UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
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AVALIAÇÃO DO EFEITO ANTI-INFLAMATÓRIO DA SILIBININA SOBRE MONÓCITOS DE SANGUE PERIFÉRICO DE INDIVÍDUOS SAUDÁVEIS, INFECTADOS *IN VITRO* COM CEPA VIRULENTA DE *Paracoccidioides brasiliensis*

CAMILA FERREIRA BANNWART

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Orientadora: Profa Titular Maria Terezinha Serrão Peraçoli

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Introdução

A silimarina, um extrato padronizado obtido de frutos e sementes de *Silybum marianum (L.) Gaertn.* (Asteraceae - Compositae) é extraído de uma das mais antigas e tradicionais ervas medicinais *Silybum marianum* e vêm sendo utilizado como hepatoprotetor há mais de dois mil anos. Atualmente é reportado como a preparação mais freqüentemente utilizada por pacientes voluntários com câncer (Singh et al., 2004). Originalmente nativa do sul da Europa até a Ásia na região do Mediterrâneo e atualmente encontrada em todo o mundo, é constituída por 70% de flavonóides, sendo eles: *silybin* (60-70%), *silychristin* (20%), *silydianin* (10%) *e isosilybin* (5%) e outros 30% de uma fração quimicamente indefinida, contendo compostos polifenólicos poliméricos e oxidados (Rui, 1991; Kren & Walterova, 2005; Pradhan & Girish, 2006). Tem como componente mais ativo e abundante a silibinina (Fig 1A), que, assim como a silimarina, apresenta interessantes atividades de citoproteção, anti-inflamatórias, antifibróticas e anticarcinogênicas (Kren & Walterova, 2005).

Flavonóides são um dos mais importantes grupos de compostos polifenólicos presentes, abundantemente, em dietas humanas com um consumo diário de aproximadamente 1g (Aherne & O´brien, 2002). Milhões de flavonóides são identificados em frutas, verduras e extratos de plantas como chás ou suco de uva e associados a uma ampla variedade de propriedades farmacológicas e químicas benéficas à saúde.

A silimarina é considerada extremamente segura devido a sua baixa interferência com a farmacocinética de terapias de câncer em doses menores que 5 g/dia (Gurley et al., 2004). Estudos farmacológicos, realizados anteriormente, indicam que a silimarina não é tóxica, mesmo quando utilizada em altas doses, no tratamento de cirrose hepática alcoólica (Ferenci et al., 1989). Resultados preliminares de nosso laboratório, avaliando a viabilidade de monócitos humanos tratados *in vitro* com altas concentrações de silibinina, variando de 1,5 a 500 ug/mL confirmam que não há citotoxicidade celular causada por esse flavonóide (Bannwart et al., 2009).

Os mecanismos moleculares envolvidos nos efeitos da silimarina/silibinina ainda não estão bem esclarecidos, mas parecem envolver a supressão do fator de transcrição nuclear kappa B (NF-kB), descoberto há mais de 20 anos como uma proteína capaz de se ligar à região promotora (GGGACTTTCC) do gene que codifica a cadeia leve kappa

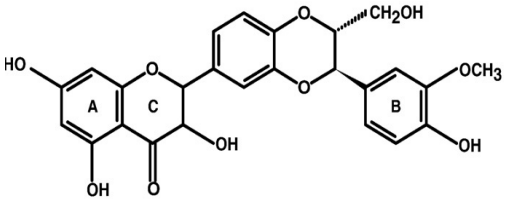
de imunoglobulinas em linfócitos B maduros e células plasmáticas (Sen & Baltimore, 1986). Hoje se sabe que está presente em todas as células do organismo e, em mamíferos, esse fator de transcrição nuclear é formado pelas proteínas da família Rel: p50, p52, p65 (RelA), c-Rel e RelB. Isso indica que diferentes tipos de combinações podem ocorrer nos hetrodímeros de NF-kB, sendo então capazes de interferir em diferentes genes (Baeuerle et al., 1996). Sua composição mais conhecida e estudada é formada pelas subunidades: p50 e p65, capaz de se ligar ao DNA (Fig 1B) e agir como um fator de transcrição em vários genes envolvidos nas respostas imune e inflamatória e em processos carcinogênicos (Manna et al., 1999; Kang et al., 2002). O NF-kB é ativado por uma variedade de componentes microbianos que sinalizam através de receptor de TNF-α (TNFR), de interleucina-1 (IL-1R), de células B (BCR), de células T (TCR), de linfotoxina β (LT βR), de fator ativador de célular B (BAFFR) e especialmente receptores toll-like (TLR), iniciando a transcrição de genes associados à resposta inflamatória (Gloire et al., 2006; Lin & Karin, 2007).

Em células não estimuladas, NF-kB encontra-se no citoplasma ligado a proteínas inibitórias da família I-kappaB (IkB), sendo elas: IkB-α, IkB- β, p105/IkB-γ, p100/IkB-d e IkB-ε (Baeuerle & Baltimore, 1996). O estímulo da célula por agentes pró-inflamatórios, como lipopolissacáride (LPS) e fator de necrose tumoral-alfa (TNF-α) inicia um processo dependente da kinase - IKK, que podem ser do tipo: IKKα, IKKβ e IKKγ ou NEMO. É bem conhecido, na via de ativação clássica de NF-kB, através da indução de estímulos por receptores como TLR e TNFR, que a proteína do tipo IKK leva à fosforilação de IkB. Uma vez fosforilada, IkB é poliubiquitinizada e rapidamente degradada pelo proteosssoma, dissociando então o heterodímero p50/p65. Isso permite que o NF-kB migre para o núcleo e ligue-se à região promotora dos genes, especialmente regiões que apresentem a sequência regulatória: GGGACTTTCC, levando a um aumento da expressão do gene alvo (Lu & Wahl, 2005; Echeverri & Mockus, 2008). Essa ligação ao DNA ocorre de uma maneira específica através de *zinc finger*, membros de uma família que contém pequenos motivos de reconhecimento de DNA compostos por resíduos de aminoácidos e um íon zinco (Freifelder, 1987).

De maneira geral, a silibinina parece interferir na cascata de transdução controlada pelo NF-kB, suprimindo a fosforilação e degradação de IkB e diminuindo assim a translocação de NF-kB para o núcleo. (Zandi et al., 1997; Kren & Walterova,

2005; Comelli et al., 2007). A inibição desse fator de transcrição tem sido ligada à atenuação de atividades inflamatórias e apoptose celular (Luo et al., 2005).

Os genes alvos de NF-kB são: citocinas como IL-1, IL-2, IL-6, IL-8, TNF- α, enzimas antioxidantes como: superóxido dismutase (SOD), catalase (Cat) e glutationa (GSH), algumas moléculas de adesão: selectina E, molécula de adesão intercelular 1 e de adesão vascular 1, além de algumas moléculas imunorregulatórias como o óxido nítrico sintase induzível (iNOS), complexo principal de histocompatibilidade (MHC), além de proteínas de ciclo celular como A20 e p53 (Beyaert et al., 2000; Zhou et al., 2001; Xu et al., 2002; Pham et al., 2004). O tempo de ativação varia segundo a via de sinalização, sendo de apenas alguns minutos para a via clássica, resultante de estímulos via receptores como TLR e, em torno de 90 min, para as vias alternativas, resultantes de estresse e dano celular (Echeverri & Mockus, 2008).



Silybin

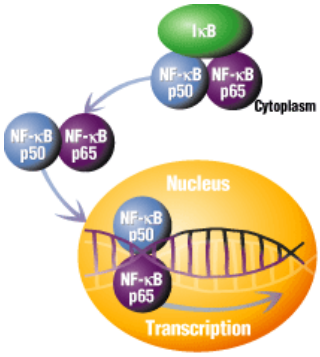


Figura 1A: Estrutura molecular da silibinina (Huber et al., 2008)

Figura 1B: Estrutura e atividade de NF-kB

Estudos *in vitro* e *in vivo* indicam que silibinina e silimarina protegem o fígado de estresse oxidativo e do aumento de processos inflamatórios, especialmente dirigidos por espécies reativas de oxigênio (ROS) e citocinas. Manna et al. (1999) sugerem que a silibinina protege as membranas das células hepáticas contra agentes tóxicos, melhorando sua função, tanto em animais como em humanos. Está claro que o mecanismo de ação citoprotetora da silibinina ocorre por inibição da peroxidação lipídica, uma vez que ela exerce ação estabilizadora na membrana celular, prevenindo ou inibindo a via da 5-lipoxigenase (Dehmlow et al., 1996). Essa inibição protege contra a toxicidade hepática, causada por uma grande variedade de agentes como radicais livres, tetracloreto de carbono, tolueno ou xileno (Mereish et al., 1991; Carini et al., 1992; Wellington & Jarvis, 2001). A peroxidação lipídica, que leva ao aparecimento

dos sintomas clínicos, está ligada à formação de prostaglandinas (PGs), um mediador que, quando induzido pela enzima constitutiva cicloxigenase-1 (COX-1), implica em regulação vascular e hemostasia. No entanto, a enzima COX-2, normalmente expressa em baixos níveis, é induzida em decorrência de estímulos de agentes pró-inflamatórios como citocinas, LPS e fatores de crescimento, resultando no aumento da produção de prostaglandinas (PGs) que desempenham um importante papel em condições inflamatórias (MacMicking et al., 1997). PGE_2 é o maior metabólito da COX-2 e atua no aumento da produção de citocinas Th1. É sintetizada pela ação coletiva de fosfolipase A2 e COX em ácido araquidônico liberado de fosfolipídeos de membrana celular após vários estímulos (Fitzpatrick & Soberman, 2001). Já foi demonstrado que os componentes da silimarina podem suprimir a formação desse mediador, juntamente com a decomposição dos lipídeos de membrana, sendo provavelmente esta a ação hepatoprotetora desse flavonóide (Wellington & Jarvis, 2001).

Estudos avaliando PGE_2, COX-2 e iNOS têm sido realizados para a determinação de efeitos anti-inflamatórios de produtos terapêuticos e a importância do NF-kB na patogênese de inflamação vêm sendo sugerida pela inibição da sua via de ativação, podendo ser, portanto, um efetivo alvo no tratamento dessas doenças.

A silimarina mostrou ser capaz de inibir a produção de óxido nítrico (NO) e a expressão da enzima óxido nítrico sintase induzível em macrófagos peritoneais de camundongos estimulados com LPS. Esses efeitos foram atribuídos à ação inibidora da silimarina sobre a atividade do fator de transcrição nuclear NF-kB, que regula vários genes envolvidos na resposta imune e na reação inflamatória. A concentração utilizada desse flavonóide no experimento foi 100 vezes menor que a concentração do salicilato, que também bloqueia NF-kB, sugerindo que a silimarina em doses substancialmente livres de efeitos tóxicos (Kang et al., 2002). A inibição da atividade de NF-kB em células de hepatoma (HepG2) estimuladas com LPS foi obtida pelo emprego de silimarina na concentração de 25uM (Saliou et al., 1998). Essas mesmas células apresentaram uma significativa inibição desse fator de transcrição quando tratadas com silibinina, o componente mais ativo da silimarina, nas concentrações de 12,5 e 25 uM (Bremmer & Heinrich, 2002).

Mediadores inflamatórios pleiotrópicos (NO e PGE_2) são altamente produzidos e estão envolvidos em inflamações crônicas e infecções (Dubois et al., 1998; Hurley et al., 2002; Ban et al., 2009). NO é sintetizado a partir da L-arginina pela enzima óxido

nítrico sintase. Três isoformas dessa enzima têm sido identificadas: endoltelial (eNOS), neuronal (nNOS) e induzível (iNOS). Enquanto as duas primeiras são constitutivamente expressas, a última isoforma citada é expressa em resposta a estímulos pró-inflamatórios como LPS, TNF- α e IFN-γ (Guzik et al., 2003). A expressão de iNOS apresenta um papel crítico na sobrevivência de hospedeiros contra infecção com *Mycobacterium tuberculosis* (Ban et al., 2009) e o aumento da produção de NO está envolvido na patogênese de muitas doenças (Guzik et al., 2003; Gupta et al., 2007).

Em modelo experimental de inflamação aguda, a administração oral de silimarina reduziu o abscesso de coxim plantar de ratos, além de inibir o acúmulo de leucócitos no infiltrado inflamatório peritoneal após inoculação de carragenina, diminuindo principalmente o número de neutrófilos, demonstrando que exerce importante ação anti-inflamatória *in vivo.* (De La Puerta et al., 1996).

Consistente com suas propriedades antioxidantes e anti-inflamatórias, alguns estudos relataram uma alta eficiência da silimarina como agente preventivo contra uma variedade de promotores de tumores, incluindo luz ultra-violeta (UV), 7,12-dimetillbenz(a)antraceno (DMBA), phorbol 12-myristate 13-acetate (PMA) e outros (revisto por Agarwal et al., 2006). A proteção contra danos celulares que a silibinina/silimarina oferece em fígado e em muitos outros tecidos e seu potencial em pacientes com câncer que recebem terapias complementares ao tratamento já estão bem estabelecidos (Comelli et al., 2007). O emprego de silibinina como adjuvante em pacientes com câncer de próstata e em células da linhagem DU145 provou que esse flavonóide é capaz de inibir fortemente a atividade de NF-kB (Dhanalakshmi et al., 2002). Assim, esse flavonóide parece corrigir o desbalanço entre a sobrevivência da célula e a apoptose através da interferência com o ciclo celular. Seu papel na apoptose está relacionado à capacidade de inibir a atividade constitutiva de NF-kB e ativar as vias de caspase 3 e caspase 9 (Yoo et al., 2004).

Contudo, estudos atuais tentam aumentar sua biodisponibilidade e eficácia terapêutica devido à sua baixa solubilidade. O uso do complexo silibinina-vitamina E e fosfolipídeos em pacientes com doenças crônicas não-alcoólicas de fígado mostrou ser mais eficaz que a silibinina, inibindo fortemente TNF- α, IFN- γ, IL-2 e IL-4 e TGF-β1 (Loguercio et al., 2007).

Em conjunto, os trabalhos da literatura sugerem importante papel antiinflamatório da silimarina/ silibinina. Entretanto, os estudos sobre a ação desse

flavonóide em células humanas ainda são escassos, não havendo relatos desse efeito sobre a atividade de monócitos humanos. Em trabalho anterior, avaliando o papel da silibinina sobre o metabolismo oxidativo de monócitos humanos, demonstramos que esse flavonóide exerce efeito antioxidante, inibindo, de maneira dose-dependente, a liberação de peróxido de hidrogênio por monócitos estimulados com PMA (Bannwart et al., 2009).

O estudo de substâncias naturais que possuem efeito sobre fases crônicas de inflamação e infecções fortemente relacionadas com a produção de citocinas inflamatórias como TNF- α, IL-1b e IL-6, de mediadores como NO e PGE_2 por células do sistema mononuclear fagocitário e envolvidas na patogênese de diferentes doenças humanas como câncer (Ikemoto et al., 2000; Lelli et al., 2003), pré-eclampsia (Faas & Schuiling, 2001) e doenças infecciosas (Dooley et al., 1994; Peraçoli et al., 2003), poderia auxiliar no estabelecimento de novas alternativas terapêuticas para essas patologias.

O reconhecimento inicial de microrganismos é mediado por receptores celulares, expressos em células da imunidade inata. A interação entre moléculas de superfície do parasita e receptores homólogos, presentes na membrana celular de macrófagos, modulam a fagocitose e a ativação da célula (Gordon et al., 1988; Ohman et al., 1989). Portanto, monócitos e macrófagos são células da imunidade inata que expressam receptores de superfície para manose, CD14, componentes do sistema complemento, porção Fc de moléculas de imunoglobulinas e receptores semelhantes a Toll (TLR, *Toll-like receptor*) capazes de reconhecer produtos microbianos, levando à estimulação da fagocitose, atividade microbicida e produção de citocinas (Abbas et al, 2008; Underhill & Ozinsky, 2002). As propriedades fagocíticas e microbicidas dessas células podem ser moduladas pelos receptores específicos de membrana, envolvidas na interação com os microrganismos (Popi et al., 2002). Entre esses receptores de superfície dos macrófagos, os melhores caracterizados são os receptores de manose e para o componente C3 do sistema complemento, ambos capazes de mediar a fagocitose e a morte intracelular de microrganismos patogênicos (Linehan et al., 2000). CD14 é uma glicoproteina ligada a glicofosfatidilinositol, rica em leucina, que reconhece produtos microbianos, produzidos por bactérias Gram-positivas e Gram-negativas, vírus e fungos, desempenhando papel essencial na resposta celular a patógenos (Landmann et al., 2000). Receptores semelhantes ao *Toll* são uma família de proteínas de transmembrana, evolutivamente

conservadas entre insetos e humanos (Anderson, 2000) que foram primeiramente identificados como moléculas essenciais para padrão embriogênico em *Drosophila* e, posteriomente como chave na imunidade antifúngica (Lamaitre et al., 1996). TLR4 e seu co-receptor MD-2, reconhecem LPS de bactérias Gram-negativas bem como polissacáride de *C. neoformans* (Shoham et al., 2001). Por outro lado, TLR2 medeia resposta celular a peptidoglicanos de bactérias, lipoproteinas e zimosan, em cooperação com TLR1 ou TLR6 (Ozinsky et al., 2000). A resposta imune inata a uma espécie de microrganismo pode refletir a integração das respostas de vários TLRs para diferentes moléculas produzidas pelo microrganismo (Abbas et al., 2008).

Todos esses receptores contêm repetições ricas em leucina, flanqueadas por motivos ricos em cisteina em suas regiões extracelulares e um domínio de homologia ao receptor Toll/IL-1R em suas regiões citoplasmáticas, o que é essencial para a sinalização (Abbas et al., 2008). Todo TLR sinaliza através de uma proteína adaptadora MyD88, que também contém um domínio Toll/IL-1R, resultando na translocação do fator de transcrição NF-kB e subseqüente transcrição de genes para citocinas pro-inflamatórias (Takeda & Akira, 2004).

Estudos *in vitro* envolvendo células fúngicas têm mostrado que *C. neoformans, C. albicans* e *A. fumigatus*, podem interagir com TLRs, particularmente TLR2, TLR4 e TLR9 presentes em células da imunidade inata (Shohan et al., 2001; Jouault et al., 2003; Meier et al., 2003; Neteaet al., 2003; Bellocchio et al, 2004).

A paracoccidioidomicose é uma micose sistêmica, endêmica na América Latina, que tem como agente etiológico o fungo dimórfico *Paracoccidioides brasiliensis* (Wanke & Londero, 1994). As manifestações clínicas da micose são de doença granulomatosa crônica, comprometendo especialmente tecidos pulmonares, mucosas e o sistema fagocítico mononuclear, com disseminação para fígado, baço, adrenais e outros órgãos (Franco et al., 1989; 1993; Mendes et al., 1999). A doença, em geral, pode ser controlada eficazmente por agentes antifúngicos, porém as recidivas são freqüentes e podem deixar seqüelas anatômicas, funcionais ou até mesmo ocasionar a morte (Mendes, 1994).

Os indivíduos acometidos pela paracoccidioidomicose apresentam mal-estar generalizado, caracterizado por anorexia, astenia, dispnéia, febre e perda de peso, que pode ser grave, levando o indivíduo a um quadro de caquexia (Mendes, 1994). A febre, em muitos processos infecciosos sistêmicos e inflamatórios, é associada à elevação de

pirógenos endógenos como TNF- α e IL-1β. e. PGE_2 (ABBAS et al., 2003). Níveis séricos elevados de TNF- α já foram descritos em pacientes com paracoccidioidomicose e associados à gravidade da doença (Mendes et al., 1991; Silva et al., 1995; Peraçoli et al., 2003). A produção de TNF- α, em quantidades elevadas, parece ser responsável pelos efeitos sistêmicos e não-protetores como indução de febre, anorexia, diarréia e estado de caquexia (Tracey & Cerami, 1994), sendo já descritos na coccidioidomicose (Dooley et al., 1994), tuberculose (Rook et al. 1987) e na septicemia durante infecção por *Neisseria meningitidis* (Brandtzaeg et al., 1996).

A maioria dos fungos causadores de micoses sistêmicas apresenta estreita relação com monócitos ou macrófagos nas fases iniciais da infecção ou no decorrer da doença (Iwatani et al.,1993). Segundo Deepe & Bullock (1990), no confronto entre o fungo e os fagócitos, é importante considerar o processo de reconhecimento e ligação desse patógeno à superfície dessas células. A ligação se estabelece devido a inúmeras moléculas presentes na superfície do macrófago, que facilitam a ligação a receptores específicos entre o hospedeiro e a célula fúngica. Na paracoccidioidomicose experimental de hamster, o estudo da produção de citocinas inflamatórias mostrou que o TNF- α, produzido em altos níveis durante a infecção pelo *P. brasiliensis,* poderia ser responsável não só pelo mecanismo de defesa contra o fungo nas primeiras semanas de infecção, como também estar envolvido na patogênese da doença. Nas fases mais tardias da doença, os altos níveis de TNF- α podem desempenhar um papel lesivo, sendo provavelmente responsável pelas alterações do estado geral apresentado pelos animais, culminando com a morte dos mesmos. (Parise-Fortes et al., 2000). Relatos sobre produção *in vitro* de TNF- α em culturas estimuladas por *P. brasiliensis* foram anteriormente descritos em estudos envolvendo macrófagos peritoneais de camundongos (Figueiredo et al., 1993), bem como monócitos humanos (Calvi et al., 2003; Kurokawa et al., 2007).

A produção de citocinas como TNF- α, IL-1β e IL-6 foi observada em estudos *in vitro* após estímulo de macrófagos e monócitos humanos com *Coccidioides immitis* (Dooley et al., 1994), *Cryptococcus neoformans* (Vecchiarelli et al., 1995), *Candida albicans* (Joualt et al., 1994), *Malassezia* (Kesavan et al, 1998) e *P. brasiliensis* (Calvi et al., 2003), demonstrando que essas células são fontes importantes de citocinas inflamatórias.

Na paracoccidioidomicose, não se conhecem claramente os mecanismos envolvidos no confronto fungo-fagócito, nem os fatores produzidos pela célula fagocitária na interação inicial hospedeiro - *P. brasiliensis*. Entretanto, Peraçoli et al. (2003) demonstraram que monócitos de pacientes com a forma ativa de paracoccidioidomicose, apresentavam-se ativados, produzindo níveis elevados de IL-1b, IL-6, IL-8, IL-10, TNF- α e TGF- β1, o que sugere o estado inflamatório intenso dessas células no curso da doença, sendo capazes de produzir tanto citocinas pró-inflamatórias como anti-inflamatórias na tentativa de não só garantir a maior eficiência na eliminação do parasita, mas também de regular a resposta imune do hospedeiro. Esses resultados confirmam relatos anteriores de que TNF- α, IL-1 e IL-6, detectados em níveis elevados no soro (Mendes et al.,1991; Silva et al., 1995) e em sobrenadante de cultura de monócitos de pacientes (Parise-Fortes et al., 2006) parecem ter importante papel na patogênese da paracoccidioidomicose.

Estudos recentes demonstram que TLR2, TLR-4 e dectina-1 atuam no reconhecimento de *P. brasiliensis*, internalização e consequente ativação da resposta imune contra o fungo. A cepa virulenta (Pb18) induziu fortemente a produção de citocina pró-inflamatórias como TNF- α, enquanto a cepa pouco virulenta mostrou equilíbrio entre TNF- α e a citocina anti-inflamatória IL-10. Células fúngicas também induziram uma elevada produção de PGE_2 por monócitos e neutrófilos, mostrando o potencial de *P. brasiliensis* em provocar uma resposta inflamatória intensa (Bonfim et al., 2009). A participação de TLR2 parece ser essencial no desenvolvimento da doença, uma vez que a expressão do gene para TLR-2 está aumentada após infecção em camundongos suceptíveis e não em resistentes (Ferreira et al., 2007).

Certo grau de immunocomprometimento de pacientes com paracoccidioidomicose é caracterizado por linfócitos que não respondem ao principal antígeno do fungo, a glicoproteína de 43 KDa, gp43. Células mononucleares de sangue periférico de indivíduos saudáveis produziram quantidades significativamente maiores de IL-2, IFN- γ e IL-10 que pacientes com as formas aguda e crônica da doença. Esses pacientes produziram baixos níveis de IL-2 e IFN- γ, mas quantidades significativas de IL-10. Células aderentes foram a principal fonte dessa citocina. Isso sugere que o desequilíbrio na produção de citocinas de pacientes com paracoccidioidomicose

desempenha papel importante na depressão da resposta de linfócitos à gp43 e na produção não protetora de anticorpos destes pacientes (Benard et al., 2001).

O *P. brasiliensis* induz resposta imunológica altamente complexa e multifatorial, cujos componentes celulares são ativados, no sentido de desempenharem papel efetor direto contra o parasita ou de participar dos mecanismos imunoregulatórios presentes no organismo infectado (Peraçoli & Soares, 1992). Assim, a imunidade celular, mediada por linfócitos T e macrófagos desempenha papel central na resistência aos fungos. Pacientes com paracoccidioidomicose mucocutânea, avaliados antes do tratamento com sulfametoxazol-trimetoprim, apresentaram expressão de TGF- β1 em todas as lesões de pele e mucosa, com depósito de aspecto granular na derme, presentes no citoplasma de macrófagos e células gigantes multinucleadas. Nas lesões observadas após o tratamento, ocorreu aumento nos depósitos difusos de TGF- β1 nas áreas de fibrose, indicando que esta citocina pode estar envolvida no processo de cicatrização das lesões desses pacientes (Parise-Fortes et al., 2006). Na paracoccidiodiomicose as lesões pulmonares causam dano crônico ao parênquima, levando à fibrose, com conseqüente restrição da função respiratória (Franco et al., 1998). O TGF- β1 induz efeitos deletérios, quando produzido em quantidades elevadas, levando à formação de matriz extracelular, de forma exacerbada no tecido lesado, podendo causar fibrose excessiva e comprometimento da função do órgão. Já é bem conhecido que TGB-β1 é um importante indutor de fibrose hepática em processos envolvendo exposição alcoólica ou infecções por microrganismos como vírus da imunodeficiência humana, da hepatite ou *Schistosoma mansoni*, que dirigem a resposta imune para perfil Th2 de resposta. A inibição da produção de TGF- β1 por agentes anti-oxidantes e anti-fibróticos como a silimarina, parece promissora no tratamento dessas patologias (Schuppan et al., 2003).

A participação da PGE_2 na paracoccidiodomicose demonstra que monócitos humanos desafiados com o fungo produziram altos níveis desse metabólito, inibindo a atividade fungicida dessas células, associado à menor liberação de H_2O_2, um importante metabólito envolvido na morte desse parasita e de TNF- α (Bordon et al., 2007).

Trabalhos realizados previamente em nossos laboratórios demonstraram que em modelo experimental de paracoccidioidomicose há um aumento significativo da produção de NO por macrófagos peritoneais de camundongos infectados em relação aos não infectados (Dias-Melicio et al., 2007), bem como em monócitos estimulados *in*

vitro com o fungo (Carmo et al., 2006), concordando com diversos resultados na literatura (Moreira et al., 2008; Livonesi et al., 2008). A superprodução desse metabólito também foi associada com um aumento da susceptibilidade à infecção (Nascimento et al., 2002).

A gravidade que o quadro clínico da paracoccidioidomicose pode assumir, sua alta incidência no Brasil e a falta de vacinas aprovadas para a prevenção da doença justificam o interesse no estudo de novos compostos capazes de modular a resposta imune do hospedeiro e assim aumentar a eficácia do tratamento com os antifúngicos. Nesse sentido, produtos naturais como a silimarina poderiam ser uma alternativa.

Na tentativa de evitar resultados polêmicos com a utilização da silmarina, devido à composição variável do extrato da planta e suas diferentes maneiras de preparação, existe a opção do emprego de seu composto mais ativo, a silibinina. Até o momento, vários trabalhos na literatura têm demonstrado a eficácia da ação anti-inflamatória e antifibrótica da silibinina, inibindo a produção de mediadores e citocinas por células fagocíticas. Assim, o estudo *in vitro* do efeito desse flavonóide sobre a expressão e produção de citocinas inflamatórias e anti-inflamatórias por monócitos humanos obtidos de indivíduos saudáveis e infectados com *P. brasiliensis* e a capacidade de modular NF-kB, poderá contribuir para melhor compreensão do mecanismo modulador da silibinina sobre a resposta inflamatória contra o fungo. Os resultados também poderão indicar o uso desse flavonóide como alternativa terapêutica adjuvante na paracoccidioidomicose, diminuindo os efeitos deletérios da doença e o risco de fibrose que ocorre em pacientes submetidos a tratamento prolongado com os agentes antifúngicos.

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Downregulation of NF-kB pathway by silibinin results in suppression of cytokines, PGE_2 and NO production by human monocytes challenged with Para*coccidioides brasiliensis*

Camila Ferreira Bannwart [a], Erika Nakaira Takahagi [a], Marjorie Assis Golim [b], Leonardo Teixeira Lopes de Medeiros [a], Mariana Romão [a], Ingrid Cristina Weel [a], Maria Terezinha Serrão Peraçoli [a]

[a] Department of Microbiology and Immunology, Institute of Biosciences, Sao Paulo State University, Botucatu, São Paulo, Brazil

[b] Botucatu Blood Center, School of Medicine, Sao Paulo State University, Botucatu, São Paulo, Brazil

Abstract

Silibinin is the major active component of silymarin (*Silybum marianum*), a polyphenolic plant flavonoid that has anti-inflammatory, cytoprotective and anticarcinogenic effects. The modulatory effect of silibinin on monocyte function against *Paracoccidioides brasiliensis* (Pb18) has not yet been demonstrated. The present study investigated whether the effect of silibinin on NF-kB pathways may affects the production of tumor necrosis factor-alpha (TNF-α), interleukin-10 (IL-10), Transforming growth factor beta (TGF-β1), prostaglandin E_2 (PGE_2) and nitric oxide (NO), as well as the fungicidal activity of human monocytes challenged *in vitro* with Pb18. Peripheral blood monocytes from healthy individuals were treated with or without 5 uM and 50 uM silibinin and challenged with Pb18 or were stimulated with lipopolissaccharide (LPS). After 18 h of culture TNF-α, IL-10, TGF-β1 and PGE_2 were determined by ELISA, while NO release was quantified by the accumulation of nitrite in the supernatants using the standard Griess assay. Fungicidal activity of monocytes against Pb18 was assessed after cell incubation with interferon-gamma, culture with or without silibinin for 18 h and challenge with Pb18 for 4h. NF-kB activation in the cultures was evaluated by flow cytometry and ELISA after monocyte stimulation with Pb18 or LPS for 30 min. Silibinin at 50uM concentration partially inhibited p65NF-kB activation as observed by reduction in the number of cells expressing this factor, that was confirmed by low concentration detection of p65NF-kB in the nucleus. This silibinin effects resulted in suppression of TNF-α, IL-10, TGF-β1 and PGE_2 and NO production, but did not affect fungicidal activity of monocytes against Pb18. The modulatory effect of silibinin on the monocytes inflammatory response against *P.*

brasiliensis might be important to establish an efficient and beneficial immune response of the host against the fungus, and contribute to the establishment of new adjuvant therapeutic alternatives for this mycosis.

Introduction

Standardized extracts from fruits and seeds of milk thistle, *Silybum marianum* have been employed for treatment of diseases with different etiologies in humans (Pradhan & Girish, 2006). The interest in the potential benefits of this flavonoid originates in antiquity, being one of the first documented examples of plant used for maintenance of health and treatment of disease (Voinovich et al., 2008). The most prevalent component of silymarin complex is silibinin (50-70%) which is the active phytochemical responsible for the claimed benefit of this extract, and comprise the mixture of two diastereomers A and B in approximately 1:1 proportion (Dixit et al., 2007). Besides silibinin, other flavonolignans are present in this complex, namely silycristin (20%), silydianin (10%), isosilibinin (5%) and a few flavonoids, mainly taxifolin (Saller et al., 2001; Dixit et al., 2007).

The use of complementary drugs or alternative medicine for the treatment of chronic diseases is emerging in many countries. It has been recently suggested that in the future a therapeutic approach to chronic disease will consist in a number of "complementary" therapeutic approaches considering the multitude of the pathogenic mechanisms (Gurley et al., 2004). *In vitro* and *in vivo* studies indicate that silymarin and silibinin protect the liver from oxidative stress and sustained inflammatory processes, mainly driven by reactive oxygen species (ROS) and cytokines. Oxidative stress and inflammation are also involved in cellular damage of many other tissues, and their role in the development and toxic reactions in patients receiving cancer therapies is established (Agarwal et al., 2006). Monocytes cultured with high doses of silibinin (50 and 100 ug/mL) presented viability above 95%. Others studies shown that silymarin effects on mouse peritoneal macrophages in lower or equal concentrations of 50 ug/mL do not have a significant effect on the viability of such cells or on the murine macrophage cell line RAW 264.7 (Kang et al., 2002).

Silibinin possesses antioxidant and anti-inflammatory properties on human monocytes due to inhibitory activity on H_2O_2 release and TNF-α production by these

cells stimulated with LPS, and this effect is more efficient than in silymarin treatment (Bannwart et al., 2009). The protective effects of silibinin, demonstrated in various tissues suggest a clinical application in cancer patients as an adjunct to established therapies, to prevent or reduce their toxicity (Comelli et al., 2007). Consistent with their antioxidant and anti-inflammatory activities, silibinin had been demonstrated to inhibit NF-kB activation through suppression of IkB phosphorylation and degradation, decrease of p65 subunit nuclear transcription. Silymarin at 10 to 100 uM range, dose-dependently inhibit the activation of NF-kB and related kinases. The concentrations of this bioflavonoid were about 100- fold lower than salicylate concentrations, suggesting that such a potent action can be exerted at concentrations substantially free of toxic effects (Manna et al., 1999; Kang et al., 2002). The NF-kB/Rel family of transcriptors factor is comprised of several structurally-related proteins that form homodimers and heterodimers that includes p50, p65 (Rel A); p52, Rel B and c-Rel. These heterodimer complexes are present in an inactive form in cytoplasm, bound to an inhibitory protein IkB. Certain stimuli results in the phosphorylation, ubiquitination and subsequent degradation of IkB proteins thereby enabling translocation of NF-kB into the nucleus and then binds to kB DNA sites and initiates gene transcription (Karin, 1999; Schulze-Luehrmann & Ghosh, 2006). This nuclear transcription factor pathway is activated by a variety of microbial components that signal through innate immune toll-like-receptors (TLR) and initiate the transcription of genes associated with spectrum of inflammatory response (Linn & Karin, 2007). The most common dimer in mammalian contains p50/p65 (RelA) heterodimers and is specifically called NF-kB. One of the target genes activated by NF-kB is the encoding IkBa. This feedback mechanism allows newly-synthesized IkBa to enter the nucleus, remove NF-kB from DNA and transport it back to the cytoplasm thereby restoring its inactive state. The importance of Rel/NF-kB transcription factors in human inflammation and certain diseases makes them attractive targets for potential therapeutics (Gilroy et al., 2004, Arkan et al., 2005; Maeda et al., 2005).

The anti-inflammatory effects of silymarin are mediated through suppression of NF-kappaB-regulated gene products, including COX-2, PGE_2, inducible nitric oxide synthesis (iNOS), TNF-α and IL-1. These inflammatory mediators have been widely used to determine the anti-inflammatory effects of potential therapeutic products (Manna et al., 1999; Kang et al., 2002). The important role of NF-kB in the

pathogenesis of inflammation suggests that inhibitors of NF-kB pathway could be an effective target in treating human inflammatory and chronic diseases.

Paracoccidioidomycosis is the most prevalent systemic human disease in Latin America caused by the dimorphic fungus *Paracoccidioides brasiliensis* (Wanke & Londero, 1994). The infection starts with the inhalation of fungal propagules that undergo differentiation into yeast cells, the infective form of *P. brasiliensis* (Restrepo & Tobón, 2005). This infectious disease mainly affects the lungs from where it disseminates to other organs producing secondary injuries to the mucosa, skin and others organs (Mendes et al., 1994). Patients with severe forms of the mycosis present generalized malaise, fever, anorexia, and weight loss, at times so intense that may lead to cachexia (Mendes, 1994). These clinical symptoms may be attributed to the overproduction of inflammatory mediators during the fungus-host interaction. Previous studies showed that monocytes from patients with active paracoccidioidomycosis are an important source of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α (Peraçoli et al., 2003). Besides, *P. brasiliensis* induced higher levels of pro-inflammatory and anti-inflammatory cytokines (Kurokawa et al., 2007) as well as elevated production of PGE_2 by human monocytes and neutrophils showing their potential to provoke an intense inflammatory response (Bordon et al., 2007; Bonfim et al., 2009). This chronic monocyte activation and the complex imbalance of the cytokines they produce may contribute to the pathogenesis of paracoccidioidomycosis (Peraçoli et al., 2003). The current chemotherapy treatment of paracoccidioidomycosis is based on sulfonamides, amphotericin B, and azole derivatives, mainly itraconazole. Because of the toxicity of antifungal drugs, and the identification in *P. brasiliensis* of genes involved in transport-mediated azole resistance with a significant frequency of relapsing disease, new treatment approaches are needed (Gray et al., 2003; Bozzi et al., 2007). Thus, the employment of natural products having anti-inflammatory properties in association with the conventional chemotherapy in paracoccidioidomycosis might be an alternative to overcome the deleterious effects of the systemic inflammatory response detected in patients with the mycosis.

On the basis of our previous results suggesting that silibinin has anti-inflammatory activity over human monocytes, the present study investigated whether the effect of this flavonoid on NF-kB pathway may affects cytokines (TNF- α, IL-10 and TGF-β1) as well as inflammatory mediators (PGE_2 and NO) production and

fungicidal activity against *P. brasiliensis* on human monocytes challenged *in vitro* with the fungus.

Material and methods

Materials

Medium culture utilized for monocyte culture was RPMI 1640 purchased from Sigma-Aldrich, Inc., (St Louis, MO. USA). Human detection antibodies for ELISA: anti-TNF-α, anti-IL-10, anti-TGF-β1 and PGE_2 were acquired from R&D Systems (Minneapolis, MN, USA). Antibodies for flow cytometry,: anti-NF-kB p65 conjugated with PE (2 ug/10^6 células), isotype control-PE was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-CD14-PerCP, isotype control – FITC, PerCP from BD Biosciences (Franklin Lakes, NJ USA). PBS 10% endotoxin free FBS was purchased from BioWest (Miami, FL, USA) and PBS/PAF 4% from BD Biosciences Discovery Labware (Bedford, MA, USA); nuclear extraction kit and NF-kB p65 transcription factor assay kit were supplied by Cayman Chemical Company, (Michigan, USA).

Fungus

Paracoccidioides brasiliensis strain 18 (Pb 18) was maintained in yeast-like form cells at 35 ^{o}C on 2% glucose, 1% peptone, 0.5% yeast extract, and 2% agar medium (GPY medium) all from Gibco Laboratories, Grand Island, NY, USA) and used on the sixth day of growth culture. Yeast cells were washed and suspended in 0.15 M phosphate-buffered saline (PBS-pH 7.2). In order to obtain individual cells, the fungal suspension was homogenized with glass beads in a Vortex homogenizer (three cycles of 10 seconds) (Peraçoli et al., 1999). Yeast viability was determined by phase-contrast microscopy (Soares et al., 2001). Fungal suspensions containing more than 95% viable cells were used for the experiments.

Healthy individuals

Twenty healthy blood donors were recruited from the University Hospital, Botucatu Medical School, São Paulo State University, Brazil, aged 20–50 years (mean age 32.5 ± 10.2 years). The study was approved by Botucatu Medical School Ethics Committee, and informed consent was obtained from all the blood donors.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by density gradient centrifugation on Histopaque [density (d) = 1.077] (Sigma-Aldrich). Briefly, 5 ml of heparinized blood was mixed with an equal volume of RPMI-1640 tissue culture medium (Gibco Laboratories, Grand Island, NY, USA) containing 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 20 mM HEPES, and 40 lg/ml gentamicin (complete medium). Samples were layered over 5-ml Histopaque in a 15-ml conical plastic centrifuge tube. After centrifuging at 400 g for 30 min at room temperature, the interface layer of PBMC was carefully aspirated and washed twice with PBS containing 0.05 mM ethylenediaminetetraacetic acid (PBS-EDTA) and once with complete medium at 300 g for 10 min. Cell viability, as determined by 0.2% Trypan Blue dye exclusion, was > 95% in all experiments. Monocytes were counted using neutral red (0.02%) and the mononuclear cells were suspended at a concentration of 2 x 10^6 monocytes/ml in complete medium, and was dispensed into 100 ul/well in 96-well flat-bottom plates (Nunc, Life Tech. Inc., MD, USA) for fungicidal activity and nitric oxide (NO) production. A suspension of 1 x 10^6 monocytes/ml was dispensed into 24-well flat-bottomed plates (Nunc) and employed to determine cytokine and prostaglandin production in supernatant culture, and measurement of NF-kB.

Determination of cytokines and PGE_2 concentration

Cytokines and PGE_2 levels were detected in supernatant of monocyte cultures treated with silibinin (5 uM and 50 uM) and challenged with Pb 18 or stimulated with 10 ug/mL lipopolysaccharide from *Escherichia coli* $O_{55}B_5$ (LPS) for 18 h. Cytokine

concentrations were determined in culture supernatants by enzyme-linked immunosorbent assay (ELISA), using Quantikine ELISA kits (R&D Systems) for TNF-α, IL-10 and TGF-β_1 according to the manufacturer's instructions. Before TGF-β_1 evaluation samples were acidified with 1 M HCl and neutralized with 1.2 N NaOH and 0.5M EDTA. Assay sensitivity limit was 10 pg/mL for TNF-α and TGF-β1 and 7.5 pg/mL for IL-10.

PGE_2 concentrations were measured by ELISA using the Prostaglandin E_2 High Sensitivity Immunoassay Kit (R&D Systems) according to the manufacturer's instructions. The sensitivity limit of the assay was 7.8 pg/mL.

Nitrite measurement in monocyte culture

Monocytes were plated at 2 x 10^6 cells/mL and treated or not with silibinin (5 and 50 uM) and challenged with suspension of *P. brasiliensis* (Pb18) containing 4 x 10^4 yeasts/ml at a ratio of 50 monocytes per one fungal cell prepared in complete medium plus 10% fresh human AB serum in 5% CO_2 at 37ºC. The cells also were stimulated with LPS. Nitrite accumulation, as indicator of NO production, was measured in culture medium (Eigler et al., 1995) after 18h on Pb18-chalenged or LPS-stimulated monocytes (10 ug/mL). Briefly, 100 uL of the supernatant were mixed with an equal volume of Griess reagent [1% sulfanilamide (Sigma-Aldrich) and 0.1% Naphthyl ethylenediamine (Sigma-Aldrich) in 2% phosphoric acid] in 96-well plates monocyte cultures and incubated for 10 min at room temperature. Nitrite concentration was determined by measuring the absorbance at 540 nm with a micro-plate reader (MD5000, Dynatech Laboratories, Inc., Chantilly, VA, USA). Conversion of absorbance to micromolar concentrations of NO was obtained using a standard curve of a known concentration of $NaNO_3$ diluted in distilled H_2O. All measurements were performed in triplicate and expressed as micromolar concentrations of NO.

Fungicidal activity

Monocytes (2 x 10^6/mL) were stimulated or not with 100 UI/mL human recombinant IFN-γ (R&D Systems) for 18h at 37°C and 5% CO_2 and then treated with silibinin (5 and 50 uM) and challenged during 4 hours in 5% CO_2 at 37°C with 100 uL

of a *P. brasiliensis* suspension, containing 4 x 10^4 yeasts/mL in a ratio of 50 monocytes per one fungus cell prepared in complete medium plus 10% fresh human AB serum, as the source of complement for yeast opsonization. Co-cultures with monocytes and fungus were harvested by aspiration with sterile distilled water to lyse monocytes. Each well washing resulted in a final volume of 2.0 mL and 0.1 mL was plated on supplemented brain-heart infusion (BHI) agar medium (Difco Laboratories, Detroit, Mich., U.S.A.) plates containing 0.5% of gentamicin, 4% horse normal serum and 5% *P. brasiliensis* strain 192 culture filtrate (v/v), the latter being the source of growth-promoting factor (Singer-Vermes al., 1992). Inoculated plates, in triplicate of each culture, were incubated at 37°C in sealed plastic bags to prevent drying. After 10 days, the number of colony forming units (CFU) per plate was counted. The inoculum used for the challenge was also plated according to the same conditions. The plates containing the material obtained from the monocyte-fungus cocultures were considered as experimental plates and those plated with the fungus inoculum alone and counted at time zero, were used as controls. Fungicidal activity percentage was determined by the following formula:

% Fungicidal Activity = [1 - (mean CFU recovered on experimental plates / mean CFU recovered on control plates)] x 100

Detection of NF-kB by flow cytometry

After 15, 30 and 45 min of Pb18-challenge or LPS-stimulation and treatment with silibinin (5 uM and 50 uM) monocytes were washed with wash buffer containing 10% endotoxin-free fetal bovine serum (FBS) (BioWest) and fixed with saline buffer plus paraformaldehyde 1% (PBS/PAF 1%) (Sigma-Aldrich) at room temperature for 20 min. Monocytes were labeled by PerCP monoclonal anti-CD14 antibody (5 ug/10^6 cells) at 4°C for 45 min. After two washes, 100 uL of reagent A from kit Fix & Perm® containing 0.03% saponin (Sigma-Aldrich) were add to monocytes at room temperature for 15 min. After two washes with wash buffer, the cells were suspended in reagent B from kit Fix & Perm® and stained with PE labeled-anti-pNF-κB p65 antibody (2 ug/10^6 cells) (Santa Cruz Biotechnology) for 60 min at room temperature. Then, after washing, human monocytes were stored in room temperature and dark for posterior analyze by flow cytometry with a FACSCalibur (BD Biosciences). The software used was

CellQuest ProTM (BD Biosciences). A total of 10^4 events were recorded for each sample. Experiments were reproduced in order to set up conditions allowing fine reproducibility of the technique with limited inter-individual variation for a given culture condition.

Detection of NF-kB in nuclear extract by ELISA

After monocyte treatment with silibinin and stimulation as described above for 15, 30 and 45 min of incubation, the cells were washed with PBS containing 10% endotoxin-free FBS on ice. Washed cells were then treated with a nuclear extract kit (Cayman Chemical) according to the manufacturer's instructions. A nuclear extract for each culture condition were obtained and conserved at -80°C until further assays. Proteins were measured by Lowry method (Lowry et al., 1951). Phosphorylated NF-κB (pNF-κB) p65 subunit levels in nuclear extracts from human monocytes were determined by ELISA for each monocyte culture condition. pNF-κB (assay sensitivity = 0.2 ug/well) was detected using a transcription factor ELISA kit (Cayman Chemical). Nuclear extract was incubated in 96-well plates containing a consensus (5'-GGGACTTTCC-3') binding site for the p65 subunit of NF-κB. pNF-κB binding to the target oligonucleotide was detected by incubation with a primary antibody specific for the activated form of p65, and then visualized by incubation with anti-IgG horseradish peroxidase conjugate at optimal concentrations and a developing solution as described by the manufacturer, and quantified at 450 nm with a reference wavelength of 655 nm (Multiskan EX, Labsystem, VWR International, Strasbourg, France).

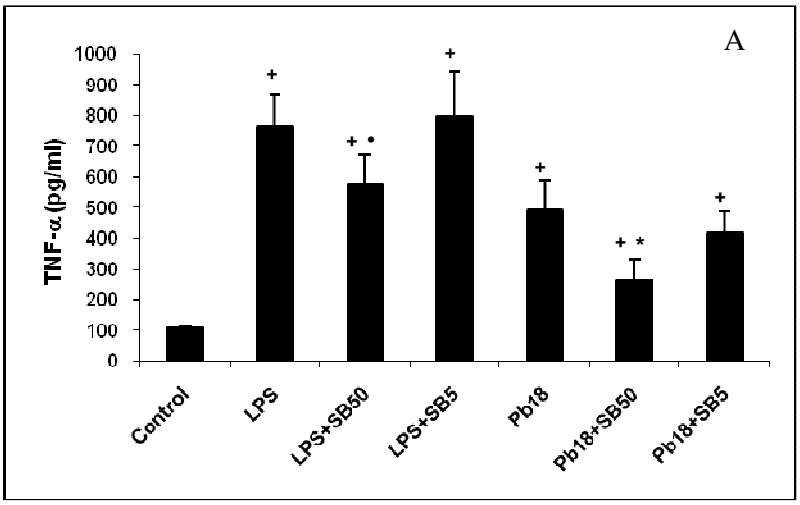
Statistical analysis

The results are presented as mean ± standard deviation. The data were evaluated by analysis of variance (ANOVA) followed by the Tukey test using INSTAT 3.05 software (GraphPad San Diego, Calif., U.S.A.). A p value < 0.05 was considered significant.

Results

Effect of silibinin on cytokines production

Human monocytes from healthy individuals were cultured with 5 uM and 50 uM of silibinin concentrations and challenged with *P. brasiliensis* or stimulated with LPS. After 18h of culture, pro-inflammatory and anti-inflammatory cytokines were determined in the supernatant of monocytes cultures. Silibinin showed a significant inhibitory effect on TNF- α production by monocytes challenged with Pb 18 or stimulated with LPS, employed as a positived control stimulus to evaluate cytokine productioncompared with cultures non-treated with silibinin. The significant inhibitory effect was observed at the concentration of 50 uM silibinin (Fig. 1A). TNF-α, IL-10 and TGF-β1 levels detected in supernatant of monocytes cultured in the absence of silibinin and stimulated with the fungus or LPS were significantly higher in relation to the control cultures. Similar suppression on IL-10 secretion was detected when the highest concentration of silibinin was employed in cultures stimulated with Pb18 and LPS. It can be observed that silibinin caused inhibitory effect of 25% and 41%, respectively, in relation to cells without treatment of silibinin (Fig. 1B). Monocytes cultured with silibinin presented a significant inhibitory effect in dose of the 50 uM on TGF- β1 production in human monocytes challenged with the fungus *P. brasiliensis*. The same pattern was observed in LPS-stimulates and treated monocytes with equal concentration of this flavonoid (Fig. 1C). These results demonstrated that silibinin interfere with pro- and anti-inflammatory secretion by monocytes.



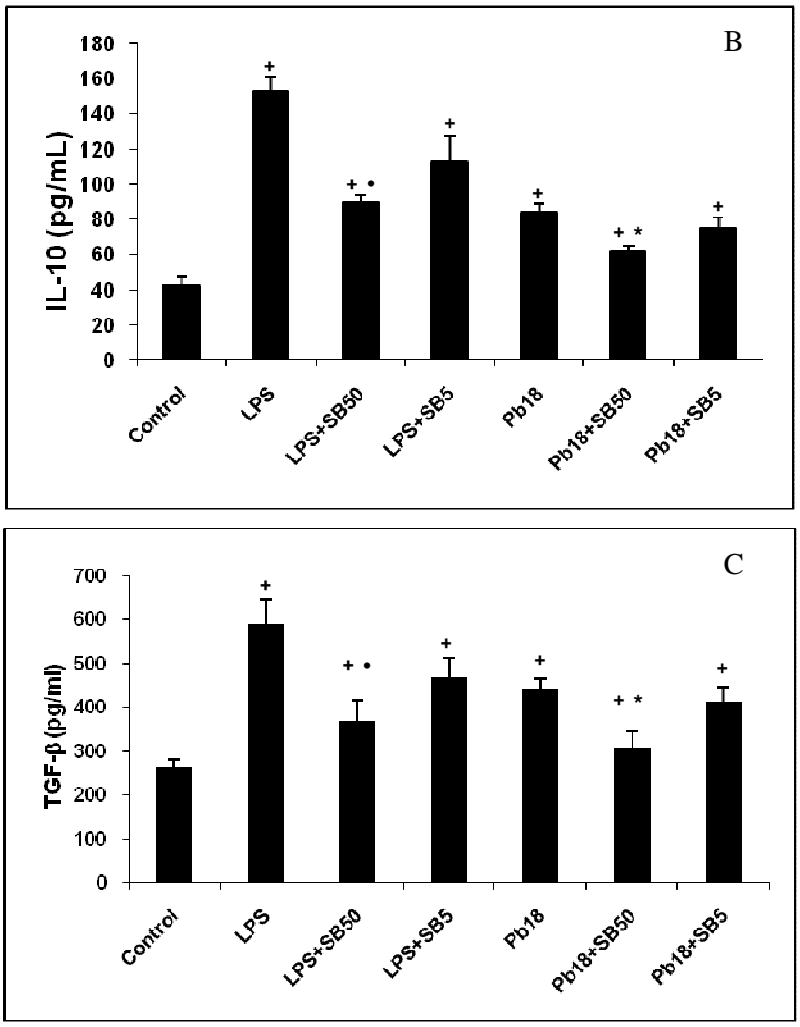


Figure 1- Effect of silibinin (SB) on the cytokines production. TNF-α (A), IL-10 (B) and TGF-β1 (C). Monocytes were cultured with or without silibinin (5 and 50 uM) plus *P. brasiliensis* (Pb18) or 10 ug/mL of LPS for 18 h at 37ºC. Cytokines levels were determined in the supernatant of monocyte cultures. Results are expressed as the mean ± SD of 20 healthy individuals.
+ $p < 0.05$ vs Control
* $p < 0.05$ vs Pb18
• $p < 0.05$ vs LPS

Effect of silibinin on inflammatory mediators production

PGE2 and NO release from human monocytes treated with 5 uM and 50 uM of silibinin and stimulated with *P. brasiliensis* or LPS and analyzed by Quantikine ELISA kit and Griess reaction, respectively, were significantly inhibited by 50 uM of silibinin. We can observe that silibinin induced 31% suppression on PGE2 production when the cells were challenged with the fungus and 30% when stimulated with LPS (Fig. 2A).

Monocytes treated with 50 uM silibinin and challenged with *P. brasiliensis* or stimulated with LPS showed 12% and 37% suppression of NO production, respectively, detected by nitrite accumulation in Griess reaction.

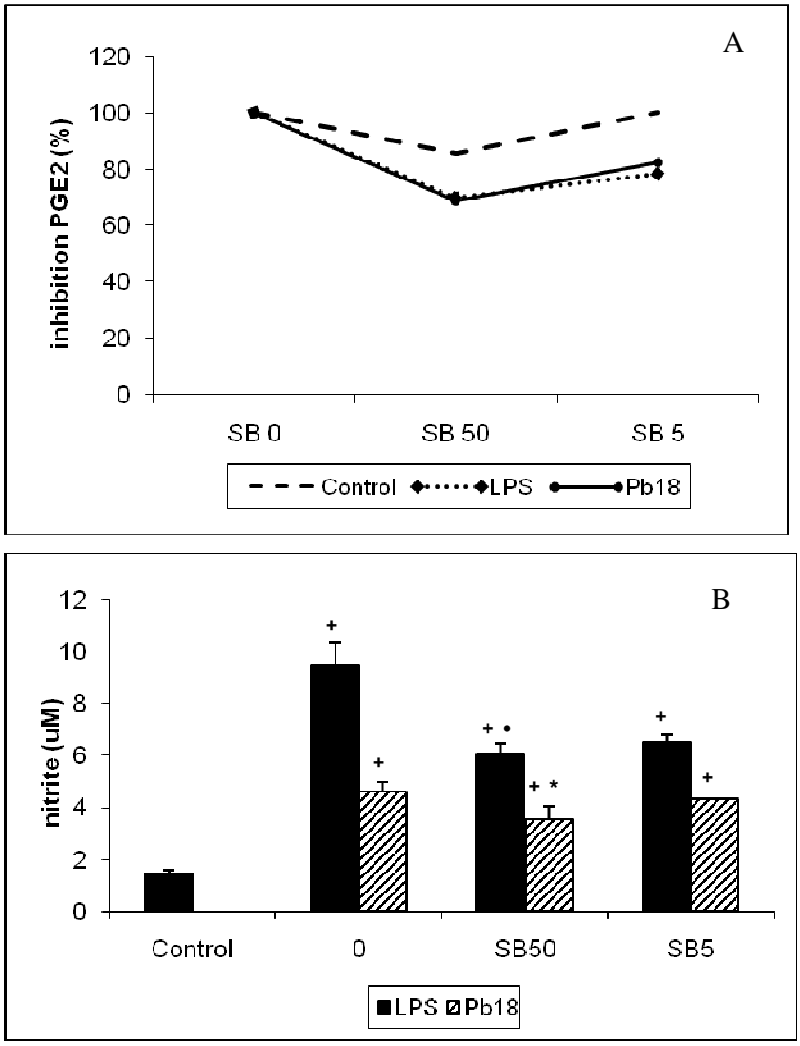


Figure 2 - Effect of silibinin (SB) on the inflammatory mediators production. Prostaglandin E2 (PGE2) (A) and nitric oxide (NO) (B). Monocytes were cultured with or without silibinin (5 and 50 uM) plus *P. brasiliensis* (Pb18) or 10 ug/mL of LPS for 18 h at 37ºC. PGE2 levels were determined in the supernatant of monocyte cultures and NO release by Griess reaction. Results are expressed as the mean ± SD of 15 healthy individuals.
+ $p < 0.05$ vs Control
* $p < 0.05$ vs Pb18
• $p < 0.05$ vs LPS

Effect of silibinin on fungicidal activity

In order to understand whether silibinin exerts effects on the microbicidal activity of monocytes, these cells were treated with or without silibinin in concentrations of 5 uM and 50 uM and then challenged with the Pb18 strain. Therefore, to better evaluate the effect of silibinin on the fungicidal activity of these cells against *P. brasiliensis*, the monocytes were first activated with IFN- γ (100UI) and treated with silibinin at doses of 5 and 50 uM. The results showed that the fungicidal activity was significant more efficient when monocytes were a pre-stimulated with IFN- γ compared with cells cultured without IFN-γ. Treatment with silbinin did not affect the monocytes fungicidal activity, even after cell activation with IFN- γ.

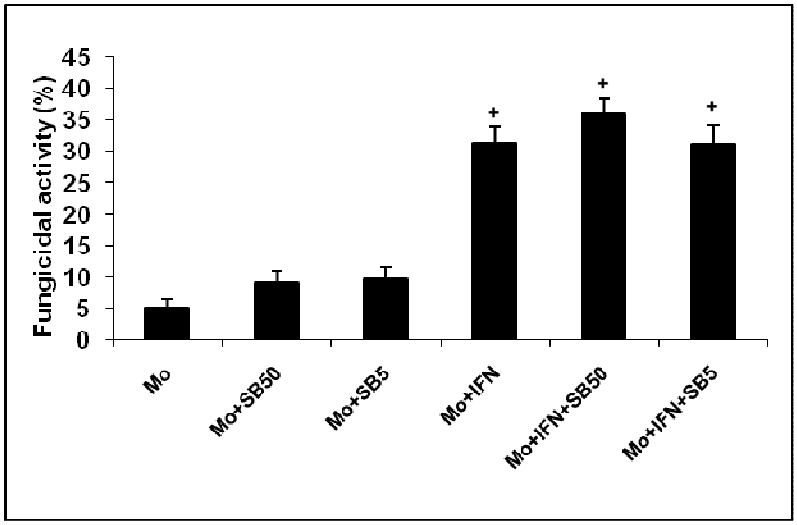
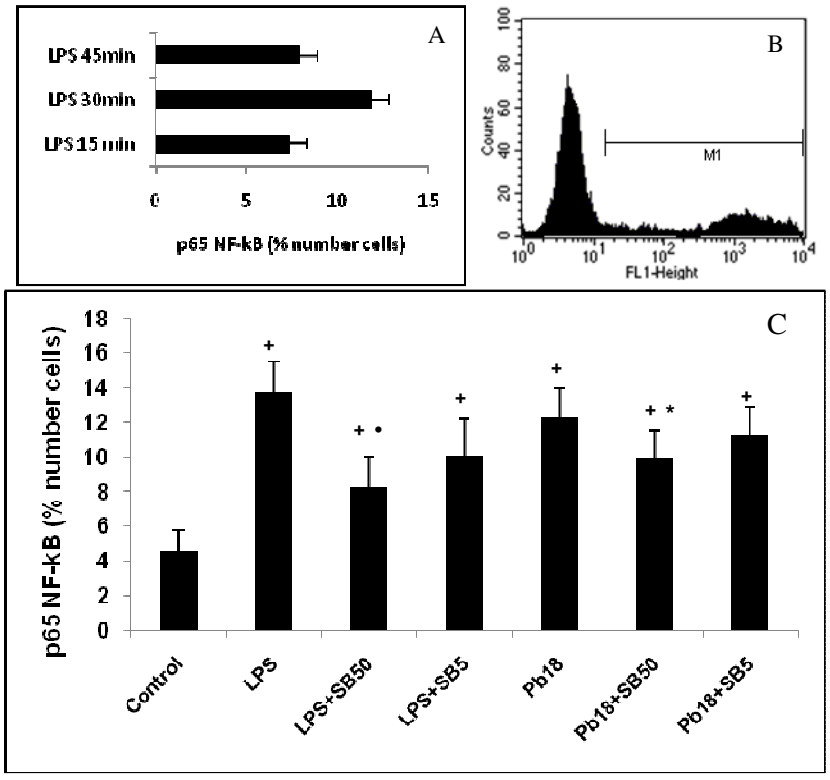


Figure 3. Effect of silibinin (SB) on fungicidal activity of human monocytes stimulated or not with 100 U/mL IFN-γ and challenged with *P. brasiliensis*. Monocytes were treated with or without silibinin (5 and 50 uM) for 18 h at 37ºC. Results are expressed as the mean percentage ± SD of 20 healthy individuals. Mo= monocytes
+ $p < 0.05$ vs Mo without IFN- γ

Effect of silibinin on p65 NF-kB signaling pathway

Evaluation of NF-kB intracellular activation in human monocytes by flow cytometry showed that 30 min of cell stimulation with LPS was the best time for this nuclear transcription factor detection (Fig. 4A). Significant NF-kB p65 activation was observed in cells challenged with Pb18 or stimulated with LPS determined at 30 min of culture. The percentage of these cells exhibiting NF-kB activation was significant higher than in control non-stimulated cultures. Treatment with 50 uM silibinin in

cultures challenged with the fungus led to significant reduction (20%) in the number of monocytes expressing p65 NF-kB, the active form of this nuclear transcription factor. In LPS-stimulated cells and treated with the same concentration of this flavonoid was observed a suppression of 40% in p65 NF-kB expression (Fig 4C). The employment of a technique that evaluates the concentration of p65 NF-kB active form found in the nucleus (ELISA) showed the same profile detected by flow cytometry. Monocytes treated with 50 uM silibinin presented 28% suppression in p65 NF-kB migration to the nucleus after challenge with *P. brasiliensis*. A significant reduction too was detected in 50 uM silibinin treated and LPS-stimulated cells (Fig. 4D).



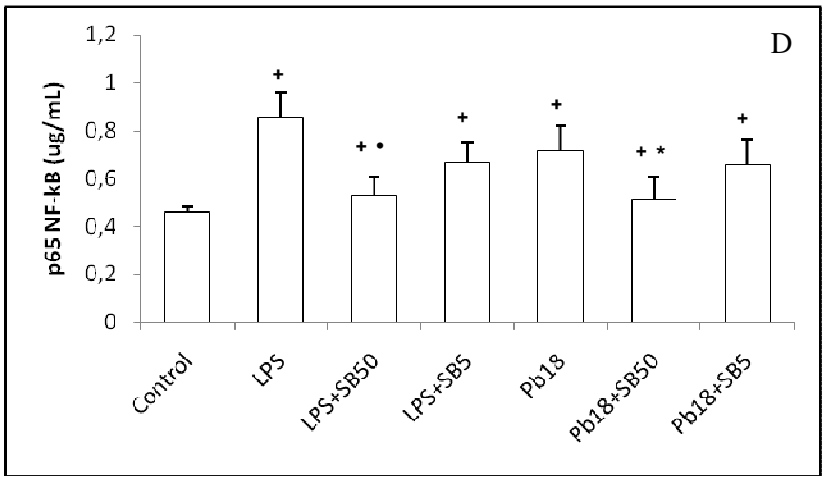


Figure 4 - Effect of silibinin (SB) on the NF-kB activity. Figure A represents LPS-stimulated monocytes with different times available by flow cytometry. Figure B shows a representative histogram of LPS-stimulated monocytes. Cells were cultured with or without silibinin (5 and 50 uM) for 30 min plus *P. brasiliensis* (Pb18) or 10 ug/mL of LPS at 37ºC. Figure C represents the percentage of cells expressing p65 NF-kB detected by flow cytometry and Figure D the concentration of p65 NF-kB in the nucleus. Results are expressed as the mean ± SD of 10 healthy individuals.
+ $p < 0.05$ vs Control
* $p < 0.05$ vs Pb18
• $p < 0.05$ vs LPS

Discussion

In this study we described an in vitro anti-inflammatory effect of silibinin on human monocytes challenged with *P. brasiliensis*, the etiologic agent of paracoccidioidomycosis. It has been known that in chronic infections caused by intracellular microorganisms such as *P. brasiliensis* the host develops an inflammatory response that may be deleterious and frequently involved in the pathogenesis of the mycosis (Peraçoli et al., 2003). In human as well as in experimental paracoccidioidomycosis the systemic overproduction of inflammatory cytokines may be responsible for the non-protective effects and constitutional symptoms of fever, anorexia and weight loss described in severe forms of the disease (Silva et al., 1995; Parise-Fortes et al., 2000; 2006; Peraçoli et al., 2003). Besides, in a previous study, we demonstrated that monocytes from patients with active paracoccidioidomycosis are an important source of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α (Peraçoli et al., 2003). High levels of these cytokines might be produced by monocyte stimulation through interaction with fungi during the active disease or are released when these cells are infected in vitro with *P. brasiliensis* (Kurokawa et al., 2007; Siqueira et al., 2009).

Thus, understanding the mechanisms involved in cytokine production by monocytes challenged with *P. brasiliensis,* and to propose therapeutic alternatives to minimize the deleterious effects of the inflammatory response is or utmost importance in paracoccidioidomycosis study. This way line, the employment of a natural product, like slibinin with anti-inflammatory and anti-oxidant properties in *ex vivo* experiments with human monocytes may bring important information in this knowledge area.

In the present work we evaluate the effect of the flavonoid silibinin over pro-inflammatory (TNF-α) and anti-inflammatory (IL-10 and TGF-β1) cytokines as well as on inflammatory mediators (PGE_2 and NO) production by monocytes challenged with *P. brasiliensis.* The mechanism involving the silibinin interference with the nuclear transcription factor in these responses was also evaluated. Human monocytes from healthy individuals were cultured with or without concentrations 5 uM and 50 uM silibinin and challenged with *P. brasiliensis* or stimulated with LPS for 18 hours. Silibinin at 50 uM showed a significant inhibitory effect on TNF-α production by monocytes challenged with Pb 18 or stimulated with LPS, employed as a positive control stimulus to evaluate cytokine production compared with cultures non-treated with silibinin. These results confirmed ours previous data showing that silibinin suppresses TNF- α production by monocyte cultures stimulated with LPS in a concentration-dependent effect. The most accentuated inhibitory effect was detected with higher concentrations of this flavonoid, such as 50 ug/mL and 100 ug/mL (Bannwart et al., 2009). Other authors related that mice treatment with silymarin inhibited biologically active TNF- α produced by peritoneal macrophages and the low production of this cytokine was detected in concentrations ranging 12.5 to 50 ug/ml (Zhang et al., 1996).

In supernatant of monocyte cultures challenged with *P. brasiliensis* or stimulated with LPS we observed higher levels of IL-10 and TGF- β1 that decreased when the cells were treated with 50 uM silibinin. Together with TNF- α levels obtained the results demonstrated that silibinin interferes with pro- and anti-inflammatory secretion by monocytes. This modulatory effect of silibinin over cytokine production may be important for the balance of pro- and anti-inflammatory cytokines produced during the host interaction with the fungus. Healthy *P. brasiliensis*-sensitized subjects produce IL-2, IFN-γ and IL-10 in response to the glycoprotein of 43 kDa (gp43) the main antigen of

the fungus, probably reflecting the well-balanced and effective anti- *P. brasiliensis* immune response in these subjects (Benard et al., 2001). Thus, IL-10 may have important regulatory effects on immunological and inflammatory responses by inhibiting the overproduction of pro-inflammatory cytokines by monocytes (De Waal Malefyt et al., 1991). However, excessive production of this cytokine has been considered as an important evasion mechanism from the host during intracellular infections by microorganisms (Redpath et al., 2001). This same process has been considered to *P. brasiliensis* since high concentrations of this cytokine were detected in patients' serum (Fornari et al., 2001), and in peripheral blood cell cultures supernatants (Benard et al., 2001; Oliveira et al., 2002). Moreover, monocytes from patients spontaneously release high concentrations of IL-10 *in vitro* (Peraçoli et al., 2003; Parise-Fortes et al., 2006) and, in experimental models of PCM infection, susceptibility was associated with high levels of IL-10 (Calich & Kashino, 1998). Therefore, the lower concentrations of IL-10 detected in monocyte cultures stimulated with LPS or *P. brasiliensis* and treated with silibinin, observed in the present study, might result from the inhibitory effect of this flavonoid on TNF- α production by these cells. Some authors suggest that TNF- α and PGE_2 are key molecules that induce IL-10 by monocytes stimulated with LPS, and TNF- α may directly stimulate IL-10 mRNA production by mechanism independent of PGE_2 (Niho et al.1998).

The inhibitory effect of silibinin on TGF- β1 production may be explained by their anti-fibrotic activity. Jeong et al. (2005) demonstrated that silymarin inhibit TGF-b β1 production by hepatocytes in CCL4 and diethylnitrosamine-induced experimental model of fibrosis. Higher intensity of TGF- β1 expression was detected in fibrotic tissues of patients with paracoccidioidomycosis by immunohistochemistry (Parise-Fortes et al., 2006), and the involvement of TGF- β1 in pulmonary fibrosis generation was demonstrated in experimental paracoccidioidmycosis induced by intranasal inoculation of *P. brasiliensis* conidia in Balb/c mice (Franco et al., 1998). Thus, by its anti-fibrotic effect silibinin may be considered important for treatment of pulmonary forms of paracoccidioidomycosis, since pulmonary fibrosis is one of the most frequent sequelar effects of the disease (Mendes et al., 1994).

Silibinin also inhibited PGE_2 and NO release from human monocytes stimulated with Pb18 and LPS. While the flavonoid suppresses at the same level 31% and 30%

PGE_2 production after Pb18 and LPS stimuli, respectively, suppression of NO release was higher after LPS-stimulation (37%) compared with Pb 18 challenge (12%). This low effect of silibinin on NO production by monocytes challenged with Pb 18 is in contrast with other studies showing an efficient inhibitory effect of silibinin on NO production. In this context Dehmlow et al. (1996) detected a 50% inhibitory effect on NO release by isolated rat Kupper cells employing 80 uM of silibinin concentration. Our results might be explained by the low concentration of the flavonoid employed (50 uM) and other target cells, since there are no studies reporting silibinin effects on NO production by monocytes. The inhibitory effect of silibinin on PGE_2 production by human monocytes are in accordance with other previous studies showing that the anti-inflammatory effect of silibinin was attributed to inhibitory effect on prostaglandins and leukotrienes production by human monocytes stimulated with LPS and granulocytes activated with zymosan (Dehmlow et al., 1996). Kang et al. (2004) reported that silymarin dose-dependently suppressed the LPS-induced production of IL-1β and PGE_2 in isolated mouse peritoneal macrophages. The authors suggested that the flavonoid has a protective effect against endotoxin-induced sepsis.

Our results did not allow us to correlate TNF- α and NO production with fungicidal activity of monocytes against *P. brasiliensis*, once lower levels of these molecules were detected in silibinin-treated cultures, which showed high fungicidal activity. The results suggest that treatment with silibinin did not affect the fungicidal activity of the monocytes probably by the potent effect of IFN-γ on monocyte activation. This effect may be observed when the killing of Pb 18 obtained by monocytes stimulated or not with IFN- γ were compared. Monocytes stimulated with IFN- γ had significantly higher fungicidal activity than the unstimulated cultures. Previous studies on fungicidal activity oh human monocytes against *P.brasiliensis* showed that IFN- γ plays an important role in monocyte and macrophage activation leading to inhibition of the fungus multiplication in these cells (Soares et al., 2001; Nascimento et al., 2008). To kill the fungus effectively, monocytes need an initial activation signal induced by IFN- γ to stimulate the cells to produce TNF- α, which is involved in the final phase activation process, suggesting that the synergic effect of these cytokines is necessary for efficient fungicidal activity against *P. brasiliensis* (Calvi et al., 2003). Although monocyte treatment with silibinin partially inhibited

TNF- α production by these cells it is possible that even the low levels of TNF- α produced were sufficient to up regulate the fungicidal activity induced by treatment with IFN- γ. It has been well established that a specific protector response against *P. brasiliensis* in experimental and human paracoccidioidomycosis depends on a Th1 pattern with IFN- γ, TNF- α and IL-12 being the main cytokines involved (Cano et al., 1995; Arruda et al., 2002; Moreira et al., 2007) in cell activation and release products of oxidative metabolism that were toxic to the fungus such as hydrogen peroxide and NO (Carmo et al., 2006; Moreira et al., 2007).

Another possibility that might explain the efficient fungicidal activity of monocytes treated with silibinin and IFN- γ against the challenge with *P. brasiliensis* is the inhibitory effect of this flavonoid on PGE_2 production by monocytes infected with the fungus. Previous studies demonstrated that PGE_2 produced by monocytes infected with high virulence strain of *P. brasiliensis* plays an inhibitory role on fungicidal activity against the fungus, and the cell treatment with indomethacin and IFN- γ restores the effective killing of the fungus (Bordon et al., 2007).

Anti-oxidant and anti-inflammatory properties of silibinin and silymarin have been attributed to their ability of inhibiting NF-kB activation through suppression of IkBa phosphorilation and degradation, decreasing p65 subunit nuclear transcription (Manna et al., 1999; Kang et al., 2002; Comelli et al., 2007). It is well known that NF-kB plays an essential role in the expression genes involved in immune and inflammatory response. LPS induce the activation of NF-kB resulting in the transcription of multiple inflammatory mediators including iNOS, COX-2 and TNF- α (Mazzeo et al., 1998; Surh et al., 2001; Lappas et al., 2002). Thus, NF-kB and enzymes involved in its activation can therefore be a target for anti-inflammatory functions by suppressing NF-kB activation (Kohno et al., 2008). Although the effect of silibinin on NF-kB in cells stimulated with LPS has been established, the effect of this flavonoid on this pathway in monocytes challenged with *P. brasiliensis* has not been studied. This is the first study wich evaluated whether silibinin inhibits inflammatory cytokines and other mediators via NF-kB signaling pathway in human monocytes challenged with *P. brasiliensis*. We employed a flow cytometric method described to detect NF-kB activation in granulocytes and mononuclear cells of peripheral blood by endotoxin (Foulds, 1997) to analyze the intracellular active form of p65 NF-kB in human

monocytes. The results showed that these cells challenged with Pb 18 or stimulated with LPS presented the best intracellular NF-kB activation at 30 min of stimulation. Treatment with 50 uM silibinin led to a 20% inhibition in the number of cells with NF-kB activation during 30 min of Pb 18 challenge. This inhibitory effect was confirmed by other ELISA assay employing nuclear extract of monocytes to detect the active form of NF-kB concentration in the nucleus. In this method monocytes treated with 50 uM silibinin presented 28% suppression of p65 NF-kB after challenge with *P. brasiliensis*. A similar and significant reduction on NF-kB activation was observed in LPS-stimulated and silibinin treated cells.

In conclusion the present study demonstrated that silibinin exerts anti-inflammatory and anti-fibrotic effect on CD14+ human monocytes challenged with *P. brasiliensis* via inhibition of p65 NF-kB activation, resulting in suppression of TNF- α, IL-10, TGF-β1, PGE_2 and NO, without effect on fungicidal activity against the fungus. Considering the established safety demonstrated by the long-standing use of silibinin to treat human diseases, the basis of actions exerted at cellular level, its pharmacokinetic properties and the existing clinical and experimental data the application of silibinin as a adjuvant therapeutic in patients with paracocidioidomycosis can be suggested.

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