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Respostas transcricionais de *Paracoccidioides* ao estresse nitrosativo

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Dissertação apresentada ao Programa de Pós-Graduação em Biologia do Instituto de Ciências Biológicas da Universidade Federal de Goiás, como requisito parcial para obtenção do título de Mestre em Biologia.

Área de concentração: Biologia Celular e Molecular

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RESUMO

Paracoccidioides é um fungo termodimórfico causador da Paracoccidioidomicose (PCM), uma doença endêmica na América Latina. Células do sistema imune humano possuem uma variedade de mecanismos de defesa contra patógenos, como a produção e liberação de óxido nítrico (NO), uma das espécies reativas de nitrogênio (RNS). RNS reagem com os componentes celulares, o que resulta em danos ao DNA e membranas, inibição da respiração e inativação de enzimas celulares. Para entender como *Paracoccidioides* responde ao estresse nitrosativo, esse trabalho tem como objetivo identificar genes que possam contribuir para esta resposta. O crescimento de células de levedura de *Paracoccidioides* foi avaliado na presença de várias concentrações de nitrito de sódio ($NaNO_2$). O estresse nitrosativo foi confirmado através da quantificação de nitrito no sobrenadante das culturas e da inibição da atividade da enzima citocromo c oxidase (Complexo IV). As análises transcricionais foram feitas a partir do sequenciamento da biblioteca de cDNA construída utilizando-se mRNA obtido após incubação de células leveduriformes de *Paracoccidioides* na presença de 500µM de $NaNO_2$ durante 1 hora. Os resultados mostram a indução de transcritos relacionados a várias vias celulares, incluindo genes da cadeia mitocondrial transportadora de elétrons. A expressão de alguns desses transcritos foi confirmada por PCR quantitativo em tempo real. Adicionalmente, a atividade mitocondrial, avaliada através do método do XTT, apresentou-se reduzida na presença de $NaNO_2$ durante as primeiras 2 horas de tratamento. A razão $NADP^+$/NADPH foi avaliada, apresentando-se menor durante o estresse nitrosativo. Os resultados sugerem que a indução de transcritos relacionados à produção de energia durante o estresse nitrosativo possa refletir um efeito compensatório sobre a inibição de enzimas que desempenham esta função metabólica, provavelmente para assegurar a produção de energia e/ou contribuir para o balanço redox através da geração de NADPH.

ABSTRACT

Paracoccidioides is a thermal dimorphic fungus that causes Paracoccidioidomycosis (PCM), an endemic disease in Latin America. Immune cells have a variety of defense mechanisms against pathogens, such as the production and release of nitric oxide (NO), one of the reactive nitrogen species (RNS). RNS react with cellular components, resulting in damage to DNA and membranes, inhibition of repiration and inactivation of cellular enzymes. To understand how *Paracoccidioides* responds to nitrosative stress, this study aims to identify genes that might contribute to this response. The *Paracoccidioides* yeast cells grouth was evaluated in the presence of various concentrations of sodium nitrite ($NaNO_2$). The nitrosative stress was confirmed by quantification of nitrite in the cultures supernatant and by the inhibition of the cytochrome c oxidase (complex IV) activity. The transcriptional analyzes were performed by sequencing a cDNA library constructed with *Paracoccidioides* mRNA obtained after incubation of fungal cells with 500 µM $NaNO_2$ during 1 h. The results show the induction of transcripts related to several cellular pathways, including genes of mitochondrial electron transport chain. The expression of selected transcripts was confirmed by quantitative real time RT-PCR. Moreover, mitochondrial activity measured by XTT method was reduced in the presence of $NaNO_2$ during the first 2 hours of treatment. Additionally, the $NADP^+$/NADPH ratio is lower during nitrosative stress, as demonstrated. The results suggest that the induction of transcripts associated with energy production during nitrosative stress may reflect a compensatory effect on the inhibition of enzymes that carry out this metabolic function, probably to ensure the production of energy and/or contribute to the redox balance by generation of NADPH.

1. INTRODUÇÃO

1.1- Aspectos gerais do fungo *Paracoccidioides*

Paracoccidioides é um fungo patogênico, originalmente descrito por Adolpho Lutz em 1908, que tem por classificação taxonômica: filo Ascomycota, ordem Onygenales, família Onygenaceae e gênero *Paracoccidioides* (Almeida, 2003; Bagagli, 2008). O fungo aqui denominado *Paracoccidioides* até então era classificado como uma espécie única – *Paracoccidioides brasiliensis* – de seu gênero até se propor a existência de pelo menos 4 espécies filogenéticas: S1 (espécie 1 com 38 isolados), PS2 (espécie filogenética 2, com 6 isolados), PS3 (espécie filogenética 3, com 21 isolados) e *Pb*01-like (incluindo o isolado *Pb*01 e isolados filogeneticamente similares). PS3 é um grupo geograficamente restrito à Colômbia; PS2 é encontrado predominantemente no Brasil, nos Estados de São Paulo e Minas Gerais e também na Venezuela; S1 está distribuída no Brasil, Argentina, Paraguai, Peru e Venezuela (Matute *et al.*, 2006). Os isolados *Pb*01-like são agrupados em uma nova espécie dentro do gênero (Teixeira *et al.,* 2009). O sequenciamento dos genomas estruturais dos isolados *Pb*01, *Pb*03 e *Pb*18, permitiu identificar a presença de cinco cromossomos. As análises permitiram verificar que o isolado *Pb*01 apresenta 9132 genes e 32,94 Mb, apresentando um maior número de genes quando comparado aos isolados *Pb*03 e *Pb*18. O isolado *Pb*03 apresentou um genoma de 29,06 Mb com 7.875 genes e Pb18 um genoma de 29,95 Mb, contendo 8.741 genes (Desjardins *et al.*, 2011).

Paracoccidioides é considerado um fungo termodimórfico por apresentar duas formas: micélio, encontrado à temperatura ambiente de 23 °C, e levedura, encontrado em tecidos do hospedeiro à temperatura de 37 °C. Tal transição, no momento da infecção, implica na capacidade do fungo de se ajustar rapidamente ao ambiente, a fim de sobreviver e invadir o

hospedeiro (San-Blas & Niño-Veja, 2008). Esta transição morfológica é determinada pela temperatura do ambiente e está intimamente relacionada à invasão e estabelecimento do patógeno nos tecidos do hospedeiro (Ramos-e-Silva *et al*., 2008). Através da utilização de técnicas, como microarranjo, construção de bibliotecas de ESTs e PCR quantitativo em tempo real, alguns estudos identificaram proteínas e genes que constituem as primeiras abordagens para lidar com as diferenças no padrão de expressão encontradas durante o processo de transição. Entre os genes identificados, se encontram fatores de virulência, proteínas relacionadas ao processo de transição e à transdução de sinal e sequencias de função desconhecida (Felipe *et al.*, 2003; Nunes *et al.*, 2005; Bastos *et al.*, 2007; Garcia *et al.*, 2010). A forma miceliana, quando examinada ao microscópio de luz, apresenta uma morfologia de hifas finas e septadas, contendo vários núcleos (Franco *et al.*, 1989). A morfologia das células leveduriformes apresenta-se oval ou alongada, contendo múltiplos núcleos (Lacaz *et al.*, 1991). A forma miceliana produz propágulos infectantes, denominados conídios, que quando inalados, entram em contato com os pulmões, onde transitam para a forma leveduriforme, constituindo sua forma parasitária nos tecidos do hospedeiro (Brummer *et al*., 1993 e McEwan *et al*., 1987).

Micélio e conídios provavelmente podem crescer saprobioticamente no solo, na água e em plantas, à temperatura ambiente e são consideradas as formas infectivas do fungo (Restrepo *et al*., 2001). O local exato e a condição na qual *Paracoccidioides* ocorre na natureza são pouco conhecidos, em virtude do raro isolamento do fungo do ambiente e seu prolongado período de latência no hospedeiro humano (Bagagli *et al*., 2006). No entanto, estudos mostram que o fungo pode ser isolado da matéria orgânica proveniente de áreas endêmicas (Ferreira, 2009). Características ambientais como temperatura média entre 24-

27°C, quantidade de chuvas anuais entre 1750-2500 mm, presença de florestas tropicais em terras altas e de vários cursos de água podem favorecer a presença de *Paracoccidioides* no ambiente (Restrepo *et al*., 2001). Estudos feitos por Terçarioli *et al.* (2007) indicam que *Paracoccidioides* possa ocorrer, preferencialmente, em solos provenientes de lugares protegidos, como no interior de tocas de tatus. Até recentemente, o homem era considerado o único hospedeiro de *Paracoccidioides*, porém outros animais como cachorros e tatus foram encontrados naturalmente infectados (Ferreira, 2009). Características encontradas em tatus, como baixa temperatura corporal, entre 32.7°C e 35.3°C, sistema imune frágil e constante contanto com o fungo no solo favorecem a aquisição do patógeno repetidamente, podendo o mesmo ser isolado desses animais regularmente (Bagagli *et al*., 2008). Grose & Tamsitt (1965) conseguiram isolar células de *Paracoccidioides* a partir do trato intestinal de morcegos na Colômbia. Richini-Pereira *et al*. (2008), através de técnicas moleculares, detectaram a presença de *Paracoccidioides* em diversos órgãos de tatus e porco-espinho, sugerindo que a infecção por esse fungo em animais selvagens em áreas endêmicas possa ser um evento comum.

1.2- A Paracoccidioidomicose (PCM)

O fungo *Paracoccidioides* é o agente etiológico da infecção sistêmica Paracoccidioidomicose (PCM), também denominada doença de Lutz, blastomicose sulamericana, blastomicose brasileira, moléstia de Lutz Splendore-Almeida e micose de Lutz, que é endêmica na América Latina, com alta prevalência em países como Brasil, Argentina, Colômbia e Venezuela (Figura 1). No Brasil, a PCM é considerada a maior causa de mortalidade entre as doenças fúngicas infecciosas, com uma taxa de 1,45 casos por milhão de

habitantes (Coutinho *et al.*, 2002; Prado *et al.*, 2009). A maior incidência da doença ocorre nos estados de São Paulo, Rio de Janeiro e Minas Gerais (Shikanai-Yasuda *et al.*, 2006).

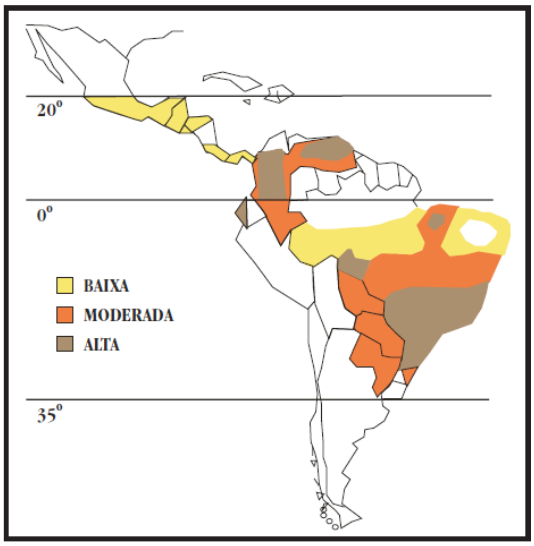

Figura 1 – Distribuição geográfica da Paracoccidioidomicose com prevalência na América Latina (Shikanai-Yasuda *et al.*, 2006).

Estima-se que mais de 10 milhões de indivíduos estejam infectados, sendo que cerca de 2% destes desenvolvem a doença (Restrepo *et al.*, 2001). Estudos apontam a PCM como oitava causa de mortalidade por doença infecciosa predominantemente crônica entre as doenças infecciosas e parasitárias, obtendo a taxa mais alta entre as micoses sistêmicas existentes (Shikanai-Yasuda *et al.*, 2006). A PCM é considerada uma micose profunda, geralmente com sintomatologia cutânea importante e grave (Santo, 2008). Estudos revelam que ambos os sexos adquirem a infecção na mesma taxa, porém a progressão da doença é mais frequente em homens, principalmente na terceira e quarta década de vida, elevando a taxa de homens com PCM em relação às mulheres para 13:1 no Brasil. (Brummer *et al.*, 1993; Pinzan *et al.*, 2010; Bonifaz *et al.*, 2011). A alta prevalência da PCM em homens pode ser devido ao papel protetor do hormônio feminino 17-β-estradiol que inibi a transição morfológica do fungo, o que é essencial para a sua patogênese (Restrepo *et al.*, 1984;

Marchiori *et al.*, 2011). Acredita-se que a interação do hormônio com uma EBP (Estradiol Binding Protein), identificada em *Paracoccidioides*, iniba a transição morfológica do fungo, explicando a baixa incidência da PCM em mulheres (Felipe et al., 2005; Shankar et al., 2011). Esta micose é endêmica entre populações de áreas rurais, está relacionada à atividade agrícola e afeta trabalhadores rurais com idades entre 30 e 60 anos, levando, em muitos casos, a sequelas irreversíveis que impossibilitam o indivíduo para o trabalho (Wanke & Aidê, 2009; Maluf *et al.*, 2003). Um levantamento feito na Colômbia sobre áreas de incidência natural de *Paracoccidioides* revelou que estas são restritas a regiões de florestas úmidas de grande altitude e pluviosidade. Não é completamente elucidado se tais circunstâncias favorecem a sobrevida do fungo no solo ou favorecem a prática da agricultura e, consequentemente, o contato de trabalhadores rurais com propágulos infectantes do fungo (Restrepo *et al.*, 2001). O contato com o patógeno ocorre, geralmente, via trato respiratório, podendo também ocorrer através de feridas na pele e membranas de mucosas (Bonifaz *et al.*, 2011). Uma vez que os conídios produzidos pela forma miceliana atingem os pulmões, podem transformar-se em células leveduriformes, causando lesões pulmonares e, subsequentemente, a doença se dissemina para outros órgãos e tecidos através das vias linfática e hematogênica (Borges-Walmsley, 2002; Ramos-e-Silva, 2008). Lesões secundárias são freqüentes nas membranas mucosas, nódulos linfáticos, pele e glândulas adrenais. Em hospedeiros com defesas imunes inatas ou adquiridas normais o patógeno pode ser eliminado. Apenas uma baixa porcentagem desses indivíduos desenvolve a infecção, que progride para a manifestação da doença, que pode se apresentar sob duas formas clínicas: (i) aguda ou juvenil, mais severa e de progressão rápida, (ii) e crônica ou adulta, de progressão lenta (Brummer *et al.*, 1993; Paniago *et al*., 2005). A forma aguda representa 3 a 5% dos casos descritos, sendo a maioria dos pacientes constituídos por crianças, adolescentes ou adultos jovens. É a forma clínica menos frequente e

o período de incubação é de semanas até meses (Barrozo *et al.*, 2009). A forma crônica é a forma encontrada em adultos e é a mais frequente (90%), acometendo principalmente homens com mais de 30 anos de idade. O período de latência pode variar desde alguns meses ou até décadas, sendo que o quadro clínico apresenta um desenvolvimento lento com comprometimento pulmonar evidente (Brummer *et al.*,1993). A doença caracteriza-se por um desenvolvimento lento e por marcante envolvimento de órgãos como baço, fígado, gânglios linfáticos e medula óssea. As manifestações clínicas dependem de fatores como a virulência do tipo de isolado de *Paracoccidioides*, o grau e o tipo da resposta imune e fatores relacionados ao hospedeiro, como sexo e imunidade (Fortes *et al.*, 2011). O tratamento da PCM é prolongado e se dá por meio de várias drogas como sulfonamidas, anfotericina B e azólicos (Ferreira, 2009). Complicações e sequelas funcionais e anatômicas ocorrem em pelo menos 20% dos pacientes, incluindo fibrose pulmonar e subsequente insuficiência respiratória (Martinez, 2010). As complicações causadas pela PCM acarretam em altos custos sociais e econômicos, caracterizando-a como uma doença de alta importância na saúde pública (Maluf *et al.*, 2003). Estudos a respeito da PCM e seu agente causador, *Paracoccidioides*, têm evoluído rapidamente através de ferramentas moleculares e químicas, abrindo caminhos para uma melhor compreensão deste problema de saúde pública que afeta a população rural da América Latina (San-Blas & Niño-Veja, 2008).

O controle imunológico da infecção por *Paracoccidioides* depende da produção de interleucinas 2 e 12 (IL_2, IL_{12}) e interferon gama (IFN-ɣ) que ativam macrófagos e linfócitos, levando à formação de granulomas compactos (Ferreira, 2009). A organização desta resposta imune celular permite o controle da multiplicação do fungo, mas formas quiescentes podem persistir no interior do granuloma (Shikanai-Yasuda *et al.*, 2006). Entre os mecanismos de controle descritos para a PCM, os macrófagos desempenham o papel principal, constituindo a

primeira linha de defesa do organismo (Brummer *et al*., 1990). Estudos de microscopia confocal revelaram que células de levedura de *Paracoccidioides* são internalizadas por macrófagos, onde se multiplicam (Brummer *et al*., 1989).

1.3- Óxido nítrico e o Estresse Nitrosativo

Células do sistema imune possuem uma variedade de mecanismos de defesa contra patógenos. Dois dos mais importantes, são as vias responsáveis pela produção dos radicais superóxido (O_2^-) e óxido nítrico (NO) através das enzimas NADPH oxidase de fagócitos e óxido nítrico sintase (iNOS), respectivamente. O_2^-, juntamente com outros intermediários derivados de oxigênio, são denominados espécies reativas de oxigênio (ROS), enquanto que o NO e seus derivados são denominados espécies reativas de nitrogênio (RNS) ou intermediários reativos de nitrogênio (RNI) (Fang, 2004). NADPH oxidase de fagócitos e iNOS são expressas tanto em leucócitos polimorfonucleares quanto em macrófagos, porém a produção de ROS é maior em neutrófilos, enquanto que os macrófagos, geralmente, produzem mais RNS (Nathan & Shiloh, 2000; Fang, 2004). O óxido nítrico, um dos RNIs, é um radical livre que age como um mediador biológico em vários processos, incluindo vasodilatação, citotoxicidade, atividades antimicrobianas e antitumorais. Em baixas concentrações, o NO desempenha um papel de sinalização celular em vários processos fisiológicos, porém, em altas concentrações, torna-se tóxico para as células (Hromatka *et al.*, 2005). Sua formação ocorre a partir da oxidação do aminoácido L-arginina por duas reações sequenciais catalizadas por uma isoforma induzível da enzima óxido nítrico sintase (iNOS ou NOS2) (Bogdan, 2001). iNOS cataliza a oxidação do aminoácido L-arginina, formando N^{ω}-OH-L-arginina como intermediário, o qual é posteriormente oxidado para formar uma molécula de NO e outra de L-citrulina (MacMicking *et al*., 1997) (Figura 2). Marletta *et al.* (1988) apontam o óxido

nítrico como um dos intermediários reativos de nitrogênio da via de formação de nitrito (NO_2), nitrato (NO_3) e L-citrulina a partir do aminoácido L-arginina.

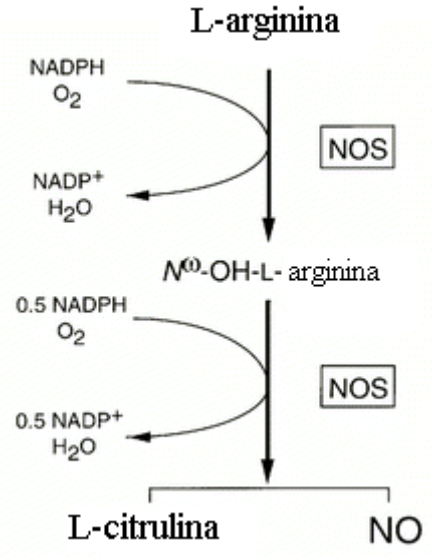

Figura 2 – Via de produção de NO em células de mamíferos. –Adaptado de MacMicking *et al*. (1997)

Estudos indicam que NO funciona como uma das moléculas responsáveis pela citotoxicidade de macrófagos, pois a reação de oxidação do aminoácido L-arginina é capaz de promover a inibição da proliferação da célula-alvo por macrófagos ativados (Alspaugh & Granger, 1991). A inibição da divisão celular em patógenos pode estar relacionada à habilidade de RNI em promover a nitrosilação de proteínas, incluindo enzimas da cadeia transportadora de elétrons e aconitase, o que leva à perda celular de ferro e torna essas enzimas inativas (Drapier & Hibbs, 1988)

Mamíferos expressam três genes (NOS1, NOS2 e NOS3) que codificam para a enzima iNOS. A isoforma NOS2, que pode produzir grandes quantidades de NO, foi a primeira enzima de macrófagos desta classe a ser clonada e apresentou sua expressão induzida durante

processos de inflamação e infecção (MacMicking *et al*., 1997). A indução de iNOS pode ser influenciada por produtos derivados de patógenos, como lipopolissacarídeos e ácido lipoteicóico, por citocinas pró-inflamatórias como IFN-ɤ, TNF-α, IL-1 e IL-2 e pela disponibilidade do aminoácido L-arginina (Nishikaku *et al.,* 2009; Fang, 1997).

Em doenças infecciosas, o NO pode desempenhar um amplo espectro de atividades com efeitos antivirais, antimicrobianos, proinflamatórios, anti-inflamatórios, citotóxicos e citoprotetivos (Bogdon, 2001). De acordo com Bocca *et al.* (1998), camundongos infectados com células leveduriformes de *Paracoccidioides* apresentaram uma aumento na produção de IFN-ɤ e TNF-α e uma alta taxa de síntese de NO, sugerindo que a produção endógena dessa molécula está envolvida no controle da infecção. Estudos realizados por Gonzalez *et al*. (2000) demonstram que a produção de óxido nítrico em macrófagos induzida por IFN-ɤ é capaz de restringir o crescimento intracelular de *Paracoccidioides* e, consequentemente a transformação de conídio em levedura. Pavanelli *et al.* (2011), através de estudos do papel do NO em granulomas em um modelo de PCM em camundongos, sugerem que o óxido nítrico seja um fator de resistência durante a doença através do controle da produção de citocinas e da proliferação das células fúngicas, regulando a resposta inflamatória. De acordo com Nishikaku *et al.* (2009), durante a fase crônica da inflamação causada por *Paracoccidioides*, NO tem uma importante papel na diminuição da síntese de matriz extracelular (ECM), principal componente de lesões por granulomas. Alguns autores, através de modelos de PCM, sugerem uma dupla atuação do NO durante a infecção. Em hospedeiros resistentes, essa molécula teria um efeito protetor através do controle da multiplicação fúngica, enquanto que seu efeito deletério é demonstrado em hospedeiros suscetíveis, onde a superprodução de NO inibe a produção de TNF, que é essencial para o controle da disseminação fúngica (Nascimento *et al.*, 2002). A morte de patógenos mediada por NO é descrita para alguns

fungos, incluindo *Aspergillus fumigatus*, C*andida. albicans* e *Criptococcus neoformans* (Brown *et al.,* 2009). Essa molécula também é descrita por estar envolvida na defesa do hospedeiro contra outros patógenos intracelulares, como *Leishmania*, micobactérias e *Salmonella* (Fang, 1997).

Estímulos inflamatórios induzem a expressão de iNOS, o que resulta em grandes quantidades de NO, que por ser uma molécula relativamente apolar, atravessa rapidamente a membrana celular do patógeno e interage com inúmeros alvos dentro da célula, incluindo tióis, metais, tirosinas de proteínas, bases de nucleotídeos e lipídios (Fang, 1997; De Groote & Fang, 1999; Nathan *et al.,* 2000) (Figura 3). Tais interações podem causar danos no DNA e na membrana, inibição de enzimas mitocondriais, alteração de proteínas por S-nitrosilação ou ADP-ribosilação, inativação de enzimas celulares pela ruptura de grupos Fe-S, dedos de zinco ou grupos heme ou por peroxidação de lipídios da membrana (De Groote & Fang, 1999; Nittler *et al*., 2005). Um importante mecanismo de citotoxicidade do NO é a inibição da respiração celular através da inativação de aconitases mitocondriais e componentes da cadeia transportadora de elétrons (Cassina & Radi, 1996). Na presença de oxigênio, NO pode ser convertido em um potente oxidante através da reação com superóxido ($O_{2^{\cdot}}$-), produzindo o ânion peroxinitrito (ONOO-). Tal molécula reage com enzimas mitocondriais como ATP sintetase (Complexo V), succinato deidrogenase (Complexo II) e NADH ubiquinona oxidorredutase (Complex I) (Brown, 1999).

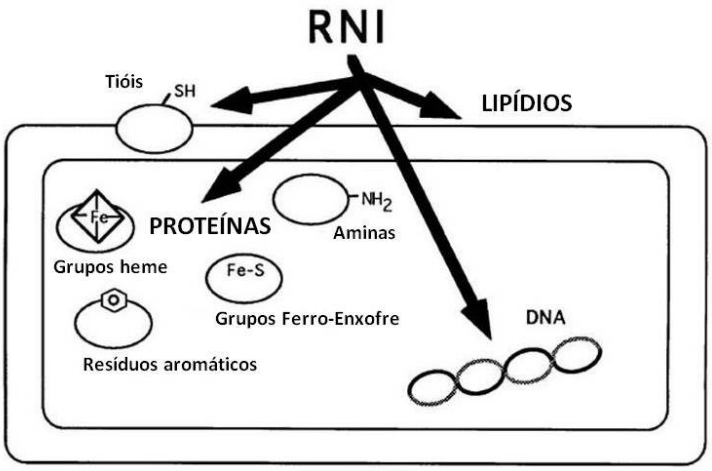

Figura 3 – Alvos dos RNI nas células de patógenos. Tal molécula pode modificar o DNA, proteínas e lipídeos – Adaptado de Fang (1997).

Para estabelecer a infecção, os patógenos precisam invadir o sistema imune e iniciar respostas aos diferentes tipos de estresses encontrados (Missall *et al.*, 2006). De acordo com Brown *et al.* (2007), tais respostas podem incluir mudanças metabólicas e rearranjos da parede celular. Estudos do perfil de expressão de genomas têm sido utilizados para avaliar os efeitos de estresses, como o nitrosativo, em eucariotos e procariotos (Vicente *et al.*, 2009). Alguns mecanismos de defesa importantes contra o estresse nitrosativo incluem enzimas como glutationa (GSH), superóxido dismutase (SOD), glicose-6-fosfato deidrogenase (G6PDH), catalase (Cat), alquil hidroperóxido redutase (Ahp), glutationa redutase (Gor), tirosina fosfatase (Yop), endonuclease IV (Nfo) e homocisteína (HC) (Fang, 1997) (Figura 4).

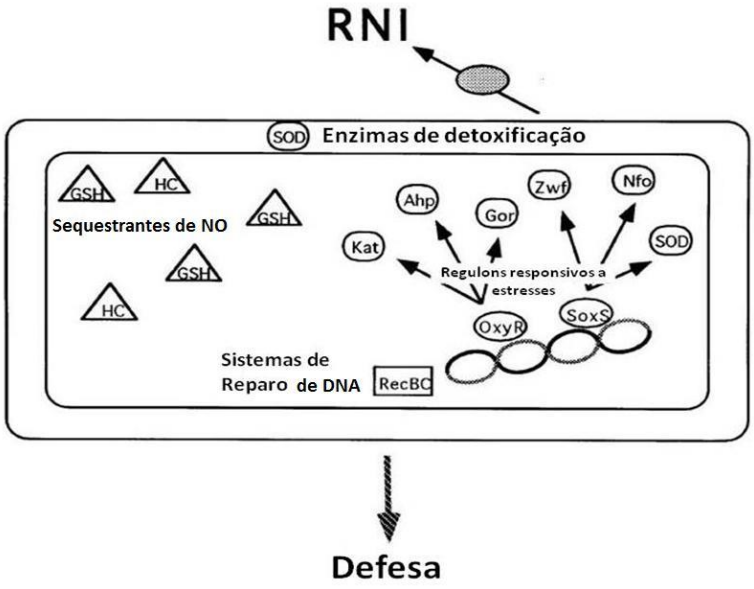

Figura 4 – Defesas de células de patógenos contra RNI – Adaptado de Fang (1997).

Células fúngicas possuem mecanismos de respostas a efeitos adversos do NO que reparam danos moleculares e ajudam estes a sobreviverem ao contato inicial com o sistema imunológico do hospedeiro, o que é essencial para o estabelecimento da doença (Brown *et al*., 2009). Dentre estes mecanismos de resposta, algumas enzimas descritas para *C. neoformans* podem detoxificar o NO como flavohemoglobina denitrosilase e NADPH deidrogenase, e enzimas como chaperonas, oxidorredutases e tiorredoxina redutase podem reparar danos produzidos por RNI (Missall *et al*., 2006). Estudos do transcritoma de células de *Histoplasma capsulatum* expostas a RNI revelam a expressão de genes envolvidos na degradação e reprocessamento de proteínas, como chaperonas e poliubiquitina, e na detoxificação, como óxido nítrico redutase, arginase e catalase (Nittler *et al*., 2005). Estudos feitos com uma variedade de fungos patogênicos descrevem vias relacionadas ao estresse nitrosativo como a via Mpk1 MAPK em um mecanismo dependente da proteína quinase C (Pkc1) em *C. neoformans* (Gerik *et al.*, 2008) e via de sinalização por cAMP em C. *albicans* e *Saccharomyces cevevisiae* (Bahn *et al.*, 2007; Wilson *et al.*, 2007). Alguns fatores de

transcrição que conferem resistência ao estresse nitrosativo também foram descritos, como o fator Cta4 em *C. albicans* e o fator Yap1 em *C. neoformans* (Missall *et al.*, 2004; Chiranand *et al.*, 2008).

1.4- Estudos do transcritoma de fungos patogênicos

A adaptação de células a mudanças ambientais acontece prioritariamente através da alteração do padrão de expressão de genes e proteínas. Assim, quando as condições ambientais mudam abruptamente, é necessário um rápido ajuste no programa de expressão gênica para permitir a adaptação às novas condições. Estudos do transcritoma de organismos têm sido utilizados como uma ferramenta útil na descoberta de marcadores moleculares e mecanismos funcionais que respondem às essas mudanças.

Em patógenos, a medida de mudanças na expressão de genes e proteínas sob condições que mimetizam o ambiente no hospedeiro pode ajudar a determinar fatores de virulência e resistência. Assim, análises do genoma de vários fungos patogênicos têm sido utilizadas como importantes ferramentas na compreensão da função de genes envolvidos em tais processos, levando a novas estratégias para o tratamento de doenças. Nesse sentido, a análise de sequências de etiquetas expressas (ESTs) provenientes de bibliotecas de cDNA de diferentes condições tem sido uma das abordagens eficientes no estudo comparativo de vários genomas (Andrade *et al.*, 2005). O mapeamento dos genes expressos por *Paracoccidioides* em diferentes condições tem sido objetivo de vários projetos transcritomas deste fungo. O Projeto Genoma Funcional e Diferencial foi desenvolvido visando uma melhor compreensão do metabolismo de *Paracoccidioides*, isolado Pb01, na fase de micélio e levedura. Nos transcritomas relativos às fases leveduriforme e miceliana foram sequenciados um total de 6.022 ESTs, apresentando transcritos de genes relacionados à virulência como quitina

deacetilase, isocitrato liase e α-1,3-glicana sintase, e a componentes das vias de sinalização como MAPKs e AMPc/proteína quinase (Felipe *et al*., 2003; Felipe *et al*., 2005)

Utilizando esse enfoque, perfis transcricionais de células de levedura de *Paracoccidioides* durante a interação com o hospedeiro têm sido descritos (Bailão *et al*., 2006; Pereira *et al.*,2009; Borges *et al*., 2011). Costa *et al*. (2007) avaliaram as respostas transcricionais de células leveduriformes de *Paracoccidioides*, isolado *Pb*01, recuperadas de fígado de camundongos infectados. Um total de 4.934 ESTs foram sequenciadas e anotadas, apresentando transcritos envolvidos no metabolismo anaeróbico da glicose e alguns responsáveis pelo metabolismo de lipídios.

Com o objetivo de identificar as respostas adaptativas de *Paracoccidioides* durante a infecção, Bailão e colaboradores (2006) utilizaram a técnica de RDA (Análise Diferencial Representacional), para analisar células leveduriformes de *Paracoccidioides*, provenientes de modelo animal e células leveduriformes do fungo incubado em sangue humano. Entre os transcritos induzidos predominantes durante esta condição, destacam-se aqueles relacionados à síntese e ao remodelamento da parede celular, como gliceraldeído-3-fosfato-desidrogenase (GAPDH), uma adesina que se liga aos componentes da matriz extracelular e é capaz de mediar a aderência e internalização de *Paracoccidioides* em cultura de células *in vitro*.

Análises do perfil transcricional de células leveduriformes de *Paracoccidioides* após incubação com plasma humano detectaram a indução de genes relacionados à síntese de proteínas, resposta celular à mudança de osmolaridade do meio, remodelamento celular e defesa, sugerindo que esses possam ser essenciais para a adaptação do fungo ao ambiente do hospedeiro (Bailão *et al.*, 2007). Estudos de Nunes *et al.* (2005), avaliaram a expressão gênica de *Paracoccidioides* durante transição de micélio para levedura, identificando um total de 2583 genes diferencialmente expressos nesta condição. Entre os genes induzidos

identificados, destacam-se aqueles relacionados ao metabolismo de aminoácidos, à transdução de sinal, à síntese proteica, metabolismo da parede celular, à resposta ao estresse oxidativo, ao controle do crescimento e ao desenvolvimento. Tal indução pode estar relacionada às mudanças bioquímicas e morfológicas associadas à transição para a forma patogênica. Parente *et al.* (2008) descrevem genes positivamente regulados durante a transição dimórfica relacionados à síntese, glicosilação e controle do processamento de proteínas. O perfil transcricional de *Paracoccidioides* durante a diferenciação morfológica de micélio para levedura foi descrito por Bastos *et al.* (2007), revelando transcritos induzidos relacionados a síntese de proteínas de membrana e de parede celular durante a diferenciação transitória de micélio para levedura após 22 horas. Em adição, genes relacionados às vias de transdução de sinal apresentaram níveis de expressão aumentados, propondo que esses sejam controlados na adaptação e sobrevivência do fungo durante os estágios iniciais da transição.

Análises da expressão gênica de células de *Paracoccidioides* internalizadas por macrófagos murinos foram realizadas com o objetivo de elucidar os mecanismos responsáveis pela sua sobrevivência e multiplicação. Entre os transcritos induzidos nesta condição, foram encontrados aqueles que codificam proteínas referentes ao transporte de elétrons mitocondrial, às subunidades ribossomais, ao remodelamento da cromatina e aos estresses oxidativo e nutricional, incluindo enzimas como Cu/Zn superóxido dismutase e cistationina beta-liase (Tavares *et al*., 2007).

Células respondem a uma variedade de mudanças ambientais através da reprogramação da expressão de conjuntos de genes que permitem a adaptação às novas condições. Em leveduras, como *S. cerevisiae*, esta resposta tem sido extensivamente estudada para uma série de estresses (Gasch *et al.*, 2000; Castells-Roca *et al.*, 2011). Estudos de perfis globais de expressão gênica têm sido amplamente utilizados para avaliar os efeitos dos

estresses oxidativo e nitrosativo em procariotos e eucariotos (Thuma & Bauersachs, 2007). A adaptação de patógenos como *Mycobacterium tuberculosis* e *Entamoeba histolistica* aos ROS e RNS tem sido descrita em estudos de expressão gênica diferencial, fornecendo um grande número de genes identificados na resistência a esses estresses. Entre os genes relacionados à resposta aos estresses oxidativo em *E. histolistica*, destacam-se proteínas de choque térmico, enzimas de conjugação da ubiquitina, proteínas quinases e GTPases. Genes que codificam para estas famílias de proteínas também foram induzidos em resposta ao estresse nitrosativo e grande parte destes genes responde aos dois tipos de estresses, refletindo uma sobreposição dos mecanismos regulatórios destas condições. Em *M. tuberculosis*, a resistência a esses estresses está relacionada à uma combinação de fatores, como a resistência oferecida pela parede celular, a expressão de genes que codificam proteínas sequestrantes de ROS e RNS, do reparo de proteínas oxidadas e mecanismos de reparo do DNA (Vicente *et al*., 2009; Voskuil *et al*., 2011).

Estudos do perfil de expressão gênica de fungos patogênicos fornecem informações relevantes sobre as respostas ao estresse nitrosativo e as consequências no metabolismo celular, nas estratégias de defesa utilizadas pelas células e nos genes envolvidos na resistência e patogênese (Horan *et al.*, 2006). Visando fazer uma análise das respostas transcricionais aos RNS, vários trabalhos têm sido realizados com o objetivo de identificar genes candidatos e vias celulares de patógenos que contribuem para esta resposta. Em fungos patogênicos como *H. capsulatum* e *C. albicans*, técnicas de microarranjo foram empregadas para examinar o perfil transcricional em resposta ao estresse nitrosativo, revelando mudanças na expressão de mais de 100 genes. Em *C. albicans*, nove genes apresentaram altos níveis de expressão durante o estresse nitrosativo, revelando produtos como flavohemoglobina (*YHB1*), oxidases alternativas, proteínas da superfície celular ligantes de heme, e transportadores de ferro,

sulfito e cobre (Hromatka *et al*., 2005; Nittler *et al.*, 2005). Em *S. cerevisiae*, os efeitos da exposição às RNS sobre o crescimento celular e na expressão gênica foram monitorados em mutantes para o gene *YHB1*, mostrando uma alteração na expressão de mais de 700 genes. Entre os genes induzidos identificados, encontram-se aqueles relacionados à detoxificação, como a enzima glutationa, ao sistema de reparo do DNA, à homeostase de ferro e ao transporte celular (Horan *et al.*, 2006). Estudos do proteoma e do transcritoma de células de *C. neoformans* submetidas ao estresse nitrosativo demonstraram que a resposta ao estresse por óxido nítrico envolve mudanças na regulação transcricional, traducional e pós traducional. As análises revelaram mudanças na expressão de genes de resposta a estresse, envolvidos na organização da parede celular, na respiração, na transdução de sinal, no transporte, no controle da transcrição e no metabolismo (Missall *et al.*, 2006).

2- JUSTIFICATIVA

Fungos patogênicos são expostos a vários tipos de estresses nos seus nichos ecológicos e no ambiente do hospedeiro, como temperatura, pH, hipóxia, deprivação de nutrientes, agentes tóxicos, como espécies reativas de oxigênio e nitrogênio, entre outros (Brown *et al.*, 2007). Portanto, respostas a esses estímulos se tornam necessárias para a sobrevivência desses patógenos. Durante o processo de infecção, células leveduriformes de *Paracoccidioides* são internalizadas por macrófagos ativados, que produzem intermediários reativos de nitrogênio (RNI). RNI causam uma série de danos moleculares nas células fúngicas, como danos no DNA, proteínas e lipídios. Tal condição, leva à expressão de transcritos que reparam tais danos, viabilizando a sobrevivência do patógeno ao seu contato inicial com o sistema imune do hospedeiro, o que é crucial para o estabelecimento da doença. Nenhuma análise do transcritoma de *Paracoccidioides* foi realizada em condições de estresse nitrosativo. Portanto, a identificação e análise dos transcritos produzidos nesta condição se tornam importantes, pois possibilitam o estudo de genes responsáveis pela resposta à presença de NO e de vias metabólicas que possam desempenhar papéis vitais na sobrevivência do fungo à esta condição.

3-OBJETIVOS

3.1- Objetivo geral

Devido à importância em elucidar as respostas do fungo *Paracoccidioides* a estresses no ambiente do hospedeiro, o objetivo geral desse trabalho foi identificar genes de *Paracoccidioides* que são diferencialmente expressos durante o estresse nitrosativo.

3.2- Objetivos específicvos

- ✓ Testar o efeito do nitrito de sódio sobre o crescimento de células leveduriformes de *Paracoccidioides*;
- ✓ Realizar o estresse nitrosativo utilizando um dos reagentes descrito na literatura como liberador de óxido nítrico: Nitrito de sódio ($NaNO_2$) (SIGMA-ALDRICH);
- ✓ Construir bibliotecas de cDNA a partir de RNAs de células leveduriformes de *Paracoccidioides* submetidas a estresse nitrosativo;
- ✓ Utilizando ferramentas de bioinformática, comparar a expressão de transcritos em condições de estresse nitrosativo com os bancos de ESTs de *Paracoccidioides* (Felipe *et al.*, 2005);
- ✓ Análise de transcritos por RT-PCR em tempo real para a confirmação dos níveis de expressão;
- ✓ Avaliar a taxa de $NADP^{+/}$NADPH durante o estresse nitrosativo.

Transcriptional responses of *Paracoccidioides* to nitrosative stress

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ABSTRACT

Background: *Paracoccidioides* is a thermal dimorphic fungus that causes Paracoccidioimycosis (PCM), an endemic disease in Latin America. Immune cells have a variety of defense mechanisms against pathogens, such as the production and release of nitric oxide (NO), one of the most important molecules among the reactive nitrogen species (RNS). RNS react with cellular components, resulting in damage to DNA and membranes, inhibition of repiration and inactivation of cellular enzymes. In response to nitrosative stress, human pathogenic fungi such as *Paracoccidioides* possess defense mechanisms against adverse effects of NO that help them survive the initial contact with the host immune system. To understand how *Paracoccidioides* responds to nitrosative stress, this study aims to identify genes that might contribute to this response.

Results: Growth of *Paracoccidioides* yeast cells was evaluated in the presence of various concentrations of sodium nitrite ($NaNO_2$). The nitrosative stress was confirmed by quantification of nitrite in the cultures supernatant and by the inhibition of the cytochrome c oxidase (complex IV) activity. The transcriptional analyzes were performed by sequencing a cDNA library constructed with *Paracoccidioides* mRNA obtained after incubation of fungal cells with 500 µM $NaNO_2$. The results show the induction of transcripts related to several cellular pathways, including genes of mitochondrial electron transport chain and those related to energy production. The expression of selected transcripts was confirmed by quantitative real time RT-PCR. Moreover, mitochondrial activity measured by XTT method was reduced in the presence of $NaNO_2$ during the first 2 hours of treatment. Additionally, the $NADP^+$/NADPH ratio is lower during nitrosative stress, as demonstrated.

Conclusion: The results suggest that the induction of transcripts associated with energy production during nitrosative stress may reflect a compensatory effect on the inhibition of enzymes that carry out this metabolic function, probably to ensure the production of energy and/or contribute to the redox balance by generation of NADPH.

Keywords: *Paracoccidioides*, nitrosative stress, transcriptome.

Background

The genus *Paracoccidioides* is composed by at least four phylogenetic groups: PS1, S2, S3 and *Pb*01-like [1, 2]. The fungus that is thermally dimorphic causes paracoccidioidomycosis (PCM) a systemic mycosis with broad distribution in Latin America [3]. The fungus exists as a saprobe in the soil producing conidia and mycelia fragments that are inhaled by the host and converted to the yeast phase in the lungs [4].

Immune cells present a large variety of defense mechanisms against pathogens. Two important pathways are those responsible for the production of superoxide radicals (O2-) and nitric oxide (NO) through the NADPH oxidase enzyme of phagocytes and nitric oxide synthase (iNOS), respectively [5]. NO is a free radical that plays role as mediator in several biological processes, including vasodilation, signaling, cytotoxicity, immunoregulation and antimicrobial activities [6, 7]. Macrophages induce the expression of the inducible nitric oxide synthase (NOS2, also known as iNOS), which catalyzes the oxidation of the amino acid L-arginine by two sequential reactions to produce NO [8]. The iNOS induction may be influenced by products derived from pathogens, such as lipopolysaccharide and lipoteichoic acid, by pro-inflammatory cytokines such as IFN-gamma,

TNF-α, IL-1 and IL-2 also by the availability of the amino acid L-arginine [6, 9]. NO is a relatively polar molecule that readily crosses the cell membrane of the pathogen and interacts with a large number of targets in the cell, including thiols, metals, protein tyrosine bases, nucleotides and lipids leading to DNA and membrane damage, inhibition of cellular respiration, and inactivation of other cellular enzymes [6, 10, 11]. Several innate immune mechanisms such as the activation of complement system, microbicidal activity of natural killer (NK) cells and macrophages act significantly in the defense against pathogenic fungi. Innate response cells, such as NK cells, neutrophils, monocytes and macrophages play a central role in the resistance to *Paracoccidioides* [12]. According to Longhi et al. (2012) [13], NK cells participate actively in the immune response against *Paracoccidioides* infection either by directly destroying yeast cells or by recognizing and killing infected cells. Inflammatory reaction and antifungal activity are induced by *Paracoccidioides* cells during their interaction with phagocytes [14]. Nitric oxide production by enzymatic action of iNOS represents one of the major microbicidal mechanisms of macrophages against pathogens [11]. Gonzalez et al. (2000) [15] demonstrated that nitric oxide production in macrophages induced by IFN-gamma is able to restrict the intracellular growth of *Paracoccidioides* and the conidia to yeast transition. According to Nishikaku et al. (2009)[9] NO has an important role in modulating chronic inflammatory response induced by *Paracoccidioides*, favoring tissue degradation and/or decreasing extracellular matrix components (ECM), by controlling inflamatory and immune mediators, as cytokines and matrix-degrading enzymes, such as matrix metalloproteinases (MMPs).

To establish infection, pathogens must be able to invade the immune system and to respond to different stresses in the host. Genome expression profile studies have been used to evaluate

the effects of nitrosative stress in eukaryotes and prokaryotes [16]. In general, the organisms responses are the induction of genes encoding proteins related to antioxidants and DNA repair systems, stress response, cellular transporters, metabolism, cell wall organization and signal transduction [7, 17, 18, 16, 19].

Here, we constructed a cDNA library from *Paracoccidioides* yeast cells submitted to nitrosative stress caused by $NaNO_2$. Our analysis revealed 163 transcripts modulated in response to nitrosative stress, when compared to ESTs (expressed sequence tags) derived from control yeast cells [20]. Data analysis allowed us to identify genes and gene sets whose induction might contribute to the response of *Paracoccidioides* to nitrosative stress. Transcripts encoding proteins from the electron transport chain were positively regulated as well as transcripts encoding proteins related to energy generation. Since the RNS are described to inhibit enzymes of the respiratory chain, we evaluated the activity of mitochondrial electron transport chain upon NO exposure. Our results suggest adaptive mechanisms used by *Paracocccidioides* to deal with stress caused by nitric oxide and its effects upon mitochondrial function.

Results and discussion

Effect of NO on growth of P*aracoccidioides* yeast cells

Under acidic conditions, $NaNO_2$ forms nitrous acid, which in the presence of oxygen generates reactive nitrogen intermediates (RNI), such as nitrogen trioxide, nitrogen dioxide and nitric oxide. In vitro studies demonstrate that $NaNO_2$ in acid conditions has antimicrobial properties against a variety of organisms such as bacteria, fungi and protozoa [21, 22]. To determine the appropriate concentration of $NaNO_2$ enough to cause a transcriptional response

without interfering with cell growth, we analyzed the effects of various concentrations of sodium nitrite (0 to 1 mM) under acidic conditions (pH 4.0) on the growth of *Paracoccidioides* yeast cells (Fig. 1). Cells grown at pH 4.0 in the absence of $NaNO_2$ showed the same phenotype of growth in all periods, when compared to pH 7.0. There were no significant effects on cell growth at 100 µM and 250 µM $NaNO_2$ in any of the incubation times. At 500 µM, $NaNO_2$ inhibits cell growth at 6 h of incubation. Higher concentrations of $NaNO_2$, such as 750 µM and 1 mM, severely inhibit cell growth. For transcriptional analysis, cells were exposed for 1 h to 500 µM $NaNO_2$.

Effect of nitrosative stress on cytochrome c oxidase activity and NO quantification

One important mechanism by which nitric oxide exerts cytotoxicity is by the inhibition of the cellular respiration. In particular, NO mediates inhibition of components of the electron transport, such as complex I, II and IV [23]. Cytochrome c oxidase (cytochrome aa 3, complex IV), located in the mitochondrial inner membrane, is the terminal complex of the mitochondrial respiratory chain, essential for energy production in cells [24]. Such enzyme catalyses the oxidation of cytochrome c^{2+} to cytochrome c^{3+} and the reduction of oxygen to water, a reaction coupled to proton translocation across the inner mitochondrial membrane [25, 26]. According to Brown (2001) [25], NO specifically and reversibly inhibits cytochrome c oxidase (complex IV). Such inhibition is competitive with oxygen and NO binds with higher affinity to the oxygen binding site of cytochrome c oxidase [27]. Because nitric oxide has been known to inhibit cytochrome c oxidase, we analyzed its activity in *Paracoccidioides* yeast cells during $NaNO_2$ treatment. Cells were exposed to 500µM $NaNO_2$ during 1, 3 and 6 h. After 1 h, enzyme activity upon NO exposure was similar to the control condition at pH 7.0 (Fig. 2A). We observed a significant inhibition on cytochrome c oxidase activity after 3 h of

treatment. After 6 h, cytochrome c oxidase activity was completely inhibited by the treatment. Additionally, we quantified nitrite levels in the culture supernatant, which is an indicator of nitric oxide generation, in order to confirm the nitrosative stress condition. As shown in Fig. 2 B, nitrite levels were similar in all time points of treatment, which confirms the release of nitric oxide by 500 μM $NaNO_2$ under acidic conditions. The results suggest the NO generation in the medium and its effect in the intracellular environment by the inhibition of cytochrome c oxidase activity.

cDNA library construction, DNA sequencing and sequence annotation

To investigate the transcriptional response of *Paracoccidioides* to nitric oxide, a cDNA library was constructed. We performed the sequencing of 4800 randomly selected clones. Of these, 4746 gave readable sequences. From those, 2460 sequences remained after vector and low quality sequences were removed. All sequences were arranged into 1325 singletons and 403 contigs with two or more ESTs.

For differential gene expression analysis, sequences were submitted to statistical test by using Audic and Claverie method [28], from which were generated 148 transcripts that displayed modulation during nitrosative stress, when compared to the EST database derived from *Paracoccidioides* control yeast cells [20]. The redundancy of the transcripts was calculated as the number of ESTs related to each transcript. MIPS functional categories were used to classify the 148 ESTs into 14 major groups (Additional File 1). The major MIPS categories represented included metabolism (14.81%), cellular transport (11.48%), protein synthesis (20.94%), protein fate (6.75%) and unclassified proteins (18.91%). In addition, 80 sequences (54.05% of the total) represented ESTs of *Paracoccidioides* that were not detected in the *in*

vitro-cultivated *Paracoccidioides* yeast cells transcriptome [20]. A total of 118 transcripts (79.72% of the total) and 30 transcripts (20.27% of the total) corresponded to overexpressed and repressed sequences, respectively, when compared to *in vitro*-cultivated *Paracoccidioides* yeast cells transcriptome (Table 1, Table 2).

Description of the transcripts induced during nitrosative stress

A direct analysis allowed the detection of up- and down- regulated genes involved in a variety of cellular processes, as demonstrated in Tables 1 and 2, respectively. Transcripts involved in carbohydrate metabolism were induced during the treatment. Transaldolase (EC: 2.2.1.2) is described to be important to oxidative stress resistance in organisms, such as *Sacharomyces cerevisiae* [29, 30]. Proteomic studies in *S. cerevisiae* yeast cells exposed to hydrogen peroxide revealed changes in carbohydrate metabolism to redirect carbohydrates to pathways that regenerate NADPH from glycolysis, such as the pentose phosphate pathway. In addition, gene expression changes encoding enzymes related to carbon metabolism are observed in *Candida albicans* and *Histoplasma capsulatum* in response to nitric oxide [7, 17].

Genes related to stress response were induced. These included copper and zinc-containing superoxide dismutase (Cu/ZnSOD) and cytochrome c peroxidase (CCP). CCPs act as mitochondrial antioxidant that catalyze the degradation of hydrogen peroxide by converting it to water and are described to protect against damage caused by ROS in cells of fungi, such as *Paracoccidioides* and *Cryptococcus neoformans* [31, 32]. Superoxide dismutase (SOD) dismutates the superoxide radical ($O2^{\bullet-}$) into molecular oxygen and H_2O_2 and is a primary antioxidant defense against ROS [33]. The importance of Cu,Zn SOD in fungal virulence and viability has been addressed in *C. albicans* in which cells lacking sod1, which encodes the

cytoplasmatic localized SOD, were unable to survive the fungicidal attack of a macrophage cell line and have attenuated virulence in a mouse model of infection [34]. Both CCP and SOD are dependent on NADPH for function and are important for nitrosative stress resistance in *C. neoformans* [35]. Studies has demonstrated that the cytosolic SOD and the pentose phosphate pathway have overlapping roles in protecting *S. cerevisiae* from oxidative stress and that both systems are critical for maintaining the intracellular redox state [36].

NO reacts directly with membrane proteins, such as ion channels and transport proteins, thus disturbing ion homeostasis [16]. Several of the up-regulated transcripts during nitric oxide stress were related to transport genes (11.86%), exhibiting homology to iron, zinc, lipid, protein and phosphate transporters (Table 1). Upregulation of transporter genes was also observed in *C. albicans*, *C. neoformans* and *H. capsulatum* during nitrosative stress [7, 17, 18]. Two products containing ATP-binding cassette (ABC transporters) and a transporter of the MFS family (Major Facilitator Superfamily), which are also implicated in cell defense and drug resistance [37, 38], were detected. Additionally, a gene encoding a membrane transporter protein responsible for efflux of sulfite, Ssu1, was induced [39]. *S. cerevisiae* cells submitted to nitrosative stress showed increased expression of SSU1at transcript and protein levels. Furthermore, the presence of SSU1 gene conferred a growth advantage after exposure to nitric oxide [40].

RNI can react with specific amino acids, which can damage protein structural arrangements and folding, leading to inhibition or inactivation of protein function [16]. Some genes involved in protein folding and degradation were also induced, such as mitochondrial inner membrane translocase Tim9 and TCP1, described to act as chaperones in fungi [41, 42] and peptidyl-prolyl cis-trans isomerase A2, which responds to protein damage in *Entamoeba*

histolistica during oxidative stress [16]. Genes related to the ubiquitin-proteasome pathway were also induced and are often described to be responsive to various types of cellular stresses, including those generated by RNS in pathogenic organisms, such as *H. capsulatum* and *E. histolistica* [17, 16]. In *Mycobacterium tuberculosis*, the degradation of proteins nitrated or nitrosated by the proteasome pathway is important in NO resistance [43]. Induction of these genes likely reflects the cell`s attempt to deal with unfolded proteins generated by nitrosative stress in *Paracoccidioides.*

Genes involved in electron transport and respiration (8.47%) were also induced upon RNS exposure (Table 1). It was observed induction of subunits of the enzymes NADH-ubiquinone oxidoreductase and a NADH dehydrogenase FAD- containing subunit, which are components of the electron transport chain (complex I) used to produce ATP for cellular processes. We observed induction of the gene encoding a bifunctional P-450:NADPH-P450 reductase, involved in the electron transfer from NADP to cytochrome P450, and a 2Fe-2S iron-sulfur cluster binding domain-containing protein, also involved in electron transport. Many of these metabolic enzymes induced upon NO exposure have an iron-sulfur cluster critical to its enzymatic action. Nitric oxide has been shown to interact with iron-sulfur centers of mitochondrial proteins removing iron, which inhibits the enzyme activity [24]. Other components of the electron transport chain, such as NADH dehydrogenase (ubiquinone) 1 alpha sub complex assembly factor 3 and mitochondrial respiratory complex I chaperone were also induced. Two genes that encode mitochondrial carrier proteins were induced and can be related to solute transport across the mitochondrial membrane necessary for the electron transport chain activity. One of these carriers, a mitochondrial carrier protein, have a calcium-binding site and is related to the transport of ion, especially Ca^{+} (data not shown). In *C.*

neoformans and *H. capsulatum*, genes involved in mitochondrial respiration were highly induced during nitric oxide stress. In *H. capsulatum* such genes include those related to mitochondrial transport, such as phosphate and succinate/furamate transporters [17, 18]. Two genes related to the mitochondrial electron transport chain were also up regulated in *Paracoccidioides* cells internalized by murine macrophages, such as cytochrome oxidase c subunit VIII and mitochondrial copper transporter [44].

Description of the transcripts repressed during nitrosative stress

A small number of genes had decreased expression upon exposure to $NaNO_2$ (Table 2). The largest category of genes was those involved in protein synthesis (50%). The repression of ribosomal genes was also observed upon phagocytosis of *C. albicans* by macrophages [45] and has also been observed in *S. cerevisiae* upon exposure to various stresses characterizing a part of the general stress response in that organism [46].

Analysis of the expression of genes induced during nitrosative stress by quantitative real- time RT-PCR

Genes induced during nitrosative stress were selected for analysis of expression levels by qRT-PCR. Those transcripts comprise relevant functional categories, such as cellular transport, electron transport, metabolism and response to stress. Total RNA from *Paracoccidioides* yeast cells cultured with 500 µM $NaNO_2$ at pH 4.0 and in the absence of $NaNO_2$ at pH 7.0 was obtained as previously described in independent experiments from those performed for the construction of the cDNA library. The genes include NADH-ubiquinone oxidureductase 21 kDa subunit, transaldolase, cytochrome c peroxidase and

vacuolar iron transporter (Fig. 3). The expression levels of transcripts changed similarly to the description in the ESTs analysis, which confirms their induction upon NO exposure.

Measurement of the Intracellular $NADP^+$/NADPH concentrations

We quantified the intracellular concentrations of NADPH, NADP and NADP/NADPH ratio in *Paracoccidioides* yeast cells grown cultured in pHs 7.0 and pH 4.0 and pH 4.0 in the presence of sodium nitrite (Fig. 4). Total NADP(H) and NADPH were extracted from whole-cell lysate, quantified by NADP cycling enzyme mix and measured by absorbance at 450 nm. Amounts were quantified by comparison to a standard curve of $NADP^+$, and the data were processed as a ratio of $NADP^+$ to NADPH. The ratio of of $NADP^+$ to NADPH is indicative of the demand for NADPH by the cell. Nitrosative stress decreased the $NADP^+$ content and $NADP^+$/NADPH ratio compared to the pH 7.0 and pH 4.0 (Fig.4). The decrease of the $NADP^+$/NADPH ratio during nitrosative stress is caused by decreased $NADP^+$ content, which could have been used extensively for NADPH production. In stress resistance, NADPH acts as electron donor for reduction of the main oxidant defense systems in most organisms, including the thioredoxin and glutathione systems. Such systems are often described acting as essential antioxidants in response to oxidative stress in *S. cerevisiae* and *Paracoccidioides* [47].

Quantitative determination of mitochondrial activity during nitrosative stress

We evaluated the mitochondrial activity during nitrosative stress. For this purpose, we used the colorimetric method of the yellow tetrazole salt XTT, which is cleaved mainly by mitochondrial dehydrogenases and reductases through direct reaction with electron carries, such as NADH or NADPH. We observed a reduction in mitochondrial activity at 1h and 2h

after treatment with 500µM $NaNO_2$ (Fig. 5). There was no significant difference between control conditions at pH 4.0 and pH 7 .0 in the treatment times. The results suggest a decrease of mitochondrial dehydrogenases activity during the first two hours of $NaNO_2$exposure.

Conclusion

The ability of the fungal pathogen *Paracoccidioides* to survive within macrophages, neutrophils and other phagocytic cells is presumably due to is capacity to protect itself from toxic reactive oxygen and nitrogen species. In this study, we characterized the transcriptional and biochemical response of *Paracoccidioides* yeast cells to nitric oxide stress. Of particular interest were the striking changes in the expression of energy-respiration-related genes after $NaNO_2$ exposure. Analysis of mitochondrial activity by the XTT method showed a decrease in mitochondrial function during the first two hours of $NaNO_2$ exposure and the cytochrome c oxidase (complex IV) activity was inhibited at 3 h of treatment. Because RNS can inhibit protein function, some genes may be induced as a compensatory mechanism, since proteins are directly compromised by RNS-induced modifications, such as *S*-nytrosilation. This hypothesis may explain why we observed induction of components of the electron transport chain. During nitrosative stress, it was observed a decrease in $NADP^+$ content, which could have been used for NADPH generation. NADPH production contributes to generate a reducing environment which favors the restoration of redox balance of the cells. Our results suggest adaptive mechanisms used by *Paracocccidioides* to deal with stress caused by nitric oxide and its effects upon mitochondrial function.

Methods

Culture and cell viability

*Paracoccidioides, Pb*01 (ATCC MYA – 826) was used in all experiments. The yeast phase was maintained *in vitro* by sub culturing at 36°C in Fava Netto's semisolid medium [1% (w/v) peptone; 0.5% (w/v) yeast extract; 0.3% (w/v) proteose peptone; 0.5% (w/v) beef extract; 0.5% (w/v) NaCl; 4% (w/v) agar] (Fava-Netto, 1955) every 7 days. A total of 10^5cells/mL was transferred to 50 mL of liquid Fava-Neto's medium pH 7.2 and grown for 72 h at 36 °C at 180 rpm. The number of viable cells was monitored by using the trypan blue dye 0.4%, (w/v).

Assessment of cell growth upon $NaNO_2$ exposure

A total of 10^8cells/mL were incubated in Fava-Netto`s medium pH 4.0 at 36 ° C at 180 rpm in the following concentrations of sodium nitrite ($NaNO_2$): 0.1, 0.25, 0.5, 0.75 and 1 mM during 1, 2, 4, 6 and 8 h. The acid pH was used, as previously described, since nitric oxide is generated by $NaNO_2$ in this condition [18]. The treated cells were washed in sterile 1X PBS. Samples containing 10^6, 10^5 and 10^4 cells were spotted onto the solid Fava-Netto`s medium supplemented with 2% (w/v) glucose and grown at 36°C. Control experiments were prepared in the absence of $NaNO_2$ at pHs 4.0 and 7.0. All tests were performed in triplicate. The plates were incubated for seven days at 36 ° C and photographed.

Nitrosative stress and nitrite quantification

Paracoccidioides yeast cells were incubated for 1 h in liquid Fava-Netto`s medium pH 4.0 containing $NaNO_2$ at a final concentration of 500 µM. Cells were centrifuged, washed with

1X PBS and used for RNA extraction. In order to investigate nitric oxide formation it was measured the nitrite (NO_2^-) formation, which is one of two primary, stable and nonvolatile breakdown products of NO [48]. The concentration of nitrite (NO_2) in the culture supernatant, as an indicator of NO generation, was measured with the Griess reagent [1% (v/v) sulfanilamide in 5% (v/v) phosphoric acid, 0.1% (v/v) naphthylethylenediamine dihydrochloride and 0.1 M sodium nitrite in water] according to the manufacturer's instructions (PROMEGA, Madison, USA). Briefly, aliquots of 50 µL of culture supernatants were transferred to 96-well microplates. Because we used 500 µM $NaNO_2$ in our stress condition, supernatants were diluted 5 x in 1X PBS to adjust the absorbance values to the standard curve, which was linear between 0 and 100 µM sodium nitrite. A volume of 50 µL of Griess reagent was mixed with 50 µL of each culture supernatant. After 30 min, the absorbance was measured in a microplate reader for 96 wells (Thermo Plate TP- Reader, EQUIPAR, Curitiba, Brazil) at 540 nm and nitrite concentration was determined from the standard curve. Nitrite concentration was determined by average absorbance value of each experimental sample and their comparison to the $NaNO_2$ standard curve.

Quantification of cytochrome c oxidase activity

Cytochrome c oxidase (CCO) activity was measured by using a Cytochrome c Oxidase Assay Kit according to the manufacturer's instructions (Sigma-Aldrich, Saint Louis Missouri, USA). After 500 µM $NaNO_2$ exposure, total protein extracts from 10^7 *Paracoccidioides* cells/mL inocullum were obtained. Briefly, extraction buffer (20 mM Tris-HCl, pH 8.8, 2 mM $CaCl_2$) was added to the cells. Glass beads were added and the sample blended in a Bead-Beater (Bio-Spec Products, Bartesville, USA) three times for 1 min at 4 ° C. The mixture was

centrifuged at 15.000 *g* at 4°C, for 20 min, the supernatant was sampled and used for quantification of CCO activity.

Control experiments were prepared in the absence of $NaNO_2$ at pH 4.0 and 7.0. Protein concentrations were determined using the Bradford reagent (Sigma-Aldrich) and bovine serum albumin (BSA) was utilized as a standard. The colorimetric assay in the kit is based on the oxidation of ferrocytochrome c by cytochrome c oxidase. The increase in absorbance levels is directly proportional to the cytochrome c oxidase activity. The linearity of the reaction was determined by plotting the absorbance values at 550 nm. The activity of the enzyme was means of three independent determinations and statistical comparisons were performed using Student´s T test. Samples with *p*-values ≤ 0.05 were considered statistically significant.

RNA extraction, mRNA purification and construction of the cDNA library

Total RNA from *Paracoccidioides* yeast cells incubated with 500µM $NaNO_2$ for 1h was extracted by using TRIZOL reagent (Invitrogen™ Life Technologies, Carlsbard, CA) according to the manufacturer's instructions. Purification of mRNA was performed using the Oligotex Direct mRNA Mini Kit (Qiagen - Sample & Assay Technologies, Hilden, Germany). mRNAs were used to construct double-stranded cDNAs by using the high capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). The cDNA library was constructed following the protocols of the SuperScript Plasmid System with Gateway Technology for cDNA Synthesis & Cloning (Invitrogen™ Life Technologies, Carlsbard, CA). The cDNA was cloned into the pCMV•SPORT6 plasmid vector, which were transformed into *E. coli* (XL1blue) cells.

cDNA sequencing and ESTs generation

The transformants were plated at approximately 200 colonies per plate (150 mm Petri dish). Colonies were randomly selected and transferred to a 96-wells polypropylene Deep Well plates containing Circle grow medium and grown under aeration and agitation for 22 h. Plasmid DNAs were isolated and purified. cDNA inserts were sequenced from the 5' end by employing a standard fluorescence labeling DYEnamic ET dye terminator kit (GE Healthcare Life Sciences, Uppsala, Sweden) with the T7 flanking vector primer. Automated sequence analysis was performed in a MegaBACE 1000 DNA sequencer (GE Healthcare Life Sciences, Uppsala, Sweden). EST sequences were pre-processed by PHRED [49] and Crossmatch (http://www.genome.washington.edu/UWGC/analysistools/Swat. cfm) algorithms. The sequences of at least 50 nucleotides with PHRED quality > 20 were considered for the assembly and formation of clusters. A total of 2460 ESTs were selected by these inclusion criteria.

EST processing pipeline, annotation and differential expression analysis

The assembly of the ESTs was performed using CAP3 algorithm [50] and clustered generating 403 contigs and 1325 singlets which were analyzed. PHRED, Crossmatch and CAP3 tools were integrated in a specific pipeline (http://www.lbm.icb.ufg.br/pipelineUFG/). For ESTs annotation, sequences were compared against the GenBank non-redundant (nr) database from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and the nucleotide database generated from *Paracoccidioides* structural genome (http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/MultiHome.htm. The database sequence matches were considered significant at e-values $\leq 10^{-5}$. To elucidate

ESTs functions, the InterPro's databases (http://www.ebi.ac.uk/InterProScan/) was used to obtain information about protein domains and classification. The Munich Information Center for Protein Sequences (MIPS) (http://mips.gsf.de/) database was used to assign functional categories. We used the enzyme nomenclature database ExPASy (http://www.expasy.org/) and Kyoto Encyclopedia of Gene and Genomes (KEGG) (http://www.kegg.com/) to access Enzyme Commission (EC) numbers and metabolic pathways, respectively. Additionally, sequences were grouped into functional categories through PEDANT 3 database (http://pedant.helmholtz- muenchen.de/index.jsp).

To assign a differential expression profile, the contigs from cDNA library were statistically evaluated by comparison with yeast ESTs previously generated in control conditions [20] using the Audic and Claverie's method [28]. Obtained data generated a heat map. It was considered induced genes in our cDNA library those as determined with a 95% confidence rate.

Real-time RT-PCR

After treatment with DNAse, the first strands of cDNAs were synthesized from total RNA using the high capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) according to the supplier's instructions. Transcripts were quantified by qRT-PCR using a SYBR green PCR master mix (Applied Biosystems Step One Plus PCR System). The PCR thermal cycling program consisted of 40 cycles of 95°C for 15s; 60°C for 1min. The SYBR green PCR master mix (Applied Biosystems) was used for each reactions that was supplemented with 20ρmol of the each gene-specific oligonucleotide pair and 40 ng of template cDNA in a final of 20 μL. A melting curve analysis was performed to confirm the amplification of single PCR product [51]. The data were normalized with *Paracoccidioides*

α-tubulin [52]. The standard cDNA was serially diluted 1:5, and a standard curve was generated by using four samples from the pooled cDNA. Up regulated genes were selected and validated by qRT-PCR.

Quantitative determination of mitochondrial activity

Mitochondrial function of *Paracoccidioides* cells upon $NaNO_2$ exposure was analyzed by using the colorimetric method of XTT (sodium3'-[1 - (fenilaminocarbonil) – 3,4-tetrazole]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) (Roche Applied Science, Mannheim, Germany) according to the manufacturer`s instructions. XTT method is based on the cleavage of the yellow tetrazole salt XTT by metabolically active cells to form the orange formazan dye that was quantified in this work by using a spectrophotometer microplate reader for 96 wells. A total of 10^7cells/mL was inoculated into 10 mL of Fava-Netto liquid medium, pH 4.0, supplemented with 2% (w/v) glucose in the presence of 500 µM $NaNO_2$ and incubated at 36 °C during 8 h. Controls were obtained. All tests were done in triplicate. The cells were washed with 150 µL of 1x PBS to remove the $NaNO_2$ and transferred to a 96-well microplate. 50 µL of XTT reagent and 1 µL of Electron-coupling reagent (N-methyl dibenzopyrazine methyl sulfate in phosphate buffered saline sterile) were added in each sample. The samples were incubated for 18 h at 36 ° C protected from light. After this period, the formation of formazan dye was quantified in a microplate reader for 96 wells at 450 nm. The increase in absorbance shows the increase in cleavage of the XTT by mitochondrial dehydrogenases and formation of formazan dye, which is proportional to the increase in mitochondrial activity. Statistical comparisons were performed using Student´s T test and samples with p-values ≤ 0.05 were considered statistically significant.

Measurement of the Intracellular $NADP^+$/NADPH

Intracellular $NADP^+$, NADPH and $NADP^+$/NADPH concentrations were measured by using NADP/NADPH Assay Kit (Abcam®, Cambridge, UK) according to the manufacturer's instructions. Briefly, the *Paracoccidioides* cells were collected and washed with 1 mL of cold PBS. The cells were resuspended in a reaction buffer and the supernatants were used to perform the NADP/NADPH quantification. The kit enzymes (NADP cycling enzyme mix) quantificate total NADP(H) and NADPH. NADP is quantified by subtracting NADPH content from total NADP(H). The quantifications were performed in 96 wells microplate reader at 450 nm wave. The concentrations of the pyridine nucleotides were obtained in three independent determinations and statistical comparisons were performed by using Student´s T test. Samples that present p-values ≤ 0.05 were considered statistically significant.

Additional material

Additional file 1: Overview of ESTs from the *Paracoccidioides* transcriptome during nitrosative stress. Classification of the ESTs based according to the functional categories of the PEDANT functional annotation scheme.

Additional file 2: Supplementary table S1 shows the oligonucleotide primers used in Real Time RT-PCR.

Authors' contributions

PECN prepared the cDNA library, performed the DNA sequencing, the validation experiments, contributed to gene annotation and classification and supported the preparation of the figures and tables. MP contributed to the analysis of the raw sequences. WSM contributed to the analysis of the raw sequences and to the construction of the EST database.

JAP contributed to the construction of the cDNA library, to the results discussion and to the manuscript preparation. CMAS designed the project, contributed to the data analysis and to the preparation of the manuscript.

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References

1. Matute DR, Mcewen JG, Puccia R, Montes BA, San-Blas G, Bagagli E, Rauscher JT, Restrepo A, Morais F, Niño-Veja G, Taylor JW: **Cryptic speciation and recombination in the fungus *Paracoccidioides brasiliensis* as revealed by gene genealogies.** *Mol Biol Evol* 2006, **23:** 65-73.

2. Teixeira MM, Theodoro RC, Carvalho MJA, Fernandes L, Paes HC, Hahn RC, Mendonza L, Bagagli E, San-Blas G, Felipe MSS: **Phylogenetics analysis reveals a high level of speciation in the *Paracoccidioides genus*.** *Mol Phylogenet Evol* 2009, **52:** 273-283.

3. Restrepo A, McEwen JG, Castaneda E: **The habitat of *Paracoccidioides brasiliensis*: how far from solving the riddle?** *Med Mycol* 2001, **39:** 233-241.

4. Franco M: **Host-parasite relationship in paracoccidioimycosis.** *J Med Vet Mycol* 1987, **25:** 5-18.

5. Fang FC: **Antimicrobial reactive oxygen and nitrogen species: concepts and controversies.** *Nat Rev Microbiol* 2004, **2:** 820-832.

6. Fang FC: **Mechanisms of Nitric Oxide–related Antimicrobial Activity in: Perspectives Series: Host/Pathogen Interactions.** *J Clin Invest* 1997, **99:** 2818–2825

7. Hromatka BS, Noble SM, Johnson AD: **Transcriptional Response of *Candida albicans* to Nitric Oxide and the Role of the YHB1 Gene in Nitrosative Stress and Virulence.** *Mol Biol Cell* 2005, **16:** 4814–4826.

8. Bogdan C: **Nitric oxide and the immune response.** *Nature Immunol* 2001, **2:** 907-916.

9. Nishikaku AS, Molina RFS, Ribeiro LC, Scavone R, Albe BP, Cunha CS, Burger E: **Nitric oxide participation in granulomatous response induced by *Paracoccidioides brasiliensis* infection in mice.** *Med Microbiol Immunol* 2009, **198:**123–135.

10. De Groote MA: **Antimicrobial Properties of Nitric Oxide.** In *Nitric Oxide and Infection.* Edited by Fang FC. New York: Kluwer Academic / Plenum Publishers; 1999, 231–261.

11. Nathan C, Shiloh MU: **Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens.** *Proc Natl Acad Sci* 2000, **97:** 8841-8848.

12. Fortes MRP, Miot HA, Kurokawa CS, Marques MEA, Marques,AS: **Immunology of paracoccidioidomycosis.** *An Bras Dermatol* 2011, **86:**516-25.

13. Longhi LN, da Silva RM, Fornazim MC, Spago MC, de Oliveira RT, Nowill AE, Blotta MH, Mamoni RL: **Phenotypic and Functional Characterization of NK Cells in Human**

Immune Response against the Dimorphic Fungus *Paracoccidioides brasiliensis*. *J Immunol* 2012, **189:** 935-45.

14. Pagliari C, Pereira NV, Kanashiro L, Stegun FW, Croda J, Duarte MI, Sotto MN: **Characterization of cytotoxic immune response in skin and mucosal lesions of paracoccidioidomycosis.** *J Cutan Pathol* 2009, **37:** 565-70.

15. Gonzalez A, Gregori W, Velez D, Restrepo A, Cano LE: **Nitric Oxide Participation in the Fungicidal Mechanism of Gamma Interferon-Activated Murine Macrophages against *Paracoccidioides brasiliensis* Conidia.** *Infect Immun* 2000, **68:** 2546-2552.

16. Vicente JB, Ehrenkaufer GM, Saraiva LM, Teixeira M, Singh U: ***Entamoeba histolytica* modulates a complex repertoire of novel genes in response to oxidative and nitrosative stresses: implications for amebic pathogenesis.** *Cel Microbiol* 2009, **11(1):** 51-69.

17. Nittler MP, Hocking-Murray D, Foo CK, Sil A: **Identification of *Histoplasma capsulatum* transcripts induced in response to reactive nitrogen species.** *Mol Biol Cell* 2005, **16:** 4792– 4813.

18. Missall TA, Pusateri ME, Donlin MJ, Chambers KT, Corbett JA, Lodge JK: **Posttranslational, Translational, and Transcriptional Responses to Nitric Oxide Stress in *Cryptococcus neoformans*: Implications for Virulence.** *Eukaryot Cell* 2006, **5:** 518–529.

19. Voskuil MI, Bartek IL, Visconti K, Schoolnik GK: **The response of *Mycobacterium tuberculosis* to reactive oxygen and nitrogen species.** *Front Microbiol* 2011, **2:**105.

20. Felipe MS, Andrade RV, Arraes FB, Nicola AM, Maranhao AQ, Torres FA, Silva-Pereira I, Pocas-Fonseca MJ, Campos EG, Moraes LM, Andrade PA, Tavares AH, Silva SS, Kyaw CM, Souza DP, Pereira M, Jesuino RS, Andrade EV, Parente JA, Oliveira GS, Barbosa MS, Martins NF, Fachin AL, Cardoso RS, Passos GA, Almeida NF, Walter ME, Soares CMA, Carvalho MJ, Brigido MM: **Transcriptional profiles of the human pathogenic fungus**

Paracoccidioides brasiliensis **in mycelium and yeast cells.** *J Biol Chem* 2005, **280:** 24706-24714.

21. Phillips R, Kuijper S, Benjamin N, Wansbrough-Jones M, Wilks M, Kolk AHJ: **In Vitro Killing of *Mycobacterium ulcerans* by Acidified Nitrite.** *Antimicrob Agents and Chemother* 2004, **48:** 3130–3132.

22. Heaselgrave W, Andrew PW, Kilvington S: **Acidified nitrite enhances hydrogen peroxide disinfection of Acanthamoeba, bacteria and fungi.** *J Antimicrob Chemother* 2010, **65:** 1207–1214.

23. Cassina A, Radi R: **Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport.** *Arch Biochem Biophys* 1996, **328:** 309-316.

24. Brown GC: **Nitric oxide and mitochondrial respiration.** *Biochim Biophys Acta* 1999, **1411:** 351-369.

25. Brown GC: **Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase.** *Biochim Biophys Acta* 2001, **1504:** 46-57.

26. Sarti P, Forte E, Mastronicola D, Giuffrè A, Arese M: **Cytochrome c oxidase and nitric oxide in action: Molecular mechanisms and pathophysiological implications.** *Biochim Biophys Acta* 2012, **1817:** 610–619

27. Brown GC: **Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase.** *FEBS Letters* 1995, **369:** 136-139.

28. Audic S, Claverie J M: **The significance of digital gene expression profiles.** *Genome Res* 1997, **7:** 986-995.

29. Juhnke H, Krems B, Kotter P, Entian KD: **Mutants that show increased sensitivity to hydrogen peroxide reveal an important role for the pentose phosphate pathway in protection of yeast against oxidative stress.** *Mol Gen Genet* 1996, **252:** 456–464.

30. Vatanaviboon P, Varaluksit T, Seeanukun C, Mongkolsuk S: **Transaldolase exhibits a protective role against menadione toxicity in *Xanthomanas campestris pv. phaseoli*.** *Biochem Biophys Res Commun* 2012, **297:** 968–973.

31. Giles SS, Perfect JR, Cox GM: **Cytochrome *c* peroxidase contributes to the antioxidant defense of *Cryptococcus neoformans*.** *Fungal Genet Biol* 2005 , **42:** 20-29.

32. Dantas AS, Andrade RV, Carvalho MJ, Felipe MSS, Campos EG: **Oxidative stress response in *Paracoccidioides brasiliensis*: assessing catalase and cytochrome c peroxidase.** *Mycol Res* 2008, **112:** 747-756.

33. Halliwell B: **Antioxidant defence mechanisms: from the beginning to the end (of the beginning).** *Free Radic Res* 1999, **31:** 261-272.

34. Hwang CS, Rhie GE, Oh JH, Huh WK, Yim HS, Kang SO: **Copper and zinc-containing superoxide dismutase (Cu/ZnSOD) is required for the protection of Candida albicans against oxidative stresses and the expression of its full virulence.** *Microbiology* 2002, **148:** 3705-3713.

35. De Jesus-Berrios A, Liu L, Nussbaum JC, Cox GM, Stamler JS, Heitman J: **Enzymes that counteract nitrosative stress promote fungal virulence.** *Curr Biol* 2003, **13:**1963–1968.

36. Slekar KH, Kosman DJ, Culotta VC: **The yeast copper/zinc superoxide dismutase and the pentose phosphate pathway play overlapping roles in oxidative stress protection.** *J Biol Chem* 1996, **271:** 28831-6.

37. Ghannoum MA, Rice LB: **Antifungal agents: mode of action, mechanism of resistance and correlation of these mechanisms with bacterial resistance.** *Clin Microbiol Rev* 1999, **12:** 501–517.

38. Lamping E, Baret PV, Holmes AR, Monk BC, Goffeau A, Cannon RD: **Fungal PDR transporters: phylogeny, topology, motifs and function.** *Fungal Genet Biol* 2010, **47:** 127.

39. Park H, Bakalinsky, AT: **SSU1 mediates sulphite efflux in *Saccharomyces cerevisiae*.** *Yeast* 2000, **16:** 881–888.

40. Sarver A, DeRisi J: **Fzf1p regulates an inducible response to nitrosative stress in *Saccharomyces cerevisiae*.** *Mol Biol Cell* 2005, **16:** 4781–4791.

41. Miklos D, Caplan S, Mertens D, Hynes G, Pitluk Z, Kashi Y, Harrison-Lavoie K, Stevenson S, Brown C, Barrel B, Horwich AL, Willison K: **Primary structure and function of a second essential member of the heterooligomeric TCP1 chaperonin complex of yeast, TCP1β.** *Proc Nati Acad Sci* 1994, **91:** 2743-2747.

42. Vial S, Lu H, Allen S, Savory P, Thornton D, Sheehan J, Tokatlidis K: **Assembly of Tim9 and Tim10 into a Functional Chaperone.** *J Biol Chem* 2002, **277:** 36100–36108.

43. Darwin KH, Ehrt S, Gutierrez-Ramos JC, Weich N, Nathan CF: **The Proteasome of *Mycobacterium tuberculosis* Is Required for Resistance to Nitric Oxide.** *Science* 2003, **302:** 1963-1966.

44. Tavares AH, Silva SS, Dantas A, Campos EG, Andrade RV, Maranhao AQ, Brigido MM, Passos-Silva DG, Fachin AL, Teixeira SM, Passos GA, Soares CMA., Bocca AL, Carvalho MJ, Silva-Pereira I, Felipe MS: **Early transcriptional response of *Paracoccidioides brasiliensis* upon internalization by murine macrophages.** *Microbes Infect* 2007, **9:** 583-590.

45. Lorenz MC, Bender JA, Fink GR: **Transcriptional response of *Candida albicans* upon internalization by macrophages.** *Eukaryot Cell* 2004, **3:**1076–1087.

46. Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz GS, Botstein D, Brown PO: **Genomic Expression Programs in the Response of Yeast Cells to Environmental Changes.** *Mol Biol Cell* 2000, **11:** 4241– 4257.

47. Campos EG, Jesuino RSA, Dantas AS, Brígido MM, Felipe MSS: **Oxidative stress response in *Paracoccidioides brasiliensis*.** *Genet Mol Res* 2005, **4:** 409-429.

48. Griess P: **Bemerkungen zu der abhandlung der H.H. Weselsky und Benedikt "Ueber einige azoverbindungen."** *Chem Ber* 1879, **12:** 426–8.

49. Ewing B, Green P: **Base-calling of automated sequencer traces using Phred. II, Error probabilities.** *Genome Res* 1998, **8:** 186 -194.

50. Huang X, Madan A: **CAP3: A DNA sequence assembly program.** *Genome Res* 1999, **9:** 868-877.

51. Bookout AL, Cummins CL, Mangelsdorf DJ, Pesola JM, Kramer MF: **High-** 2006, **15:**1–28.

52. Borges CL, Bailao AM, Bao SN, Pereira M, Parente JA, Soares, CMA: **Genes potentially relevant in the parasitic phase of the fungal pathogen *Paracoccidioides brasiliensis*.** *Mycopathologia* 2011, **171(1):** 1-9.

Figure Legends

Figure 1 Growth of *Paracoccidioides* yeast cells in the presence of sodium nitrite. A total of 10^8 cells/mL were grown in liquid medium and treated with $NaNO_2$. Samples were harvested 2, 4, 6 and 8 h after treatment and transferred to plates containing solid medium. Controls were performed at pHs 7.0 and 4.0. All experiments were done in triplicate. The plates were incubated for 7 days at 36 °C and photographed.

Figure 2 Nitrosative stress as determined by cytochrome c oxidase activity and nitrite measurements. A- Inhibition of cytochrome c oxidase (CCO) activity upon nitric oxide exposure. *Paracoccidioides* yeast cells were grown during 1, 3 and 6 h in Fava Neto's liquid medium pH 7.0, pH 4.0 and in the presence of 500 µM $NaNO_2$ at pH 4.0. Protein extracts from 10^7cells/mL were used for CCO enzyme activity measurements. B-Measurement of nitrite concentration in *Paracoccidioides* yeast cells during $NaNO_2$ treatment. The nitrite concentration was monitored in cells grown in the presence of 500 µM $NaNO_2$ at 0-6 h. Values of nitrite concentration was calculated. Controls performed at pH 4.0 and pH 7.0 did not show absorbance values. Significant difference (* $p < 0.05$ compared with control - two-tailed Student`s T test).

Figure 3 Validation of the expression of genes. Quantitative Real Time RT-PCR analyzes were carried out with specific sense and antisense oligonucleotide primers as described in Additional File 2 (Suplementary Table S1). RNAs obtained from nitrosative stress and control conditions were used to obtain the cDNAs used to perform quantification of vacuolar iron transporter, transaldolase, NADH-ubiquinone oxidureductase 21 kDa subunit and cytochrome

c peroxidase genes. Reactions were performed in triplicate and normalized by using α-tubulin expression. Asterisk denotes values statistically different from control ($p \leq 0.05$) (two-tailed Student`s T test).

Figure 4 Intracellular concentrations of *Paracoccidioides* yeast cells pyridine nucleotides. The pyridine nucleotides dosage was performed using *Paracoccidioides* proteins extracts obtained after incubation of yeast cells in the nitrosative stress condition. Controls were performed at pHs 7.0 and 4.0 without sodium nitrite. Nitrosative stress growth conditions indicates a significant (* $p < 0.05$ compared with control - two-tailed Student`s T test) decrease of the $NADP^+$/NADPH ratio in *Paracoccidioides*. Error bars indicate standard errors of the means).

Figure 5 Effect of nitrosative stress on mitochondrial activity of *Paracoccidioides* yeast cells. A total of 10^7cells/mL was grown in Fava-Netto liquid pH 4.0 in the presence of 500µM $NaNO_2$ for 1, 2, 3, 4, 5 and 6h. The nitrosative stress led to a significant ($p < 0.05$* compared with control - two-tailed Student`s T test) decrease of the mitochondrial activity during the first two hours. Control conditions were performed at pH 7.0 and pH 4.0. Absorbance was obtained at 450 nm.

Figures and Tables

Table 1 *Paracoccidioides* transcripts induced during nitrosative stress.

Accession number [a] / Functional classification	Gene product	ESTs number[b]		*E*-value	Annotated function[c]	EC number
		E	C			
Amino acid metabolism						
PAAG_07626	Cobalamin-independent synthase *	3	0	$6e^{-52}$	Cysteine and methionine metabolism	2.1.1.14
PAAG_04925	Homoserine O-acetyltransferase	4	1	0	Cysteine and methionine metabolism	2.3.1.31
PAAG_00285	Imidazole glycerol phosphate synthase hisHF *	2	0	1e-^{45}	Histidine biosynthesis	2.4.2
Fungal-type cell wall biogenesis						
PAAG_07698	D-alanine-poly(phosphoribitol) ligase subunit 1 *	2	0	$5e^{-46}$	D-alanine metabolism	6.1.1.13
PAAG_01456	Dolichol phosphate mannosyltransferase subunit 3 *	3	0	$1e^{-33}$	N-glycan biosynthesis	2.4.1.83
PAAG_00850	Glucosamine-fructose-6-phosphate aminotransferase	4	4	$7e^{-41}$	Cell wall chitin biosynthetic process	2.6.1.16
PAAG_03297	Cell wall alpha-1,3-glucan synthase mok11	4	1	$2e^{-49}$	Alpha-glucan biosynthetic process	2.4.1.183
PAAG_06210	ER glucosidase I *	2	0	$1e^{-67}$	Glycan metabolism	3.2.1.106
PAAG_02629	Alpha-1,2-galactosyltransferase gmh3 *	3	0	$5e^{-76}$	N-directed glycosylation/deglycosylation	2.4.1.256
Lipid metabolism						
PAAG_02401	Glycerophosphodiester phosphodiesterase GDE1	4	2	$1e^{-51}$	Glycerophospholipid metabolism	3.1.4.46
PAAG_01575	Sterol 24-C-methyltransferase *	2	0	$8e^{-17}$	Ergosterol biosynthesis	2.1.1.41
Carbohydrate metabolism						
PAAG_08449	Malate dehydrogenase	3	1	$6e^{-10}$	Glyoxylate cycle	1.1.1.37
PAAG_02011	Phosphoglucomutase	3	2	0	Glycolisys/Gluconeogenesis	5.4.2.2
PAAG_02548	Hydroxyacylglutathione hydrolase	4	1	$1e^{-71}$	Pyruvate metabolism	3.1.2.6
PAAG_04166	Transaldolase * †	2	1	$5e^{-37}$	Pentose phosphate pathway	2.2.1.2
PAAG_05416	NADP-dependent leukotriene B4 12 hydroxydehydrogenase	4	2	$9e^{-64}$	Leukotriene B4 metabolism	1.3.1.48
PAAG_01076	Acylphosphate phosphohydrolase *	2	0	$7e^{-49}$	Pyruvate metabolism	3.6.1.7
Nucleotide metabolism						
PAAG_04974	Adenylosuccinate lyase *	2	0	$3e^{-61}$	Purine biosynthesis	4.3.2.2
Protein synthesis						
PAAG_07450	Mitochondrial 40S ribosomal protein MRP2 *	2	0	$3e^{-62}$	Structural constituent of ribosome	-
PAAG_05704	40S ribosomal protein S13-1	3	1	$3e^{-56}$	Structural constituent of ribosome	-
PAAG_06743	Ribosomal protein L23 *	2	0	$4e^{-51}$	Structural constituent of ribosome	-
PAAG_06569	60S ribosomal protein L43	4	2	$1e^{-40}$	Structural constituent of ribosome	-
PAAG_07484	54S ribosomal protein L9	4	4	$6e^{-146}$	Structural constituent of ribosome	-
PAAG_01435	40S ribosomal protein S1	3	2	$3e^{-07}$	Structural constituent of ribosome	-
PAAG_08153	50S ribosomal protein L6	3	1	0	Structural constituent of ribosome	-
PAAG_03322	40S ribosomal protein S20	4	3	$2e^{-54}$	Structural constituent of ribosome	
PAAG_01050	Cytosolic large ribosomal subunit protein L30	4	3	$2e^{-56}$	Structural constituent of ribosome	-
PAAG_03028	Elongation factor 1-beta	5	5	$1e^{-45}$	Elongation of translation	
PAAG_03556	Elongation factor 1-gamma 1 *	2	0	$9e^{-42}$	Elongation of translation	-
PAAG_08348	Eukaryotic translation initiation factor 3 39 kDa subunit *	2	0	$3e^{-24}$	Translation initiation	-
PAAG_08716	Tyrosyl-tRNA synthetase *	2	0	$3e^{-39}$	Tyrosyl-tRNA aminoacylation	6.1.1.1
PAAG_05015	Leucyl-tRNA synthetase *	2	0	$1e^{-47}$	Leucyl-tRNA aminoacylation	6.1.1.4
PAAG_01777	Alanyl-tRNA synthetase	6	3	0	Alanyl-tRNA aminoacylation	6.1.1.7
PAAG_02820	GTP-dependent nucleic acid-binding protein engD	4	4	$2e^{-40}$	Regulation of translation	-
Transcription						
PAAG_06965	ISWI chromatin-remodeling complex ATPase ISW1 *	2	0	$4e^{-12}$	Transcription regulation	-
PAAG_08223	Small nuclear ribonucleoprotein Sm D3	3	2	$3e^{-51}$	Transcription initiation	-
PAAG_04982	Transcription initiation factor IIE subunit beta *	2	0	$9e^{-21}$	mRNA synthesis and processing	-
PAAG_02522	Sm-like (Lsm) protein (LSM3)	3	1	$1e^{-11}$	mRNA processing	-
PAAG_08183	Heterochromatin protein HP1 *	1	0	$7e^{-34}$	Chromatin regulation	-
PAAG_07234	NAP family protein	3	1	$2e^{-92}$	Transcription regulation	-
Response to stress						
PAAG_03502	Cytochrome c peroxidase * †	2	0	$3e^{-60}$	Hydrogen peroxide catabolic process	1.11.1.5
PAAG_09044	SYM1 Mvp17/PMP22 family protein *	2	0	$8e^{-43}$	Maintain mtDNA integrity and stability	-
PAAG_05142	10 kDa heat shock protein, mitochondrial	3	2	$1e^{-51}$	Unfolded protein response	-
PAAG_04164	Superoxide dismutase Cu-Zn	6	5	$9e^{-56}$	Oxygen and radical detoxification	1.15.1.1
Detoxification by export						
PAAG_06017	Pleiotropic ABC efflux transporter of multiple drugs	3	1	0	Substrates transport	3.6.3.44

PAAG_03746	ATP-dependent permease PDR12	4	4	$2e^{-67}$	Substrates transport	-
PAAG_00607	MFS transporter *	2	0	$3e^{-15}$	Substrates transport	-
Cellular transport						
PAAG_07313	Zinc-regulated transporter 1 Zrt1	3	1	$1e^{-20}$	High-affinity zinc transport	-
PAAG_07762	Vacuolar iron transporter Ccc1 †	5	3	$1e^{-75}$	Cellular iron transport	-
PAAG_05120	Coatomer subunit gamma-1 *	2	0	$2e^{-29}$	Protein transport ER to Golgi	-
PAAG_05584	Peroxisomal adenine nucleotide transporter 1 *	2	0	$4e^{-49}$	Uniport of ATP and adenine nucleotide hetero-exchange transport	-
PAAG_04529	Synaptobrevin	3	1	$2e^{-16}$	Golgi to plasma membrane transport	-
PAAG_06533	Peptide transporter PTR2	3	2	$1e^{-34}$	Uptake of small peptides	-
PAAG_08469	Inorganic phosphate transporter	67	12	$4e^{-28}$	Phosphate transport	-
PAAG_06677	RTA1 domain-containing protein PriAC*	2	0	$1e^{-14}$	Lipid/fatty acid transport	-
PAAG_07524	Peroxin 8 *	3	0	$8e^{-46}$	Protein import to peroxissomal matriz	-
PAAG_05443	BAP31 domain-containing protein *	2	0	$3e^{-17}$	Export of membrane proteins from ER to the secretory pathway	-
PAAG_00568	Sulfite transporter Ssu1	6	1	$9e^{-84}$	Efflux of free sulfite	-
Electron transport/Respiration						
PAAG_08352	NADH-ubiquinone oxidoreductase *	4	0	$1e^{-66}$	Complex I of respiratory chain	1.6.5.3
PAAG_02266	NADH-ubiquinone oxidoreductase 21 kDa subunit	5	1	$2e^{-60}$	Complex I of respiratory chain	1.6.5.3
PAAG_01494	NADH-ubiquinone oxidoreductase 21 kDa subunit †	3	1	$9e^{-13}$	Complex I of respiratory chain	1.6.5.3
PAAG_07251	NADH dehydrogenase, FAD-containing subunit *	2	0	$9e^{-08}$	Energy production and conversion	1.6.99
PAAG_04259	2Fe-2S iron-sulfur cluster binding domain-containing protein *	2	1	$1e^{-62}$	Electron transport	-
PAAG_06351	Bifunctional P-450:NADPH-P450 reductase *	3	0	$1e^{-84}$	Electron transfer from NADP to cytochrome P450	1.6.2.4
PAAG_00999	Mitochondrial respiratory complex I chaperone *	2	0	$6e^{-19}$	Accessory protein of electron transport	-
PAAG_03930	Mitochondrial carrier protein *	2	0	$6e^{-71}$	Mitochondrial cation (Ca^{+}) transport	-
PAAG_08433	Mitochondrial carrier protein	3	2	$1e^{-57}$	Mitochondrial solute transport	-
PAAG_03772	Mitochondrial import inner membrane translocase subunit tim9	3	1	$1e^{-40}$	Protein import into mitochondrial inner membrane	-
Cell cycle and DNA processing						
PAAG_01758	ELMO/CED-12 family protein *	3	0	$2e^{-17}$	Cytoskeleton reorganization	-
PAAG_07050	Phosphoesterase *	2	0	$6e^{-49}$	Cell cycle checkpoints	3.1.4.1
PAAG_07993	Minichromosome loss protein *	2	0	$1e^{-36}$	DNA synthesis, replication and repair	-
PAAG_03025	Sas10/Utp3 family protein	3	2	$2e^{-29}$	DNA conformation/modification	-
Cell growth/differentiation						
PAAG_02547	Cell morphogenesis protein Las1	3	1	0	Budding, cell polarity and filament formation	-
PAAG_01193	DUF21 and CBS domain protein (Mam3)*	2	0	$2e^{-31}$	Mitochondrion organization	-
Signal transduction						
PAAG_04561	Serine/threonine-protein kinase Sgk2*	3	0	$2e^{-54}$	Intracellular protein kinase cascade	2.1.11.1
PAAG_00108	cAMP-dependent protein kinase catalytic subunit pkaC*	6	0	$2e^{-158}$	Regulation of protein phosphorylation	2.7.11.11
Protein fate (folding, modification, destination)						
PAAG_02773	Ubiquitin-conjugating enzyme variant MMS2	3	1	$8e^{-64}$	Modification by ubiquitination	6.3.2.19
PAAG_03642	Ubiquitin-like protein*	2	0	$6e^{-54}$	Modification by ubiquitination	-
PAAG_08119	Ubiquitin fusion degradation protein*	2	0	$2e^{-28}$	Modification by ubiquitination	-
PAAG_00448	Ubiquitin C-terminal hydrolase family protein*	2	0	$5e^{-37}$	Modification by ubiquitination	3.1.2.15
PAAG_01941	Proteasome component C7-alpha*	2	0	$8e^{-40}$	Proteasomal degradation	3.4.25.1
PAAG_04282	UBX domain-containing protein	3	1	$1e^{-44}$	Ubiquitin/proteasome protein degradation	-
PAAG_06132	FAD-linked sulfhydryl oxidase ALR ERV1	3	2	$5e^{-152}$	Biogenesis of cytosolic Fe/S proteins	1.8.3.2
PAAG_05788	Peptidyl-prolyl cis-trans isomerase A2*	3	0	$2e^{-59}$	Protein folding and stabilization	5.2.1.8
PAAG_04205	Vacuolar aminopeptidase	3	2	0	Cytoplasmic and nuclear protein degradation	3.4.11.22
PAAG_06068	T-complex protein 1 subunit beta (TCP1)	3	1	$4e^{-171}$	Protein folding and stabilization	-
Protein of unknown function						
PAAG_06570	PHD and RING finger domain-containing protein*	2	0	$4e^{-18}$	-	6.3.2.19
PAAG_07840	Vacuolar membrane PQ loop repeat protein	3	1	$1e^{-83}$	-	-
PAAG_01423	HHE domain-containing protein	4	2	$8e^{-63}$	-	-
PAAG_02808	DUF636 domain-containing protein	3	1	$2e^{-42}$	-	-
PAAG_08313	L-PSP endoribonuclease family protein (Hmf1)	5	5	0	-	-
PAAG_08446	Protein of unknown function (DUF2423) *	2	0	$1e^{-22}$	-	-
PAAG_03707	Protein of unknown function localised to mitochondria *	2	0	$4e^{-21}$	-	-
Unclassified proteins						
PAAG_07106	Conserved hypothetical protein *	4	0	$6e^{-18}$	-	-
PAAG_08800	Conserved hypothetical protein	6	2	$9e^{-157}$	-	-
PAAG_05911	Conserved hypothetical protein	4	4	$9e^{-82}$	-	-
PAAG_08752	Conserved hypothetical protein	3	1	$6e^{-90}$	-	-
PAAG_07736	Conserved hypothetical protein *	2	0	$7e^{-63}$	-	-
PAAG_08640	Conserved hypothetical protein *	2	0	$9e^{-47}$	-	-

PAAG_01717	Conserved hypothetical protein *	2	0	$5e^{-47}$	-	-
PAAG_06213	Conserved hypothetical protein *	2	0	$2e^{-48}$	-	-
PAAG_04801	Conserved hypothetical protein *	2	0	$9e^{-22}$	-	-
PAAG_07960	Conserved hypothetical protein	10	15	$2e^{-169}$	-	-
PAAG_06301	Conserved hypothetical protein	4	2	$8e^{-63}$	-	-
PAAG_08115	Conserved hypothetical protein *	2	0	$6e^{-71}$	-	-
PAAG_01388	Conserved hypothetical protein *	2	0	$2e^{-36}$	-	-
PAAG_02639	Conserved hypothetical protein	4	1	$2e^{-35}$	-	-
PAAG_08758	Conserved hypothetical protein	3	1	$6e^{-31}$	-	-
PAAG_04796	Conserved hypothetical protein	3	1	$3e^{-19}$	-	-
PAAG_01737	Conserved hypothetical protein *	2	0	$1e^{-61}$	-	-
PAAG_01880	Hypothetical protein *	2	0	$1e^{-65}$	-	-
PAAG_04617	Hypothetical protein	4	1	$5e^{-41}$	-	-
PAAG_04935	Hypothetical protein	8	6	$3e^{-36}$	-	-
PAAG_08979	Hypothetical protein	3	2	$3e^{-175}$	-	-
PAAG_07597	Hypothetical protein *	13	0	$2e^{-17}$	-	-
PAAG_07025	Hypothetical protein *	2	0	$2e^{-26}$	-	-
PAAG_02483	Hypothetical protein *	2	0	$4e^{-10}$	-	-
PAAG_00167	Hypothetical protein *	2	0	$2e^{-49}$	-	-

[a] Accession number at Broad Institute (http://www.broadinstitute.org).

[b] ESTs from experimental condition (E) and control (C). Control corresponds to yeast cells ETSs previously generated in control conditions [20].

[c] Metabolic function from PEDANT automated annotation scheme (http://pedant.helmholtz-muenchen.de/index.jsp).

*Transcripts not detected in the yeast transcriptome library [20].

†Transcripts confirmed by qRT-PCR analysis.

Table 2- *Paracoccidioides* transcripts repressed during nitrosative stress.

Accession number [a] / Functional classification	Gene product	ESTs number [b]		*E*-value	Annotated function[c]	EC number
		E	C			
Metabolism						
PAAG_03624	Arp2/3 complex subunit Arc16	1	33	1e-^{47}	Chitin metabolism	3.5.1.41
PAAG_00771	Enolase*	0	15	1e^{-120}	Glycolisys/Gluconeogenesis	4.2.1.11
PAAG_05249	Aldehyde dehydrogenase*	0	32	0	C-2 compound and organic acid catabolism	1.2.1.3
PAAG_01570	Choline sulfatase*	0	15	9e^{-160}	Sulfur metabolism	3.1.6.6
PAAG_07003	Glutamine synthetase*	0	22	3e^{-97}	Glutamate metabolism	6.3.1.2
PAAG_04291	Nucleoside diphosphate kinase	5	51	5e^{-121}	Nucleotide metabolism	2.7.4.6
Protein synthesis						
PAAG_00205	60S ribosomal protein L24	1	53	1e^{-32}	Structural constituent of ribosome	-
PAAG_01433	40S ribosomal protein S14	1	36	3e^{-31}	Structural constituent of ribosome	-
PAAG_05017	40S ribosomal protein S10-A	4	71	8e^{-33}	Structural constituent of ribosome	-
PAAG_04691	Ribosomal protein l P2	3	102	6e^{-17}	Structural constituent of ribosome	-
PAAG_09043	40S ribosomal protein S2	4	45	7e^{-69}	Structural constituent of ribosome	-
PAAG_03664	Ribosomal L28e protein *	0	23	5e^{-65}	Structural constituent of ribosome	-
PAAG_07955	60S ribosomal protein L18 *	0	22	3e^{-85}	Structural constituent of ribosome	-
PAAG_07841	60s acidic ribosomal protein P1 *	0	41	2e^{-28}	Structural constituent of ribosome	-
PAAG_05882	Translation factor SUI1 *	0	15	9e^{-31}	Translation initiation factor	-
PAAG_00088	60S ribosomal protein L3 *	0	16	0	Structural constituent of ribosome	-
PAAG_04965	60S ribosomal protein L31 *	0	17	7e^{-64}	Structural constituent of ribosome	-
PAAG_02111	40S ribosomal protein S0 *	0	20	1e^{-144}	Structural constituent of ribosome	-
PAAG_01785	40S ribosomal protein S3 *	0	37	2e^{-106}	Structural constituent of ribosome	-
PAAG_07649	Ribosomal protein S36 *	0	18	1e^{-36}	Structural constituent of ribosome	-
PAAG_00206	Ribosomal protein S30 *	0	20	2e^{-22}	Structural constituent of ribosome	-
Cellular transport						
PAAG_03385	Non-classical export protein Nce102	3	44	6e^{-53}	Cellular export and secretion	-
PAAG_05643	Endoplasmic reticulum and nuclear membrane proteinc Npl4	10	98	6e^{-44}	Protein and mRNA transport	-
PAAG_02817	Stomatin family protein	1	23	1e^{-19}	Ion channel mediated signalling pathway	-
Electron transport/Respiration						
PAAG_04570	ATP synthase D chain, mitochondrial	1	32	1e^{-37}	Energy production and conversion	3.6.3.14
PAAG_05879	Complex I intermediate-associated protein *	0	17	0	Assembly of mitochondrial complex I	-
Response to stress						
PAAG_05679	Heat shock protein *	0	23	9e^{-110}	Unfolded protein response	-
Unclassified proteins						
PAAG_04913	Conserved hypothetical protein	1	35	1e^{-50}	-	-
PAAG_04707	Conserved hypothetical protein *	0	21	2e^{-50}	-	-
PAAG_02996	Hypothetical protein *	0	15	2e^{-30}	-	-

[a] Accession number at Broad Institute (http://www.broadinstitute.org).
[b] ESTs from experimental condition (E) and control (C). Control corresponds to the yeast cells ETSs previously generated in control conditions [20].
[c] Metabolic function from PEDANT automated annotation scheme (http://pedant.helmholtz-muenchen.de/index.jsp).
*Transcripts not detected in the yeast cells transcriptome [20].

Suplementary Table S1- Oligonucleotide primers used in Real Time PCR.

Sequence name	Forward primer (5'→3')	Reverse primer (3'→5')
Cytochrome c peroxidase	CCGCTCGAGCGGTCAGATATTCCACTCGC ACTTC	GGCGCGCCCACCCTCAGGACCA GAAGCA
NADH- ubiquinone oxidureductase 21 kDa subunit	CAACACATCATGTAAACGTTTCT	TAAGGCGAGAGTGACGACTC
Transaldolase	TGACCATTTCGCCCAACCTC	GATGTAGGTGCGCTTGGGAA
Vacuolar iron transporter	GGTGCGAAGAGTGAACTAGAA	GGGAGACTGTACGGAGCAAA
α-tubulin	ACAGTGCTTGGGAACTATACC	GGGACATATTTGCCACTGCC

Figure 1

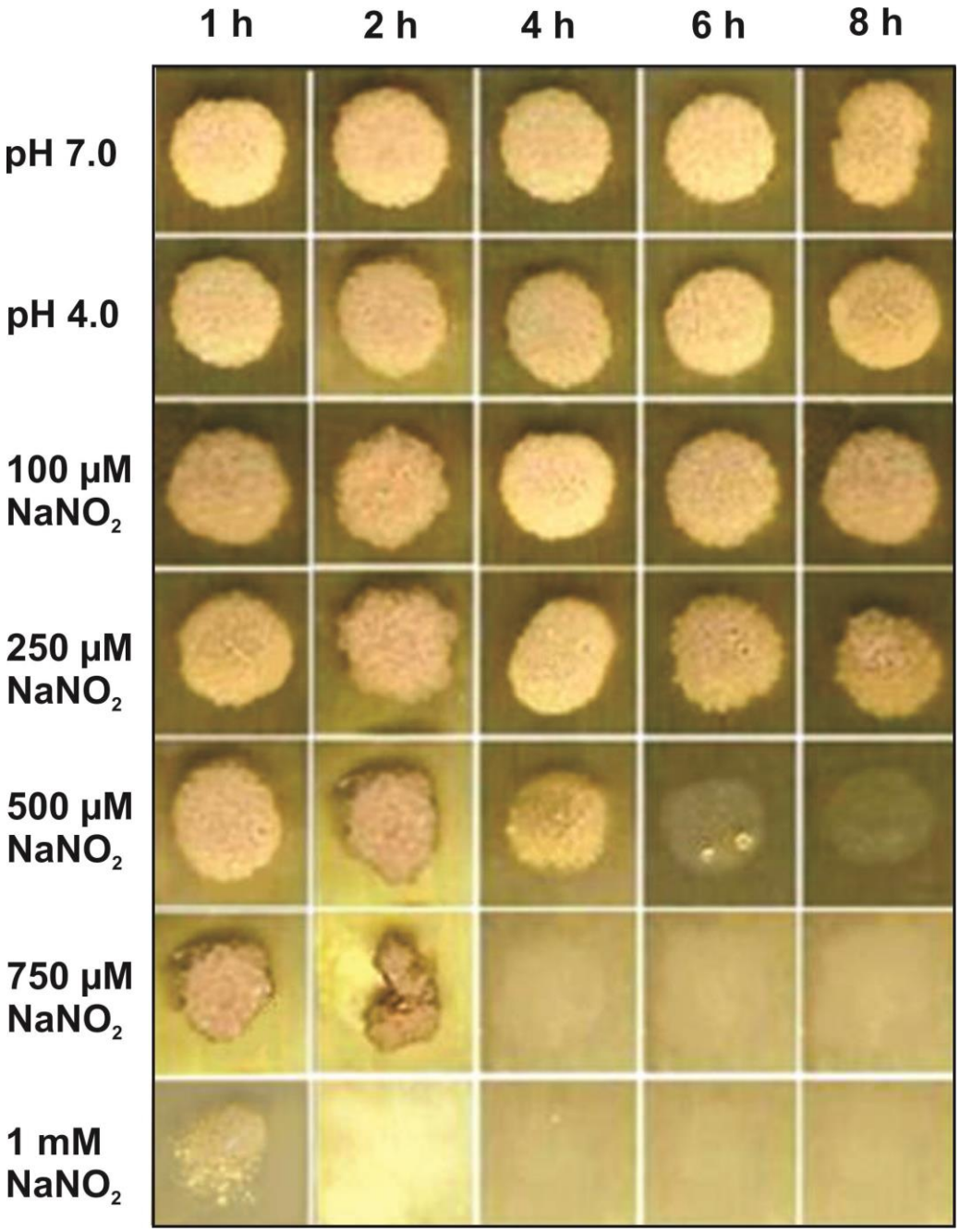

1 h
2 h
4 h
6 h
8 h
pH 7.0
pH 4.0
100 µM NaNO2
250 µM NaNO2
500 µM NaNO2
750 µM NaNO2
1 mM NaNO2

Figure 2

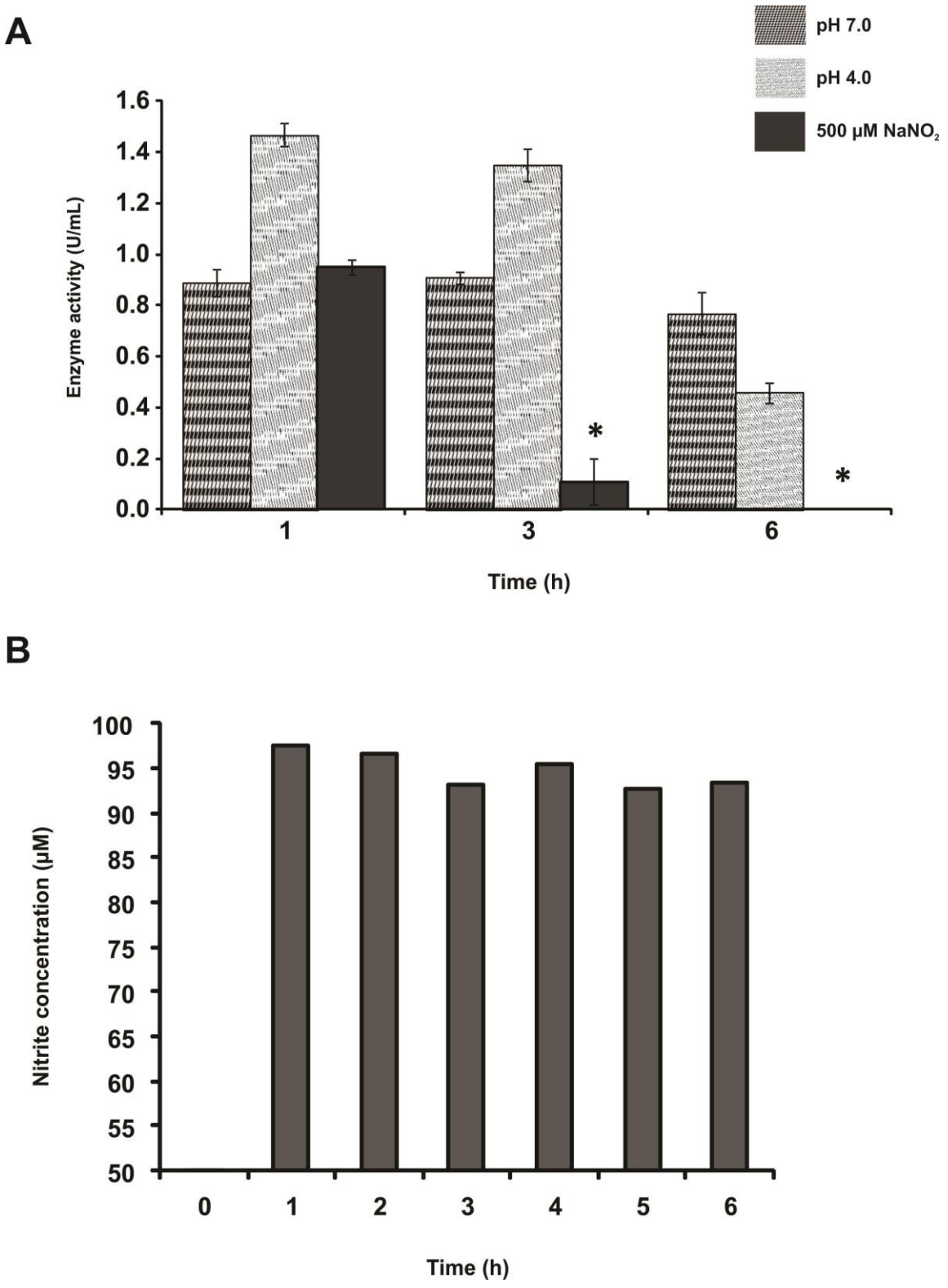

A
pH 7.0
pH 4.0
500 µM NaNO2
Enzyme activity (U/mL)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
1
3
6
Time (h)
B
Nitrite concentration (µM)
100
95
90
85
80
75
70
65
60
55
50
0
1
2
3
4
5
6
Time (h)

Figure 3

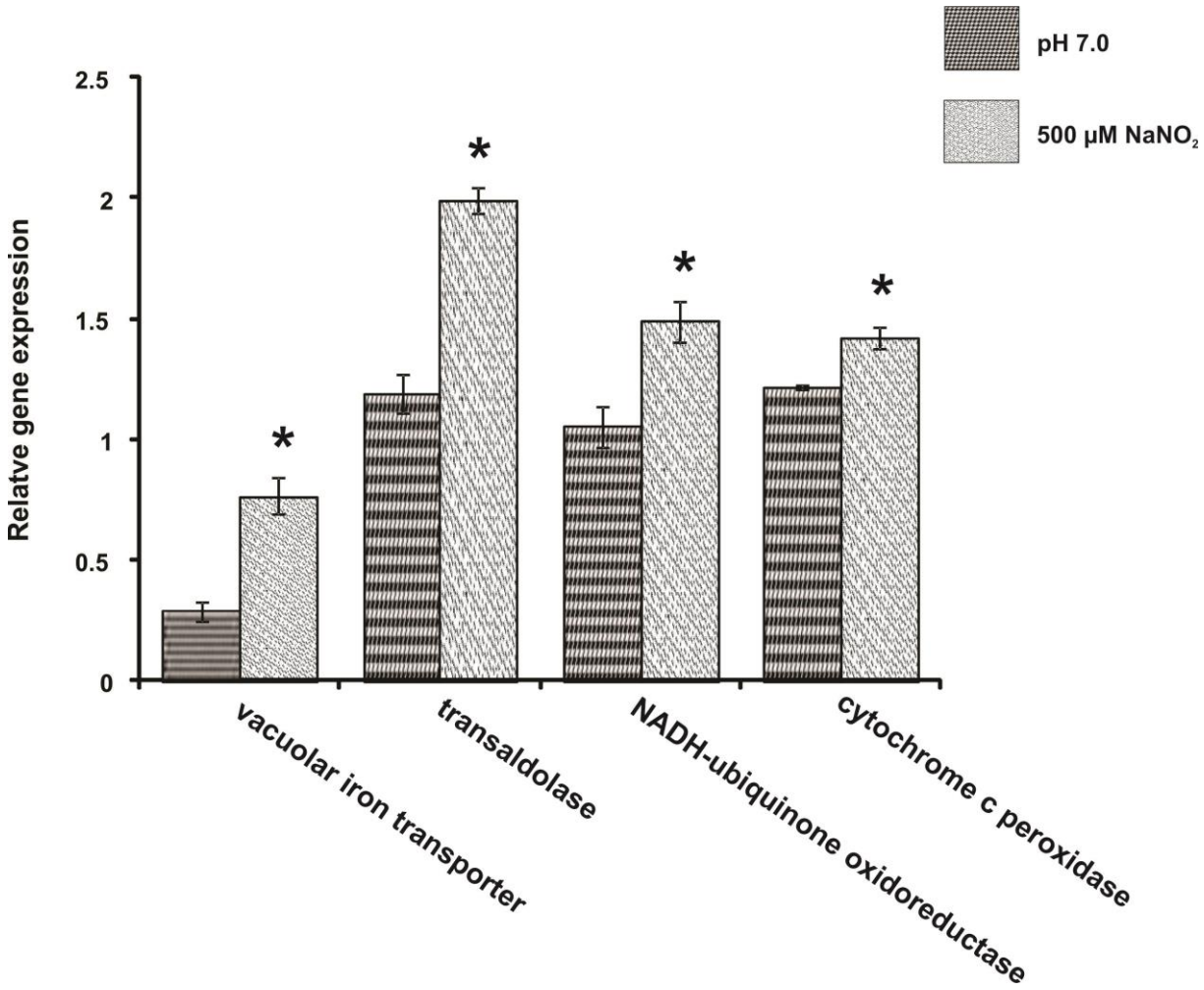

pH 7.0
500 µM NaNO2
Relatve gene expression
2.5
2
1.5
1
0.5
0
*
*
*
*
vacuolar iron transporter
transaldolase
NADH-ubiquinone oxidoreductase
cytochrome c peroxidase

Figure 4

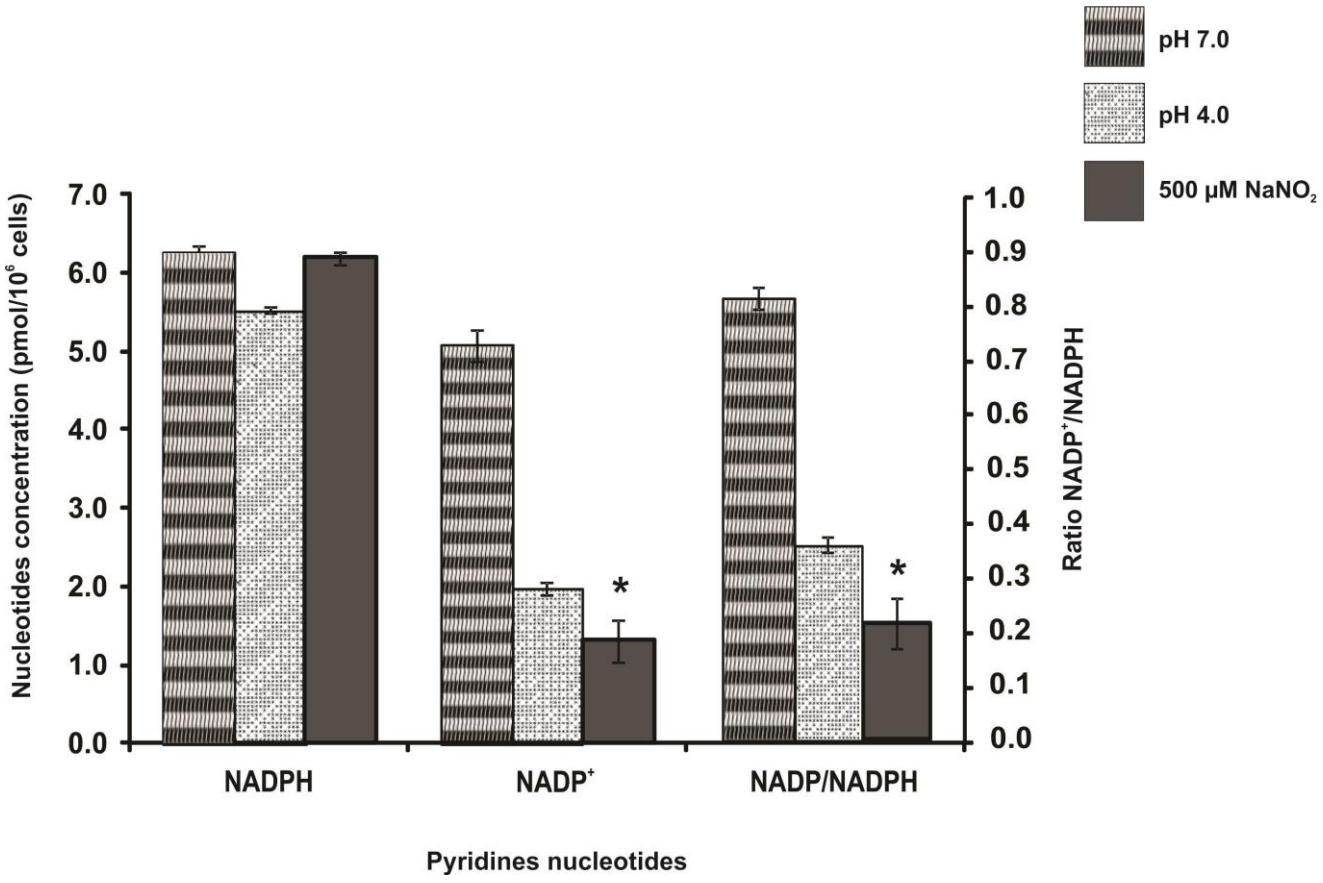
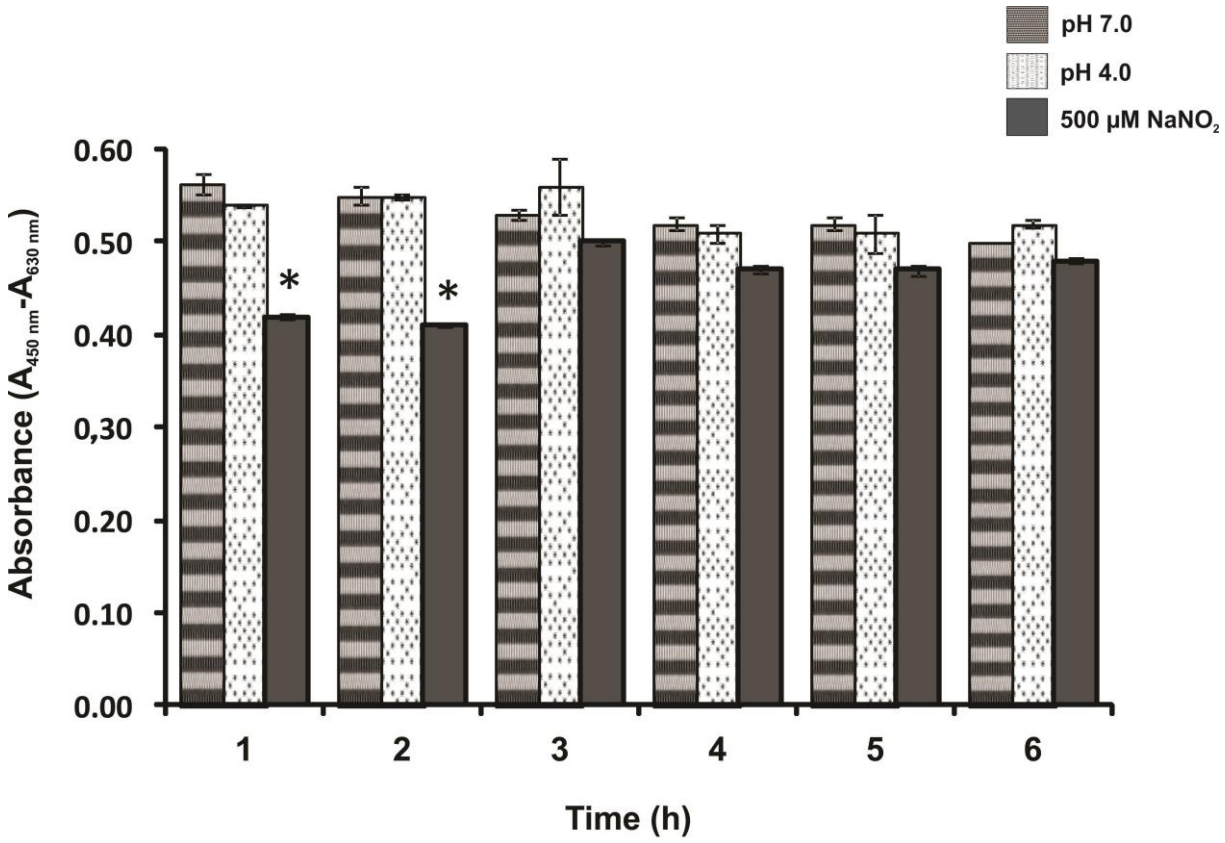
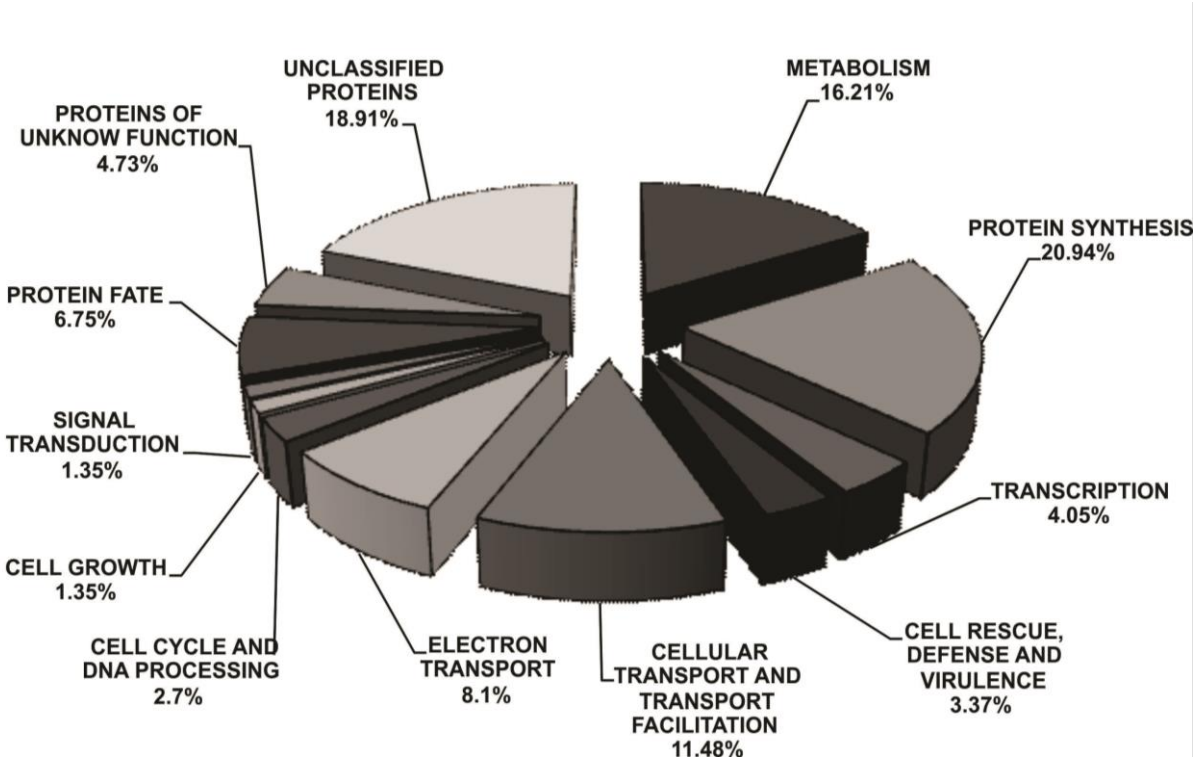

pH 7.0
pH 4.0
500 µM NaNO2
Nucleotides concentration (pmol/10^6 cells)
Ratio NADP+/NADPH
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
NADPH
NADP+
NADP/NADPH
Pyridines nucleotides

Figure 5


pH 7.0
pH 4.0
500 µM NaNO2
Absorbance (A450 nm-A630 nm)
0.60
0.50
0.40
0,30
0.20
0.10
0.00
*
*
1
2
3
4
5
6
Time (h)

Additional File 1



5- Perspectivas

i – Realizar um estudo proteômico da resposta ao estresse nitrosativo causado por $NaNO_2$ para futura análise comparativa com o transcritoma de *Paracoccidioides*;

ii – Realizar um estudo proteômico das proteínas mitocôndriais isoladas de células de levedura de *Paracoccidioides* submetidas ao estresse nitrosativo causado por $NaNO_2$;

iii – Realizar análises do transcritoma de *Paracoccidioides* em resposta ao estresse nitrosativo causado por outro agente liberador de óxido nítrico, S-Nitrosoglutathione (GSNO), para futura análise comparativa com a resposta transcricional ao $NaNO_2$;

iv– Selecionar mutantes sensíveis ao estresse nitrosativo e caracterizá-los.

6. Referências Bibliográficas

Almeida OP, Junior JJ. Paracoccidioidomycosis of the mouth: an emerging deep mycosi. Crit Rev Oral Biol Me 2003, 14: 268-274.

Alspaugh JA, Granger DL. Inhibition of *Cryptococcus neoformans* Replication by Nitrogen Oxides Supports the Role of These Molecules as Effectors of Macrophage-Mediated Cytostasis. Infect Immun 1991, 59: 2291-2296.

Andrade RV, da Silva SP, Torres FAG, Poças-Fonseca MJ, Silva-Pereira I, Maranhão AQ, Campos EG, Moraes LMP, Jesuíno RSA, Pereira M, Soares CMA, Walter MEMT, Carvalho MJA, Almeida NF, Brígido MM, Felipe MSS. Overview and perspectives on the transcriptome of *Paracoccidioides brasiliensis*. Rev Iberoam Micol 2005, 22: 203-212.

Bagagli E, Bosco SM, Theodoro RC, Franco M. Phylogenetic and evolutionary aspects of *Paracoccidioides brasiliensis* reveal a long coexistence with animal hosts that explain several biological features of the pathogen. Infect Genet Evol 2006, 6: 344-351.

Bagagli E, Theodoro RC, Bosco SM, McEwen JG. *Paracoccidioides brasiliensis*: phylogenetic and ecological aspects. Mycopathologia 2008, 165: 197-207.

Bahn YS, Geunes-Boyer S, Heitman J. Ssk2 mitogen-activated protein kinase kinase kinase governs divergent patterns of the stress-activated Hog1 signaling pathway in *Cryptococcus neoformans*. Eukaryot Cell 2007, 6: 2278-89.

Bailão AM, Schrank A, Borges CL, Dutra V, Molinari-Madlum EEWI, Felipe MSS, Mendes-Giannini MJS, Martins WS, Pereira M, Soares CMA. Differential gene expression by *Paracoccidioides brasiliensis* in host interaction conditions: representational difference analysis identifies candidate genes associated with fungal pathogenesis. Microbes Infect 2006, 8: 2686-2697.

Bailão AM, Shrank A, Borges CL, Parente JA, Dutra V, Felipe MS, Fiuza RB, Pereira M, Soares CMA. The transcriptional profile of *Paracoccidioides brasiliensis* yeast cells is influenced by human plasma. FEMS Immunol Med Microbiol 2007, 51: 43-57.

Barrozo LV. Climate and acute/subacute paracoccidioidomycosis in a hyper-endemic area in Brazil. Int J Epidemiol 2009, 38: 1642–1649.

Bastos KP, Bailão AM, Borges CL, Faria FP, Felipe MSS, Silva MG, Martins WS, Fiúza RB, Pereira M, Soares CMA. The transcriptome analysis of early morphogenesis in *Paracoccidioides brasiliensis* mycelium reveals novel and induced genes potentially associated to the dimorphic process. BMC Microbiol 2007, 7: 29.

Bocca AL, Hayashi EE, Pinheiro AG, Furlanetto AB, Campanelli AP, Cunha FQ, Figueiredo F. Treatment of *Paracoccidioides brasiliensis*-Infected Mice with a Nitric Oxide Inhibitor Prevents the Failure of Cell-Mediated Immune Response. J Immunol 1998, 161: 3056-3063.

Bogdan C. Nitric oxide and the immune response. Nature Immunol 2001, 2: 907-916.

Bonifaz A, Vázquez-González D, Perusquía-Ortiz AM. Endemic systemic mycoses: coccidioidomycosis, histoplasmosis, paracoccidioidomycosis and blastomycosis. J Dtsch Dermatol Ges 2011. 9: 705-14.

Borges CL, Bailao AM, Bao SN, Pereira M, Parente JÁ, Soares CMA. Genes potentially relevant in the parasitic phase of the fungal pathogen *Paracoccidioides brasiliensis*. Mycopathologia 2011, 171: 1-9.

Borges-Walmsley MI, Chen D, Shu X, Walmsley AR. The pathobiology of *Paracoccidioides brasiliensis*. TRENDS in Microbiology 2002, 10: 80-87.

Brown AJP, Haynes K, Quinn J. Nitrosative and oxidative stress responses in fungal pathogenicity. Curr Opin Microbiol 2009, 12: 384–391.

Brown GC. Nitric oxide and mitochondrial respiration. Biochim Biophys Acta 1999, 1411: 351-369.

Brown SM, Campbel LT, Lodge JK. *Cryptococcus neoformans*, a fungus under stress. Curr Opin Microbiol 2007, 10: 320–325.

Brummer E, Castaneda E, Restrepo A. Paracoccidioimycosis: na Update. Clin Microbiol Rev 1993, 6: 89-117.

Brummer E, Hanson LH, Restrepo A, Stevens DA. Intracellular Multiplication of *Paracoccidioides brasiliensis* in Macrophages: Killing and Restriction of Multiplication by Activated Macrophages. Infect Immun 1989, 57: 2289-2294.

Brummer E, Sun SH, Harrison JL, Perlman AM, Philpott DE, Stevens DA. *Ultrastructure of phagocytosed Paracoccidioides brasiliensis* in nonactivated or activated macrophages. Infect Immun 1990, 58: 2628-36.

Cassina A, Radi R. Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. Arch Biochem Biophys 1996, 328: 309-316.

Castells-Roca L, García-Martínez J, Moreno J, Herrero E, Bellí G, Pérez-Ortín JE. Heat Shock Response in Yeast Involves Changes in Both Transcription Rates and mRNA Stabilities. PLoS One 2011, 6: e17272.

Chiranand W, McLeod I, Zhou H, Lynn JJ, Vega LA, Myers H, Yates III JR, Lorenz MC, Gustin MC. CTA4 Transcription Factor Mediates Induction of Nitrosative Stress Response in *Candida albicans*. Eukaryot Cell 2008, 7: 268–278.

Coutinho ZF, Silva D, Lazera M, Petri V, Oliveira RM, Sabroza PC, Wanke B. Paracoccidioidomycosis mortality in Brazil (1980-1995). Cad Saude Publica 2002, 18: 1441-1454.

Costa M, Borges CL, Bailao AM, Meirelles GV, Mendonca YA, Dantas SF, de Faria FP, Felipe MS, Molinari-Madlum EE, Mendes-Giannini MJ, Fiuza RB, Martins WS, Pereira M, Soares CMA. Transcriptome profiling of *Paracoccidioides brasiliensis* yeast-phase cells recovered from infected mice brings new insights into fungal response upon host interaction. Microbiology 2007, 153: 4194-4207.

De Groote MA, Fang FC. Nitric Oxide and Infection. Kluwer Acad 1999, 231–261.

Desjardins CA, Champion MD, Holder JW, Muszewska A, Goldberg J, Bailão AM, Brigido MM, Ferreira MES, Garcia AM, Grynberg M, Gujja S, Heiman DI, Henn MR, Kodira CD, León-Narváez H, Longo LVG, Ma L, Malavazi I, Matsuo AL, Morais FV, Pereira M, Rodríguez-Brito S, Sakthikumar S, Salem-Izacc SM, Sykes SM, Teixeira MM, Vallejo MC, Walter MEMT, Yandava C, Young S, Zeng Q, Zucker J, Felipe MS, Goldman GH, Haas BJ, McEwen JG, Nino-Vega G, Puccia R, San-Blas G, Soares CMA, Birren BW, Cuomo CA. Comparative genomic analysis of human fungal pathogens causing Paracoccidioidomycosis. Plos Genet 2011, 7: e1002345.

Drapier JC, Hibbs JBJr. Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine-dependent inhibition of mitochondrial iron-sulfur enzymes in the macrophage effector cells. J Immunol 1988, 140: 2829-38.

Fang FC. Mechanisms of Nitric Oxide–related Antimicrobial Activity in: Perspectives Series: Host/Pathogen Interactions. J Clin Invest 1997, 99: 2818–2825.

Fang FC. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat Rev Microbiol 2004, 2: 820-832.

Felipe MS, Andrade RV, Arraes FB, Nicola AM, Maranhao AQ, Torres FA, Silva-Pereira I, Pocas-Fonseca MJ, Campos EG, Moraes LM, Andrade PA, Tavares AH, Silva SS, Kyaw CM, Souza DP, Pereira M, Jesuino RS, Andrade EV, Parente JA, Oliveira GS, Barbosa MS, Martins NF, Fachin AL, Cardoso RS, Passos GA, Almeida NF, Walter ME, Soares CMA, Carvalho MJ, Brigido MM. Transcriptional profiles of the human pathogenic fungus *Paracoccidioides brasiliensis* in mycelium and yeast cells. J Biol Chem 2005, 280: 24706-24714.

Felipe MSS, Andrade RV, Petrofeza SS, Maranhão AQ, Torres FAG, Albuquerque P, Arraes FBM, Arruda M, Azevedo MOA, Baptista J, Bataus LAM, Borges CL, Campos EG, Cruz MR, Daher BS, Dantas A, Ferreira MASV, Ghil GV, Jesuino RSA, Kyaw CM, Leitão L, Martins CR, Moraes MPL, Neves EO, Nicola AM, Alves ES, Parente JA, Pereira M, Poças-Fonseca MJ, Resende R, Ribeiro BM, Saldanha RR, Santos SC, Silva-Pereira I, Silva MAS, Silveira E, Simões IC, Soares RBA, Souza DP, De-Souza MT, Andrade EV, Xavier MAS, Veiga HP, Venancio EJ, Carvalho MJA, Oliveira AG, Inoue MK, Almeida NF, Walter MEMT, Soares CMA, Brígido MM. Transcriptome characterization of the dimorphic and pathogenic fungus *Paracoccidioides brasiliensis* by EST analysis. Yeast 2003, 20: 263–271

Ferreira MS. Paracoccidioimycosis. Pediatr Respir Rev 2009, 10: 161-165.

Fortes MRP, Kurokawa CS, Marques SA, Miot HA, Marques MEA. Immunology of paracoccidioidomycosis. An Bras Dermatol 2011, 86: 516-525.

Franco M, Sano A, Kera K, Nishimura K, Takeo K, Miyaji M. Chlamydospore formation by *Paracoccidioides brasiliensis* mycelial form. Rev Inst Med Trop 1989, 31(3): 151-7.

Garcia AM, Hernandez O, Aristizabal BH, De Souza Bernardes LA, Puccia R, Naranjo TW, Goldman GH, Goldman MH, Cano LE, Restrepo A, McEwen JG. Gene expression analysis of *Paracoccidioides brasiliensis* transition from conidium to yeast cell. Med Mycol 2010, 48: 1-9.

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz GS, Botstein D, Brown PO. Genomic Expression Programs in the Response of Yeast Cells to Environmental Changes. Mol Biol Cell 2000, 11: 4241– 4257.

Gerik KJ, Bhimireddy SR, Ryerse JS, Specht CA, Lodge JK. PKC1 is essential for protection against both oxidative and nitrosative stresses, cell integrity, and normal manifestation of virulence factors in the pathogenic fungus *Cryptococcus neoformans*. Eukaryot Cell 2008, 7: 1685-98.

Gonzalez A, Gregori W, Velez D, Restrepo A, Cano, LE. Nitric Oxide Participation in the Fungicidal Mechanism of Gamma Interferon-Activated Murine Macrophages against *Paracoccidioides brasiliensis* Conidia. Infect Immun 2000, 68: 2546-2552.

Grose E, Tamsitt JR. *Paracoccidioides brasiliensis* recovered from the intestinal tract of three bats (Artibeus lituratus) in Colombia, S.A. Sabouraudia 1965, 4: 124-125.

Horan S, Bourges I, Meunier B. Transcriptional response to nitrosative stress in *Saccharomyces cerevisiae*. Yeast 2006, 23: 519–535.

Hromatka BS, Noble SM, Johnson AD. Transcriptional Response of *Candida albicans* to Nitric Oxide and the Role of the YHB1 Gene in Nitrosative Stress and Virulence. Mol Biol Cell 2005, 16: 4814 – 4826.

Lacaz CS, Martins JEC. Paracoccidioidomicosis. In Micologia Médica. 8. edition. São Paulo; Sarvier; 1991: 248-26.

MacMicking J, Xie Q, Nathan C. Nitric Oxide and Macrophage Function. Annu Rev Immunol 1997, 15: 323-350.

Maluf MLF, Pereira SRC, Takahachi G, Svidzinski TIE. Prevalence of paracoccidioidomycosis infection determined by sorologic test in donors' blood in the Northwest of Paraná, Brazil. Rev. Soc Bras Med Trop 2003, 36: 11-16.

Marchiori E, Valiante PM, Escuissato DL, Souza JrAS, Mano CM, Zanetti G, Capone D. Paracoccidioidomycosis: High-Resolution Computed Tomography-Pathologic Correlation. Eur J Radiol 2011, 77: 80-84.

Marletta MA, Yoon PS, Iyengar R, Leaf CD, Wishnok JS. Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. Biochemistry 1988, 27: 8706-8711.

Martinez R. Paracoccidioidomycosis: the dimension of the problem of a neglected disease. Rev Soc Bras Med Trop 2010, 43: 480.

Matute DR, Mcewen JG, Puccia R, Montes BA, San-Blas G, Bagagli E, Rauscher JT, Restrepo A, Morais F, Niño-Veja G, Taylor JW. Cryptic speciation and recombination in the fungus *Paracoccidioides brasiliensis* as revealed by gene genealogies. Mol Biol Evol 2006, 23: 65-73.

McEwen JG, Bedoya V, Patiño MM, Salazar ME, Restrepo A. Experimental murine paracoccidioidomycosis induced by inhalation of conidia. J Med Vet Mycol 1987, 25: 165-175.

Missall TA, Cherry-Harris JF, Lodge JK. Two glutathione peroxidases in the fungal pathogen *Cryptococcus neoformans* are expressed in the presence of specific substrates. Microbiology 2006, 151: 2573–2581.

Missall TA, Lodge JK, McEwen JE. Mechanisms of Resistance to Oxidative and Nitrosative Stress: Implications for Fungal Survival in Mammalian Hosts. Eukaryot Cell 2004, 3: 835–846.

Nascimento FR, Calich VLG, Rodrigues D, Russo M. Dual role for nitric oxide in paracoccidioidomycosis: essential for resistance, but overproduction associated with susceptibility. J Immunol 2002, 168: 4593-4600.

Nathan C, Shiloh MU. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc Natl Acad Sci 2000, 97: 8841-8848.

Nishikaku AS, Molina RFS, Ribeiro LC, Scavone, R, Albe BP, Cunha CS, Burger E. Nitric oxide participation in granulomatous response induced by *Paracoccidioides brasiliensis* infection in mice. Med Microbiol Immunol 2009, 198: 123–135.

Nittler MP, Hocking-Murray D, Foo CK, Sil A. Identification of *Histoplasma capsulatum* transcripts induced in response to reactive nitrogen species. Mol Biol Cell 2005, 16: 4792–4813.

Nunes LR, Oliveira RC, Leite DB, da Silva VS, Marques ER, Ferreira MES, Ribeiro DCD, Bernardes LAS, Goldman MHS, Puccia R, Travassos LR, Batista WL, Nóbrega MP, Nobrega FG, Yang D, Pereira CAB, Goldman GH. Transcriptome Analysis of *Paracoccidioides brasiliensis* Cells Undergoing Mycelium-to-Yeast Transition. Eukaryot Cell 2005, 4: 2115–2128.

Paniago AM, de Freitas AC, Aguiar ES, Aguiar JI, da Cunha RV, Castro AR and Wanke B. Paracoccidioidomycosis in patients with human immunodeficiency virus: review of 12 cases observed in an endemic region in Brazil. J Infect 2005, 51: 248-252.

Parente JA, Borges CL, Bailão AM, Felipe MSS, Pereira M, Soares CMA. Comparison of transcription of multiple genes during mycelia transition to yeasts cells of *Paracoccidioides brasiliensis* reveals insights to fungal differentiation and pathogeneses. Mycopathologia 2008, 165: 259-273.

Pavanelli WR, Silva JJN, Panis C, Cunha TM, Costa IC, Menezes MCND, Oliveira FJA, Lopes LGF, Cecchini R, Cunha FQ, Watanabe MAE, Itano EM. Experimental Chemotherapy in Paracoccidioidomycosis Using Ruthenium NO Donor. Mycopathologia 2011, 172: 95–107.

Pereira M, Bailão AM, Parente JA, Borges CL, Salem-Izacc, SM, Soares CMA. Preferential transcription of *Paracoccidioides brasiliensis* genes: host niche and time-dependent expression. Mem Inst Oswaldo Cruz 2009, 104: 486-491.

Pinzan CF, Ruas LP, Casabona-Fortunato AS, Carvalho FC, Roque-Barreira MC. Immunological basis for the gender differences in murine *Paracoccidioides brasiliensis* infection. PLoS ONE 2010, 5: 10757.

Prado M, da Silva MB, Laurenti R, Travassos LR, Taborda CP. Mortality due to systemic mycoses as a primary cause of death or in association with AIDS in Brazil: a review from 1996 to 2006. Mem Inst Oswaldo Cruz 2009, 104: 513-521.

Ramos-e-Silva M, Saraiva LES. Paracoccidioimycosis. Dermatol Cli 2008, 26: 257-269.

Restrepo A, McEwen JG, Castaneda E. The habitat of *Paracoccidioides brasiliensis*: how far from solving the riddle? Med Mycol 2001, 39: 233-241.

Restrepo A, Salazar ME, Cano LE, Stover EP, Feldman D, Stevens DA. Estrogens inhibit mycelium-to-yeast transformation in the fungus *Paracoccidioides brasiliensis*: implications for resistance of females to paracoccidioidomycosis. Infect Immun 1984, 46: 346-53.

Richini-Pereira VB, Bosco Sde M, Griese J, Theodoro RC, Macoris SA, da Silva RJ, Barrozo L, Tavares PM, Zancope-Oliveira RM, Bagagli E. Molecular detection of *Paracoccidioides brasiliensis* in road-killed wild animals. Med Mycol 2008, 46: 35-40.

San-Blas G, Niño-Vega G, Iturriaga T. *Paracoccidioides brasiliensis* and paracoccidioidomycosis: molecular approaches to morphogenesis, diagnosis, epidemiology, taxonomy and genetics. Med Mycol 2008, 40: 225-42.

Santo AH: Paracoccidioidomycosis-related mortality trend, state of São Paulo, Brazil: a study using multiple causes of death. Rev Panam Salud Publica 2008, 23: 313-24.

Shankar J, Restrepo A, Clemons KV, Stevens DA. Paracoccidioidomycosis: hormones and the resistance of women. Clinic Microbiol Rev 2011, 24: 296-313.

Shikanai-Yasuda MA, Telles Filho Fde Q, Mendes RP, Colombo AL, Moretti ML. Guidelines in paracoccidioidomycosis. Rev Soc Bras Med Trop 2006, 39: 297-310.

Tavares AH, Silva SS, Dantas A, Campos EG, Andrade RV, Maranhao AQ, Brigido MM, Passos-Silva DG, Fachin AL, Teixeira SM, Passos GA, Soares CMA, Bocca AL, Carvalho MJ, Silva-Pereira I, Felipe MS. Early transcriptional response of *Paracoccidioides brasiliensis* upon internalization by murine macrophages. Microbes Infect 2007, 9: 583-590.

Thuma T, Bauersachs J. Microarray-based gene expression profiling to elucidate cellular responses to nitric oxide - A review from an analytical and biomedical point of view. J Chromatogr B Analyt Technol Biomed Life Sci 2007, 1-2: 3-11.

Teixeira MM, Theodoro RC, Carvalho MJA, Fernandes L, Paes HC, Hahn RC, Mendonza L, Bagagli E, San-Blas G, Felipe MSS. Phylogenetics analysis reveals a high level of speciation in the *Paracoccidioides* genus. Mol Phylogenet Evol 2009, 52: 273-283.

Terçarioli GR, Bagagli E, Reis GM, Theodoro RC, Bosco SMG, Macoris SAG, Richini-Pereira VB. Ecological study of *Paracoccidioides brasiliensis* in soil: growth ability, conidia production and molecular detection. BMC Microbiol 2007, 7: 92.

Vicente JB, Ehrenkaufer GM, Saraiva LM, Teixeira M, Singh U. *Entamoeba histolytica* modulates a complex repertoire of novel genes in response to oxidative and nitrosative stresses: implications for amebic pathogenesis. Cel Microbiol 2009, 11: 51-69.

Voskuil MI, Bartek IL, Visconti K, Schoolnik GK. The response of *Mycobacterium tuberculosis* to reactive oxygen and nitrogen species. Front Microbiol 2011, 2:105.

Wanke B, Aidê MA. Chapter 6 – Paracoccidioimycosis. J. Bras. Pneumol 2009, 35: 1245-1249.

Wilson D, Tutulan-Cunita A, Jung W, Hauser C, Hernandez R, Williamson T, Piekarska K, Rupp S, Young T, Stateva L. Deletion of the high-affinity cAMP phosphodiesterase encoded by PDE2 affects stress responses and virulence in *Candida albicans*. Mol Microbiol 2007, 65: 841–856.