damage cells (BUUR-RASMUSSEN & BROSEN, 1999; SWARTZ, 2001).

Antipsychotics may enhance oxidative stress, although atypical antipsychotics produce less damage, clozapine is a potential oxidant (AGOSTINHO et al., 2007). Reports demonstrate that chronic exposure to clozapine resulted in significant reduction of antioxidant enzymes SOD and CAT, as well as producing oxidative damage in different structures of rat brain (POLYDORO et al., 2004; REINKE et al., 2004).

One of hypotheses involving oxidative stress and CZP is the potential mechanism involving metabolic activation of CZP by free radicals generated by peroxidase, or genetic factors (GASZNER, MAKKOS & KOSZA, 2002). Studies reported that activation of metabolites CZP can cell death induced by oxidative stress (PIRMOHAMED & PARK, 1997; HUSAIN et al., 2006; PEREIRA & DEAN, 2006; FLANAGAN & DUNK, 2008).

Lipid peroxidation has been implicated in many toxic effects of many drugs and tissue injury and disease processes (DAL-PIZZOL et al., 2001). It has been suggested that the reactive oxygen species may be involved in neuronal damage, inducing an increase lipid peroxidation. In addition, lipid peroxidation can be responsible to loss of membrane permeability (DAL-PIZZOL et al., 2000).

Advances in nanotechnology over the past three decades have had significant impact on clinical diagnosis and therapy (SALATA, 2004). The nanocapsules have the advantage of their small size that when combined with the use of polymers can vectorize drugs to target tissue and/or cell, improved oral bioavailability, controlled release, and protection against enzymatic degradation (HAIXIONG et al., 2002).

However, security aspects of nanosystems are not well defined. Changes in DNA can occur by direct interaction when the nanocapsules cross cell membranes obtaining direct access to the core, or indirect damages owing oxidative damage and inflammatory responses.

These materials may remain accumulated in the cells, which can trigger severe responses such as mutagenesis and carcinogenesis (LANDSIEDEL et al., 2009).

However, the aim this study was to evaluate oxidative damage of biomolecules lipids, proteins and DNA in the brain of rats after treatment with nanosystems containing clozapine.

2 Material e methods

2.1 Materials and reagents

All chemicals were of analytical grade. All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 Experimental animals

This study was approved by the Ethics Committee on Animal Use (CEUA), Federal University of Pampa (UNIPAMPA) under Protocol nº. 034/2012, which is affiliated to the Brazilian College of Animal Experimentation (COBEA). Since the experiments were conducted in accordance with the ethical and technical principles of animal experimentation established by the National Council for the Control of Animal Experimentation (CONCEA) and Law nº. 11.794 of 08 October 2008 which establishes procedures for the scientific use of animals (BRASIL, 2008; 2013).

Were used 48 adult Wistar rats, weighing about 250g, coming from the Bioterio the Federal University of Santa Maria. The animals remained in the Bioterio the Federal University of Pampa, Campus Uruguaiana, under standard environmental conditions, maintained in cabinets with dark/light cycle of 12 hours. They were fed *ad libitum* diet, this being appropriate in quantity and quality to maintain their health, with free access to drink water, *ad libitum*.

The animals were divided into eight experimental groups consisting of 6 animals each. The groups were treated as follows: saline solution NaCl 0.9% 1.0 mL/Kg (SAL), free clozapina 25 mg/Kg (CZP), blank uncoated nanocapsules 1.0 mL/Kg (BNC), clozapine-loaded uncoated nanocapsules 25 mg/Kg (CNC), blank chitosan-coated nanocapsules 1.0 mL/Kg (BCSN), clozapine-loaded chitosan-coated nanocapsules 25 mg/Kg (CCSN), blank polyethyleneglycol-coated nanocapsules 1.0 mL/Kg (BPEGN), clozapina-loaded

polyethyleneglycol-coated nanocapsules 25 mg/Kg (CPEGN). The animals received the formulation once daily for seven consecutive days and euthanized on the eighth day.

The dose of administration of the solutions containing clozapine used was 25 mg/Kg in a volume of 1.0 mL/Kg and route of administration was intraperitoneal (CANADIAN COUNCIL ANIMAL CARE, 1993; POLYDORO et al., 2004; REINKE et al., 2004). The animals received the formulations once a day, always at the same time, during seven consecutive days. In the eighth day, the rats were euthanized, after euthanasia the brain was collected each animal and immediately the organ homogenate was processed for further analysis.

2.3 Preparation of the suspensions of nanocapsules

The nanocapsules (NC) were prepared using the interfacial precipitation of the preformed polymer method. The organic phase was constituted with poli(ε-caprolactone), TCM, Lipoid S45® and CZP dissolved in acetone kept under heating and stirring. This phase was poured in aqueous phase with polysorbate 80. After the formation of suspension of NC, the acetone and part of the water are evaporated (1.5 mg/mL of CZP). To obtain the formulation covered with PEG, this was added to the aqueous phase of the suspension. For the covering with CS, aqueous acid solution of polysaccharide was added to the NC solution and kept under constant stirring for a period an hour. Unloaded NC were prepared (BNC).

2.4 Physico-chemical characterization of nanocapsules

The formulations were characterized by the diameter, specific surface area (SPAN) (Mastersizer, Malvern), Zeta potential (Zetasizer, Malvern), pH, drug content and encapsulation efficiency (EE) (HPLC-PDA) (BIENIEK et al., 2011).

2.5 Oxidative parameters

2.5.1 Lipid peroxidation

As an index of production of reactive species were measured by the spectrophotometric method the formation of thiobarbituric acid reactive substance (TBARS) during an acid-heating reaction, which is widely adopted as a sensitive method for measuring

lipid peroxidation (OHKAWA et al., 1979). The results are expressed as equivalents of malondialdehyde (MDA) (nmolMDA/mL). All assays were performed in triplicate.

2.5.2 Carbonylation proteins

Oxidative damage of proteins was assessed by the spectrophotometric method for the determination of carbonyl groups based on the reaction with DNPH (LEVINE et al., 1990). All assays were performed in triplicate.

2.5.3 Comet assay

The assessment of DNA damage index was performed by comet assay (SINGH et al. 1988).

2.6 Statistical analysis

Data were expressed as mean ± standard deviation (SD). Comparisons between groups were performed using a two-way analysis of variance (ANOVA), followed by post hoc of Bonferroni for multiple comparison tests. Results were considered statistically significant when $p<0.05$. The statistical analysis was performed using the software GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

3 Results

The formulations containing CZP showed a pH higher than the formulations without drug. BNC and CNC group showed a similar diameter of 139 ± 1 and 137 ± 2 nm respectively. BPEGN and CPEGN group showed different values, 140 ± 1 and 142 ± 1 nm, respectively, the same was observed to BNCS and CCSN group, 135 ± 2 and 141 ± 1, respectively. All formulation showed SPAN values inferior to 1.5. The zeta potential was negative for all formulation, except those covered with CS. CNC and CPEGN group presented drug content near 100% and the EE was above 95%, but around 70% for CCSN (Table 1).

TABLE 1

Values (mean ± SD) of particle mean diameter (D[4,3], nm), SPAM, zeta potential (mV) and pH of clozapine nanoformulations (n = 3 batches)

Formulation	D[4,3] Nm	SPAN ± DP	Zeta potential (mV)	pH ± DP
BNC	139±1	1.2 ± 0.2	-33.2 ± 0.5	5.5 ± 0.01
CNC	137 ± 2	1.3 ± 0.1	-20.7 ±1.6 [a]	7.1 ± 0.01 [a]
BCSN	135 ± 2	1,1 ± 0.1	7.2 ±1.5	4.3 ± 0.005
CCSN	141 ± 1[b]	1.4 ± 0.001	29 ± 0.8[b]	4.5 ± 0.005 [b]
BPEGN	140 ± 1	1.3 ± 0.1	-22 ± 0.6	5.2 ± 0.005
CPEGN	142 ± 1	1,2 ± 0.1	-10.6 ± 1.3[c]	6.9 ± 0.005 [c]

[a] Difference between BNC x CNC ($p < 0.05$)
[b] Difference between BCSN x CCSN ($p < 0.05$)
[c] Difference between BPEGN x CPEGN ($p < 0.05$)

In Figure 1 are showed the values obtained for oxidative stress parameters in the brain of Wistar rats. The evaluation of lipid peroxidation showed a significant increase ($p < 0.05$) induced by CZP and CCSN groups, other groups treated with the drug indicating the lipid membrane damage, CNC and CPEGN groups showed a reduction of lipid peroxidation, indicating protective character of nanosystems, a fact confirmed by blank nanocapsules groups that were equivalent SAL group (Figure 1A). The carbonylation proteins were induced by CZP and CCSN groups, while other groups- treated showed a significant improvement nanosystems these levels suggesting protective character nanosystems to protein damage (Figure 1 B). The CZP was able to induce oxidative genetic damage, while nanocapsules conferred a protective character causing less damage to DNA (Figure 1C).

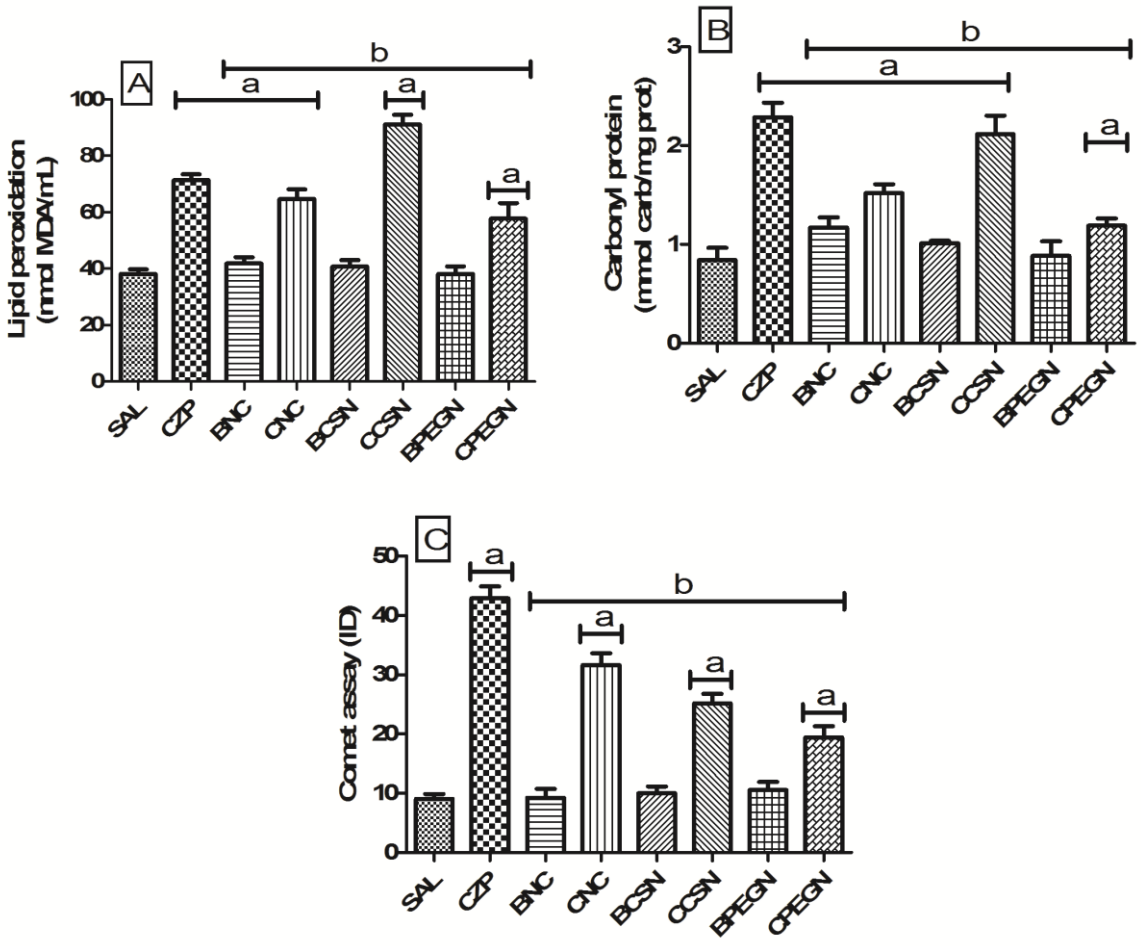

FIGURE 1 - Oxidative damage markers in brain in Wistar rats exposed to different nanosystems treatments. In A: Lipid peroxidation levels; B: Carbonyl protein contents; C: Comet assay. SAL: saline solution; CZP: clozapine free; BNC: blank uncoated nanocapsules; CNC: clozapine-loaded uncoated nanocapsules; BCSN: blank chitosan-coated nanocapsules; CCSN: clozapine-loaded chitosan-coated nanocapsules; BPEGN: blank polyethyleneglycol-coated nanocapsules; CPEGN: clozapina-loaded polyethyleneglycol-coated nanocapsules. [a] Significantly different SAL ($p < 0.05$); [b] Significantly different CZP ($p < 0.05$).

4 Discussion

The findings showed that different coatings can act protecting target tissues and/or cell decreasing lipid, protein and genetic damage. These results indicate that CZP linked to nanosystems is able of attenuating the effects of oxidative stress parameters. This is probably owing vectoring of the drug through the nanocapsules, the drug not undergo extensive hepatic metabolism and thus generating less toxic metabolites. Similar findings were obtained with haloperidol nanoencapsulado demonstrated the beneficial effects of nanocapsules the engine system, and resulting in lower oxidative damage in brain regions compared with free drug (BENVEGNÚ et al., 2012).

The results related to brain cells showed a reduction of greater damage in groups treated with PEG and uncoated nanocapsules in comparison with chitosan, leading to a neuroprotective action related type of coating.

Studies with antipsychotic showed that effects of haloperidol when nanocapsuladed when was maintained for a longer time and more effectively, have been reduced motors side effects compared to free drug (BENVEGNÚ et al., 2011). Many studies have shown that drug-loaded nanoparticles are an efficient tool for drug delivery, enhancing the therapeutic effect and reduce adverse side effects (BECK et al., 2005; 2006; BERNARDI et al., 2009; FONTANA et al., 2011; WU et al., 2008). Moreover, these nanosystems are able to promote the permeation of drugs across the hematoencephalic barrier, as has been demonstrated by several authors, suggesting its use as an alternative to drug delivery in the brain (BERNARDI et al., 2009; 2010; XIN -HUA et al., 2011; WANG et al., 2009).

5 Conclusion

Our findings indicate oxidative damage membranes of lipids, proteins and genetic material brain cells when exposed to clozapine. When the drug was administered through the nanocapsules has been reduced damage. Regarding the type of coating, uncoated and coated nanocapsules with PEG have a more and efficient protective activity compared CS-coated. The nanosystems have potential to provide clinical benefit as an effective therapy for refractory schizophrenia.

Conflicts of interest statement

All authors report no conflict of interest.

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PARTE III

4 CONCLUSÃO

De acordo com os resultados apresentados nesta dissertação pode-se inferir que os nanossistemas:

- Melhoram os parâmetros hematológicos como a contagem total de hemácias (RBC), hemoglobina e hematócrito, bem como os demais parâmetros hematimétricos, sendo que os revestimentos QTS e PEG obtiveram melhor desempenho nos parâmetros avaliados;

- Aprimoram os parâmetros hematológicos da série branca (WBC), contudo, as nanocápsulas revestidas com QTS apresentaram leucocitose severa sugerindo um processo inflamatório. Este é caracterizado por linfopenia devido a depressão do sistema imune, confirmado pela monocitose e basofilia;

- Não houve significado clínico para a contagem global de plaquetas e MPV;

- Reduzem os níveis dos marcadores de função cardíaca CK, CK-MB e homocisteína, sendo que a QTS obteve melhor desempenho como revestimento;

- Reduzem os níveis dos marcadores de função hepática TGO e TGP, sendo que o revestimento QTS obteve melhor desempenho;

- Não se mostraram nefrotóxicos, mantendo os marcadores de função renal dentro dos limites da normalidade, a exceção das nanocápsulas revestidas com QTS as quais tiveram os níveis de ureia significativamente aumentados;

- Reduzem o dano tecidual verificado através da análise histopatológica dos órgãos coração, fígado e rim;

- Reduzem o dano lipídico em plasma, a exceção das nanocápsulas revestidas com QTS;

- Reduzem o dano em proteínas plasmáticas, a exceção das nanocápsulas revestidas com QTS;

- Reduzem o dano oxidativo no material genético (DNA) em sangue total, sendo que o revestimento PEG mostrou melhores resultados;

- Aumentam a atividade das enzimas antioxidantes CAT, SOD e GPx em eritrócitos, sendo o melhor desempenho obtido pelo revestimento QTS;

- Aumentam os níveis de GSH em eritrócitos, sendo o revestimento QTS o com melhor desempenho;

- Reduzem o dano oxidativo em biomoléculas lipídios, proteínas e DNA no homogenato do cérebro de ratos Wistar após o tratamento com os nanossistemas contendo clozapina, a exceção das nanocápsulas revestidas com QTS que induziram dano a lipídeos de membrana e proteínas.

Estes resultados evidenciam os efeitos positivos dos nanossistemas, portanto, a nanoencapsulação da clozapina é uma ferramenta terapêutica promissora, capaz de atenuar os efeitos nocivos do medicamento, minimizando o estresse oxidativo, tornando-a um fármaco mais seguro aos pacientes.

5 PERSPECTIVAS

Este trabalho tem como perspectivas futuras:

- Realizar estudo com nanocápsulas lipídicas com diferentes tipos de revestimento;

- Verificar a eficácia e o perfil hematológico, bioquímico e histopatológico dos diferentes tratamentos com nanossistemas;

- Avaliar vias de estresse oxidativo e parâmetros inflamatórios (PCR, IL-1B, IL-6, IL-10 e TNF-α).

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ANEXO A - Protocolo de aprovação do projeto pelo CEUA-UNIPAMPA

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

Pró-Reitoria de Pesquisa

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

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PROTOCOLO Nº 034/2012

Título: EFEITO DO TRATAMENTO COM NANOSSISTEMAS CONTENDO CLOZAPINA SOBRE PARÂMETROS DE ESTRESSE OXIDATIVO EM RATOS WISTAR

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Após a análise detalhada do projeto de pesquisa a relatoria da CEUA-Unipampa emite parecer **FAVORÁVEL** para o cadastro do protocolo e execução do referido projeto.

Luiz E. Henkes
Professor Adjunto
Coordenador do CEUA/Unipampa