Centro de Ciências Agrárias – CCA
Depto. de Ciência e Tecnologia de Alimentos
Programa de Pós-Graduação em Ciência de Alimentos

METODOLOGIA OTIMIZADA PARA EXTRAÇÃO DA β-GLUCANA DE *SACCHAROMYCES CEREVISIAE* E AVALIAÇÃO DO POTENCIAL IMUNOMODULADOR E ANTIGENOTÓXICO EM PACIENTES COM CÂNCER DE PRÓSTATA AVANÇADO

MARCIANE MAGNANI

Londrina - PR
2010

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Tese apresentada ao Programa de Pós-Graduação em Ciência de Alimentos, nível Doutorado, da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Doutor em Ciência de Alimentos.

Aluna: Marciane Magnani

Orientador: Prof. Dr. Raúl Jorge Hernan Castro-Gómez

Londrina - PR
2010

COMISSÃO EXAMINADORA

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Prof. Dr. Raúl Jorge Hernan Castro-Gómez
Universidade Estadual de Londrina, Centro de Ciências Agrárias
Departamento de Ciência e Tecnologia de Alimentos
(Presidente – Orientador)

_____

Profª Drª Roberta Losi Guembarovski
Universidade Estadual de Londrina, Pós Graduação em Patologia Experimental
Laboratório de Imunologia e Genética Molecular
(Membro)

_____

Prof. Dr. Cláudio Takeo Ueno
Universidade Tecnológica Federal do Paraná
Campus de Londrina
(Membro)

_____

Profª Drª Daniele Sartori
Universidade Estadual de Londrina, Centro de Ciências Biológicas
Departamento de Biologia Geral
(Membro)

_____

Prof. Dr. Hevenilton José Matiazi
Empresa Biorigin/ São Paulo
(Membro)

Londrina, 13 de agosto de 2010.

Ao meu marido Fábio Augusto com amor ofereço, pois nem todas as palavras que conheço, nem um lindo texto escrito com elas poderia descrever meu amor por ti.

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Magnani, M.

MAGNANI, Marciane. Metodologia otimizada para extração da β-glucana de *Saccharomyces cerevisiae* e avaliação do potencial imunomodulador e antigenotóxico em pacientes com câncer de próstata. 2010. Tese (Doutorado em Ciência e Tecnologia de Alimentos) – Universidade Estadual de Londrina.

RESUMO

No presente estudo uma metodologia otimizada foi desenvolvida para extração da β-glucana de células de *Saccharomyces cerevisiae* , envolvendo sonicação e tratamento enzimático, com rendimento de 11,08 ± 0,19 %. A partir da β-glucana extraída foi obtido o carboximetil-derivado correspondente, que não mostrou, *in vitro*, quaisquer efeitos citotóxicos, genotóxicos e na modulação da viabilidade celular. Os efeitos da administração oral da Carboximetil-glucana (CM-G) foram avaliados em um ensaio clínico envolvendo 30 pacientes com câncer de próstata (PCa) avançado sob hormonioterapia. Após 28 dias de administração da CM-G houve aumento significativo nas contagens de leucócitos totais, hemáceas, plaquetas, hemoglobina e hematócrito, sem associação com hábitos do estilo de vida dos pacientes. Não foram observados efeitos colaterais e ou alterações nas funções renal e hepática associados a CM-G. As análises do genótipo dos pacientes para receptor de quimiocina CCR5, envolvido na resposta imune celular, revelaram o genótipo selvagem Wt/Wt em 80% dos pacientes e o genótipo heterozigoto Wt/delta32 em 20%. Após a administração da CM-G foi constatado um aumento significativo de linfócitos T $CD3^+$, $CD4^+$ e $CD8^+$ nos pacientes Wt/Wt, não relacionado a idade, ou ao tempo de hormonioterapia, sugerindo uma interação entre a imunomodulaçao pela CM-G e o receptor CCR5. Na avaliação dos efeitos de proteção contra danos no DNA, em 20 pacientes com PCa, foi constatada uma redução de danos média de 59%, não relacionada ao aumento do número de leucócitos do sangue periférico. Os resultados do presente estudo evidenciam o estímulo da hematopoiese e da resposta imune pela CM-G, bem como efeitos de proteção contra danos no DNA, em pacientes com PCa. Estes dados mostram que o uso da CM-G como adjuvante no tratamento de PCa pode melhorar parâmetros importantes para a saúde dos pacientes.

Palavras-chave: β-glucana, *Saccharomyces cerevisiae*, carboximetil-glucana (CM-G), imunomodulador, câncer de próstata (PCa)

MAGNANI, Marciane. Optimizated methodology for extraction of *Saccharomyces cerevisiae* β-glucan and evaluation of immunomodulator and antigenotoxic potential patients with prostate cancer. 2010. Tese (Doutorado em Ciência e Tecnologia de Alimentos) – Universidade Estadual de Londrina.

ABSTRACT

In the present study an optimized methodology for extraction of β-glucan from *Saccharomyces cerevisiae* cells involving sonication and enzyme treatment was developed, with a yield of 11.08 ± 0.19%. From the β-glucan extracted the corresponding carboxymethyl-derivative was obtained, which did not reveal, *in vitro*, any cytotoxic or genotoxic effects or influences on cell viability. The effects of oral CM-G administration were evaluated in a clinical trial with 30 patients with advanced prostate cancer (PCa) under hormone-therapy. After 28 days of CM-G administration, a significant increase of total leukocyte, red cells, platelet, hemoglobin and hematocrit was observed, without association with the patients' lifestyle habits. No side effects or changes in hepatic or renal functions were observed after CM-G administration. The analyses of chemokine receptor CCR5 genotypes of the patients, involved on immune cell response, revealed the wild type Wt/Wt genotype for 80% of the patients and the heterozygotic Wt/delta32 genotype for 20%. After CM-G administration a significant increase in $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes was observed in Wt/Wt patients, with no association with age or hormone-therapy period, suggesting an interaction between immunomodulation by CM-G and the CCR5 receptor. In the evaluation of the protective effect of CM-G against DNA damage, in 20 patients with PCa, an average DNA damage reduction rate of 59% was found, with no relation with the increased number of peripheral blood leukocytes**.** The results of the present study showed the enhanced of the hematopoiesis and of the immune response by CM-G, as well as its protective effects against DNA damage in advanced PCa patients. This data shows that the use of CM-G as an adjuvant to PCa treatment may improve important parameters for health of cancer patients.

Key words: β-glucan, *Saccharomyces cerevisiae*, carboxymethyl-glucan (CM-G), immunomodulation, prostate cancer (PCa),

LISTA DE ILUSTRAÇÕES

Artigo publicado na Carbohydrate Polymers

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Artigo submetido na Natural Product Research

Artigo submetido na Genetics and Molecular Biology

LISTA DE TABELAS

Artigo publicado na Carbohydrate Polymers

Artigo publicado na Experimental and Therapeutic Medicine

Artigo submetido na Natural Product Research

Artigo submetido na Genetics and Molecular Biology

SUMÁRIO

INTRODUÇÃO

β-glucanas são polissacarídeos constituintes estruturais da parede celular de leveduras, fungos e alguns cereais, que se diferenciam pelo tipo de ligação entre as unidades de D-glicose da cadeia principal e pelas ramificações que se conectam a essa cadeia. Uma importante fonte de β-glucana é a parede celular de leveduras, particularmente de *Saccharomyces cerevisiae*, levedura de fermentação amplamente empregada na indústria de panificação, sucroalcoleira e cervejaria. Em *S. cerevisiae* a β-glucana é constituída por um esqueleto linear central de unidades de glicose ligadas na posição β(1-3), com cadeias laterais unidas em β(1-6), que ocorrem em diferentes intervalos e têm tamanhos variados.

Classificada como um modificador da resposta biológica (MRB), a β-glucana de *S. cerevisiae* é capaz de alterar a resposta de defesa do hospedeiro pelo estímulo do sistema imune, com efeitos na imunidade inata e adaptativa. Esta habilidade da β-glucana, de ativar mecanismos de defesa no hospedeiro, em vertebrados, ocorre após seu reconhecimento pelo sistema imune por receptores celulares específicos, preferencialmente expressos em células imunes. Além da imunomodulação, propriedades antigenotóxica, antinflamatória, antitumoral, hipocolesterolêmica hipoglicêmica, e de proteção contra infecções já foram comprovadas para a β-glucana. Sua atividade biológica está relacionada, entre outras características, ao peso molecular, tipo de ligações glicosídicas presentes, solubilidade em água e grau de polimerização. Dessa forma, o processo de extração influencia diretamente a bioatividade da β-glucana e geralmente sua obtenção a partir da parede celular de *S. cerevisiae* envolve lavagens ácidas e alcalinas, que degradam parte das cadeias de glicose. Consequentemente, o rendimento do processo é pouco satisfatório e ocorre a geração de resíduos nocivos ao meio ambiente, o que torna a busca de métodos alternativos para extração de β-glucana um importante objetivo para pesquisa científica.

Substâncias bioativas e imunomoduladoras, como a β-glucana de *S. cerevisiae*, tem sido propostas como adjuvantes em tratamentos contra o câncer especialmente pela ausência de efeitos adversos.

O câncer de próstata (PCa) é o sexto tipo de câncer mais comum no mundo, e representa cerca de 10% do total de câncer diagnosticado. No Brasil, sem considerar o câncer de pele não melanoma, o de próstata é mais incidente no sexo masculino, mais frequente em todas as regiões do país e o segundo tipo de câncer com maior taxa de mortalidade. Cerca de três quartos dos casos de PCa no mundo ocorrem a partir dos 65 anos, e em estágio metastático, permanece incurável. Nesta fase, as metas da medicina são aumentar a curta sobrevida e, sobretudo melhorar qualidade de vida, comumente comprometida com a evolução da doença e complicações decorrentes do tratamento crônico. Fatores adicionais como a idade avançada e hábitos do estilo de vida, como tabagismo e alcoolismo, tornam estes pacientes ainda mais vulneráveis.

Pelo exposto, avaliar os efeitos da administração da β-glucana de *S. cerevisiae* em pacientes com PCa avançado, que exibem um quadro clínico agravado pela idade, tratamento, e estilo de vida, é de suma importância, pois a utilização de um adjuvante bioativo, que não causa efeitos colaterais, pode representar uma importante estratégia para melhorar a qualidade da curta sobrevida destes pacientes.

2 OBJETIVOS

2.1 Objetivo geral

Desenvolver uma metodologia para extração da β-glucana insolúvel da parede celular de *S. cerevisiae* e avaliar o potencial imunomodulador e antigenotóxico do carboximetil-derivado correspondente em pacientes com PCa avançado.

2.2 Objetivos específicos

- Desenvolver uma metodologia otimizada para extração da β-glucana de *S. cerevisiae* livre de proteínas, utilizando tratamentos pouco agressivos à estrutura do polímero e sem a geração de efluentes nocivos ao meio ambiente;

- Obter, a partir da β-glucana insolúvel de *S. cerevisiae,* o carboximetil-derivado correspondente com grau de substituição considerado ótimo para atividade biológica;

- Avaliar *in vitro* a carboximetil-glucana (CM-G) obtida quanto à citotoxicidade, genotoxicidade e modulação da viabilidade celular em células epiteliais de ovário de hamster chinês (CHO-k1);

- Avaliar os efeitos da administração oral da CM-G na celularidade do sangue periférico e nas funções renal e hepática de pacientes com PCa avançado sob hormonioterapia;

- Conhecer o genótipo para o receptor de quimiocina CCR5 dos pacientes e analisar a frequência do genótipo mutado CCR5delta32;

- Analisar a relação entre o genótipo CCR5 e os efeitos da CM-G nas subpopulações de linfócitos T $CD3^+$, $CD4^+$ e $CD8^+$ dos pacientes;

- Avaliar os efeitos antigenotóxicos da CM-G nas células do sangue periférico dos pacientes;

3 REVISÃO DE LITERATURA

β-glucana de *Saccharomyces cerevisiae*: constituição, bioatividade e obtenção.

Publicada na Revista Semina - Ciências Agrárias

β-glucana de *Saccharomyces cerevisiae:* constituição, bioatividade e obtenção

β-glucana from *Saccharomyces cerevisiae:* constitution, bioactivity and obtaining

Marciane Magnani[1*]; Raul Jorge Hernan Castro-Gómez[2]

Resumo

β-glucanas são polissacarídeos constituintes estruturais da parede celular de leveduras, fungos e alguns cereais, que se diferenciam pelo tipo de ligação presente entre as unidades de glicose. Uma importante fonte destes polissacarídeos é a parede celular de *Saccharomyces cerevisiae*, uma levedura amplamente empregada em processos industriais de fermentação. A β-glucana é considerada um modificador da resposta biológica devido ao seu potencial imunomodulador, pois ao ser reconhecida por receptores celulares específicos tem habilidade de realçar a resposta imune do hospedeiro. Outros efeitos benéficos como anticarcinogênico, antimutagênico, hipocolesterolêmico e hipoglicêmico também têm sido relacionados à β-glucana Esta revisão de literatura teve por objetivo agregar conhecimentos científicos sobre a constituição e bioatividade da β-glucana, incluindo seu reconhecimento pelo sistema imune, bem como, a obtenção a partir da parede celular de *S. cerevisiae*.
Palavras-chave: β-glucana, *Saccharomyces cerevisiae*, bioatividade, imunomodulador, sistema imune

Abstract

β-glucans are polysaccharides that constitute the structure of the cell wall of yeast, fungi and some cereals, which differs each other by the linkages between glucose units. An important source of these polymers is the *Saccharomyces cerevisiae* cell wall, which is a yeast widely used in industrial processes of fermentation. The β-glucan is considered to be a modifier of biological response due to its immunomodulator potential. When it is recognized by specific cellular receptors, have the ability to enhance the host's immune response. Other beneficial effects such as anticarcinogenic, antimutagenic, hypocholesterolemic and blood sugar reduction have also been related to the β-glucan. The aim of this literature review was expand scientific knowledge about the constitution and bioactivity of β-glucan, including its recognition by the immune system, as well as its obtaining from *S. cerevisiae* cell wall.
Key words: β-glucan, *Saccharomyces cerevisiae*, bioactivity, immunomodulator, immune system

[1] Doutoranda em Ciência de Alimentos, DCTA, CCA; UEL. E-mail: magnani2@bol.com.br
[2] Professor doutor titular do DCTA; CCA; UEL.
[*] Autor para correspondência

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Introdução

β-glucanas são polissacarídeos constituintes estruturais da parede celular de leveduras, fungos e alguns cereais, que se diferenciam pelo tipo de ligação entre as unidades de glicose da cadeia principal e pelas ramificações que se conectam a essa cadeia. Nas últimas décadas estes polímeros vem recebendo especial atenção por sua bioatividade, principalmente no que se refere a imunomodulação. Além disso, inúmeros efeitos benéficos como antitumoral, antinflamatório, antimutagênico, hipocolesterolêmico e hipoglicêmico têm sido relacionados à β-glucanas. Uma importante fonte de β-glucana é a parede celular de *Saccharomyces cerevisiae*, também conhecida como levedura de fermentação, que é amplamente empregada nas indústrias de panificação, cervejaria e sucroalcoleira. Em *S. cerevisiae*, a β-glucana é constituída por um esqueleto linear central de unidades de glicose ligadas na posição β(1-3), com cadeias laterais unidas em β(1-6), que ocorrem em diferentes intervalos e têm tamanhos variados. A β-glucana é designada como um modificador da resposta biológica pois, ao ser reconhecida pelo organismo desencadeia uma série de eventos na resposta imune. Em vertebrados este reconhecimento ocorre através de receptores específicos de superfície celular e em seguida o sistema imune é estimulado pela β-glucana em resposta a inúmeras situações prejudiciais ao indivíduo. A modulação da β-glucana inclui a ativação de macrófagos e linfócitos polimorfonucleares, além da indução da expressão de diversas citocinas. O mecanismo de ação parece estar relacionado ao peso molecular, tipo de ligações glicosídicas, resíduos presentes, solubilidade em água, conformação espacial e grau de polimerização da β-glucana. Nos últimos anos, inúmeras pesquisas tem sido realizadas visando à otimização do processo de obtenção da β-glucana a partir da parede celular de *S. cerevisiae*. Além disso, há um crescente interesse na identificação das características estruturais e biológicas da β-glucana e seus derivados, na expectativa de viabilizar seu uso, aproveitando seu potencial imunoestimulante. Esta revisão teve como objetivo aprofundar conhecimentos sobre este importante biopolímero, enfatizando sua obtenção a partir de *S. cerevisiae*, pois representa um potencial adjuvante na profilaxia e terapia de inúmeras doenças.

Parede celular de *Saccharomyces cerevisiae*

Designada comumente como levedura de cervejaria ou de panificação, *Saccharomyces cerevisiae* é um microrganismo eucariótico unicelular, que apresenta forma variada. Dentre suas características, destaca-se a capacidade de ajustar-se metabolicamente para a fermentação de açúcares em presença ou ausência de oxigênio, produzindo álcool ou gás carbônico (TORTORA; BERDELL; CASE, 2000).

O desenvolvimento de uma camada de proteção externa é crítico para o crescimento e sobrevivência das células fúngicas (DURÁN; NOBLEGA, 2004). A parede celular é uma organela comum em leveduras e fungos filamentosos, localizada na interface entre o microrganismo e o meio ambiente. Nas últimas décadas, sua organização e composição vêm sendo amplamente estudadas (FIRON; LESSAGE; BUSSEY, 2004).

A parede celular é uma estrutura forte, responsável pela proteção física e estabilidade osmótica da célula que através de permeabilidade seletiva permite o transporte de nutrientes para o citoplasma (SMITH et al., 2000; KLIS et al., 2002). Neste contexto, Kapteyn et al. (1997) relataram que a parede é determinante para a integridade da célula, pois sustenta a forma e oferece proteção mecânica, além de promover o metabolismo e crescimento celular pela modulação da seletividade às macromoléculas. Além disso, para preservar a integridade da célula, quando ocorrem perturbações na parede celular mecanismos de reparo reorganizam a estrutura molecular (KLIS et al., 2002).

Em *S. cerevisiae*, a parede celular representa de 15 a 30% do peso seco total da célula, sendo que seus componentes são sintetizados e unidos em sincronia com o crescimento e a divisão celular (CID et al., 1995).

Estudos sobre a composição da parede de células de *S. cerevisiae* revelaram a presença de glucana (48-60%), que é um polímero de unidades de glicose com ligações β(1-3) e β(1-6), manoproteínas (20-23%), quitina (0,6-2,7%), que é composta por β(1-4) N-acetilglicosamina e uma pequena porção de lipídios (FLEET, 1985; HARTLAND et al., 1994; KLIS, 1994). A distribuição destes componentes está organizada em duas camadas principais, sendo a externa composta de manoproteínas e, a interna de glucana e quitina, em uma estrutura interconectada por ligações covalentes (HA et al., 2002; KLIS et al., 2002).

A camada externa de manoproteínas retém as proteínas periplasmáticas, conferindo resistência às células vivas de leveduras e limitando o acesso de enzimas externas, como aquelas elaboradas por outros microrganismos (ZIOTNIK et al., 1984). Em contrapartida, a camada de glucana entrelaçada por fibrilas de quitina, adjacente a membrana plasmática, mantém a rigidez e a forma da célula (KOPECKÁ; PHAFF; FLEET, 1974; KAPTEYN et al., 1997). Conforme Kollár et al. (1997), na camada interna, os componentes ligados covalentemente oferecem força e resistência pela formação de complexos macromoleculares.

A arquitetura molecular da parede celular de *S. cerevisiae* não é fixa e durante o ciclo celular sofre consideráveis ajustes na composição e estrutura (SUZUKI et al., 2004). Conforme Firon, Lesage e Bussey (2004) a parede da célula é alvo de inúmeras mudanças, e características como plasticidade e força de tensão são continuamente alteradas durante o ciclo celular, para permitir o crescimento e divisão. Além disso, também apresenta alterações em resposta à disponibilidade de nutrientes, oxigênio, e à condições de temperatura e pH, formando complexos específicos de proteínas e polissacarídeos (KAPTEYN; VAN OEN ENOE; KLIS, 1999). Em recente estudo, Karreman et al. (2007) relataram que a Hsp12p, uma proteína hidrofílica de resposta ao stress, influencia na rigidez e permeabilidade, além de aumentar a flexibilidade da parede celular de *S. cerevisiae*. A regulação para expressão desta proteína é vinculada a condições de aumento da temperatura, pressão osmótica e concentração salina ou ainda, perturbações na parede da célula.

Kim e Yun (2006) estudaram a influência da composição do meio e das condições de cultivo na composição da parede celular de *S. cerevisiae*. As alterações no conteúdo de β-glucana, durante o cultivo em batelada e fermentação contínua, evidenciaram que a composição da parede celular é afetada pela composição do meio, principalmente na fase estacionária, quando a taxa de crescimento é baixa e a parede apresenta mudanças inclusive nas características de força e resistência.

Morris et al. (1996) relataram que a parede celular, em *S. cerevisiae*, é altamente elástica, pois as células podem diminuir rapidamente seu volume em soluções hipertônicas como resposta à pressão osmótica, e retornar ao volume original em condições ambientais favoráveis. Segundo Rees et al. (1982), a propriedade de elasticidade pode ser atribuída às cadeias de β(1-3)glucana organizadas numa espécie de mola flexível de extensão variada. Esta organização na parede permite o aumento ou redução do volume celular em resposta às condições externas e explica, em parte, a maior permeabilidade em células vivas do que na parede isolada (De NOBEL et al., 1990; De NOBEL; BARNETT, 1991). Já, a β(1-6)glucana serve de âncora para a estrutura e integridade da camada de manoproteínas (ROEMER et al., 1994), sendo sua síntese vinculada a expressão de genes de proteínas específicas e características da parede celular (SIMONS; EBERSOLD; HELENIUS, 1998).

β-glucanas: constituição e características

As β-glucanas são moléculas altamente ordenadas, que se diferenciam pelo tipo de ligação entre as unidades de D-glicose (anéis tipo β-D-glucopiranose) da cadeia principal e, pelas ramificações que se conectam a essa cadeia (BROWN; GORDON, 2001).

Uma importante fonte de β-glucana é a parede celular de leveduras, particularmente da levedura de fermentação *S. cerevisiae* (DIJKGRAAF; LI; BUSSEY, 2002). Isto porque, o componente presente em maior quantidade na parede celular de *S. cerevisiae* é uma β-glucana constituída por um esqueleto linear central de unidades de glicose ligadas na posição β(1-3), com cadeias laterais de tamanhos variados, também de glicose porém, unidas em β(1-6). As cadeias laterais podem ocorrer em diferentes intervalos ao longo do esqueleto central (MANNERS et al., 1973; Di LUZIO et al., 1979).

Amplamente distribuídas na natureza, as β-glucanas estão presentes em fungos, leveduras, algas, bactérias e plantas superiores apresentando diferentes estruturas (MIURA et al., 2003). Em cereais, como cevada e aveia, a β-glucana da parede do endosperma apresenta, além de β(1-3), ligações β(1-4), formando polímeros complexos de polissacarídeo-proteína (BHATTY, 1993). De modo geral, embora ligações β(1-4), ou β(1-6) possam estar presentes na estrutura, além de β(1-3), os estudos relatam estes polímeros somente como glucana ou β-glucana (FREIMUND et al., 2003). Em seu estudo, Pelosi et al. (2003) comentaram que também o nome genérico β(1-3)glucana é utilizado para referência a glucanas contendo somente ligações β(1-3), ou além destas, porção variável de β(1-4) ou β(1-6) conectadas a cadeia principal.

Quanto a solubilidade em álcali, duas frações de β(1-3)glucana são encontradas na parede celular de *S. cerevisiae*, sendo uma solúvel e outra insolúvel. A porção insolúvel contém de 3 a 6% de ramificações unidas em β(1-6) e representa o maior componente da parede. A porção solúvel, que representa de 15 a 20% tem estrutura semelhante a insolúvel, porém com maior número de ramificações β(1-6) (MANNERS et al., 1973; SHAHINIAN; BUSSEY, 2000). Conforme Kapteyn et al. (1997) os heteropolímeros β(1-3)/β(1-6)glucana identificados como alcalino-solúveis em *S. cerevisiae*, tornam-se alcalino-insolúveis através da ligação entre o resíduo terminal reduzido β(1-4) da quitina e o terminal não reduzido da β(1-3)glucana.

As β(1-3)glucanas têm tamanho estimado de 1500 resíduos de glicose e as β(1-6)glucanas apresentam de 150 a 200 resíduos e, na parede de *S. cerevisiae* são rearranjadas por ramificações introduzidas por glicosiltransferases (STRATFORD, 1994). Segundo Lee et al. (2002), a solubilidade em água depende do número de resíduos de glicose unidos em β(1-6) das cadeias laterais, e do grau de polimerização. E, de acordo com Klis et al. (2002) o grau de polimerização da β(1-3)glucana pode variar com as condições em que a levedura se encontra. Neste contexto, sabe-se que proteínas essenciais para a síntese da β-glucana são dependentes de condições ambientais (CABIB; DRGANOVÁ; DRGON, 1998).

Considerando as estruturas tri-dimensionais já observadas, β(1-3)glucanas com a mesma composição química podem apresentar conformação de hélice simples, tripla hélice ou em forma de espiral (YOUNG; JACOBS, 1998). As hélices são compostas por uma, ou três cadeias de polissacarídeos que permanecem unidas por pontes de hidrogênio (LIPKE, 1998), formando uma estrutura helicoidal. Aparentemente, pelo menos uma porção da β(1-3)glucana assume estrutura helicoidal (KRAINER et al., 1994).

Dentre as conformações já descritas, a hélice simples e a tripla hélice são consideradas biologicamente ativas. Porém, a conformação em tripla hélice é sugerida como aquela que apresenta maior atividade biológica (HA et al., 2002; KO; LIN, 2004).

Bioatividade da β-glucana

Alguns polissacarídeos, como β-glucanas obtidas de fungos, bactérias e leveduras pertencem a uma classe de substâncias conhecidas como modificadores da resposta biológica (MRBs), pois alteram a resposta no hospedeiro pelo estímulo do sistema imune (BOHN; BeMILLER, 1995). Esses polímeros ativam a resposta imune via sistema complemento, diretamente ou, com auxílio de anticorpos, e produzem fatores quimiotáticos que induzem a migração de leucócitos para o sítio da infecção (NICHOLAS; SHAUN, 2001).

As propriedades biológicas de preparações brutas de β(1-3)glucanas vem sendo estudadas desde a década de 50 (PILLEMER et al., 1956) após a obtenção do zymosan, que é o extrato insolúvel da parede celular de *S. cerevisiae* (HASSID; JOSLYN; McCREADY, 1941). Descrita por DiCarlo e Fiore (1958) a composição do zymosan inclui proteínas, quitina, β-glucana, mananas e lipídeos, sendo a β-glucana o constituinte biologicamente ativo (FITZPATRICK; DiCARLO, 1964).

Utilizado como um modificador da resposta biológica, o zymosan é reconhecido como ativador de uma via alternativa do sistema complemento (HIDA et al., 2006). Desde sua descoberta diversos estudos das funções imunes, in vivo e in vitro, envolvendo β-glucanas foram realizados (BROWN; GORDON, 2003).

A atividade biológica da β-glucana vem sendo estudada com atenção nos últimos anos especialmente, pela habilidade em ativar mecanismos de defesa no hospedeiro (YUN et al., 2003; KO; LIN, 2004; KIM et al., 2006). Além disso, outros efeitos, como antitumoral, antinflamatório, antimutagênico, hipocolesterolêmico, hipoglicêmico e proteção contra infecções vêm sendo avaliados e comprovados (KOGAN, 2000; LIN et al., 2004; BEHALL et al., 2006; KIM et al., 2006). A β-glucana é considerada um candidato promissor para uso como agente imunoestimulante em pacientes imunocomprometidos, pois estes ficam mais propensos à infecção por bactérias multiresistentes a drogas (KULICKE; LETTAU; THIELKING, 1997).

E, conforme Kim e Yun (2006), a β-glucana obtida da parede celular de leveduras parece ser mais efetiva do que aquelas obtidas de outras fontes.

Atividade imunomodulatória: reconhecimento da β-glucana pelo sistema imune

A β-glucana é designada como um modificador da resposta biológica, pois, ao ser reconhecida pelo organismo possui a capacidade de desencadear uma série de eventos na resposta imune (BOHN; BeMILLER, 1995). Seu reconhecimento pelo sistema imune de vertebrados foi primeiramente descrito em estudos envolvendo patógenos fúngicos (ADEREM; ULEVITCH, 2000). Neste aspecto, é sabido que alguns fungos patogênicos mascaram sua β-glucana, ou pelo menos parte dela, evitando o reconhecimento pelas células de defesa do hospedeiro e, assim minimizando significativamente as respostas pró-inflamatórias para a infecção (HERRE et al., 2004). Essa camuflagem da β-glucana, que é resultado da expressão de genes sabidamente conservados em fungos, também favorece a colonização comensal de fungos oportunistas, que por sua vez tornam o hospedeiro mais propenso a doenças (WHEELER; FINK, 2006).

As respostas à β-glucana em vertebrados têm início com o reconhecimento por receptores presentes na superfície celular (BROWN; GORDON, 2005). Estes receptores já foram identificados em células imunes como macrófagos/monócitos, neutrófilos e células natural killer (NK). Além disso, também foram descritos em células não imunes como endoteliais, fibroblastos, do epitélio alveolar e de Langerhans (BROWN; GORDON, 2003). Conforme Tokunaka et al. (2002), o reconhecimento da β-glucana pelos receptores pode ser influenciado pela solubilidade do polissacarídeo. Também a conformação do polímero em solução foi sugerida por Chorvatovicová, Machová e Sandula

(1996) como um importante fator para a ligação aos receptores de uma dada atividade biológica.

A estrutura que permite o reconhecimento pelo sistema imune está associada a padrões moleculares (PAMPs), que normalmente são essenciais para sobrevivência de patógenos microbianos. Entre as PAMPs mais conhecidas estão as β-glucanas que desencadeiam respostas para proteger o hospedeiro contra invasão de patógenos, caracterizando a imunidade inata de organismos superiores (BROWN; GORDON, 2005).

Inúmeros receptores estão relacionados ao reconhecimento da β-glucana em vertebrados, incluindo dectin-1, Receptor do Sistema Complemento 3 (CR3), lactosilceramida e scavenger receptors. O mecanismo de resposta parece ser mediado pela combinação destes (BATTLE et al., 1998), porém o papel melhor esclarecido é o da dectin-1.

Caracterizada como uma glicoproteína transmembrana tipo II, a dectin-1 possui um domínio extracelular CDR responsável pelo reconhecimento de carboidratos e, uma cauda citoplasmática com o imunoreceptor ITAM (tirosine-based activating motif). O imunoreceptor pode ser fosforilado pelo estímulo da molécula de β-glucana e, ao que tudo indica, esta fosforilação está envolvida na produção de superóxido pelos macrófagos em resposta de defesa (BROWN; GORDON, 2001).

A expressão da dectin-1 é predominante em monócitos, macrófagos, linhagens de neutrófilos e células dendríticas, sendo significativamente influenciada por citocinas e produtos microbianos (TAYLOR et al., 2002; WILLMENT; GORDON; BROWN, 2001). Embora seja expressa de forma heterogênea nos tecidos, dectin-1 é um receptor que tem distribuição consistente com a tarefa de vigilância contra patógenos (REID et al., 2004). Esta glicoproteína reconhece β-glucanas solúveis e particuladas, incluindo partículas fúngicas intactas e também possui um ligante para células T (ARIIZUMI et al., 2000; STEELE et al., 2003).

A dectin-1 pode fazer a mediação de diversas respostas celulares incluindo fagocitose, endocitose e burst oxidativo. Adicionalmente, em colaboração com receptores toll-like, dectin-1 pode induzir a produção de citocinas e quimiocinas pró-inflamatórias como Fator de Necrose Tumoral Alfa (TNF-α), Proteína Inflamatória de Macrófagos-2 (MIP-2) e Interleucina-12 (IL-12) (BROWN; GORDON, 2003; GANTNER et al., 2003; HERRE et al., 2004). De acordo com Balloy et al. (2005) os receptores celulares toll-like disparam no sistema imune uma complexa sinalização em cascata após o reconhecimento de partículas fúngicas.

Outro receptor envolvido no reconhecimento e resposta imune para a β-glucana é o CR3, uma integrina heterodimérica expressa em células mielóides, NK e alguns linfócitos. CR3 possui um sítio de ligação para carboidratos, localizado no carbono terminal, e funciona como molécula de adesão celular, além de receptor fagócito para a β-glucana (THORNTON et al., 1996; XIA; ROSS, 1999). Este receptor estimula a secreção de citocinas em células NK, principalmente na presença de patógenos (ROSS et al., 1999) contudo, seu mecanismo de ação não é conhecido em detalhes (GANTNER et al., 2003).

Encontrado em diversos tipos de células, o receptor de superfície lactosilceramida é um glicoesfingolipídio que forma microdomínios na membrana plasmática. A interação deste receptor com a β-glucana realça o burst oxidativo e as funções antimicrobianas, induz a ativação da citocina Fator Nuclear kappa B (NFκB) e a produção da MIP-2 nas células do epitélio alveolar (WAKSHULL et al., 1999; HAHN et al., 2003).

Existe também um grupo heterogêneo de moléculas, chamado Scavenger receptors que reconhece lipoproteínas modificadas de baixa densidade (PEISER, MUKHOPADHYAY, GORDON, 2002) e que é capaz de reconhecer a estrutura básica da β-glucana. Sua expressão ocorre em algumas células mielóides e endoteliais e parece

que estas moléculas estão envolvidas na homeostase e imunidade, porém, sua afinidade para carboidratos é significativamente afetada pela quantidade de polímeros (RICE et al., 2002).

Atividade imunomodulatória da β-glucana

O efeito imunomodulador das β-glucanas está envolvido tanto na imunidade humoral quanto na celular (SOLTÝS; QUINN, 1999, TOKUNAKA et al., 2002; TAKAHASHI et al., 2001; KUBALA et al., 2003). O sistema imune do hospedeiro é estimulado pela β-glucana em resposta à formação de tumores (KOGAN et al., 2002; KHALIKOVA et al., 2005) e contra infecções (TZIANABOS, 2000). Existem diversos relatos que comprovam o efeito imunomodulatório em infecções de origem bacteriana (TZIANABOS; CISNEROS, 1996; LIANG et al., 1998), viral (REYNOLDS et al., 1980; JUNG et al., 2004), fúngica (MEIRA et al., 1996) e parasitária (HOLBROOK; COOK; PARKER, 1981).

Os mecanismos envolvidos na resposta imune desencadeada pela β-glucana incluem estímulo da hematopoiese (HOFER; POSPISIL, 1997), ativação de macrófagos, neutrófilos e de células NK (LEE et al., 2001; BROWN; GORDON, 2001; XIAO; TRINCADO; MURTAGH, 2004). Neste contexto, Kubala et al. (2003) relataram que a modulação da atividade celular da β-glucana inicia com a ativação de macrófagos, células endoteliais e dendríticas, células B e T, e, linfócitos polimorfonucleares. Além disso, envolvem a resposta imune específica pela indução da expressão de diversas citocinas como TNF-α, IL-6, IL-8 e IL-12 (Di RENZO; YEFENOF; KLEIN, 1991; LEE et al., 2001; TSIAPALI et al., 2001; XIAO; TRINCADO; MURTAGH, 2004; MOON et al., 2005). A relação entre a resposta de defesa desencadeada pela β-glucana e a produção de mediadores específicos vem sendo estudada na busca de esclarecimento dos mecanismos envolvidos. Sob este aspecto, Berner et al. (2005) sugeriram a possibilidade de sinergismo entre a β-glucana e a citocina Interferon Alfa (IFN-α) na diminuição do limiar de sensitividade da resposta imune inata para patógenos fúngicos.

Como biomodulador, a β-glucana apresenta ainda habilidade para deprimir respostas pró-inflamatórias associadas à septicemia bacteriana. Isto ocorre através da remoção de óxido nítrico e células produtoras de citocinas do tipo TNF-α, IL-1 e IL-6 dos locais de inflamação, e aumento dos níveis de mediadores antinflamatórios, como IL-10 e Proteína Quimiotática de Monócitos-1 (MCP-1). Dessa forma, o efeito imunomodulador diminui a possibilidade de choque endotóxico e morte do hospedeiro (TZIANABOS, 2000). Em seu estudo, Luhm et al. (2006) relataram que a imunomodulação pela β-glucana em respostas pró-inflamatórias envolve após a ligação do receptor com a β-glucana, uma sinalização em cascata mediada pela IL-1 para que a reação de defesa pró-inflamatória seja alterada para uma resposta imune antiinflamatória. Pelo exposto, conhecer a ação de um imunomodulador é importante, pois permite identificar aspectos que melhoram a resposta imune do hospedeiro durante o curso de uma infecção e, adotar estratégias para sua prevenção (TZIANABOS, 2000).

O mecanismo de ação imunomodulatória das β-glucanas ainda não está totalmente esclarecido, mas parece estar relacionado ao peso molecular, tipo de ligações glicosídicas, resíduos presentes, solubilidade em água, conformação espacial e grau de polimerização (OHNO et al., 1996; TOKUNAKA et al., 2002; FREIMUND et al., 2003; KUBALA et al., 2003). Conforme Manners (1973), variações estruturais em uma molécula como ramificações, tipo de ligações presentes e peso molecular podem definir seu mecanismo de ação, que é fundamental para a modulação do sistema imune.

O grau de ramificação pode influenciar na bioatividade da β-glucana. Conforme relatado por Chorvatovicová, Machová e Sandula (1996) a solubilidade da β-glucana depende do grau de ramificação, sendo que aquelas altamente ramificadas

são solúveis em água. Então, supostamente, β-glucanas com elevado grau de ramificações e, consequentemente solúveis, seriam mais eficientes na ativação da resposta imune do hospedeiro.

Outro fator que pode contribuir para a atividade biológica da β-glucana é sua longa permanência nos sistemas de mamíferos, em virtude da ausência de β-glucanases (BROWN; GORDON, 2003). Isto permite que o polímero fique acumulado no fígado, baço e outros órgãos do sistema reticuloendotelial sem sofrer mudanças estruturais significativas. A metabolização da β-glucana é muito lenta e ocorre principalmente por oxidação (NONO et al., 1991; SUDA et al., 1992; MIURA et al., 1998), ou pela secreção através de filtração glomerular (SUDA et al., 1996). Um estudo realizado por Ohno et al. (1999) sugeriu relação entre a longa permanência da β-glucana no organismo dos mamíferos e sua atividade imunoestimulante. Após obter uma β-glucana solubilizada por oxidação, com estrutura possivelmente similar àquela depositada em mamíferos, a atividade biológica foi avaliada, sendo constatado estímulo para biossíntese de IL-8 e atividade antitumoral.

O peso molecular do polissacarídeo também parece estar relacionado com a atividade biológica. β-glucanas de elevado peso molecular ativam diretamente leucócitos, estimulando a atividade fagocítica e citotóxica, bem como a produção de mediadores pró-inflamatórios como citocinas e quimiocinas (WILLIAMS; MUELLER; BROWDER, 1996; BROWN; GORDON, 2003), consideradas mediadores chave da resposta imune humoral para uma infecção (OHATA et al., 2003). Ainda no que se refere ao peso molecular, é sabido que β-glucanas de peso molecular intermediário ou baixo possuem atividade biológica in vivo, porém, seus efeitos celulares são menos conhecidos. Geralmente quando muito curtas as β-glucanas são consideradas inativas para imunomodulação (BROWN; GORDON, 2003).

Muckosová, Babicek e Pospisil (2001) relataram que propriedades fisico-químicas e a habilidade de absorção das células intestinais são fatores que podem limitar a eficiência de agentes imunomodulatórios. Estes pesquisadores analisaram os efeitos imunoestimulantes da β-glucana e de dois derivados solúveis, a carboximetilglucana (CMG) e a sulfoetilglucana (SEG), obtidos de *S. cerevisiae*, administrados via oral e intraperitoneal para camundongos estimulados com lipopolissacarídeos (LPS). As duas vias de administração promoveram melhora significativa na produção de ácido nítrico e atividade de peroxidase das células aderentes peritoniais, porém, a via intraperitoneal foi mais eficaz.

Contudo, podem ocorrer efeitos adversos da administração intraperitoneal e intravenosa de β-glucanas de alto peso molecular, pois elas exibem afinidade para inúmeras ligações, além de atividades biológicas variadas (KUBALA et al., 2003). Conforme Chorvatovicová, Machová e Sandula (1996) a administração oral pode apresentar vantagens, embora o alto peso molecular e a estrutura complexa da β-glucana possam interferir na absorção pelo trato gastrointestinal. De acordo com Tsukada et al. (2003), a administração oral de β-glucana extraída de *S. cerevisiae* realça as funções dos linfócitos intraepiteliais do intestino e, portanto, é absorvida pelo mesmo. Em revisão sobre imunomoduladores, Tzianabos (2000) considerou que a dose, a via e o sincronismo de administração de um biomodulador podem realçar ou suprimir a resposta imune do hospedeiro.

Atividade anticarcinogênica

Anticarcinogênicos são substâncias capazes de impedir, retardar ou reduzir o surgimento, ou desenvolvimento de neoplasias (KURODA; HARA, 1999). Neste aspecto, a introdução de biomoduladores combinados com quimioterapia contribuiu significativamente para a terapia antineoplásica. Dentre os compostos conhecidos como imunomoduladores, β-glucanas extraídas da parede celular de fungos e leveduras vem

sendo avaliadas por sua atividade antitumoral (MIADOKOVÁ et al., 2005).

Os estudos iniciais sobre o potencial uso da β-glucana contra o câncer foram realizados na década de 70. Já em 1979, Di Luzio et al. demonstraram que preparações de β-glucana de *S. cerevisiae* reduzem significativamente o crescimento de carcinomas mamários e melanomas. No mesmo estudo foi constatado aumento da sobrevida de camundongos com implante tumoral subcutâneo.

Atualmente, sabe-se que o mecanismo de ação antitumoral das β-glucanas, efetivo inclusive em situações de metástase, atua pela ativação e ampliação das diversas funções imunológicas do hospedeiro, constituindo-se de um tratamento que melhora a imunosobrevivência do hospedeiro ao tumor. Em adição, tem efeito antagônico à imunossupressão decorrente da quimioterapia e de tratamentos com irradiação (MOON et al., 2005). Os pesquisadores Xiao, Trincado e Murtagh (2004) relataram que os efeitos antitumorais da β-glucana são baseados principalmente, na habilidade de ativar leucócitos pelo estímulo da atividade fagocítica e produção de citocinas como o TNF-α.

Em estudo de Hofer e Pospisil (1997), preparações de β-glucana de *S. cerevisiae* foram administradas a camundongos antes e depois de regimes sub-letais de irradiação gama. Os resultados revelaram estímulo da hematopoiese em todos os camundongos, inclusive no grupo controle. Segundo os pesquisadores o estímulo da produção de células sanguíneas na medula óssea, e consequentemente o aumento das células de defesa, conferiu proteção contra os efeitos da radiação e proporcionou aumento na sobrevida dos camundongos irradiados.

Inúmeras pesquisas realizadas, principalmente no Japão, comprovaram que a β-glucana de levedura realça os efeitos do tratamento quimioterápico e melhora a qualidade da sobrevida com pacientes de câncer tratados com radiação (KIM et al., 2006). No referido país, algumas β-glucanas licenciadas para o uso terapêutico vêm sendo utilizadas nas últimas décadas como adjuvantes na terapia antitumoral.

Derivados da β-glucana solúveis em água também apresentam potencial para a prevenção e terapia do câncer (MIADOKOVÁ et al., 2005). A administração de Carboximetilglucana (CMG) obtida de levedura durante o tratamento com ciclofosfamida, para o carcinoma de pulmão, contribuiu positivamente com significativo estímulo dos macrófagos (KOGAN et al., 2002). Conforme Miadoková et al. (2005) os derivados da β-glucana pertencem a uma das mais abundantes classes de biopolímeros que podem contribuir para a prevenção e terapia do câncer.

Demir et al. (2007) realizam estudo na Turkia, onde a β-glucana de *S. cerevisiae* foi administrada oralmente, durante 14 dias, para 23 mulheres com câncer de mama em estágio avançado. Foi constatada ativação de monócitos no sangue periférico, bem como estímulo da proliferação destes. Os exames clínicos não evidenciaram quaisquer efeitos colaterais decorrentes do uso via oral da β-glucana.

Atividade antimutagênica

Qualquer substância capaz de reduzir a freqüência de mutações espontâneas ou induzidas, independente do mecanismo de ação é considerada antimutagênica (WATERS et al., 1990). A atividade antimutagênica da β-glucana de *S. cerevisiae* vem sendo investigada, in vitro e in vivo, por diversos grupos de pesquisa. Compostos potencialmente mutagênicos como peróxido de hidrogênio, doxorrubicina e ciclofosfamida têm seus efeitos prejudiciais reduzidos pela administração deste polissacarídeo (CHORVATOVICOVÁ; MACHOVÁ; SANDULA, 1996; TOHAMY et al., 2003; SLAMENOVA et al, 2003; LIN et al., 2004).

Chorvatovicová, Machová e Sandula (1996) avaliaram os efeitos antimutagênicos da CMG, obtida

da β-glucana de *S. cerevisiae*, em camundongos utilizando a ciclofosfamina como agente indutor de danos ao DNA. A análise dos resultados demonstrou que a administração da CMG, tanto via parenteral, como oral reduziu os efeitos clastogênicos da ciclofosfamina.

Estudos in vitro de Slamenová et al. (2003) comprovaram a eficácia da CMG, SEG e carboximetilquitinaglucana (CMCG) na prevenção de lesões no DNA. A quantificação dos danos ao DNA em células pulmonares de hamsters V79 revelou maior eficiência da CMG no efeito preventivo. SEG e CMCG também foram eficientes, porém em menor grau. A prevenção, segundo os pesquisadores, ocorre pela reação da β-glucana com os radicais OH-, liberados na biotransformação do peróxido de hidrogênio.

Estudo posterior ao de Slamenová, (MIADOKOVÁ et al., 2005) evidenciou que a CMG pode também reduzir os efeitos mutagênicos causados pela ofloxacina, exercendo bioproteção contra mutações pelo efeito antitóxico.

Recentemente, a pesquisa de Oliveira et al. (2007) comprovou o efeito da β-glucana de *S. cerevisiae* contra lesões no DNA, induzidas pelo metilmetano sulfonado, em células ovarianas de hamsters chineses. A β-glucana foi testada com pré, simultâneo e pós-tratamento ao agente mutagênico. Os efeitos quimiopreventivos da β-glucana foram observados em todas as situações de tratamento, com índices de redução de danos ao DNA variando de 35 a 57,3%.

Efeitos da β-glucana na dieta

Os efeitos benéficos da ingestão continuada de β-glucana podem diminuir os riscos de doenças crônicas em humanos e animais. Em estudo sobre a atividade biológica de polissacarídeos da parede celular de *S. cerevisiae* na alimentação de suínos, Kogan e Kocher (2007) ressaltaram a importância do efeito protetor da β-glucana ao organismo pelo estímulo ao sistema imune comum de mucosas, que são áreas permanentemente expostas a patógenos.

A β-glucana tem se destacado entre os ingredientes utilizados para produção de alimentos funcionais (TOKUNAKA et al., 2002; RAMESH; THARANATHAN, 2003). Fragmentos obtidos a partir desta macromolécula, os oligossacarídeos, podem atuar como prebióticos estimulando seletivamente o crescimento de bactérias do trato intestinal, e servindo de fonte energética para a microflora benéfica (PRZEMYSLAW; PIOTR, 2003). Outros aspectos positivos, como redução dos níveis de colesterol e de açúcar no sangue, pela inclusão da β-glucana na dieta também já foram comprovados em humanos (KIM et al., 2006).

Nicolosi et al. (1999) estudaram os efeitos da ingestão de β-glucana de *S. cerevisiae*, adicionada ao suco de laranja, para os níveis de lipídios plasmáticos em homens obesos hipercolesterolêmicos. No estudo foi observada queda significativa no colesterol total e redução de 8% nos níveis séricos de colesterol LDL, após 8 semanas de consumo.

Wilson et al. (2004) avaliaram duas frações de β-glucana de cevada, sendo uma de baixo e outra de elevado peso molecular, para a diminuição de colesterol sérico utilizando hamsters com dieta hipercolesterolemica como modelo experimental. Os exames não evidenciaram diferenças na atividade das frações, sendo que ambas promoveram redução nos níveis de colesterol no soro. Semelhantes observações foram descritas por Matiazi (2005) na avaliação do efeito hipocolesterolêmico da β-glucana extraída de *S. cerevisiae* administrada a camundongos em dieta hipercolesterolêmica.

Obtenção de β-glucana de *S. cerevisiae*

Desde a descoberta das propriedades benéficas da β-glucana para animais e humanos, inúmeros processos de isolamento e purificação deste polissacarídeo tem sido desenvolvidos (FREIMUND et al., 2003).

A partir de linhagens de levedura a β-glucana pode ser obtida como material solúvel ou insolúvel. Preparações de β-glucana derivada de *S. cerevisiae* geralmente envolvem processos relativamente longos com diversas etapas de extração alcalina, ácida e orgânica e sucessivas lavagens (JAMAS; EASSON; OSTROFF , 1997). Três principais frações geralmente são obtidas na extração da parede celular de *S. cerevisiae*, sendo uma rica em manoproteínas com pouca β-glucana, outra contendo quitina e β-glucana insolúvel em álcali e uma terceira contendo β-glucana solúvel em álcali (MANNERS et al., 1973; FLEET; MANNERS, 1976). Uma etapa adicional no processamento de extração da β-glucana aplicada em alguns processamentos comerciais é o tratamento com ácido acético diluído para remover o glicogênio (BACON et al., 1969; JAMAS; EASSON; OSTROFF, 1997). Embora não seja um componente da parede celular e sim um material de reserva, o glicogênio pode ficar entre as cadeias de glucana e interferir no resultado da extração (THANARDKIT et al., 2002). Enzimas hidrolíticas seletivas também representam uma alternativa para obtenção dos componentes da parede celular (SHIBATA et al., 1986).

LEE et al. (2001) utilizaram passos seqüenciais de purificação na obtenção de β-glucanas de *S. cerevisiae*. Inicialmente com extração alcalina e posterior tratamento ácido obtiveram a glucan-p1 contendo 0.8% de proteínas, que após cromatografia de alta eficiência (CLAE) resultou na glucan-p2 contendo 0.3% de proteínas. Através da cromatografia de afinidade em gel de agarose da glucan-p2 foi obtida a glucan-p3, livre de proteínas. As β-glucanas resultantes de cada passo da purificação foram avaliadas quanto à eficiência para a ativação de macrófagos. A glucan-p3 foi a mais eficaz dentre as três, evidenciando que as proteínas remanescentes do processo de extração interferem na bioatividade. Neste aspecto, Bohn e BeMiller (1995) comentaram que efeitos colaterais decorrentes do uso de extratos de β-glucana, geralmente, estão associados à presença de manoproteínas nas preparações extraídas.

O fato de ser insolúvel em água, e parte insolúvel em álcali dificulta a extração da β-glucana e, além disso, o rendimento nos processos tradicionais é pouco satisfatório (OHNO et al., 1999). Portanto, a possibilidade de simplificação e otimização dos processos de extração, sem o comprometimento das propriedades imunoestimulantes, traz inúmeros benefícios (SUPHANTHARIKA et al., 2003).

Ohno et al. (1999) obtiveram uma β-glucana de levedura, com solubilidade em solução diluída de hidróxido de sódio (NaOH), utilizando oxidação com hipoclorito de sódio (NaClO) e subseqüente extração com dimetil sulfóxido (DMSO). Conforme os pesquisadores, além do tempo reduzido e rendimento satisfatório, a extração manteve a atividade biológica da β-glucana. Posteriormente, o mesmo método foi empregado por Ishibashi et al. (2004) para extração de β-glucana de Aspergillus spp., e os resultados obtidos foram similares aos de Ohno et al. (1999).

Em seu estudo, Suphantharika et al. (2003) otimizaram a concentração de NaOH, a temperatura e o tempo de extração para a obtenção da β-glucana da parede de levedura descartada em cervejaria. Após autólise das células de levedura a 50 ºC por 24 horas, uma única etapa foi proposta para extração alcalina, tendo como condições ótimas: 5 volumes de NaOH 1N a 90ºC durante 1 hora. As propriedades imunoestimulantes da β-glucana foram comprovadas in vitro e in vivo em administração oral à camarões. Neste contexto, Jamas, Easson e Ostroff (1997) comentaram que no tratamento com álcali, em temperaturas elevadas, são hidrolizados e solubilizados proteínas celulares, mananas, frações solúveis de β-glucana, ácidos nucléicos e lipídeos polares, que ficam na fração sobrenadante, deixando as β-glucanas insolúveis na fração sólida. Conforme Thanardkit et al. (2002), as proteínas podem ser removidas na extração alcalina devido a alta concentração de NaOH e elevada temperatura , pois o uso soluções pouco concentradas e baixas temperaturas resultaria em preparações de β-glucana com conteúdo elevado de proteína residual.

Outra pesquisa de otimização foi realizada por Matiazi (2005), para as condições de pH e temperatura na extração de β-glucana de *S. cerevisiae* oriunda de usina sucroalcoleira. Utilizando NaOH e ácido sulfúrico (H2SO4) para extração, os valores que permitiram o maior rendimento de β(1-3)/(1-6)glucana foram temperatura entre 68 e 78ºC e pH entre 9,7 e 10,5.

Com o objetivo de aumentar a especificidade e o rendimento da extração de β-glucana a partir de leveduras, métodos envolvendo etapas de digestão enzimática tem sido descritos (MAGNELLI; CIPOLLO; ABEIJON, 2002; LIU et al., 2006).

Um processo envolvendo extração com água quente, homogeneização, solvente orgânico e proteases para extração de β-glucana de *S. cerevisiae* foi apresentado por Liu et al. (2006). O processo aplica etapas "suaves" que não degradam as cadeias de β-glucana, mantendo sua estrutura original, além de permitir rendimento satisfatório e pureza. Outra consideração, não menos importante, é que o método proposto para a obtenção da β-glucana não utiliza produtos químicos agressivos ao meio ambiente.

A busca de novos métodos, que priorizam etapas pouco agressivas durante a extração da β-glucana, e possibilitam manter o máximo da estrutura original é de considerável importância. Além das etapas de extração, deve-se considerar também o processo empregado para secagem, onde o polímero também pode ser danificado. Hromádková et al. (2003) avaliaram a influência de três diferentes métodos de secagem nas propriedades físicas e imunomodulatórias da β-glucana de *S. cerevisiae*. As amostras foram secas através de liofilização, extração de solvente e spray drying, sendo posteriormente analisadas quanto às características reológicas da dispersão aquosa da β-glucana, estrutura, conformação e atividade imunomodulatória. O método de secagem afetou consideravelmente a microestrutura das partículas de β-glucana, que apresentaram diferenças nas propriedades físicas. Os resultados do estudo sugerem que estas diferenças influenciam significativamente também a atividade imunomodulatória, sendo que, a β-glucana seca em spray drying apresentou atividade duas vezes maior nos ensaios imunológicos, quando comparada às amostras provenientes dos demais métodos testados.

Alterações estruturais que proporcionam o aumento da solubilidade da β-glucana podem aumentar sua atividade imunomoduladora e antitumoral (BOHN; BeMILLER, 1995). Tokunaka et al. (2002) comentaram que a solubilidade afeta o efeito imunológico, pois exerce influência na ligação com o receptor. Sob este ponto de vista, Chorvatovicová, Machová e Sandula (1996) sugeriram que a solubilidade em água e a bioatividade das glucanas estão relacionadas ao seu grau de ramificação, sendo que cadeias altamente ramificadas são solúveis.

Diferentes métodos, como hidrólise ácida parcial e alcalina, digestão enzimática, fosforilação, sulfonilação, sulfatação, carboximetilação, irradiação de ultrasson e aminação podem ser aplicados na despolimerização de biopolímeros como a β-glucana, resultando em fragmentos solúveis e de menor massa molecular (SANDULA et al., 1999).

Nos últimos anos, inúmeras pesquisas vêm sendo realizadas para identificar características estruturais e biológicas de derivados da β-glucana solúveis em água, na expectativa de viabilizar seu uso clínico (SAKURAI et al., 1997; OHNO et al., 1999; MIURA et al., 2003; ISHIBASHI et al., 2004; MIADOKOVÁ et al., 2005; HARADA et al., 2006).

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4 ARTIGO I

Optimized methodology for extraction of (1→3)(1→6)-β-D-glucan from *Saccharomyces cerevisiae* and in vitro evaluation of the cytotoxicity and genotoxicity of the corresponding carboxymethyl derivative.

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Optimized methodology for extraction of (1 → 3)(1 → 6)-β-D-glucan from *Saccharomyces cerevisiae* and *in vitro* evaluation of the cytotoxicity and genotoxicity of the corresponding carboxymethyl derivative

Marciane Magnani [a,*], Caroline M. Calliari [a], Fernando C. de Macedo Jr. [b], Mateus P. Mori [c], Ilce M. de Syllos Cólus [c], Raul J.H. Castro-Gomez [a]

[a] Departamento de Ciência e Tecnologia de Alimentos, Universidade Estadual de Londrina, Londrina, Paraná, Brazil
[b] Departamento de Química, Universidade Estadual de Londrina, Londrina, Paraná, Brazil
[c] Departamento de Biologia Geral, Universidade Estadual de Londrina, Londrina, Paraná, Brazil

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ABSTRACT

The cell wall of *Saccharomyces cerevisiae* is an important source of β-D-glucan, a glucose homopolymer with immunostimulant properties. The standard methodologies described for its extraction involve acid and alkaline washings, which degrade part of its glucose chains and reduce the final yield. In the present study, an optimized methodology for extraction of β-D-glucan from *S. cerevisiae* cells, involving sonication and enzyme treatment, with a yield of 11.08 ± 0.19%, was developed. The high-purity (1 → 3)(1 → 6)-β-D-glucan was derivatized to carboxymethyl-glucan (CM-G). *In vitro* tests with CM-G in Chinese hamster epithelial cells (CHO-k1) did not reveal any cytotoxic or genotoxic effects or influences of this molecule on cell viability. The method described here is a convenient alternative for the extraction of (1 → 3)(1 → 6)-β-D-glucan under mild conditions without the generation of wastes that could be potentially harmful to the environment.

1. Introduction

β-D-Glucans are glucose homopolymer constituents of the cell wall of cereals, fungi and yeasts, which differ by the type of glycosidic linkage between glycopyranose residues. An important source of this polysaccharide is the cell wall of *Saccharomyces cerevisiae*, which represents up to 20% of the cell dry weight. Around 60% of this total corresponds to the β-glucans, which occur as long chains with about 1500 residues of β(1 → 3) glucose (Klis, Mol, Hellingwerf, & Brul, 2002) and short chains with approximately 190 β(1 → 6) glycosidic units (Aimanianda et al., 2009). β-D-Glucan from *S. cerevisiae* has been studied extensively, especially for its immunostimulatory potential. *In vitro* and *in vivo* studies have revealed that the immunomodulatory properties of β-D-glucan are related to the structure of the polymer, its molecular weight and side chains (Bohn & BeMiller, 1985). The methods usually employed to extract β-D-glucans from the cell wall of *S. cerevisiae* involve acid and alkaline washings, which lead to degradation of glucose chains (Liu, Wang, Cui, & Liu, 2008). Its exposure to these conditions, even for short periods of time, results in unsatisfactory yields and limited purity, and generates waste potentially harmful to the environment (Freimund, Sauter, Kappeli, & Dutler, 2003). Thus, less aggressive methods have been described in an attempt to replace the harsh conditions of alkaline extraction.

Freimund et al. (2003) reported extraction with hot water and enzyme treatment as an alternative method for obtaining β-D-glucan from *S. cerevisiae*. Later, Liu et al. (2008) proposed an additional high-pressure homogenization step to aid in disrupting the yeast cell wall, and observed not only satisfactory yields, but also preservation of the glucose chains of the polymer. A limiting and possibly the most important factor in the use of β-D-glucan extracted from *S. cerevisiae* as an immunostimulating agent is its insolubility in water. According to Mantovani et al. (2008), this characteristic limits its β-glucan application and the extrapolation of experimental *in vitro* data. A technique frequently used to increase its solubility is the derivatization of the glucan molecule (Slamenová et al., 2003) to carboxymethyl-glucan (CM-G), one of its most studied derivatives. The aim of this study was to develop a method to extract (1 → 3)(1 → 6)-β-D-glucan from the cell wall of *S. cerevisiae* via optimized steps involving sonication, removal of lipids and enzyme treatment. To assess the potential for the use of the immunostimulatory potential of β-D-glucan, CM-G was obtained and evaluated *in vitro* for its cytotoxicity, genotoxicity and cell viability effects in Chinese hamster ovary epithelial cells (CHO-k1).

* Corresponding author. Address: Departamento de Ciência e Tecnologia de Alimentos, Universidade Estadual de Londrina, Rod Celso Garcia Cid, PR 445, Km 380, Campus Universitário, C.P. 6001, CEP 86051-990, Londrina, Paraná, Brazil. Tel.: +55 43 3371 4565; fax: +55 43 3371 4080.
E-mail address: magnani2@gmail.com (M. Magnani).

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2. Experimental

2.1. Materials

Saccharomyces cerevisiae cells were obtained from pressed mass of yeast, 28% on dry basis (ITAIQUARA®). The protamex enzyme was provided by Novozymes® Latin America Ltda. All reagents used were of analytical grade.

2.2. Analytical methods

2.2.1. Carbohydrate determination

Total carbohydrate content was determined via the phenol-sulfuric method according to Dubois, Gilles, Hamilton, Rebers, and Smith (1956), using glucose for the standard curve. The qualitative analysis of carbohydrates in the proteolysis optimization was achieved by thin layer chromatography (TLC), according to the methods of Moreira, Souza, and Vendruscolo (1998), in reference to glucose and mannose standards from Sigma®.

2.2.2. Protein determination

Total soluble protein content was determined via the method described by Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin (Sigma®) for the standard curve. Total nitrogen (N) was measured via the micro-Kjeldahl method (Tecnal® (TE-036/1)), using 6.25 as conversion factor.

2.2.3. Fat determination

Total fat content was determined according to the AOAC method (1990) (Soxlet Tecnal® (TE-188)), with petroleum ether as the organic solvent.

2.2.4. Analysis of macro- and micro-elements

Analyses were conducted by plasma atomic emission spectrometry (ICP-ICAP 61E, Thermo JARREL Ash Corporation), or with a Flame Photometer (Micronal®).

2.2.5. Infrared spectroscopy

The infrared spectra of β-D-glucan and CM-G were obtained in an FT-IR spectrophotometer Shimadzu model 3300. KBr pellets were used for the preparation of samples, and the readings were considered to have a deviation of ±2 cm^{-1}.

2.2.6. Nuclear magnetic resonance spectroscopy (NMR)

The ^{13}C and ^{1}H NMR spectra were obtained from 50 mg of (1 → 3)(1 → 6)-β-D-glucan dissolved in d_6-DMSO or 50 mg of CM-G in D_2O by a Gemini–Varian Spectrometer equipped with a NMR300-OXFRORD magnet, operating at 75.449 MHz for ^{13}C and 300.059 MHz for ^{1}H. The chemical shifts were expressed in ppm in reference to the peaks of the solvents (δ_H 2:49 and δ_C 39.50 ppm for d_6-DMSO and δ_H 4.78 for D_2O).

2.2.7. Statistical analysis

The results were analyzed with Statistica 7.0, with $p \leqslant .05$ representing statistically significant data, and experimental validations performed in triplicate.

2.3. Procedures

2.3.1. Extraction of (1 → 3)(1 → 6)-β-D-glucan

2.3.1.1. Autolysis and hot water treatment. Autolysis and hot water treatment were performed according to the methods of Liu et al. (2008) with modifications. A 30% (w/w) cell suspension, pH 5.0, was added to 3% NaCl (w/v) and incubated for 24 h at 55 °C in a water bath with mild agitation. Then, the material was heated to 85 °C, maintained at that temperature for 15 min, and cooled down to 25 °C; the mixture was then centrifuged at 4500*g* for 10 min. The autolysis rate was defined as the total loss of biomass during the process.

The material resulting from the autolysis was adjusted to a 30% dilution in sodium phosphate buffer, 0.02 M, pH 7.5, to which glass spheres (0.4 mm) were then added. The suspension was heated to 121 °C in an autoclave and maintained at that temperature for 4 h. The insoluble residue was separated and washed three times, with centrifugation at 4500*g* for 7 min at room temperature after each wash, and was subsequently maintained at 4 °C.

2.3.1.2. Sonication. To optimize the sonication time and concentration of the cell suspension, tests were conducted at 20 kHz and 150 W in an ice bath via a 3^2 full factorial design, with three replicates of the central point. The times studied, in minutes, were 2, 4 and 6 for cell dilutions of 10%, 15% and 20% in distilled water, respectively. After centrifugation at 4500*g* for 15 min at 10 °C, the slides were prepared and submitted to Gram-staining to differentiate intact cells (violet) from those with ruptured walls (pink) (Liu et al., 2008). The material was observed under 100× magnification using a Photonic microscope coupled to a video camera and displayed using the Motic Images Plus 2.0 program. The centesimal count of treatments was performed in three fields in triplicate to obtain the mean. Control counts were performed before sonication at each cell concentration tested. The results were submitted to ANOVA and Tukey tests, and the best conditions were experimentally validated.

2.3.1.3. Lipid extraction. After sonication, the lipids were extracted with isopropanol and petroleum ether. For extraction with isopropanol, the procedure was conducted according to methods of Liu et al. (2008). For extraction with petroleum ether, the sample was wrapped with filter paper and positioned so that the petroleum ether passed throughout the sample for 2 h under reflux. The recovered residue was washed two times with acetone 1:1 (w/v), centrifuged at 4500*g* for 5 min, and then stored at 4 °C. The tests were performed in triplicate and were analyzed using the ANOVA and Tukey tests.

2.3.1.4. Proteolysis of the cell wall. The protamex enzyme has been reported as effective for the removal of remaining proteins from the hot water extraction (Liu et al., 2008), with optimum conditions of 5 h at 55 °C and pH 7.5. However, the amount of protease that allows for the total removal of proteins during extraction of β-D-glucan was not reported. Moreover, considering the protein nature of protamex, the necessary number of washes to ensure its total removal after hydrolysis was determined.

To determine the optimum amount of enzyme necessary, duplicate tests with 0.05 U, 0.1 U, 0.2 U, 0.3 U, 0.4 U and 0.5 U of enzyme per gram of cell wall in a 20% aqueous suspension were randomly conducted. At the end of the reaction time, the enzyme was inactivated at 85 °C for 20 min. The material was cooled to 25 °C and washed with centrifugation at 4500*g* for 5 min. The supernatants were collected for detection of soluble proteins, and the precipitate of each treatment was used for total nitrogen determination. The results were analyzed via ANOVA and Tukey tests.

2.3.2. Dialysis and lyophilization

Dialysis was performed for 48 h against distilled water, under mild agitation, with frequent water replacements. After dialysis, the material was frozen at −20 °C and dried under vacuum at −80 °C. The residual moisture after lyophilization was determined by drying at 102 °C until a constant weight was achieved.

2.3.3. Carboxymethylation of β-D-glucan

The derivatization of non-dialyzed water-insoluble (1 → 3)(1 → 6)-β-D-glucan was performed as described by Sandula, Kogan, Kacuraková, and Machová (1999), using monochloro acetic acid. The substitution degree (DS) of the carboxymethylated product was determined by potentiometric titration with potassium hydroxide solution, according to the methods described by Rinaudo and Hudry-Clergeon (1967).

2.3.4. In vitro tests with (1 → 3)(1 → 6)-β-D-glucan and CM-G

The *in vitro* assays to evaluate the cytotoxicity, genotoxicity and cell viability modulation of (1 → 3)(1 → 6)-β-D-glucan and CM-G were performed with CHO-k1 cells cultivated at 37 °C in a BOD incubator in HAM-F10 and Dulbecco modified Eagle (1:1) culture media (Gibco) supplemented with 15% fetal bovine serum and antibiotics (0.01 mg/ml streptomycin and 0.005 mg/ml penicillin) (Gibco). As agent for inducing damage to DNA and negative controls, doxorubicin (DXR – 1 μg/ml) and culture medium (10 μl/ml) were used, respectively. For each test, three independent experiments were performed.

2.3.4.1. Cytotoxicity. The cytotoxicity of water-insoluble (1 → 3)(1 → 6)-β-D-glucan and CM-G was evaluated using the MTT test (Thiazolyl Blue Tetrazolium Bromide), conducted according to methods described by Mosmann (1983). CHO-k1 cells were treated with (1 → 3)(1 → 6)-β-D-glucan or CM-G at the following concentrations (in μg/ml): 6.25, 12.5, 25, 50, 75, 100, 125, 150, 175 and 200, with eight replicates per treatment. Exposure times of 3 h and 24 h were studied. The results of treatment were analyzed by ANOVA and compared to the control with the Dunnet test. Based on the results, the CM-G concentrations were defined for the genotoxicity test.

2.3.4.2. Genotoxicity. The genotoxic effects of CM-G were evaluated with the comet assay (Electrophoresis test in single-cell gel), according to the methods of Speit and Hartmann (1999) after treatment with 12.5 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml and 200 μg/ml CM-G for 24 h in medium free of fetal bovine serum. Slides of each replicate were coded, and 100 cells from each treatment were visually analyzed (Kobayashi, Sugiyama, Morikawa, Hayashi, & Sofundi, 1995) and classified according the following criteria: Class 0: no tail; Class 1: tail up to the diameter of the comet head, Class 2: intermediate-sized tail, with two times the diameter of the comet head; Class 3: long tail, longer than two times the diameter of the comet head. Cells with fully fragmented nuclei were not accounted. The scores were obtained according to Manoharan and Banerjee (1985) with modifications, as shown by Eq. (1). The results were analyzed via the ANOVA.

$$\text{Score} = (1 \times n_1) + (2 \times n_2) + (3 \times n_3) \quad (1)$$

where n = number of cells tested in each class.

2.3.4.3. Cell viability tests. The cell viability, apoptosis and necrosis indexes, after treatment with CM-G concentrations of the comet assay, were determined by differential staining with acridine orange (AO) and ethidium bromide, as described by McGahon et al. (1995). A total of 200 cells were visually examined and classified as: (I) live, with a functional membrane and uniform green color in the nucleus; (II) in an early apoptosis stage with a functional membrane, but fragmented DNA, showing a green color in the nucleus and cytoplasm with an apparent marginalization of its nuclear content; (III) in the final apoptotic stage, showing orange-stained areas in the cytoplasm and in regions where the chromatin was condensed in the nucleus, but distinct from necrotic cells; (IV) necrotic and showing a uniform orange color in the nucleus. Each examination was performed in triplicate. The means of the indexes of each treatment were analyzed and compared to controls via ANOVA and the Dunnet test.

3. Results and discussion

3.1. Extraction of water-insoluble (1 → 3)(1 → 6)-β-D-glucan

3.1.1. Autolysis and hot water treatment

The autolysis rate in this study was 53 ± 3%, higher than that reported by Liu et al. (2008) (48%), under the same conditions. However, those researchers worked with a brewer's *S. cerevisiae* slurry, which may explain this difference, since the yeast cell changes with the environment in which it is found (Kapteyn, Van Oen Enoe, & Klis, 1999).

Generally, obtaining *S. cerevisiae* β-D-glucan includes alkaline treatments for the removal of proteins that are covalently linked to mannans in the cell wall. Although alkali treatment efficiently removes the protein, it also results in loss of carbohydrates (Thanardkit, Khunrae, Suphantharika, & Verduyn, 2002). In this study, the proteins, especially the mannoproteins, were removed in the extraction with hot water, and the glass spheres helped to rupture the cells during the high-pressure agitation.

The total crude protein determined before treatment with hot water was 18.02 ± 1.28%, and after treatment, the percentage decreased to 3.25 ± 0.96%, with approximately 82% of the total protein being extracted, similar to that reported by Freimund et al. (2003).

3.1.2. Breaking of the cell wall through sonication after treatment with hot water

Saccharomyces cerevisiae cells are very resistant, and due to the thickness and rigidity of the cell wall, sonication is not enough to rupture them (Mendes-Costa & Moraes, 1999). However, the structure of the cell wall changes after the removal of mannoproteins, which contribute to its mechanical strength and confer resistance to external pressure. Additionally, after the removal of mannoproteins, the β-D-glucan swells, altering its compactness, which also leads to a decrease in the mechanical strength of the cell wall. Thus, at the end of the treatment with hot water, the cell wall is more susceptible to breakage (Liu et al., 2008). In addition to being fast and inexpensive, sonication also removes the need for chemicals potentially harmful to the environment. According to Sandula et al. (1999), sonication helps in the removal of impurities, such as the amorphous portions of (1 → 6)-β-D-glucan, which are trapped between the fibrils of the glucan in the cell wall. Analyses of the effects of cell concentration and sonication time show that both affect the disruption of the cell wall. The adjusted determination coefficient (R^2) of the model was 0.90, and the lack of fit was not significant (0.19). The coefficients derived for the cell wall breaking rate enabled the design of Eq. (2), which is adjusted for the experimental data. The best results were observed after 6 min at 10% and 15% (Table 1).

$$y = 86.57 - 4.10x_1 - 1.36x_1^2 + 5.05x_2 + 2.37x_2^2 - 1.86x_1 \cdot x_2 \quad (2)$$

The analysis of the surface response curves revealed that the increase in cellular concentration negatively affected the breakage of the cell wall through sonication of *S. cerevisiae* cells. The results suggest an increase in the rupture of the cell wall after over 6 min of sonication at a 10% concentration. However, this fact should be carefully examined, since longer sonication times have been used in the depolymerization of glucan and its derivatives (Chorvatovicová, Machová, & Sandula, 1996). In the present study, sonication is proposed to rupture the cell wall of intact cells remaining in the extraction with hot water. In the experimental validation of the 10% and 6 min conditions, values of 97 ± 0.18%

Table 1
Full factorial design (3^2) with absolute and codified values of sonication time and cell concentration, and results predicted and observed for the breaking rate of the *S. cerevisiae* cell wall.

Test	Concentration (%)	x_1	Time (min)	x_2	% Breaking rate	
					Observed[a]	Predict[b]
1	10	−1	2	−1	81	83.08
2	10	−1	4	0	90	87.97
3	10	−1	6	+1	97	96.96
4	15	0	2	−1	81	80.31
5	15	0	4	0	85	85.20
6	15	0	6	+1	94	94.08
7	20	+1	2	−1	80	78.59
8	20	+1	4	0	82	83.41
9	20	+1	6	+1	85	84.98
10	15	0	4	0	86	85.20
11	15	0	4	0	84	85.20

[a] Results observed for the experimental data.
[b] Results predicted by the model based on the equation $y = 86.57 - 4.10x_1 - 1.36x_1^2 + 5.05x_2 + 2.37x_2^2 - 1.86x_1 \cdot x_2$.

Table 2
Results observed in the cell wall proteolysis step for each test with the protamex enzyme.

Test	Enzyme units[a] (U)	x_1	% Total N[b]	Proteins[c] (μg)
1	0.05	A	4.61	318.18
2	0.05	A	4.59	316.12
3	0.1	B	4.50	361.87
4	0.1	B	4.48	364.50
5	0.2	C	4.43	380.06
6	0.2	C	4.44	375.56
7	0.3	D	3.91	511.87
8	0.3	D	4.43	506.43
9	0.4	E	0.02	536.87
10	0.4	E	0.02	542.62
11	0.5	F	0.01	542.37
12	0.5	F	0.02	537.06

[a] Units of protamex, Novozymes®.
[b] Determined by the micro-Kjeldahl method using a 6.25 conversion factor.
[c] Detected by the method of Lowry in the supernatant of the first wash after proteolysis.

were observed. However, the small difference between the 10% and 15% breaking rates suggests that higher concentrations may be used to speed up the process.

3.1.3. Extraction of lipids from the cell wall after sonication

When alkaline treatment is used, lipids are hydrolyzed into glycerol and fatty acids (Freimund et al., 2003). According to Jamas, Easson, and Ostroff (1997), polar lipids are solubilized and discarded in the supernatant after treatment with alkali; therefore, most of the β-D-glucan extraction processes do not include lipid extraction. In this study, after sonication, the precipitate contained 1.93 ± 0.61% lipids. Before the enzyme treatment, lipids should be extracted because they may interfere in the protease action (Liu et al., 2008). Several organic solvents have been proposed for extraction of lipids during the removal of the β-D-glucan from *S. cerevisiae*. Liu et al. (2008) reported extraction under reflux with isopropanol; however, Freimund et al. (2003) extracted only part of the lipids with the same solvent. Previous studies have reported that many treatments with isopropanol were required for complete delipidization (Jamas, Easson, & Ostroff, 1996). In this study, the percentage of lipids after extraction with isopropanol and petroleum ether was 0.1 ± 0.01% and 0.09 ± 0.01%, respectively, with $p \geqslant .05$, as determined by the Tukey test. Having the option to use either solvent is very interesting because it allows for the selection of the most appropriate solvent for the process flow diagram.

3.1.4. Proteolysis of the cell wall

The protamex enzyme has been widely used in the food industry and has proven to be safe (Shen, Wang, Wang, Wu, & Chen, 2008). In addition, it is inexpensive, easy to handle and easy to store, and does not invalidate the large-scale β-D-glucan extraction process.

After five washings, it was not possible to detect proteins in the supernatant of the treatments. Table 2 describes the total soluble proteins present in the supernatant of the first washing of each treatment. As expected, the largest quantities of soluble proteins were detected in the supernatant of treatments with 0.4 U and 0.5 U. The total N mean of treatments revealed differences by the Tukey test between 0.4 U of enzyme and treatments with lower amounts of the protease; however, no difference was observed between 0.4 U and 0.5 U. Such enzymatic treatments have been used in the extraction of β-D-glucan (Freimund et al., 2003; Kath & Kulicke, 1999; Liu et al., 2008), but there are no previous studies focusing on optimizing the amount of enzyme used and the washings required for its total removal.

The TLC analysis of carbohydrates from the insoluble portion of each treatment revealed a retention factor (Rf) of 0.43, corresponding to glucose, and an absence of an Rf value corresponding to mannose (Moreira et al., 1998). In the experimental validation of proteolysis with 0.4 U of enzyme 0.02 ± 0.01% total N was observed.

3.2. Experimental validation of the process, lyophilization and residual moisture

The (1 → 3)(1 → 6)-β-D-glucan obtained in optimized conditions contained 96.82 ± 0.81% total carbohydrates, higher than the 92% observed by Xiaozhong, Jie, Baogui, and Wangxiang (2000) for β-D-glucan obtained via alkaline and acid extraction.

After lyophilization the residual moisture was 4.8 ± 0.9%, which is important for avoiding the compaction of the (1 → 3)(1 → 6)-β-D-glucan during storage. These results differ from those of Hromádková et al. (2003), who reported a moisture content of 12.7% for *S. cerevisiae* β-D-glucan that was lyophilized and extracted through alkaline treatment. Considering that the structure and organization of glucan particles, which is directly related to water holding capacity, changes with the extraction process, our results showed that the methodology proposed here allows for obtaining of (1 → 3)(1 → 6)-β-D-glucan for long-term use.

3.3. Extraction yield

The extraction yield of (1 → 3)(1 → 6)-β-D-glucan was 11.08 ± 0.19% from *S. cerevisiae* cells, similar to the 11% observed by Liu et al. (2008) with similar methods, but higher than the yield related to alkaline extractions.

Considering the yield obtained in relation to the amount of total β-glucan in *S. cerevisiae*, around 94% of β-glucan was recovered. The polymer obtained was totally insoluble in water and was an ivory color. According to Mantovani et al. (2008), the solubility of β-glucan is associated with the degree of polymerization (DP), which is higher than 100 for those completely insoluble in water.

The solubility of β-glucans also depends on the degree of branching (DB), with more branched glucan being more soluble. In their study, Kogan, Alföldi, and Masler (1988) reported low degrees of branching for water-insoluble β-D-glucan from *S. cerevisiae*, which had long side chains. According to Chorvatovicová et al. (1996), modestly branched glucan has DB < 0.25 and Chen and Seviour (2007) related that DBs up to 0.33 are optimal for biological activity. However, these data are not entirely clear because several factors are involved in the immunomodulatory activity of glucan,

and considerable variations may occur due to the side chains. Moreover, in recent study, Aimanianda et al. (2009) reported that the *S. cerevisiae* (1 → 6)-β-glucan have on average branching at every fifth residue with one or two β-(1 → 3)-linked glucose units in the side chain and this can have a great influence on recognition of β-D-glucan by specific cellular receptors.

3.4. Carboxymethylation of (1 → 3)(1 → 6)-β-D-glucan

The derivatization of water-insoluble β-D-glucan facilitates and allows its use in medicine, since adverse effects, such as formation of granuloma, hypersensitivity and pain, are associated with parental use of insoluble β-D-glucan (Maeda, Watanabe, Chihara, & Rokutanda 1988). Moreover, the derivatization may increase its biological activity, and the derivatives are more efficient than β-D-glucan itself (Chen & Seviour, 2007). CM-G is one of the most studied soluble derivatives; it is commercially available in several countries and has many proven beneficial effects (Miadoková et al., 2005).

The solubility of β-D-glucan derivatives depends on and is directly proportional to the degree of substitution (DS). Water-soluble CM-G with a DS between 0.4 and 1.15 has been reported. The CM-G obtained here had a DS of 0.8, displayed a whitish color and formed an opalescent solution at a concentration of 2 mg/ml. These same characteristics were observed by Sandula, Machová, and Hrıbalová (1995) for CM-Gs with a DS between 0.56 and 0.89, which is considered an optimum DS for CM-G. The DS reached in the derivatization of this study is identical to that obtained by Miadoková et al. (2005) for CM-G with antimutagenic, anticlastogenic and antigenotoxic effects. The derivatization yield from water-insoluble β-D-glucan was 94 ± 2%, similar to the 90–95% reported by Sandula et al. (1999).

3.5. Characterization of (1 → 3)(1 → 6)-β-D-glucan and CM-G

3.5.1. Analysis of macro- and micro-elements

The values of macro- and micro-elements detected in water-insoluble (1 → 3)(1 → 6)-β-D-glucan and CM-G before and after dialysis are shown in Table 3. The (1 → 3)(1 → 6)-β-D-glucan contained total macro- and micro-nutrient concentrations of 8.82 ± 0.2 mg/g before and 1.21 ± 0.1 mg/g after dialysis (Table 3). These values are considerably lower than the 38.70 mg/g, reported by Liu et al. (2008) for non-dialyzed β-D-glucan extracted from *S. cerevisiae* spent from a brewery. Yamada, Alvim, Santucci, and Sgarbieri (2003) described which brewery by-products are rich in macro- and micro-nutrients, so that the differences in the amount of minerals may be associated with the yeast itself. Another factor that may have influenced the results is the increase in the solubility of minerals due to the number of washings proposed here. Supposedly, the low amount of minerals even before the dialysis step suggests that for a large-scale extraction, or for any application of β-D-glucan, this step can be eliminated. However, it is an important step in studies involving the biological activity of water-insoluble β-glucan, due to the interference of salts in vital processes.

In CM-G, the amount of macro- and micro-elements detected was 9.02 ± 0.11 mg/g before and 1.20 ± 0.05 mg/g after dialysis. However, the dialysis of CM-G is necessary for the removal of residual salts from the derivatization process, which can interfere with clinical performance of CM-G. However, this is the first report of analysis of macro- and micro-elements in CM-G before and after dialysis.

3.5.2. Structural characterization

Infrared spectroscopy (IR) is often applied for the structural characterization of β-glucans, and according to Sandula et al. (1999), it can provide rapid and reliable information on the quality of glucan preparations. In this study, the spectrum obtained for (1 → 3)(1 → 6)-β-D-glucan showed typical patterns, with absorption at 891 cm^{-1}, which is characteristic of β-glycosidic linkages (Hromádková et al., 2003; Schmid et al., 2001). In contrast, the absence of absorption at 841 cm^{-1} provided evidence of the absence of α linkage in the polysaccharide.

The bands at 1040 cm^{-1} and 1080 cm^{-1}, relating to the stretching of CO and CC, are described for (1 → 3)-β-D-glucan and (1 → 6)-β-D-glucan, respectively. The intense band due to the axial deformation of OH at the region from 3100 to 3600 cm^{-1}, centered at 3415 cm^{-1}, has also been reported for β-D-glucans in addition to the vibrations of the CH linkage with absorption at the region of 2925 cm^{-1}, indicating the frequencies of the functional groups of these polysaccharides (Sandula et al., 1999). There was no formation of bands in the region from 1650 to 1550 cm^{-1}, corresponding to amide CO stretching.

In the IR spectrum of the CM-G, bands at 1595 and 1421 cm^{-1} were observed, referring to the COO– stretching due to the introduction of carboxymethyl anions in the molecule during carboxymethylation. These data are in line with those of Sandula et al. (1999), who reported vibration at the region near 1600 and 1421 cm^{-1} for the IR spectrum of the CM-G from *S. cerevisiae*. Compared to the spectrum of the water-insoluble (1 → 3)(1 → 6)-β-D-glucan, a considerable decrease of the band at the region from 3100 to 3600 cm^{-1} was observed, corresponding to OH stretching, due to its link with carboxymethyl anions, forming $–OCH_2COOH$ and $–OCH_2CH_2COOH$.

The NMR spectrum revealed no evidence of the α configuration of the anomeric carbons (δ 100.0), showing only the presence of anomeric carbons in the β configuration, with signals close to 103 ppm (Schmid et al., 2001).

^{13}C NMR spectra of (1 → 3)(1 → 6)-β-D-glucan in this study (Fig. 1) are identical to those published by Kogan et al. (1988), corresponding to a (1 → 3)(1 → 6)-β-D-glucan composed of a main chain of glycosidic units linked in β(1 → 3), with branching points at position 6 and side chains with at least four glucose units binding at β(1 → 6). The observed signals corresponding to side-chain carbons, such as δ 75.51, referring to the C5 of side glycosidic units

Table 3
Values of the macro- and micro-elements detected in the (1 → 3)(1 → 6)-β-D-glucan and carboxymethyl-glucan (CM-G) before and after dialysis, in milligrams (mg) per gram (g).

Polymer		Macro- and micro-elements (mg/g)									Total
		Ca	K	P	Mg	Cu	Zn	Fe	Al	Na	
β-Glucan	BD[a]	0.91	0.15	2.94	0.10	0.05	1.51	0.25	0.91	2.00	8.82
	AD[b]	0.14	0.05	0.43	0.02	nd[c]	0.13	0.02	0.02	0.40	1.21
CM-G*	BD	0.95	0.14	2.95	0.09	0.05	1.50	0.26	nd	3.08	9.02
	AD	0.08	0.02	0.05	0.02	0.11	0.74	0.03	nd	0.15	1.20

[a] Before dialysis.
[b] After dialysis.
[c] Non-detected.
* Obtained from (1 → 3)(1 → 6)-β-D-glucan non-dialyzed.

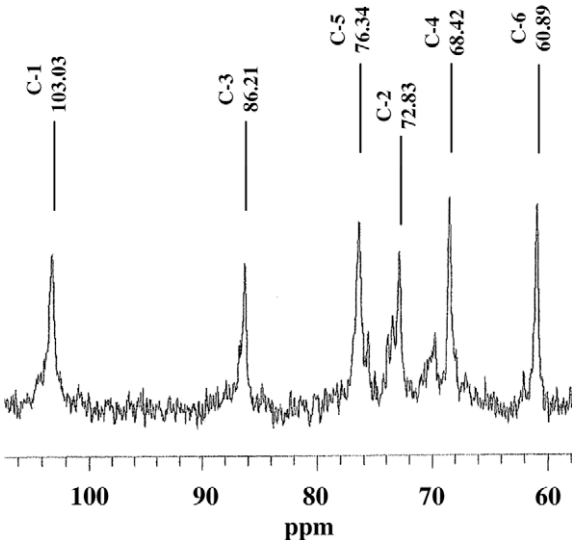

Fig. 1. ^{13}C RMN spectrum of water-insoluble (1 → 3)(1 → 6)-β-D-glucan from *S. cerevisiae* (d_6-DMSO 75.449 MHz) with indicated main chain carbons.

in a β(1 → 6) linkage, have also been previously reported (Kogan et al., 1988). Other signals assigned to the O^- substituted glucose unit that are related to the presence of side chains identified in this study are in agreement with previous reports (Barbosa, Steluti, Dekker, Cardoso, & da Silva, 2003; Schmid et al., 2001).

The assignment of signals obtained from the ^{1}H NMR spectrum agreed with the reports of Freimund et al. (2003) for β-glucan extracted from *S. cerevisiae*, under similar conditions. There was no δ_H 4:36 signal corresponding to pustulan, or any δ signal corresponding to mannoprotein carbohydrates.

Signals of the ^{13}C and ^{1}H NMR spectra of CM-G agree with those published by Machová, Kogan, Alfoldi, Soltes, and Sandula (1995), which characterized glucans carboxymethylated with different DS, even 0.8.

3.6. In vitro tests with CM-G

In vitro tests do not reflect the *in vivo* behavior, but provide valuable information on compounds with potential for use in humans (Takahashi, 2003, chap. 6). Yeast β-D-glucan is assigned as safe by the Food and Drug Administration (FDA); however, preclinical assays are important because a new methodology is proposed for its extraction here.

3.6.1. Cytotoxicity

The colorimetric MTT test is based on the reduction of yellow tetrazolium salts by mitochondrial reductases in the metabolically active cells. Intracellularly, blue crystals are formed, which are solubilized and then analyzed through UV visible spectrophotometry. Thus, the lower the MTT reduction is, the lower the spectrophotometric signal, and hence, the lower the mitochondrial activity will be (Mosmann, 1983). Fig. 2 shows the results of the MTT tests for (1 → 3)(1 → 6)-β-D-glucan and CM-G in CHO-k cells, after exposure times of 24 h.

Only 200 μg/ml (1 → 3)(1 → 6)-β-D-glucan after an exposure time of 24 h had cytotoxic effects (Fig. 2), decreasing mitochondrial activity by 36.8%. In a study with β-glucan from barley, Angeli, Ribeiro, Angeli, and Mantovani (2009) also reported cytotoxic effects for the same concentration. It is important to emphasize that after an exposure of 3 h, which is generally the time estimated in MTT assays, no cytotoxic effects were observed for all the concentrations of (1 → 3)(1 → 6)-β-D-glucan tested in this study.

In the CHO-k1 assays with CM-G, no cytotoxic effects were observed in both exposure times of 3 h and 24 h for the concentrations evaluated (Fig. 2).

3.6.2. Comet assay

The comet assay is widely used for evaluation of genotoxic effects, and its advantages include sensitivity in detecting low levels of DNA damage, collection of data at the level of individual cells, use of a small number of cells for analysis and the possibility of application in any population of isolated eukaryotic cells (Tice

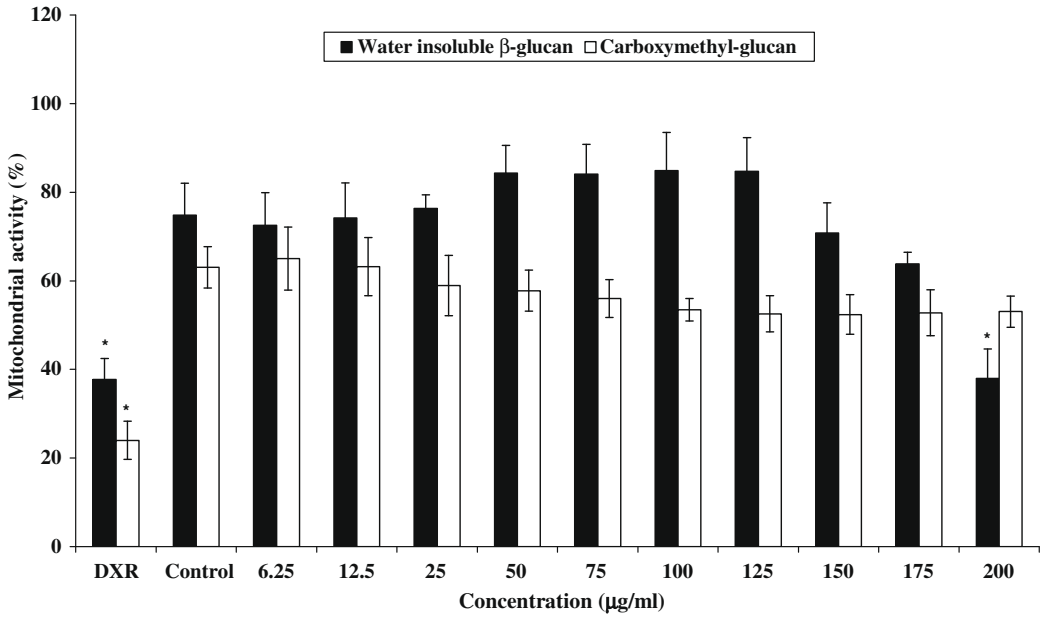

Fig. 2. MTT test in CHO-k1 cells treated with (1 → 3)(1 → 6)-β-D-glucan and CM-G for 24 h. The data are expressed as means of mitochondrial activity (%) and bars denote standard deviation corresponding to three individual experiments; DXR: doxorubicin; * denotes $p \leqslant .05$, a statistically significant difference from the control, as determined by the Dunnet test.

Table 4
Distribution of different classes of damage, scored in reference to the genotoxicity and frequency of lesioned cells observed in the comet assay, after CHO-k1 cell exposure to CM-G for 24 h.

Treatment	Class of damage ($X \pm SD$)				Score	Frequence of DC
	0	1	2	3		
Control	91.67 ± 3.21[a]	7.67 ± 2.08[a]	0.67 ± 1.15[a]	0.00[a]	9.00 ± 4.36[a]	0.08 ± 0.03[a]
DXR (1 μg/ml)	4.67 ± 2.08[b]	66.00 ± 13.89[b]	20.33 ± 10.12[b]	9.00 ± 1.73[b]	133.67 ± 11.55[b]	0.95 ± 0.02[b]
CM-G (μg/ml)						
12.5	98.33 ± 0.58[c]	1.67 ± 0.58[a]	0.00[a]	0.00[a]	1.67 ± 0.58[a]	0.02 ± 0.01[c]
25	97.67 ± 1.15[c]	2.33 ± 1.15[a]	0.00[a]	0.00[a]	2.33 ± 1.15[a]	0.02 ± 0.01[c]
50	95.67 ± 1.15[a,c]	4.33 ± 1.15[a]	0.00[a]	0.00[a]	4.33 ± 1.15[a]	0.04 ± 0.01[a,c]
100	94.67 ± 1.53[a,c]	5.33 ± 1.53[a]	0.00[a]	0.00[a]	5.33 ± 1.53[a]	0.05 ± 0.02[a,c]

DXR = doxorubicin; $X \pm SD$ = (mean ± standard deviation); DC = damage cells.
[a,b,c] Values of the same column with different cases differ statistically by Dunnet test.

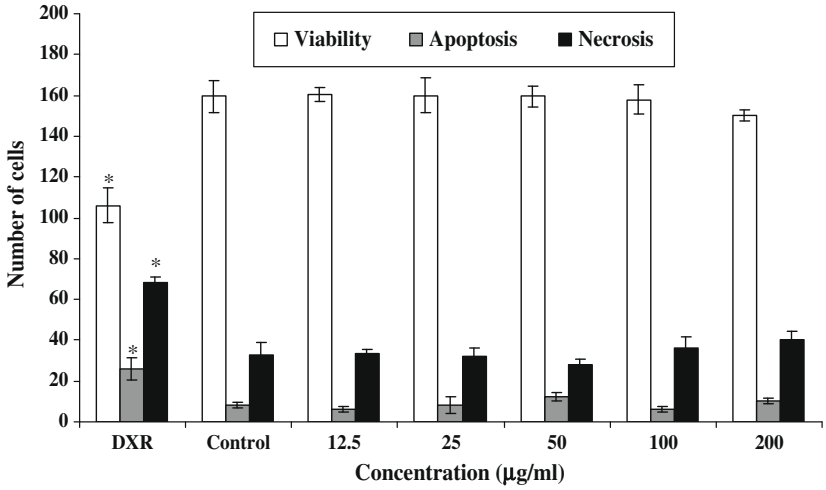

Fig. 3. Mean indexes of viability, apoptosis and necrosis of CHO-k1 cells after a 24-h exposure to CM-G; bars denote standard deviation of three individual experiments; * denotes $p \leqslant .05$, a statistically significant difference from the control, as determined by the Dunnet test.

et al., 2000). Table 4 shows the results of the comet assay for the different CM-G concentrations studied.

Statistical analyses have shown that none of the concentrations had genotoxic effects because there was no increase in the number of cells with DNA damage in relation to the control. Similar data were observed by Oliveira et al. (2007) in their genotoxicity evaluation of the *S. cerevisiae* β-D-glucan.

The values of scores and cells with damage observed after CM-G treatments were lower than those obtained in negative control groups, and denote that the administration of CM-G, a potential adjuvant in therapy of various diseases, including cancer, will not damage the DNA of patients.

3.6.3. Cell viability

Fig. 3 shows the data on cell viability, apoptosis and necrosis indexes for CHO-k1 cells treated with CM-G.

The CM-G concentrations evaluated did not interfere with the viability, apoptosis or necrosis indexes compared with the control. These results corroborate those obtained by Oliveira et al. (2007), who observed that β-D-glucan extracted from *S. cerevisiae* does not decrease cell viability or increase apoptosis or necrosis.

4. Conclusion

This study describes a high-performance optimized process that enables the extraction of 94% of β-glucans from the cell wall of *S. cerevisiae*. In several steps, it is possible to obtain the polymer with a high degree of purity without the use of drastic conditions or generation of waste potentially harmful to the environment. The carboxymethyl derivative obtained from water-insoluble (1 → 3) (1 → 6)-β-D-glucan had a DS of 0.8 and lacked any cytotoxic or genotoxic effects and did not interfere with cell viability. Due to the potential use of CM-G in therapy for various diseases, additional *in vivo* studies involving the administration of CM-G as an adjuvant in prostate cancer therapy are in progress.

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5 ARTIGO II

Effects of carboxymethyl-glucan from *Saccharomyces cerevisiae* on the peripheral blood cells of patients with advanced prostate cancer

Publicado na Revista Experimental and Therapeutic Medicine

Effects of carboxymethyl-glucan from *Saccharomyces cerevisiae* on the peripheral blood cells of patients with advanced prostate cancer

MARCIANE MAGNANI[1], RAUL HERNAN CASTRO-GOMEZ[1], MATEUS NOBREGA AOKI[2], EMERSON PEREIRA GREGÓRIO[3], FARID LIBOS Jr[3] and MARIA ANGELICA EHARA WATANABE[2]

[1]Departamento de Ciência e Tecnologia de Alimentos, and [2]Departamento de Ciências Patológicas, Universidade Estadual de Londrina; [3]Instituto do Câncer de Londrina, Londrina, Paraná, Brasil

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Abstract. Carboxymethyl-glucan (CM-G) is a water-soluble derivative of β(1-3)(1-6) glucan that is well known for its immunostimulant and hematopoiesis-enhancing activities. In this clinical trial, we assessed the effects of oral CM-G administration on the peripheral blood cells of patients with advanced prostate cancer, and examined its ability to alter hepatic and renal function. After CM-G administration, the total leukocyte count increased significantly ($p \leq 0.02$), with no associated changes in the lifestyle habits of the patients. A significant increase ($p \leq 0.001$) was also observed for red blood cell, hematocrit, hemoglobin and platelet counts. No changes were observed in hepatic or renal function after CM-G administration, and no side effects associated with its use were recorded. These results suggest that using CM-G as an adjuvant to cancer treatment may improve the health parameters of prostate cancer patients.

Introduction

The β-glucans are of particular interest to researchers because they are naturally occurring glucose polymers, are orally active when taken as food supplements, and have a long track record of safe use. Due to their immunomodulatory properties, purified β-glucans have been used clinically as part of a combination therapy for a variety of cancers (1).

Carboxymethyl-glucan (CM-G) is a water-soluble derivative of the *Saccharomyces cerevisiae* cell wall β(1-3)(1-6) glucan. It is classified as a biological response modifier and is well known for its immunomodulatory and hematopoiesis-enhancing activities (2-4). Several experimental studies *in vivo* have shown that β-glucans administered orally, intravenously or enterally enhance hematopoietic regeneration without side effects (5-8).

Prostate cancer is the sixth most common cancer in the world and represents approximately 10% of all cancers diagnosed. In Brazil, prostate cancer is the most common type of cancer in males (excluding non-melanoma skin cancer), and is prevalent in all regions of the country. More than any other type of cancer, prostate cancer is considered a disease of the elderly, since nearly three-fourths of the cases worldwide occur at the age of 65 or above (www.inca.gov.br) (9).

While a man with localized prostate cancer is more likely to die from comorbidities, the probability of death from prostate cancer exceeds other causes when clinical metastases occur. Metastatic prostate cancer remains incurable, and it is known that many parameters of immunity decline with age (10). At this stage, the medical goals are to increase the short survival period of the patients and, above all, to improve their quality of life, which often declines as the disease progesses.

Considering the beneficial properties ascribed to CM-G and its potential use as an adjuvant to cancer treatment, we assessed *in vivo* the effects of oral CM-G administration on the peripheral blood cells of patients with advanced prostate cancer (PCa) and on alterations in hepatic and renal functions.

Patients and methods

This study was conducted with the approval of the Committee on Ethical Research Involving Human Beings of the University of Londrina and the Northern State of Paraná University Hospital. CONEP 268 and 212/07 for this project are available under registration no. 0214.0.268.000-07 at the National Information on Ethics in Research Involving Human Beings, Brazil (SISNEP). Patients at the Londrina Cancer Institute with a histological diagnosis of stage T3 (n=15) and T4 (n=15) prostate adenocarcinoma according to TNM staging (www.cancerstaging.org) and undergoing androgen deprivation therapy (ADT) with goserelin acetate or leuprolide were included in this study after giving written informed consent. The age of the patients ranged from 52 to 84 years, with a median age of 69 years. The ADT treatment duration ranged

Correspondence to: Dr Marciane Magnani, Departamento de Ciência e Tecnologia de Alimentos, Universidade Estadual de Londrina, Rod Celso Garcia Cid, PR 445, Km 380, Campus Universitário, C.P. 6001, CEP 86051-990, Londrina, Paraná, Brasil
E-mail: magnani2@gmail.com

Key words: β-glucan, prostate cancer, blood cells

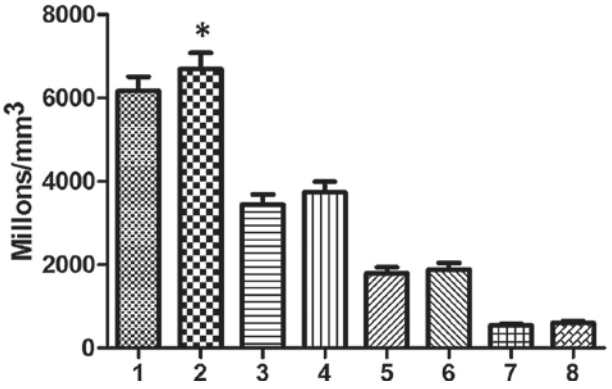

Figure 1. Leukocyte counts before and after CM-G treatment for 28 days. 1 and 2, total leukocytes before and after CM-G treatment; 3 and 4, neutrophils before and after treatment; 5 and 6, typical lymphocytes before and after treatment; 7 and 8, monocytes before and after treatment. A significant increase in total leukocytes was observed after treatment (*p≤0.02).

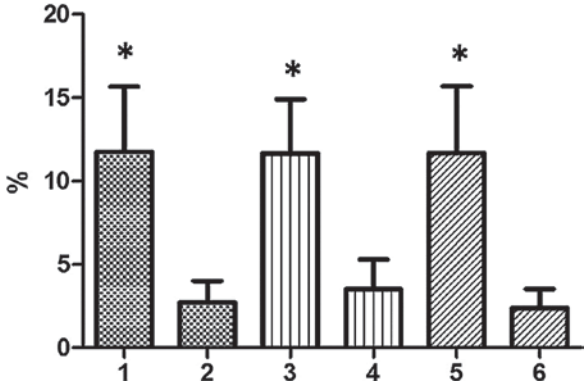

Figure 2. Variations in hematological data before and after CM-G treatment for 28 days. Patients were divided according to their initial erythrocyte, hemoglobin and hematocrit counts into groups below or above the reference limit. 1 and 2, variations in erythrocyte levels in the below and above reference limit groups; 3 and 4, variations in hemoglobin levels in the below and above reference limit groups; 5 and 6, variations in hematocrit levels in the below and above reference limit groups. Patients who were below the reference limit (*p≤0.001) had significantly elevated values for all three parameters.

from 5 to 36 months. Among the 30 patients studied, 9 underwent radiation before ADT, 8 were smokers and 8 consumed alcohol regularly. During the 28-day treatment, patients were contacted weekly in order to to monitor their conditions and to receive reports of any adverse effects associated with the CM-G treatment.

Soluble β-glucan in the carboxymethylated (CM-G) form was obtained from the cell wall of *Saccharomyces cerevisiae*, according to the protocol described by Magnani *et al* (11). The CM-G was distributed into gelatin capsules containing 20 mg of CM-G with starch as an excipient vehicle. This procedure was carried out under medical request by Vico Farma®, which is registered at the National Health Surveillance Agency (ANVISA) in Brazil under no. 136420-0.

Peripheral venous blood samples were drawn for initial values from the patients early in the morning of Day 1 before the patients ingested the first capsule of CM-G. Early every morning, before eating, the patients ingested a 20-mg CM-G capsule (12). After 28 days, the blood samples were analyzed. Both samples were collected while patients were fasting. Vaccutainer™ tubes (4.5 ml) containing EDTA were used, and all the samples were processed immediately after collection by the Abbott Cell Dyn 3200 flow cytometer.

The results obtained before and after CM-G treatment for each patient were analyzed using the Wilcoxon signed-rank test and the t-test for dependent paired samples. p≤0.05 indicated significant differences.

Results

After CM-G administration, the total leukocyte counts increased significantly (p≤0.02), averaging a 10.64% increase. In the differential count, an increasing trend for typical lymphocytes, monocytes and neutrophils was observed (Fig. 1). The lifestyle habits of patients, such as smoking and alcohol use, were not associated with increased leukocytes.

The leukocyte counts carried out before CM-G administration showed that no patients had leukopenia. However, leukocyte levels closer to the minimum reference value (3,500/mm^3) were observed among patients who had received radiation. For these patients, CM-G administration resulted in an 11% increase in the values.

Table I. Results of liver and kidney function tests performed before and after carboxymethyl-glucan (CM-G) administration for 28 days in patients with advanced prostate cancer.

	Before CM-G	After CM-G	Reference value (method)
Liver function			
Transaminase aspartate amino transferase (TGO)	20.1±2.0	20.9±2.0	11-41 U/l (automated kinetics)
Transaminase alanine amino transferase (TGP)	9.2±0.9	9.4±0.8	7-52 U/l (automated kinetics)
Albumin	4.10±0.75	4.20±0.50	3.35-5.62 g/dl (capillary electrophoresis)
Direct bilirubin	0.08±0.01	0.07±0.02	Up to 0.3 mg/dl (colorimetric)
Indirect bilirubin	0.32±0.26	0.31±0.13	Up to 0.7 mg/dl (colorimetric)
Alkaline phosphatase	40.2±12.0	39.9±9.0	27-100 U/l (automated kinetics)
Kidney function			
Urea	39±10	38±10	10-52 mg/dl (automated enzymatic)
Creatinine	0.87±0.21	0.89±0.23	1.30 mg/dl (automated kinetics)

Before CM-G administration, 9 of the 30 patients studied had red blood cell counts below the minimum reference value (4.3/mm^3), 14 patients had hemoglobin levels below normal values (12.8 g/dl) and 10 patients had hematocrit values below the minimum reference value (38.8%). After 28 days of CM-G ingestion, significant increases ($p \leq 0.001$) in red blood cell, hematocrit and hemoglobin levels were observed; this increase was more pronounced in patients who were initially below the minimum (Fig. 2).

There were no significant changes in VCM, HCM, CHCM and RDW; however, the platelet counts increased significantly after treatment with CM-G ($p=0.007$).

No changes in kidney and liver function were found in associaion with CM-G administration (Table I), and no CM-G-related side effects were observed.

Discussion

CM-G stimulates retioculoendothelial system function and modulates cellular and humoral immunity (2,13,14). In its soluble form, β-glucan acts synergistically with *in vivo* myeloid growth factors, improving hematopoietic recovery and mobilizing progenitor cells in the peripheral blood (14). According to Rice *et al* (15), orally administered soluble derivatives of β-glucan are absorbed through the gastrointestinal wall and pass into the circulation system, activating immune pathways such as Dectin-1, CR-3, SIGNR1, TLR-2/6 and -4 (16).

Cancer-induced immunosuppression is a well-known phenomenon (12), but was not observed among the patients in this study. However, the standard treatment for advanced PCa is hormone therapy (17) and not chemotherapy, which is usually associated with leukopenia. By contrast, low white blood cell counts were observed prior to CM-G treatment in patients who had received radiation therapy. CM-G has proven effective in stimulating hematopoiesis after repeated doses of radiation, and in increasing granulocytes and other hematopoietic indices (18). Several experimental models have demonstrated the ability of β-glucans and derivatives administered by different routes to raise blood cell counts after leukopenia caused by cancer treatments (2,6,19).

The increase in leukocyte counts reported in this study supports the findings of Weitberg *et al* (20) regarding increases in white blood cells after oral β-glucan administration, and those of De Felippe *et al* (5), who found an increase in leukocytes at the expense of neutrophils, lymphocytes and monocytes on the fifth day of intravenous treatment. By contrast, Demir *et al* (12) observed no changes in leukocyte counts after 14 days of oral β-glucan administration. Although only the increase in leukocytes was significant in the present study, typical lymphocytes, monocytes and neutrophils clearly showed an increasing trend with CM-G treatment.

In the present study, no side effects associated with the ingestion of CM-G were observed. These results are in agreement with the findings of Demir *et al* (12), who observed no adverse effects of the daily oral administration of 20 mg of soluble β-glucan from *S. cerevisiae* for 14 days in 20 patients with metastatic breast cancer. Weitberg (20) administered 15 mg of oral β-glucan daily to 20 patients with various types of advanced cancer for 6 months and also found no side effects.

The absence of a significant variation in the results of hepatic and renal function tests before and after CM-G treatment (Table I) are in agreement with the findings of De Felippe *et al* (5), who treated 11 septic patients with 100-240 mg of β-glucan from *S. cerevisiae* for 8 days.

Smoking is associated with increased numbers of leukocytes (21). This association was not observed among the smokers in this study, who showed no difference before or after CM-G administration compared to non-smokers.

After 28 days of treatment with CM-G, a significant increase in red blood cell, hemoglobin and hematocrit levels was confirmed. Previous studies have reported that β-glucan acts directly on the myeloid progenitors, which contribute to hematopoietic regeneration (2,22). Increased levels of hemoglobin in cancer patients treated with oral β-glucan have been reported by Wietberg (20). Moreover, Demir *et al* (12) found no significant differences in the hematocrit count after treatment with β-glucan.

Platelet numbers also increased significantly in the present study, which corresponded with the results of Weitberg (20), but not with those described by Demir *et al* (12). Differences between our results and those reported by Demir *et al* (11) may be related to the duration of β-glucan administration (28 days in the present study vs. 14 days in that of Demir *et al*).

Ever closer to clinical practice, the use of β-glucan as an adjuvant has been used effectively for decades in Japan, improving the quality of life of cancer patients and reducing side effects (23). The results of this study suggest that the use of CM-G may enhance the hematologic parameters of patients with prostate cancer, benefiting the body weakened by the disease and its treatments.

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6 ARTIGO III

Analysis of peripheral T cells and the CC Chemokine Receptor (CCR5) Delta32 polymorphism in prostate cancer patients treated with Carboxymethyl-Glucan (CM-G)

Short Communication submetida na Revista Natural Product Research

Analysis of peripheral T cells and the CC Chemokine Receptor (CCR5) Delta32 polymorphism in prostate cancer patients treated with Carboxymethyl-Glucan (CM-G)

Marciane Magnani [a*], Raul Hernan Castro-Gomez [a], Mateus Nobrega Aoki [b], Emerson Pereira Gregório [c], Farid Libos Jr. [c], Helena Kaminami Morimoto [d], Edna Maria Vissoci Reiche [d], Maria Angelica Ehara Watanabe [b].

[a]*Departamento de Ciência e Tecnologia de Alimentos,* [b]*Departamento de Ciências Patologicas, Universidade Estadual de Londrina;* [c]*Instituto do Câncer de Londrina,* [d]*Laboratório de Imunologia do Hospital Universitário da Região Norte do Paraná, Londrina, Paraná, Brasil,*

*Corresponding author. Address: Departamento de Ciência e Tecnologia de Alimentos, Universidade Estadual de Londrina, Rod Celso Garcia Cid, PR 445, Km 380, Campus Universitário, C. P. 6001, CEP 86051-990, Londrina, Paraná, Brasil.

Tel: +55-43-3371-4565; Fax: +55-43-3371-4080; E-mail: magnani2@gmail.com

Abstract

β-glucan from *Saccharomyces cerevisiae* is a biological response modifier with effects on the innate and adaptive immune responses. The CCR5 chemokine receptor is crucial for the immune cell response. In this study, the effects of the carboxymethylated form of β-glucan (CM-G) on lymphocyte population of CCR5 genotypes patients with prostate cancer (PCa), undergoing Androgen Deprivation Therapy (ADT) was assessed. It was investigated the CCR5 genotype and lymphocyte population by cytometry flow in 30 Brazilian patients with advanced PCa who were treated with CM-G for 28 days. Analysis of the CCR5 chemokine receptor revealed that the wild type genotype Wt/Wt was present in 80% of patients while the heterozygotic genotype Wt/delta32 was present in 20% of patients. After CM-G administration, a significant increase in $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes was observed in patients who displayed the wild type genotype for the CCR5 chemokine receptor. No association between patient's age or time of ADT and increased of T lymphocytes were found. The results demonstrated the ability of CM-G to stimulate $CD4^+$ and $CD8^+$ T cells in the peripheral blood of patients carrying a wild type CCR5 genotype suggesting an interaction between immunomodulation by CM-G and the CCR5 receptor.

Keywords: β-glucan, prostate cancer, CCR5, T lymphocytes

1.1. Introduction

Prostate cancer (PCa) is one of the leading causes of death of adult men. In Brazil, it is estimated that 49.530 new cases were diagnosed in 2008; and disregarding to non-melanoma skin cancer, prostate cancer is the most frequently diagnosed tumor in all regions of Brazil (INCA 2010).

The use of immunomodulatory bioactive substances as adjuvants in cancer treatments have been proposed to increase treatment efficacy, especially given the absence of adverse effects associated with this approach (Baran, Allendorf, Hong & Gordon, 2007; Liu, Gunn, Hanses & Yan, 2009). β-glucan from *Saccharomyces cerevisiae* is a glucose polymer classified as a biological response modifier that has proven effects on the innate and adaptive immune responses. A technique widely used to increase the solubility in water, the most important factor limiting β-glucan for use as an immunostimulant, which may even enhance the immunomodulatory activity, is the derivatization of the molecule. Among the soluble derivatives, carboxymethyl-glucan (CM-G) is one of the most studied forms. Commercially available in several countries, CM-G has many proven beneficial effects, including stimulation of the immune response (Falameeva et al. 2001; Chen & Seviour, 2007).

The effective immune response against cancer involves a response mediated by cytotoxic T cells that specifically attack cancer cells. $CD4^+$ T lymphocytes play a role as modulators of cell immunity, producing multiple cytokines that are essential mediators of the generation of this effective immune response involving $CD8^+$ T lymphocytes, which are essential for protection against tumor cells (Zhang, Zhang &Zhao, 2009). $CD4^+$ T cells (Th1) express assorted interleukin and chemokine receptors, among which CCR5 is crucial for the immune cell response (Sallusto, Lenig, Mackay & Lanzavecchia, 1998).

The CCR5 receptor participates in chemotaxis of memory and activated naive T cells and is required for T cell activation (Taub, Turcovski, Key, Longo & Murphy, 1996). The CCR5 gene product is a member of the

seven transmembrane G-protein-coupled receptor family, which responds to normal ß -chemokine ligands. Martinson, Chapman, Rees, Liu &Clegg (1997) characterized a mutant allele of the ß-chemokine receptor gene CCR5 bearing a 32-bp deletion in the region corresponding to the second extracellular loop of CCR5, denoted as delta CCR5 or CCR5delta32. This variant results in a nonfunctional form of the chemokine receptor that is incapable of binding ß-chemokines.(Liu et al., 1996). According to Balistreri et al. (2009), the delta 32 variant of the CCR5 receptor may confer resistance to the development of PCa.

The stimulation of the immune response by β-glucan mainly involves the cell surface receptor Dectin-1, which is primarily expressed in macrophages, neutrophils, dendritic cells and subpopulations of T lymphocytes (Demir, Klein, Mandel-Molinas & Tuzuner, 2007). It is also known that β-glucan activates macrophages and dendritic cells by increasing the release of interleukin 12 (IL-12), which primes the differentiation of T helper cells into IFN-y-secreting Th1 cells. These Th1 cells are required for the activation of completely naive $CD8^+$ cells to cytotoxic T lymphocytes (CTL) (Harnack, Eckert, Fichtner & Pecher, 2009). The activation of lymphocytes involved in antitumor immune response by β-glucan were related by Mah-Lee & Ann-Teck (2002) in an interesting model. These research had inolulated in nude mice (athymic) lymphocytes extracted from the spleens of mice that received and not received orally β-glucan; after this inoculated the same nude mice with human colon carcinoma cells. They observed a significant regression in tumor formation for the nude mice with lymphocytes from mice trated with β-glucan showed primed lymphocytes during the β-glucan immunomodulation were able to retard the development of the tumors.

The effects of β-glucan on $CD4^+$ and $CD8^+$ subpopulations of lymphocytes have been reported in animal experimental models (Tsukada et al., 2003; Wang, Shao, Guoa & Yuan, 2008; Harnack, Eckert, Fichtner & Pecher, 2009; Zhou, Zhang, Zhang, Liu & Cao, 2009); however in humans, particularly those affected by cancer, such studies are scarce. The present study assessed for the first time the changes in subpopulations of $CD3^{+,}$ $CD4^+$ and $CD8^+$ T lymphocytes from patients with advanced PCa of both Wt/Wt and Wt/delta32 genotypes for the chemokine receptor CCR5, orally administrated with CM-G from *S. cerevisiae* for 28 days.

1. 2. Results and discussion

Among the 30 patients analyzed, 50% were white, 20% brown, 17% black and 13% yellow (Table 1), based on Brazilian Institute of Geography and Statistics guidelines (IBGE, 2010). This distribution reflects the high degree of racial admixture in all ethnic groups in Brazil, also described in others studies (Vargas, Marrero, Salzano, Bortolini & Chies, 2006; Aoki et al., 2009). The duration of treatment with ADT ranged from 5 to 36 months; 57% of patients had undergone treatment for up to 6 months; 27%, between 7 and 12 months; and 16%, for more than 12 months (Table 1).

Of the total, 60% of patients reported feeling well during the CM-G administration and expressed a desire to continue with treatment upon the return of the bottles. There were no side effects reported from ingesting CM-G. These results are in accordance with Weitberg (2008), who studied patients with various types of cancer in advanced stages and did not observed adverse effects with the use of oral β-glucan from *S. cerevisiae.* Additionally, these researches related that patients who were undergoing chemotherapy reported a decreased sensation of fatigue.

Eight patients (27%) were current smokers, using the criterion “smokes at least five cigarettes per day or stopped smoking less than two years ago”. According to Oefeliein & Resnick (2004), the use of tobacco is associated in a dose-dependent way with the onset of hormone-refractory PCa, in which the patient no longer responds to treatment with ADT, and also influences the survival rate of patients undergoing ADT. Supposedly, smokers develop hormone-refractory PCa before those who previously stopped smoking and before those who had never smoked. Failures on treatment of advanced hormone-refractory PCa occur due the nature multi-drugs resistance of prostatic cells. However, the associated use of β-glucan with carmustine, a drug against hormone-refractory PCa cells, may help to improve the treatment efficacy Feskanich, Korrick, Greenspan, Rosen, & Colditz (1999).

In relation to the genotype of the CCR5 chemokine receptor, 80% of patients had the Wt/Wt genotype, and 20% possessed the Wt/delta32 genotype. Individuals homozygous for allele CCR5delta32 were not identified. The frequency of the CCR5delta32 individuals found here is greater than that reported by previous studies involving patients with PCa (Petersen et al., 2008; Balistreri et al., 2009). However, this frequency is similar to that reported in other studies involving Brazilian populations (Grimaldi et al., 2002; Vargas et al., 2006). Due to the high degree of racial miscegenation in Brazil, this variation may be related to selective pressure.

Five patients reported cases of PCa in the family. Among these, patients 11, 16, and 18 reported a single first-degree relative affected, while patients 21 and 28 reported two first-degree relatives affected. Petersen et al. (2008) evaluated six genetic variants of chemokine receptors, including CCR5delta32, which could be involved in the onset and development of PCa, and found no significant association. However, they observed that the probability ratio of developing PCa in individuals heterozygous for CCR5delta32 increases with the number of first-degree relatives affected. One of the leading risk factors for PCa is a family history of the disease, and the risks increase as the kinship degree and the number of affected individuals increase. In the present study, among the five patients who mentioned PCa in their family history, the two patients who reported two affected first-degree relatives possessed the CCR5delta32 genotype.

No relationship was established between the age of the patients and the number of $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes prior to CM-G administration. However the minimum reference values for age matched patients is 560 cells/mm^3 of $CD4^+$ T and 330 cells/mm^3 of $CD8^+$ T and it was observed values below the minimum in 60% and 43% of patients, respectively. Although no relationship between age and the number of $CD4^+$ and $CD8^+$ cells was observed in this study, it is known that many parameters of immunity decline with age (Pawelec, Lustgarten, Ruby & Gravekamp, 2009). Another important factor that may have influenced the numbers of $CD4^+$ and $CD8^+$ T cells in patients is the decrease in testosterone levels that occurs due to ADT. Recent data have shown that the drop in testosterone levels due to ADT negatively affects the counts of $CD4^+$ and $CD8^+$ T lymphocytes from the second month of treatment on (Elshaikh et al., 2009). However, in the present study the counts of $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes prior to CM-G administration were not influenced by the duration of ADT when divided into periods longer than or equal to 12 months and shorter than or equal to 12 months.

After the 28 days of treatment with CM-G a significant increase in $CD3^+$ T ($p=0.004$) and $CD4^+$ T ($p=0.01$) lymphocytes was observed, with average of 9.02% and 11.93%, respectively. In relation to the number of $CD8^+$ T lymphocytes taken before and after the treatment with CM-G the increased was 6.2%. The increase in

$CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes due to the oral administration of β-glucan has been reported in experimental models involving rats. Hanaue et al. (1989) found an increase in $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes after four weeks of treatment with β-glucan, observing a stimulation of T cells, especially T helper cells. Tsukada et al. (2003) observed an increase in intestinal intraepithelial subpopulations of $CD4^+$ and $CD8^+$ T lymphocytes following the administration of β-glucan and suggested that its oral administration may potentiate the cytotoxicity mediated by $CD8^+$ T lymphocytes.

Although it was observed significant increase for $CD3^+$ T and $CD4^+$ lymphocytes subpopulations, there were differences between Wt/Wt and Wt/delta32 individuals. Regarding the changes in $CD3^+$ T lymphocytes, in all Wt/Wt patients the $CD3^+$ T lymphocytes increased, but only in one Wt/delta32 patient. Specifically in the Wt/Wt group, $CD3^+$ T lymphocytes increased by an average of 13.54% (Figure 1). There was also a difference in number of the $CD4^+$ T lymphocytes in relation to the genotype for CCR5; between Wt/Wt patients; only one patient reduced the count of $CD4^+$ T lymphocytes, while only one Wt/delta32 patient displayed an increase in this number. After CM-G administration, an average increase in $CD4^+$ T cells of 16.25% was detected in Wt/Wt patients (Figure 1). Considering all 30 analyzed patients there was no significant difference between the scores for $CD8^+$ T lymphocytes before and after CM-G. However, none of the patients with the Wt/delta32 genotype increased the number of $CD8^+$ T cells after treatment and considering only the Wt/Wt patients, a significant increase ($p=0.03$) in $CD8^+$ T lymphocytes, averaging 12.57%, was observed. (Figure 1)

The CCR5 receptor plays an important role in recruitment of the T cells, driving an immune response toward a Th1 cytokine pattern (Loetscher et al.1998) which is crucial in the antitumor response. The CCR5delta32 deletion may alter the expression or function of the protein products (Sidoti et al. 2005), and any subsequent defect in T cell recruitment can lead to suppression of this immune response. According to Baran, Allendorf, Hong & Gordon (2007), macrophages produced IL-12 after *in vivo* stimulation with β-glucan from *S. cerevisiae*, stimulating the production of IFNy by T cells and favoring the Th1-mediated cellular immune response. As observed by these authors, β-glucan converts the immune response Th2 to Th1 cells, decreasing the production of Th2 cytokines such as IL-4 while increasing IFNy. Harnack, Eckert, Fichtner & Pecher (2009) observed in an experimental model that the oral administration of soluble β-glucan potentiated a vaccination-based immunotherapy for the treatment of lymphoma. Furthermore, mice treated with β-glucan alone displayed an early systematic differentiation of Th1 cells and subsequent activation of naive $CD8^+$ T cells.

The mean $CD4^+/CD8^+$ ratio in Wt/Wt patients was 1.70 before and 1.55 after treatment with CM-G. However, for Wt/delta32 patients, the ratio was 1.98 before CM-G and 2.21 after CM-G, indicating an increasing trend in the ratio of Wt/delta32 patients and a decreasing one in Wt/Wt patients. Wang, Shao, Guoa & Yuan (2008) similarly observed a decline in the $CD4^+/CD8^+$ ratio after 28 days of supplementation with β-glucan in pigs. A lower $CD4^+/CD8^+$ T cell ratio is desirable in patients with cancer, as it indicates a greater number of $CD8^+$ T lymphocytes directly involved in the antitumor response..

The Th1 response enhances the response of cytotoxic T lymphocytes whereas Th2 suppresses it. The Th1/Th2 balance is crucial for the mediation of cancer resistance. Cancer patients experience an imbalance between the Th1 and Th2 immune responses during the course of the disease, such that the Th2 response becomes dominant as a result of the progressive loss of the Th1 response (Baran, Allendorf, Hong & Gordon, 2007; Harnack, Eckert, Fichtner & Pecher, 2009). Zhou, Zhang, Zhang, Liu & Cao (2009) showed that the administration of β-glucan can inhibit the action of regulatory T cells (Tregs) that negatively control the cellular

immune response in addition to stimulating the Th1 response. Patients with PCa have an increase of Tregs cells in the peripheral blood (Miller et al., 2006). Therefore, the administration of β-glucan might benefit patients with PCa by preventing the suppression of the Th1 immune response, which is essential in protecting against cancer.

The results of this study showed that oral administration of CM-G increase the number of $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes in patients with PCa, stimulating the cellular immune response. However, the data hardly suggest an interaction between immunomodulation by CM-G, involving the Th1 response, and the CCR5 receptor. The immunomodulation pattern observed in these patients should be examined in detail in future studies as well the effects on disease progression in the patient population not contemplated in the present study due the short-follow term. A prospective, randomized multi-center study, conduced in Japan, involving patients with advanced PCa under hormoniochemotherapy showed higher five-year survival rate for those which received β-glucan when compared to others (Tari et al. 1994).

Therapies involved in the treatment of cancer are complex and need to be improved, particularly in ways that allow for individualization. The analysis of patient-specific variables such as the expression and subsequent translation of particular genes are factors that may allow for a more effective and satisfactory treatment of cancer patients utilizing regimens that involve both immunity mechanisms and the direct and indirect actions of the drugs and compounds used.

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Supplementary File

S1 Experimental

S1.1. Patients and methods

The protocol was approved by the institutional Human Research Ethics Committee of the State University of Londrina, Parana, Brazil. A term of free informed consent was signed by all sample donors and doctors involved. Thirty patients with a histological confirmation of prostate adenocarcinoma, stage T3 (n=15) or T4 (n=15) according to TNM classification (AJCC/UICC, 2010), undergone Androgen Deprivation Therapy (ADT) with goserelin acetate or leuprolide for a period of no less than five months were selected from medical records of Cancer Institute of Londrina (ICL) in the state of Parana, Brazil. A face-to-face questionnaire to

collected individual characteristics was conducted with each patient before blood collection., Weekly contacts, during the treatment, were performed to report any adverse effects associated with CM-G.

S1.2. Carboxymethyl-glucan (CM-G)

Soluble β-glucan in the carboxymethylated (CM-G) obtained according Magnani et al. (2009) was distributed into gelatin capsules containing 20 mg of CM-G and starch as the excipient vehicle. This procedure was carried out under medical surveillance by Vico Farma ®, which is registered in the National Health Surveillance Agency (ANVISA) in Brazil under number 136420-0. Bottles containing between 28 and 35 capsules with 20 mg of CM-G were labeled with instructions. The varying number of capsules was used to verify that patients were consuming the correct number of capsules. After the daily administration of one capsule of CM-G (Demir, Klein, Mandel-Molinas & Tuzuner 2007) for 28 days the return of the bottle was requested and should contain between zero and seven capsules, depending on the initial amount received.

S1.3. Flow Citometry

For counts of subpopulations of T $CD3^{+,}$ $CD4^{+}$ and $CD8^{+}$ lymphocytes, peripheral blood samples were collected from patients by venipuncture on day 1 of treatment, before intake of the 1st capsule CM-G, and after 28 days of daily intake. Vaccutainer ® 4.5 mL tubes containing ethylene diamine tetra acid (EDTA) were used for collection. Blood samples were always taken between 8-9 am, and the samples were processed four hours after collection. Automated flow cytometry was performed using fluorescence isothiocyanate (FITC) or phycoerythrin (PE) conjugated monoclonal antibodies (mAB) anti-CD3-FITC, anti-CD4-PE and anti-CD8-PE. Cell associated immunofluorescence was measured using a FACS Count® Flow cytometer (Becton Dickinson, San Diego, CA, USA). Results were recorded as number of cells/mm^3.

S1.4. DNA extraction and amplification of CCR5 gene PCR

DNA was extracted from whole blood in the presence of 0.2 M NaCl and 0.25% sodium dodecyl sulfate (SDS) and proteinase K for 4 h at 37 ˚C. After precipitation with absolute ethanol, the pellet was dried and resuspended in 50 μl of milli-Q water. DNA was amplified by PCR using specific primers for CCR5: (GenBank accession no.: AF009962) and the kit buffer plus 1.5 mmol/μl Taq polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA). PCR conditions were: denaturation at 94˚C for 5 min, 35 cycles of 1 min at 94˚C, 1 min at 58˚C and 1 min at 72˚C, followed by 10 min elongation at 72˚C. All assays included a positive and negative control. PCR products were analyzed by electrophoresis in a 3% agarose gel and visualized using UV fluorescence after staining with ethidium bromide. Ladder of 100-bp was used as molecular weight marker (Invitrogen Life Technologies).

S1.5. Statistical analyses

The results were analyzed with the aid of MEDCAL 9.2 software using the t-test for paired samples, Wilcoxon and Fisher exact tests suitable for analysis of data from paired samples of small populations. Values of $p \leq 0.05$ were considered as significant.

Table 1. Data from patients with advanced prostate cancer (PCa) according to ethnicity, age, stage of cancer and duration of androgen deprivation therapy (ADT).

Age	Ethnicity				TNM Stage		ADT*		
	White	**Black**	**Brown**	**Yellow**	**III**	**IV**	**until 6**	**7-12**	**> 12**
50-59	4 (100%)	0	0	0	2 (50%)	2 (50%)	3 (75%)	0	1 (25%)
60-69	5 (50%)	2 (20%)	2 (20%)	1 (10%)	6 (60%)	4 (40%)	5 (50%)	4 (40%)	1 (10%)
70-79	4 (30.8%)	2 (15.4%)	4 (30.8%)	3 (23%)	5 (38.5%)	8 (61.5%)	7 (53.8%)	3 (23.1%)	3 (23.1%)
80-89	2 (66.7%)	1 (33.3%)	0	0	2 (66.7%)	1 (33.3%)	2 (66.7%)	1 (33.3%)	0
Total	15	5	6	4	15	15	17	8	5

***** In months

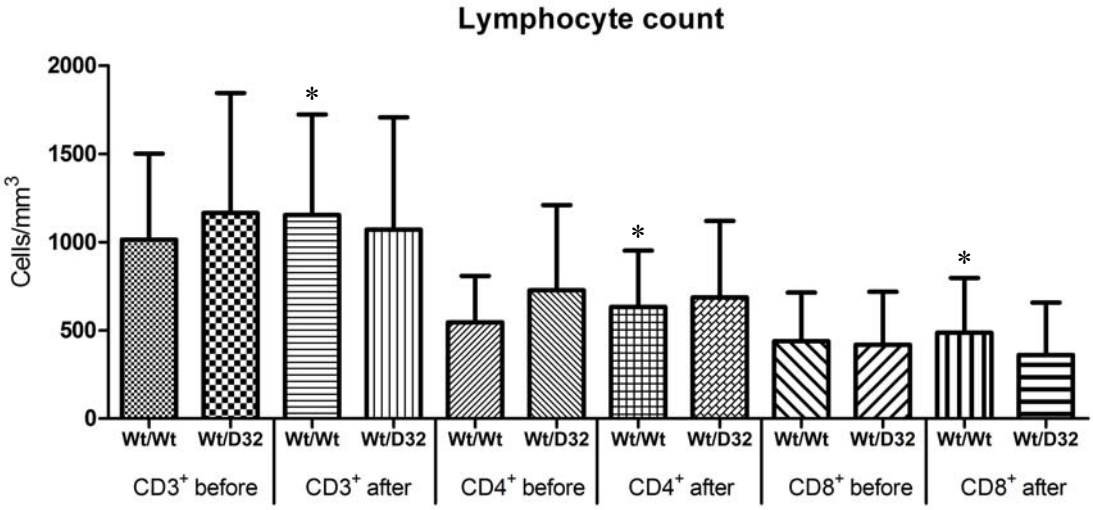

Figure 1. $CD3^+$ $CD4^+$ and $CD8^+$ T cell counts before and after oral administration of carboxymethyl-glucan (CM-G) for 28 days in patients with advanced prostate cancer of Wt/Wt and Wt/delta32 genotype for the CCR5 chemokine receptor. The signal * denotes $p \leq 0.05$, a statistically significant difference between the results before and after treatment, as determined by Wilcoxon test.

7 ARTIGO IV

Protective effect of carboxymethyl-glucan (CM-G) against DNA damage in patients with advanced Prostate Cancer

Short Communication submetida na Revista Genetics and Molecular Biology

Protective effect of carboxymethyl-glucan (CM-G) against DNA damage in patients with advanced prostate cancer

Marciane Magnani[a*], Raul Jorge Hernan Castro-Gomez[a], Mateus Prates Mori[b], Hellen Kuasne[b], Emerson Pereira Gregório[c], Farid Libos Jr.[c], Ilce Mara de Syllos Cólus[b]

[a]Departamento de Ciência e Tecnologia de Alimentos, [b]Departamento de Biologia Geral, Universidade Estadual de Londrina; [c]Instituto do Câncer de Londrina, Londrina, Paraná, Brasil

*Corresponding author. Address: Departamento de Ciência e Tecnologia de Alimentos, Universidade Estadual de Londrina, Rod Celso Garcia Cid, PR 445, Km 380, Campus Universitário, C. P. 6001, CEP 86051-990, Londrina, Paraná, Brasil.

Tel: +55-43-3371-4565; Fax: +55-43-3371-4080; E-mail: magnani2@gmail.com

Running Title: Protective effect of carboxymethyl-glucan (CM-G)

ABSTRACT

The carboxymethyl-glucan (CM-G) is a soluble derivative from *Saccharomyces cerevisiae* (1→3)(1→6)-β-D-glucan. The protective effect of CM-G against DNA damage in cells from patients with advanced Prostate cancer (PCa) undergoing Androgen Deprivation Therapy (ADT) were evaluated. The DNA damage scores were obtained by the comet assay before and after treatment with CM-G. The DNA damage reduction rates ranged from 18% to 87% to with an average of 59% and were not related to the increased number of leukocytes in the peripheral blood. The results demonstrate for the first time the protective effect of CM-G against DNA damage in patients with advanced PCa. Among the smokers, three had the highest DNA damage reduction rates after treatment with CM-G. No relation was observed between the DNA damage scores before and after CM-G treatment and age, alcoholism and radiotherapy.

Key words: antioxidant, β-D-glucan, carboxymethyl-glucan, prostate cancer

β-D-glucan from the cell wall of *Saccharomyces cerevisiae* is a polymer composed of repeating glucose units organized in a central skeleton linked by β (1 → 3) glycosidic bonds with side chains of varying size joined by β (1 → 6) linkages (Di Luzio *et al*., 1979). The benefits of the biological activity of (1→3)(1→6)-β-D-glucan are limited by its insolubility in water which has led to studies with derivatives of this polymer in an attempt to improve its availability. The soluble derivatives have advantages when administered *in vivo*, such as the absence of toxicity and adverse effects (Miadaková *et al*., 2005). Moreover, the derivatization may even enhance some biological activities, such as antioxidant and immunomodulation activities (Chen and Sevior, 2007; Xu *et al*., 2009). Another important factor is the maintenance of bioactivity, since (1→3)(1→6)-β-D-glucan soluble derivatives can cross the

gastrointestinal wall without causing damage to the digestive system, even when orally administered (Chorvatovicová *et al.*, 1996; Rice *et al*., 2005). Among the soluble derivatives of the *S. cerevisiae* (1→3)(1→6)-β-D-glucan, the carboxymethyl-glucan (CM-G), one of the most studied, shows a significant bioprotective properties such as antimutagenic, antigenotoxic, antioxidant and anticancer activities (Chorvatovicová *et al*., 1996; Babincová *et al*., 2002; Slamenová *et al*., 2003; Miadaková *et al*., 2005). The main mechanism proposed for these protective effects of CM-G is its ability to scavenge reactive oxygen species (Kogan *et al*., 2005), even at low concentrations (Babincová *et al*., 2002).

Prostate cancer (PCa) is the sixth most common cancer in the world, and represents about 10% of all cancers diagnosed. In Brazil, it is estimated that in 2009, 49.530 new cases were diagnosed. Excluding non-melanoma skin cancer, PCa is the most frequent type of cancer in males and is prevalent in all regions of the country. It has the second highest mortality rate in Brazil (National Cancer Institute of Brazil, 2010).

While a man with localized PCa is more likely to die from other comorbidities, the probability of death from PCa exceeds other causes when clinical metastases occur. At this stage, the goals of medicine are to increase the short survival period of patients and, above all, to improve the quality of life, which often declines with the disease evolution. The standard treatment of advanced PCa is Androgen Deprivation Therapy (ADT), during which the administration of hormone analogues of gonadotropin - releasing hormone (GnRH) leads to the receptor desensitization, thus inhibiting the release of LH and FSH and resulting in a chemical castration (Hellerstedt *et al*., 2002).

The complications that arise from chronic treatment in addition to advanced age and lifestyle habits make patients with advanced PCa even more vulnerable. Therefore, the use of natural bioactive compounds that cause no side effects and can improve the life's quality of these patients can be an interesting alternative.. Simulations of reactions that occur in the

human body due to the administration of CM-G has been reported in several experimental models, however evaluations in humans are scarce. Our goal in this study was to evaluate the possible protective effects of CM-G against DNA damage in the peripheral lymphocytes of patients with advanced PCa. The target population, which was not randomly selected, displays a clinical cancer description in some cases aggravated by lifestyle habits.

This study was conducted after approval from the Ethics Committee for Research involving Humans under registration number NTC406800007 in the records of the Brazilian Information on Ethics in Research involving Humans (SISNEP). Patients at the Londrina Cancer Institute (ICL) with a histological diagnosis of stage T3 (n=7) and T4 (n=13) prostate adenocarcinoma according to TNM stating (AJCC/UIAC, 2010) and undergoing androgen deprivation therapy (ADT) with goserelin acetate were included in this study after signing written informed consent. Individual information was collected by a face-to-face questionnaire (Carrano and Natarajana, 1988). The patients were followed in order to check possible side effects of the CM-G administration.

CM-G with substituition degree (DS) 0.8 was obtained from *Saccharomyces cerevisiae* insoluble β-glucan (Magnani *et al*. 2009) and distributed into gelatin capsules containing 20 mg of CM-G with starch as an excipient vehicle. This procedure was carried out by Vico Farma® (National Health Surveillance Agency in Brazil number 136420-0). The bottles were labeled with use instructions and contained between 28 and 35 capsules of CM-G. The varying number of capsules was used to help control the consumption by patients, and return of the bottle was requested after completion of treatment. After the daily administration of one capsule of CM-G (Demir et al. 2007), for 28 days, the return of the botlle should contain between zero and seven capsules, depending of initial amount received by each patient.

Peripheral venous blood samples were collected in the fasting state on day 1 of treatment before ingestion of the first capsule of CM-G and after 28 days of daily administration. The patients were distributed into couples and no more that two patients have the same day 1 or last day ingestion. Vaccutainer^{TM} tubes of 4.5 mL containing EDTA were used for collect the samples. The leukocyte counts were obtained by automated method Abbott Cell Dyn 3200. The samples to comet assay were kept under refrigeration and protected from light during the transport between the ICL and the Laboratory of Mutagenesis and Oncogenetics at the University of Londrina, Parana State, Brazil. The comet assay was conduced immediately after the arrivals of the samples in the Laboratory according Singh *et al*. (1988) with slight modifications. For the preparation of fresh blood slides, 20 μL of peripheral blood was mixed with 180 μL of 0.5% low melting temperature agarose and applied to microscope slides pre-coated with 1.5% normal melting temperature agarose. The slides were covered with a microscope coverslip and refrigerated for 5 min at 4^{o}C. Duplicate coded slides were prepared for each sample, and the samples were processed in a randomized manner. Cells attached to the agarose matrix were submitted to lysis by immersion in an ice-cold solution containing 1% Triton X-100, 10% DMSO, 2.5 M NaCl, 100 mM EDTA, and 10 mM Tris (pH 10.0) for at least 1 h. The DNA was subsequently denatured for 20 min in an alkaline buffer (300 mM NaOH and 1 mM EDTA, final pH> 13) and electrophoresed for 20 min at 25 V and 300 mA (1.25 V/cm) in the same buffer. After electrophoresis, the slides were then neutralized (400 mM Tris, pH 7.5) and fixed with 100% ethanol for 10 min. For visual analysis, the slides were coded and stained with ethidium bromide (20 mg/mL) and one hundred cells per patient were immediately scored at 400× magnification using a fluorescence microscope (Nikon-Brazil) with a blue (488 nm) excitation filter and yellow (515 nm) emission (barrier) filter. Just one person analyzed the slides throughout the study by visual scoring. The cells were classified into four categories representing different degrees of DNA

damage, ranging from no visible migration (class 0, undamaged cells) to the maximum length comet (class 3, maximally damaged cells (DC) (Kobayashi *et al*., 1995; Forchahammer *et al*., 2008). The DC frequency was obtained by determining the number of undamaged (class 0) and DC from classes 1, 2 and 3 and dividing by the total number of cells analyzed in each treatment. The total score for 100 nucleoids was obtained by multiplying the number of cells in each class by the damage class (Manoharan and Banerjee, 1985): Total score = $(1 \times n_1) + (2 \times n_2) + (3 \times n_3)$, where n = number of cells in each class analyzed. Thus, the total score could range from 0 (100 cells presenting no damage) to 300 (all cells presenting class 3 damage). The damage reduction rates for each patient were determined based in scores observed before and after CM-G.

The results were analyzed with MEDCAL 9.2 software using non-parametric Mann-Whitney test for analysis of DNA damage scores before and after CM-G treatment and Wilcoxon test for analysis of the leukocyte counts, considering significant difference $p \leq 0.05$.

After completing treatment with CM-G the number of capsules returned by the patients corresponded to the use of 28 capsules in the period and, through weekly contacts, any side effects associated with the use of CM-G were reported. The reports included typical symptoms of the PCa under chronic treatment of testosterone inhibition, like urinary symptoms and sexual dysfunction. The bone pain was reported by 90% of the patients. This effect of the ADT occurs due the loss of bone mineral density (BMD) which is worsened in these patients due to age (Diamond *et al*., 2004).

Regarding to diet, all men mentioned eating red meat and salad an average of five and three times a week, respectively. However, 65% of the patients reported unintentional weight loss. When asked about it, they responded that the reduction in food intake was due to symptoms such as taste changes, mouth dryness, vomiting, constipation and nausea. Those

symptoms persisted during the treatment with CM-G, except for two patients who reported a decrease in nausea.

Data for each patient obtained from the medical records follow-ups and the individual questionnaire are presented in Table 1 showing various individual lifestyle characteristics such as smoking, age, alcoholism, exposure to chemical and radiation, that influenced the baseline of DNA damage.

Analysis of the DNA damage scores data obtained before and after treatment with CM-G shows significant difference ($p \leq 0.05$), with DNA damage reduction rates ranging from 18% to 87% with average of 59% (Figura 1). The number of DC into classes 1, 2 and 3 have significantly decreased ($p \leq 0.05$) after the CM-G treatment. In the other hand the number of undamaged cells increases for all patients (Figure 2). Based on the fact that there are reports of increased stimulation of hematopoiesis by CM-G (Pospisil *et al*., 1991), the reduction in DNA damage could be attributed to an increase in the number of cells. In this way, the average numbers of white blood cells counted before (1789.25 ± 766.1 / mm^3) and after (1827.9 ± 757.8 / mm^3) administration of CM-G were not statistically different ($p=0.63$).

Five patients (25%) were classified as current smokers. Nicotine, the most toxic component of cigarettes, is known to cause oxidative stress by inducing the generation of reactive oxygen species (ROS) (Sener *et al*., 2007). It is known that CM-G is a potent antioxidant, which is effective even at very low concentrations, and plays an important role in protecting biological membranes against the adverse effects of free radicals (Babincová *et al*., 2002). In their study, Sener *et al*. (2007) suggested that the oral administration of soluble β-D-glucan from *S. cerevisiae* act effectively against the chronic toxicity of nicotine, reducing the oxidative damage. Our data is consistent with these findings, since among the patients evaluated, three smokers (5, 19 and 20) showed the three highest DNA damage reduction rates after treatment with CM-G.

Eight men (40%) showed alcohol dependence. Ethanol alone does not induce lesions in the DNA of human lymphocytes, but its primary metabolite, acetaldehyde, induces breaks in single- and double-stranded DNA (Singh *et al*., 1995). Together with other factors that generate DNA damage it may have contributed to some of the highest rates of damaged DNA observed in these patients 1, 5, 8 and 10 before CM-G (Table 1).

Six patients (30%) received radiotherapy before starting ADT (Table 1) with purpose of controlling the localized prostate tumor. The exposure of patients with cancer to therapeutic doses of radiation causes typical changes in the genomic DNA of lymphocytes, induced by free radicals (Olinski *et al*., 1996). Morever, the generation of free radicals in the tissues may continue after exposure for longer periods of time (Robbins and Zhao, 2004). The high radical scavenging activity of CM-G from *S. cerevisiae*, with DS 0.8, was confirmed by paramagnetic resonance spectroscopy (Kogan *et al*., 2005). Therefore, CM-G could help decrease the oxidative damage that results from radiotherapy, even after the end of its.

Of the total number of patients, 85% were occupationally exposed, for at least 10 years, to a mixture of agrochemicals (Table 1), like organophosphates, carbamates and organochlorines including DDT and BHC, the use of which has been banned in Brazil for over 20 years due to their high toxicity.

We believe that the treatment with CM-G, can protect against DNA damage promoted by mutagenic compounds through desmutagenic activity, which is able to block the action of damage-inducing agents, or by bio-antimutagenic activity, which exerts its effect by preventing damage or promoting DNA repair, since Oliveira *et al*. (2007) showed that this is a characteristic of the (1→3)(1→6)-β-D-glucan from *S. cerevisiae*.

Prostate carcinogenesis may be related to chronic and acute prostate inflammation (De Marzo et al., 2007). The possible chemoprotective effect of antioxidants may protect prostate cells from the DNA damage that occurs in the setting of inflammatory lesions (Smigel, 1998).

Thus, there is a possibility that the DNA damage generated by the typical inflammation profile has been reduced through the administration of CM-G.

The results presented here are of great importance to the scientific community and highlight the protective effect of CM-G against DNA damage, which was shown for the first time in humans. These results suggest that CM-G has the potential to be useful to improve the short-term survival of patients with advanced PCa. Studies on the other beneficial effects of CM-G administration such as those related to immunomodulation, are underway to access if the use of CM-G can improve the prognosis of these patients.

Acknowledgements

The authors wish to thank Londrina Cancer Institute for the patient's records and the National Council of Scientific and Technological Development, Brazil (CNPq), for financial support.

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Internet Resources

American Joint Committee on Cancer/ Union International Against Cancer (AJCC/UICC) http//www.cancerstaging.org. (May 10, 2010).

National Cancer Institute of Brazil (INCA) http//www.inca.gov.br (April 5, 2010).

Table 1. Data obtained from the medical reports of 20 patients of the Cancer Institute of Londrina City, Parana State and from in the questionnaire completed after formal written consent and prior to administration of carboxymethyl-glucan (CM-G).

Patient	Age (years)	Ethnicity	TNM	Goserelin use (months)	Life style		Agrochemicals[c]	Radiotherapy
					Smoking[a]	Alcoholic[b]		
1	73	African descendent	IV	6	no	yes	yes*	no
2	77	European descendent	III	6	no	no	yes	no
3	84	European descendent	IV	7	no	no	yes	yes
4	70	Asian descendent	IV	7	no	no	yes	yes
5	63	European descendent	IV	12	yes	yes	yes	no
6	71	European descendent	IV	6	no	no	yes	no
7	71	African descendent	III	8	no	no	yes*	no
8	63	African descendent	III	10	no	yes	yes	yes
9	71	European descendent	IV	19	no	no	yes	no
10	57	European descendent	IV	23	yes	yes	yes	no
11	63	European descendent	IV	6	no	no	no	no
12	79	Asian descendent	IV	36	no	no	yes	no
13	67	African descendent	IV	10	no	yes	yes	no
14	70	African descendent	III	6	yes	no	yes	yes
15	68	European descendent	III	18	no	yes	yes	no
16	63	African descendent	IV	6	no	no	no	no
17	62	Asian descendent	III	5	no	yes	yes	no
18	52	European descendent	IV	5	no	no	yes	yes
19	76	African descendent	IV	6	yes	no	yes	yes
20	80	African descendent	III	6	yes	yes	no	no

[a]Patients who had quit smoking for less than one year are considered smokers.
[b]Patients with behavior in alcohol-dependence
[c]Yes = patients who have had contact or exposure to agrochemicals for ≥ 10 years.
* Patients who reported intoxication by agrochemicals.

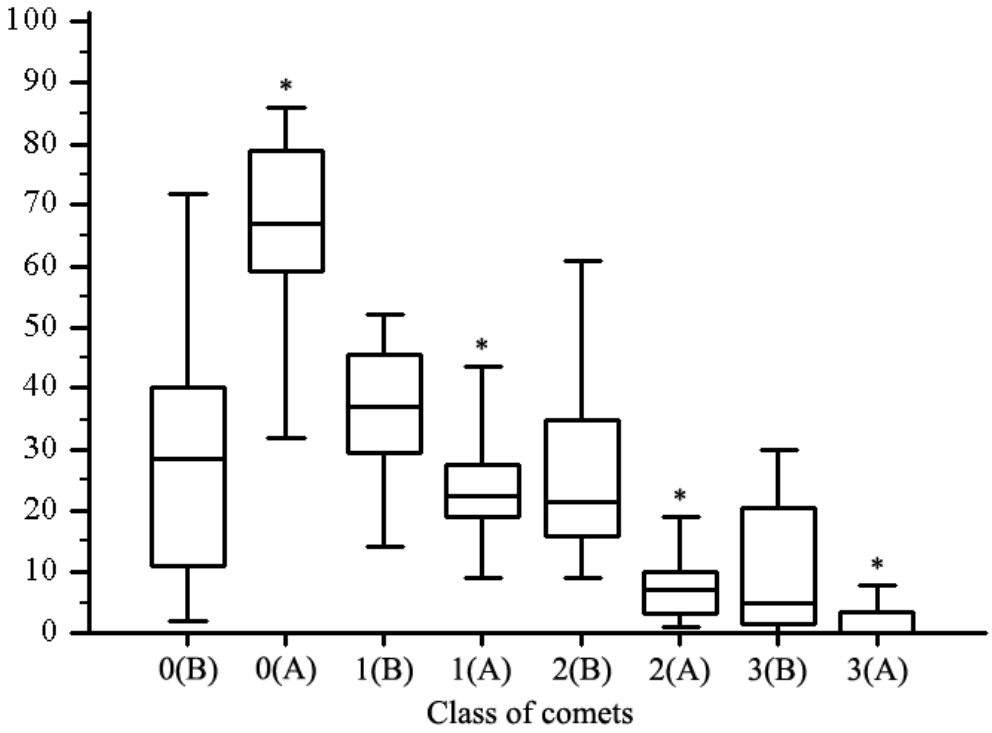

Figure 1. Distribution of the damaged cells in different classes of DNA damage, observed in the Comet assay, for patients with advanced cancer prostate before and after 28 days-treatment with carboxymethyl-glucan (CM-G); B=before and A= after; Bars denote values minimum and maximum observed for each damage class and horizontal line inside the box represents the median; * denotes $p \leq 0.05$, a statistically significant difference from the result before the treatment, as determined by Mann-Whitney test.

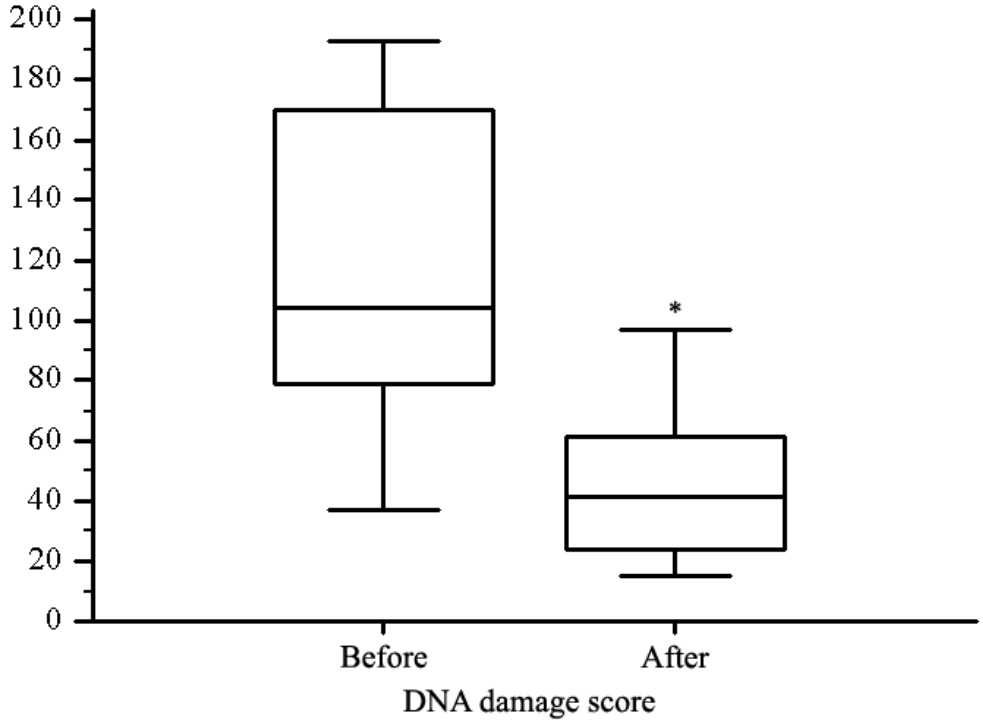

Figure 2. DNA damage scores observed in the Comet Assay for patients with advanced cancer prostate before and after 28 days-treatment with carboxymethyl-glucan (CM-G); B=before and A= after; Bars denote values minimum and maximum observed for each score and horizontal line inside the box represents the median; * denotes $p \leq 0.05$, a statistically significant difference between the results before and after treatment, as determined by Mann-Whitney test.

8 CONCLUSÕES

- A metodologia de extração otimizada no presente estudo é eficiente e permite a obtenção de 94 % da β-glucana disponível na parede celular de *Saccharomyces cerevisiae* sem o emprego de condições drásticas e, ou a geração de efluentes nocivos ao meio ambiente;

- A Carboximetil-Glucana (CM-G) obtida pela derivatização da β-glucana insolúvel, extraída pela metodologia proposta, alcança grau de substituição considerado ótimo para a atividade biológica e não apresenta quaisquer efeitos citotóxicos, genotóxicos, ou interferência na modulação da viabilidade celular de células CHO-k1;

- A CM-G administrada oralmente, durante 28 dias, em dose diária de 20mg não provoca quaisquer efeitos colaterais, e ou alterações nas funções renal e hepática de pacientes com câncer de próstata (PCa) avançado sob hormonioterapia;

- A administração oral de 20mg diários da CM-G, durante 28 dias, aumenta significativamente as contagens de leucócitos totais, hemáceas e plaquetas, níveis de hemoglobina e hematócrito de pacientes com PCa avançado sob hormonioterapia;

- A administração oral de 20mg diários da CM-G, durante 28 dias aumenta linfócitos T $CD3^+$, $CD4^+$ e $CD8^+$, em pacientes com PCa avançado sob hormonioterapia;

- O aumento distinto dos linfócitos T $CD3^+$, $CD4^+$ e $CD8^+$ nos pacientes de genótipo Wt/Wt e Wt/delta32 para o receptor de quimiocina CCR5 sugere uma interação entre a imunomodulação pela CM-G e o receptor CCR5;

- A CM-G administrada oralmente durante 28 dias, em dose diária de 20mg exerce efeito de proteção contra danos no DNA de pacientes com PCa avançado sob hormonioterapia;

ANEXO A

Ofício da aprovação do projeto de tese pelo Comitê de Ética

Universidade Estadual de Londrina
COMITÊ DE ÉTICA EM PESQUISA

COMITÊ DE ÉTICA EM PESQUISA ENVOLVENDO SERES HUMANOS
Universidade Estadual de Londrina/ Hospital Universitário Regional Norte do Paraná
Registro CONEP 268

Parecer Nº 212/07 **CAAE Nº 0214.0.268.000-07**	**Londrina, 23 de novembro de 2007.**

PESQUISADOR: **RAUL JORGE HERMAN CASTRO GOMEZ**

Ilmo Sr.

O "**Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina/ Hospital Universitário Regional Norte do Paraná**" de acordo com as orientações da Resolução 196/96 do Conselho Nacional de Saúde/MS, APROVA a execução do projeto:

"EXTRAÇÃO E CARACTERIZAÇÃO DA B-GLUCANA DE SACCHAROMYCES CEREVISIAE E AVALIAÇÃO DO POTENCIAL IMUNOMODULADOR EM HUMANOS".

Informamos que a Sr deverá comunicar, por escrito, qualquer modificação que ocorra no desenvolvimento da pesquisa, bem como deverá ser apresentado ao CEP/UEL relatório final da pesquisa.

Situação do Projeto: **APROVADO**

Atenciosamente,

Profª. Dra. ***Nilza Maria Diniz***
Coordenadora
Comitê de Ética em Pesquisa-CEP/UEL

ANEXO B

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO PARA PARTICIPAÇÃO DA AVALIAÇÃO DO POTENCIAL IMUNOMODULADOR DA β-GLUCANA (CM-G) EM CÁPSULAS

Se tiver qualquer dúvida, ou necessitar de informações adicionais, entre em contato com a Sra Marciane Magnani, pelo fone: (43) 3337 4984 ou (43) 9923 9920.

Eu, _____, R.G. _____,

aceito participar do Projeto "**Extração e Caracterização da β-glucana de *Saccharomyces cerevisiae* e avaliação do potencial imunomodulador em humanos**", como integrante do grupo que receberá cápsulas, contendo 20 mg de β-glucana na forma carboximetilada (CM-G), para ingestão em jejum, diariamente, durante 28 dias consecutivos. Estou informado que antes da ingestão da 1ª cápsula (dia zero), e um dia após a ingestão da última (28º dia) serão coletadas amostras de sangue para verificação do efeito protetor da β-glucana, valores de células de defesa e quaisquer outras informações decorrentes dos exames. Além disso, tenho conhecimento que a referida β-glucana foi extraída de *Saccharomyces cerevisiae***,** a levedura empregada pela indústria de panificação, sucroalcoleira e cervejeira, e que a β-glucana, que é um polímero de glicose com potencial imunomodulador. Fui informado que a ausência de toxicidade já foi cientificamente testada e comprovada e que a β-glucana não representa quaisquer riscos para minha saúde. Terei a tarefa de ingerir uma cápsula, pela manhã, antes do meu desjejum. Estou ciente que tenho total liberdade para deixar de integrar o grupo da pesquisa, em qualquer fase, se assim o decidir. Entendo que, ao participar, estarei colaborando no desenvolvimento de uma Tese de Doutorado, e, portanto, no treinamento e formação de um profissional.

Londrina,_____ de _____ de 200__.

Assinatura

ANEXO C

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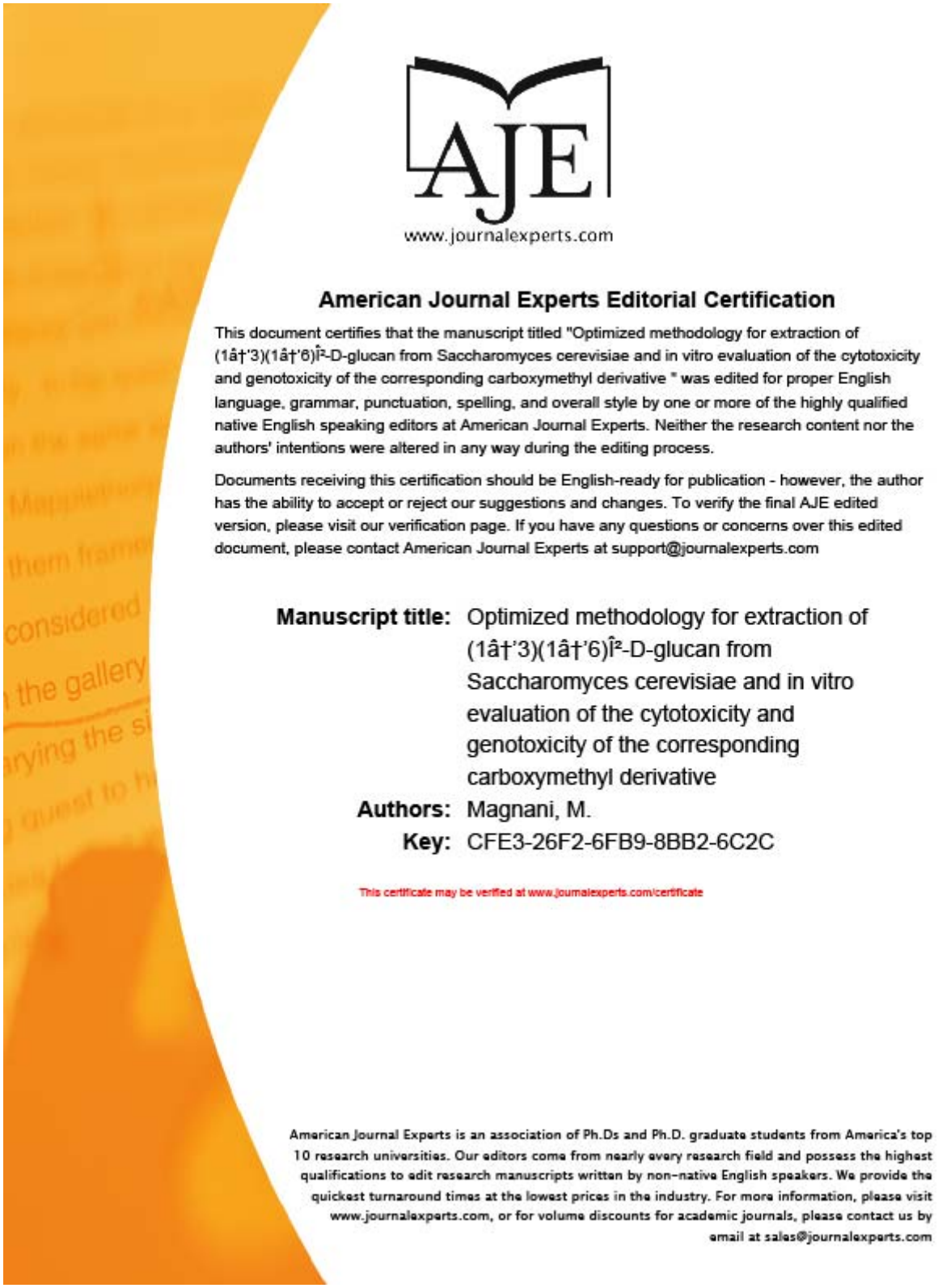

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Manuscript title: Analysis of the CC chemokine receptor (CCR5) Delta32 polymorphism and peripheral T cells in prostate cancer patients treated with CM-G

Authors: Magnani, M.

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Title page
Abstract
Keywords
1. Introduction
2. Results and discussion
3. Experimental
4. Conclusions
(then the usual, un-numbered)
Acknowledgements
References
Table(s) and figure(s) (on individual pages) with caption(s) (as a list)

Short Communications:
Title page
Abstract
Keywords

1.1. Introduction
1.2. Results and discussion
1.3. Conclusions
(then the usual, un-numbered)
Acknowledgements
References
Table(s) and figure(s) (on individual pages) with caption(s) (as a list)
'Experimental' information should be supplied as supplementary material (see 6. below)

- Advised length for research articles: 10–15 pages of 1.5-spaced manuscript pages (to include figures and tables).
- Advised length for short communications 4–9 pages of 1.5-spaced manuscript pages (to include figures and tables).
- **Authors are encouraged to submit supplementary material** (see 6. below).
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- Additional experimental data should be submitted as supplementary material which will be hosted online.
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ANEXO G

Comprovante de submissão de artigo na Natural Product Research

Natural Product Research - New Manuscript ID GNPL-2010-0425

Dear Dr Magnani:

Thank you for recently submitting your manuscript entitled "Analysis of peripheral T cells and the CC Chemokine Receptor (CCR5) Delta32 polymorphism in prostate cancer patients treated with Carboxymethyl–Glucan (CM–G)" to our new ScholarOne Manuscripts site.

Due to your submission coinciding with the launch of the site, we have had to change the manuscript ID you were assigned previously. Please note that your new manuscript ID is now GNPL-2010-0425.

This manuscript ID should be used in all future correspondence with the journal office and editors. We apologise for any inconvenience this may cause, and please do not hesitate to contact the journal office if you have any queries about your paper.

You can view the status of your manuscript at any time by checking your Author Centre after logging in to http://mc.manuscriptcentral.com/gnpl .

Thank you for submitting your manuscript to Natural Product Research.

Sincerely,

Natural Product Research Editorial Office

ANEXO H

Certificado da revisão da língua inglesa do artigo submetido na Genetics and Molecular Biology

www.journalexperts.com

American Journal Experts Editorial Certification

This document certifies that the manuscript titled "Protective effect of carboxymethyl glucan (CM-G) against DNA damage in patients with advanced prostate cancer " was edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native English speaking editors at American Journal Experts. Neither the research content nor the authors' intentions were altered in any way during the editing process.

Documents receiving this certification should be English-ready for publication - however, the author has the ability to accept or reject our suggestions and changes. To verify the final AJE edited version, please visit our verification page. If you have any questions or concerns over this edited document, please contact American Journal Experts at support@journalexperts.com

Manuscript title: Protective effect of carboxymethyl glucan (CM-G) against DNA damage in patients with advanced prostate cancer

Authors: Magnani, M.

Key: F046-7E66-6B30-E8BB-378E

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ANEXO I

Normas para publicação na Genetics and Molecular Biology

INSTRUCTIONS TO AUTHORS

Scope and policy

Genetics and Molecular Biology (formerly named Revista Brasileira de Genética/Brazilian Journal of Genetics - ISSN 0100-8455) is published by the Sociedade Brasileira de Genética (Brazilian Society of Genetics).

The Journal considers contributions that present the results of original research in genetics, evolution and related scientific disciplines.

Although Genetics and Molecular Biology is an official publication of the Brazilian Society of Genetics, contributors are not required to be members of the Society.

It is a fundamental condition that submitted manuscripts have not been and will not be published elsewhere. With the acceptance of a manuscript for publication, the publishers acquire full and exclusive copyright for all languages and countries.

Manuscripts considered in conformity with the scope of the journal, judged by the Editor in conjunction with the Editorial Board, are reviewed by the Associate Editor and two or more external reviewers. Acceptance by the Editor is based on the quality of the work as substantial contribution to the field and on the overall presentation of the manuscript.

Submission of papers

1. Manuscripts should be submitted to:

Angela M. Vianna-Morgante, Editor-in-Chief
Genetics and Molecular Biology
E-mail: editor@gmb.org.br

2. A submission package sent to the Editorial Office must contain:

a) The manuscript that must be submitted by the Corresponding Author. This is the person who will also check the page proofs, and arranges for the payment that may incur during the editorial process.

b) An accompanying cover letter, signed by the corresponding author, stating that the data have not being published and are not under consideration elsewhere, and that all authors have approved the submission of the manuscript. It must also inform the e-mail addresses of all other authors so that they can be contacted by the Editorial Office for confirmation of the submission. Possible conflicts of interest (e.g. due to funding, consultancies) must be disclosed.

c) An electronic copy of the text, tables and figures, including supplementary material to be published online only. Formats for text are Word or RTF in Windows platform. Images in TIFF or JPEG formats should be sent in separate files (For Figures, see detailed instructions in 3.1.h).

d) Manuscripts including photos or any other identifiable data of human subjects must be accompanied by a copy of the signed consent by the individual or his/her guardian.

Failure to adhere to these guidelines can delay the handling of your contribution and manuscripts may be returned before being reviewed.

3. Categories of Contribution
3.1. Research Articles

Manuscripts must be written in English in double-spaced, 12-point type throughout; formatted to A4 paper with 2.5 cm margins; marked with consecutive line and page numbers, beginning with the cover page.

The following elements must start on a new page and be ordered as they are listed below:

a) The title page must contain: a concise and informative title; the authors' names (first name at full length); the authors' institutional affiliation, including department, institution, city, state or province and country; different affiliations indicated with superscript Arabic numbers; a short running title of about 35 characters, including spaces; up to five key words; the corresponding author's name, postal address, phone and fax numbers and email address.

b) The Abstract must be a single paragraph that does not exceed 200 words and summarizes the main results and conclusions of the study. It should not contain references.

c) The text must be as succinct as possible. Text citations: articles should be referred to by authors' surnames and date of publication; citations with two authors must include both names; in citations with three or more authors, name the first author and use et al. List two or more references in the same citation in chronological order, separated by semi-colons. When two or more works in a citation were published in the same year, list them alphabetically by the first author surname. For two or more works by the same author(s) in a citation, list them chronologically, with the years separated by commas. (Example: Freire-Maia et al., 1966a, 1966b, 2000). Only articles that are published or in press should be cited. In the case of personal communications or unpublished results, all contributors must be listed by initials and last name (et al. should not be used). Numbers: In the text, numbers nine or less must be written out except as part of a date, a fraction or decimal, a percentage, or a unit of measurement. Use Arabic numerals for numbers larger than nine. Binomial Names: Latin names of genera, species and infraspecific taxa must be printed in italics; names of orders and families should appear in the Title and also when first mentioned in the text. URLs for programs, data or other sources should be listed in the Internet Resources Section, immediately after the References Section, not in the text.

The text includes the following elements:

Introduction - Description of the background that led to the study.

Material (or Subjects) and Methods - Details relevant to the conduct of the study. Statistical methods should be explained at the end of this section.

Results - Undue repetition in text and tables should be avoided. Comment on significance of results is appropriate but broader discussion should be part of the Discussion section.

Discussion - The findings of the study should be placed in context of relevant published data. Ideas presented in other publications should not be discussed solely to make an exhaustive presentation.

Some manuscripts may require different formats appropriate to their content.

d) The Acknowledgments must be a single paragraph that immediately follows the discussion and includes references to grant support.

e) The References Section: references must be ordered alphabetically by the first author surname; references with the same first author should be ordered as follows: first, as single author in chronological order; next, with only one more co-author in alphabetical order by the second author; and finally followed by references with more than two co-authors, in chronological order, independent of the second author surnames. In references with more than 10 authors only the first ten should be listed, followed by et al. Use standard abbreviations for journal titles as suggested byNCBI (http://www.ncbi.nlm.nih.gov/journals/).

Only articles that are published or in press should be included in this section. Works submitted for publication but not yet accepted, personal communications and unpublished data must be cited within the text. "Personal communication" refers to individuals other than the authors of the manuscript being submitted; "unpublished data" refers to data produced by at least one of the authors of the manuscript being submitted. Works of restricted circulation (e.g., theses not available in public databases, congress abstracts not published in regular journals or public databases) should not be listed in this section.

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Yonenaga-Yassuda Y, Rodrigues MT and Pellegrino KCM (2005) Chromosomal banding patterns in the eyelid-less microteiid lizard radiation: The X1X1X2X2:X1X2Y sex chromosome system in Calyptommatus and the karyotypes of Psilophtalmus and Tretioscincus (Squamata, Gymnophthalmidae). Genet Mol Biol 28:700-709.

Sample book citation:
Dobzhansky T (1951) Genetics and Origin of Species. 3rd edition. Columbia University Press, New York, 364 pp.

Sample chapter-in-book citation:
Crawford DC and Howard-Peebles PN (2005) Fragile X: From cytogenetics to molecular genetics. In: Gersen SL and Keagle MB (eds) The Principles of Clinical Cytogenetics. 2nd edition. Humana Press, New Jersey, pp 495-513.

Sample electronic article citation:
Simin K, Wu H, Lu L, Pinkel D, Albertson D, Cardiff RD and Van Dyke T (2004) pRb inactivation in mammary cells reveals common mechanisms for tumor initiation and progression in divergent epithelia. Plos Biol 2:194-205. http://www.plosbiology.org.

f) Internet Resources Section: this section should contain a list of URLs referring to data presented in the text, software programs and other Internet resources used during data processing. Date of consultation must be stated.
Sample Internet resource citation:
Online Mendelian Inheritance in Man (OMIM),
http://www.ncbi.nlm.nih.gov/OMIM (September 4, 2005)
LEM Software,
http://dir.niehs.nih.gov/dirbb/weinbergfiles/hybrid_design.htm (September 4, 2005)

g) Tables must be in Word format prepared with table tool, inserted at the end of the main text file, each table starting on a new page. A concise title should be provided above the table. Tables must be numbered consecutively in Arabic numerals. Each column must have a title in the box head. Footnotes typed directly below the table should be indicated in lowercase superscript numbers.

h) Figures must be numbered consecutively in Arabic numerals. Images should be in TIFF or JPEG format and provided in separate files. Identify each illustration by the first author name and the number of the respective figure. Figures in Word, PowerPoint or Excel format cannot be published. Only sequence data can be presented in Word format. Journal quality reproduction will require grayscale resolution yielding 300 dpi, color figures should be at 600 dpi. These resolutions refer to the output size of the file; if it is anticipated that images will be enlarged or reduced, the resolutions should be adjusted accordingly. Figures composed of several elements should be sent as a single panel, obeying the print size definitions of the journal (single or two columns width). Scanned figures should not be submitted. Color illustrations can be accepted.
Figure legends must be included in the main text file and should be typed on a new page that immediately follows the tables.

i) Nomenclature should adhere to current international standards.

j) Sequences may appear in text or in figure. DNA, RNA and protein sequences equal to or greater than 50 units must be entered into public databases. The accession number must be provided and released to the general public together with publication of the article.

k) Data access: reference should be made to availability of detailed data and materials used for reported studies.

l) Ethical issues: reports of experiments on live vertebrates must include a statement that the institutional review board approved the work and the protocol number must be provided. For experiments involving human subjects, authors must also include a statement that informed consent was obtained from all subjects. If photos or any other identifiable data are included, a copy of the signed consent must accompany the manuscript.

m) Supplementary Material: Data that the authors consider of importance for completeness of a study, but which are too extensive to be included in the published version, can be submitted as Supplementary Material. This material will be made available together with the electronic version. In case a manuscript contains such material, it should be appropriately identified within the text file. Supplementary material in tables should be identified as Table S1, Table S2, etc., in case of figures, they should be named accordingly, Figure S1, Figure S2. In addition, a list of this material should be presented at the end of the manuscript text file, containing the following statement: Supplementary material - the following online material is available for this article: - Table S1 < short title > - Figure S1 - < short title >
This material is available as part of the online article from http://www.scielo.br/gmb

3.2 Short Communications
Present brief observations that do not warrant full-length articles. They should not be considered preliminary communications. They should be 15 or fewer typed pages in double spaced 12-point type, including literature cited. They should include an Abstract no longer than five percent of the paper's length and no further subdivision with introduction, material and methods, results and discussion in a single section. Up to to four items (tables and/or figures) may be submitted. The title page and reference section format is that of full-length article.

3.3 Letters to the Editor
Relate or respond to recent published items in the journal. Discussions of political, social and ethical issues of interest to geneticists are also welcome in this form.

3.4 Review Articles
Review Articles are welcome. The Editor should be contacted prior to submission.

3.5 Book Reviews
Publishers are invited to submit books on Genetics, Evolution and related disciplines, for review in the journal. Aspiring reviewers may propose writing a review.

3.6 History, Story and Memories
Accounts on historical aspects of Genetics relating to Brazil.

4. Proofs and Copyright Transfer
Page proofs will be sent to the corresponding author. Changes made to page proofs, apart from printer's errors, will be charged to the authors. Notes added in proof require Editorial approval. A form of consent to publish and transfer of copyright will have to be signed by the corresponding author, also on behalf of any co-authors.

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ANEXO J

Comprovante de submissão de artigo na Genetics and Molecular Biology

Subject: MS2010/194 - Protective effect of carboxymethyl-glucan (CM-G) against DNA damage in patients with advanced Prostate Cancer

Marciane Magnani*, Raul Jorge Hernan Castro-Gomez[a], Mateus Prates Mori[b], Hellen Kuasne[b], Emerson Pereira Gregório[c], Farid Libos Jr. [c], Ilce Mara de Syllos Cólus[b]

Dear Dr. Magnani,

We acknowledge receipt of the abovementioned manuscript to which has been assigned the following number: **MS2010/194**.

All correspondence concerning this manuscript will be sent to the corresponding author.

Please refer to the manuscript number whenever you contact us.

We thank you for submitting your paper to *Genetics and Molecular Biology*.

Yours sincerely,

Angela M. Vianna-Morgante

Editor

Genetics and Molecular Biology

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