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# *Cryptococcus neoformans* and *gattii* promote DNA damage in human peripheral blood mononuclear cells

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## Abstract

Cryptococcosis, a systemic mycosis capable of disseminating to the central nervous system with frequent lethal effects, is caused by the species *Cryptococcus neoformans* and *Cryptococcus gattii*. Several infectious agents such as virus, bacteria, and parasites may be associated to DNA damage and carcinogenesis in humans. Products of the oxidative metabolism, such as NO, produced as a host defense mechanism to destroy these pathogens, have been implicated in this damage process, due to excessive production related to an established chronic inflammatory response. Here, we investigated whether *C. neoformans* and /or *C. gattii* can cause DNA damage in human peripheral blood mononuclear cells (PBMCs) and whether this process is related to NO levels produced by PBMCs. We found that both species are equally able to induce genotoxicity in PBMCs. However, an association between DNA damage and high NO levels was only detected in relation to *C. gattii*. The results point to the possibility that patients with cryptococcosis are more susceptible to the development of other diseases.

**Key words:** *Cryptococcus neoformans*, *Cryptococcus gattii*, DNA damage, NO, peripheral blood mononuclear cells.

## Introduction

Cryptococcosis, a systemic mycosis whose incidence has increased drastically in last decades, is caused by capsulated yeasts belonging to the genus *Cryptococcus*. Infection in humans is associated with two fungus species: *Cryptococcus neoformans* and *Cryptococcus gattii* that, however, affect different populations.[1] While *C. neoformans* infects individuals with a profound immune deficiency response, *C. gattii* is able to infect immunocompetent individuals.[2–4] Understanding the mechanisms involved in these differences

has been a challenge for researchers. Infection of brain and meninges by *C. neoformans* is the most important clinical form in immunosuppressed individuals, such as those with AIDS, in which cryptococcosis is listed as one of the most important causes of death.[5,6] In immunocompetent patients, *C. gattii* infection is clinically characterized by pulmonary complications and pneumonia, with or without meningitis. Symptoms may also occur in the skeletal muscles, with a tendency to present lesions with lung and/or cerebral mass, and hydrocephalus.[5]

Host protection against *Cryptococcus* is closely associated to M1 or classically activated macrophages.[7] This mechanism, in turn, depends on immune cells, including natural killer (NK) cells, $CD8^+$ T cells, and $CD4^+$ Th1 cells[8] that respond to the pathogen by secreting inflammatory cytokines, such as interferon $\gamma$ (IFN-$\gamma$), which signals macrophages to polarize toward a M1 phenotype.[8–11] These macrophages destroy pathogens mainly via the activation of the oxidative metabolism.[12] Specifically in the relation to *Cryptococcus*, the enzyme iNOS acts on the substrate L-arginine to produce NO, which has anti-fungal properties.[13–16] However, the metabolite produced primarily to attack the pathogen by nitration, oxidation, or chlorination[17] can cause, when in excess, injury to host cells inducing DNA damage leading to mutagenesis and other diseases.[18]

Studies have strongly suggested that several infectious agents such as virus, bacteria and parasites may be associated to carcinogenesis in humans.[19] Despite this, it has been very difficult to definitively identify pathogens as causative agents of cancers. In addition, studies making association of fungal diseases and DNA damage have not yet been described. This study aimed to evaluate whether *Cryptococcus neoformans* and/or *C. gattii* can cause DNA damage in human peripheral blood mononuclear cells and whether this process is related to NO levels produced by these cells.

## Methods

### Subjects

Six healthy donors of both sexes and from 18 to 40 years old, who attended to the Hemocenter of University Hospital of the School of Medicine - Botucatu, São Paulo State University – UNESP, were included in this study after signature of informed consent form. Individuals with infectious or granulomatous diseases, positive human immunodeficiency virus (HIV) diagnosis, pregnant women, and smokers were excluded. This study was approved by the Institution Research Ethics Committee (registration number: 38381014).

### Blood taken

Blood (60 ml) was collected through venipuncture (forearm vein) and by using a vacuum system (Vacutainer®). Sodium heparin was used as anticoagulant.

### Fungi

*C. neoformans* strains were obtained from the American Type Culture Collection (ATCC 28957), and *C. gattii* strains (AFLP4) were provided by the Mycology Service from the Department of Microbiology and Immunology of the Biosciences Institute - I.B.B., UNESP, Botucatu. Both strains are clinical isolates. Yeasts were cultured in 20 × 200 mm tubes containing Sabouraud culture medium (Difco, Franklin Lakes, NJ, USA) with yeast extract, at 25°C for 5 days.

### Isolation and infection of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll- Hypaque density gradient (Sigma-Aldrich, St. Louis, MO, USA) (centrifugation at 405 g for 30 minutes). The ring rich in lymphocytes and monocytes was collected and washed twice with RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA). The cells were subsequently resuspended in complete culture medium (RPMI 1640 supplemented with 2 mM L-glutamine, 40 mg/ml gentamicin and 10% inactivated fetal bovine serum). Identification of the cells and assessment of their viability were performed using Trypan Blue staining. Cells were seeded in 24-well tissue culture plates ($1 \times 10^7$/ml) infected with *C. neoformans or C. gattii* ($10^6$ yeasts/ml) during 7 days at 37°C in an atmosphere of 5% $CO_2$. After this period, culture supernatants were collected and evaluated for NO concentrations, and cells were submitted to comet assay for analyzing DNA damage. At the time of performing these assays cells viability was about 80%.

### NO quantification

NO concentrations were evaluated by measuring the accumulation of nitrite in cultures supernatants of noninfected or *C. neoformans* or *C. gattii* infected PBMCs using the standard Griess assay. Aliquots (100 ul) of the supernatants were added to equal volumes of Griess reagent constituted of 1% sulfananilamide diluted in 5% $H_3PO_4$ and 0.1% naphthylenediamine (all from Sigma-Aldrich) in 24-well tissue culture plates. Absorbance at 540 nm of the resultant reaction was determined by using an ELISA reader (BioTex, Wenoaski, Vermont, USA). Conversion of absorbance to

NO concentrations was performed according to a standard curve ($NaNO_2$), and the results were expressed in $\mu$M.

### DNA damage

DNA damage was evaluated in noninfected or *C. neoformans* or *C. gattii* infected PBMCs and was performed as described by Singh et al.[20] with some modifications. Three types of lesions were analyzed: the general lesions, the oxidized purines by using the enzyme formamidopyrimidine-DNA glycosylase (FPG), and the oxidized pyrimidines by using the enzyme endonuclease III (endoIII). Aliquots of 10 ul of PBMCs ($10^7$/ml) were added to 120 ul of 0.5% low–melting point agarose. This mixture was layered onto a slide precoated with a 1.5% normal–melting point agarose, covered with a cover slip and placed at 4°C for 10 minutes to allow agarose to solidify. After this period, the coverslip was removed and the slide submersed in freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% Triton × 100 and 10% DMSO) followed by washing in phosphate-buffered saline (PBS) ($Ca_2$ + and $Mg_2$ + free) for 5 minutes and treatment with FPG and endoIII enzymes (1:1000 dilution) at 37°C for 30 minutes. Slides were then transferred to horizontal electrophoresis tank, which was filled with freshly prepared alkaline buffer (1 mM EDTA and 300 mM NaOH, pH > 13). After 40 minutes, electrophoresis was performed (25 V for 30 minutes). The slides were placed for 15 minutes in neutralization solution (0.4 M Tris, pH 7.5), fixed with absolute ethanol, dried at room temperature, and stored at 4°C. At the time of analysis, the slides were stained with 50 ul Syber Gold solution, covered with coverslips and 100 nucleoids per slide (total of 400 nucleoids per culture) were examined under an immunofluorescence microscope (400× magnification) coupled to the Comet Assay II (Perceptive Instruments, Havervill, Suffolk, UK). The test was performed in duplicate, with slides coded and analyzed by blind test. DNA damage was measured by analyzing tail intensity (% of migrated DNA) and tail moment [the product of tail length (DNA migration) and fraction of DNA in the comet tail, i.e., % of DNA in the tail].[21]

### Statistical analysis

Analyses were performed by using the GraphPad Prism 5.0 software. Results concerning to NO evaluation were analyzed by ANOVA for independent samples followed by Tukey Kramer test. Differences among the results of DNA damage evaluation were calculated by the gamma distribution test. The level of significance was set at $P < .05$.

## Results

### DNA damage induced by *C. gattii* and *C. neoformans* in PBMC cultures

The results regarding general DNA damages triggered by both fungal species and referring to tail moment, that identifies the presence or absence of lesions, and those of tail intensity indicating the lesions intensity, are shown in Figure 1A. We found that the values after infection with *C. gatti* were significantly higher ("tail moment" and "tail intensity") as compared to control cultures ($P < .05$). The same did not occur in cultures infected with *C. neoformans*, in which the values were similar to the controls. In Figure 1B the oxidative lesions detected specifically in purines, by using the enzyme formamidopyrimidine-DNA glycosylase (FPG), are shown. Values higher than those obtained in the control cultures were detected after infection with *C. gatti* and *C. neoformans* ("tail moment" and "tail intensity"). However, the results were statistically significant only after challenge with *C. neoformans* ($P < .05$). In Figure 1C the lesions occurring specifically in the pyrimidines (enzyme ENDO III) can be analyzed. We detected values significantly higher than control cultures for both species (tail moment and tail intensity) ($P < .05$). Together, our results indicate that both *C. neoformans* and *C. gattii* are able to induce damage in the DNA of the evaluated cells.

### NO production

In order to evaluate whether the detected DNA damage was associated to NO production, levels of this metabolite were quantified in the supernatants of PBMCs infected with the two species of *Cryptococcus*. Unstimulated PBMCs produced low NO levels that were significantly increased ($P < .05$) only after infection with *C. gatti* (Fig. 2).

## Discussion

Although studies have demonstrated that some pathogens are able to induce DNA damage,[19] data about fungal genotoxicity are rare. Peripheral blood leukocytes have been used to study DNA damage caused by some factors such as infectious agents, as they can represent the lesions induced in other cells. Another advantage is the feasibility of using such cells in human studies. In turn, comet assay is a relatively simple, sensitive and powerful method to determine genotoxicity.[20] Thus, in this study the capacity of *C. neoformans* and *C. gattii* in inducing DNA damage was investigated by using human peripheral blood mononuclear cells and the comet assay. In the experiments in which the general lesions were evaluated, we found that only *C. gattii* induced

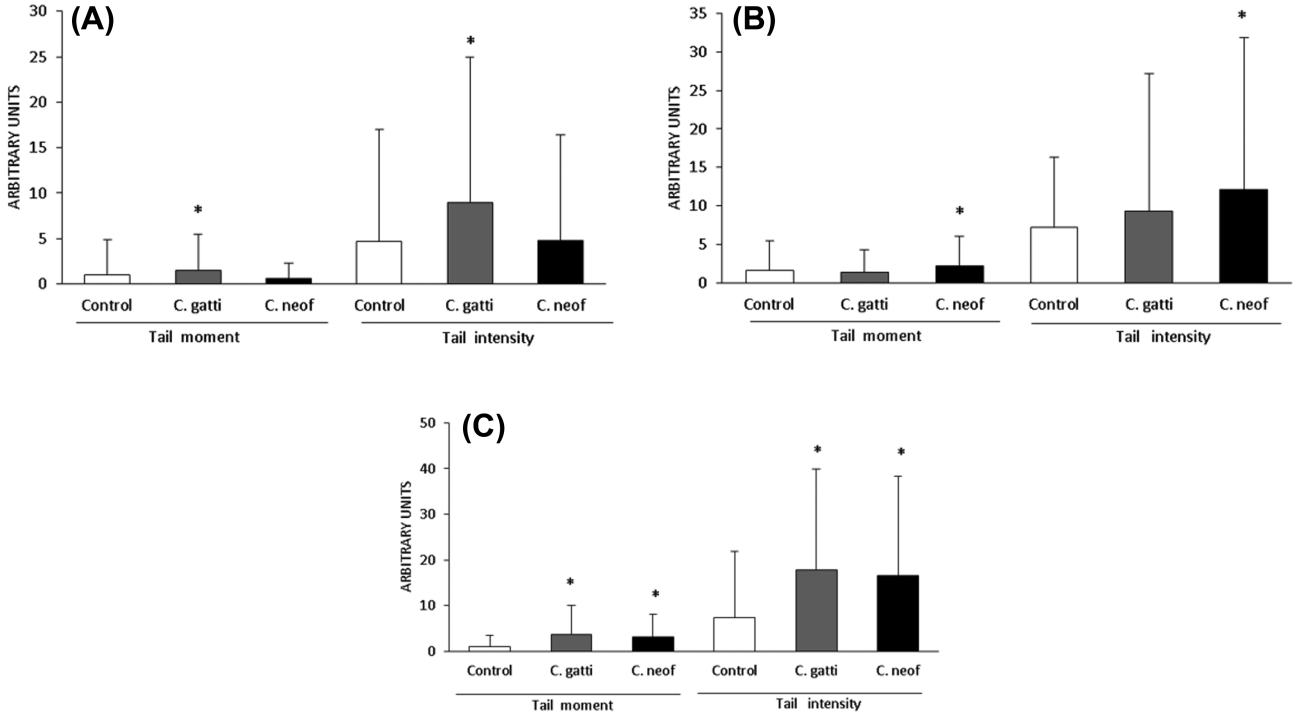


**Figure 1.** DNA damage detected by Comet Test and expressed as tail moment and tail intensity in noninfected (control) or in *C. neoformans* or *C. gattii* infected PBMCs during 7 days. *A*: general lesions; *B*: oxidative lesions detected specifically in purines, by using the enzyme formamidopyrimidine-DNA glycosylase (FPG); *C*: oxidative lesions occurring specifically in the pyrimidines (enzyme ENDO III). The results are expressed as mean ± SD of experiments performed with cells obtained from six subjects. Statistically significant differences between groups are indicated: $^{*}P < .05$ versus control.

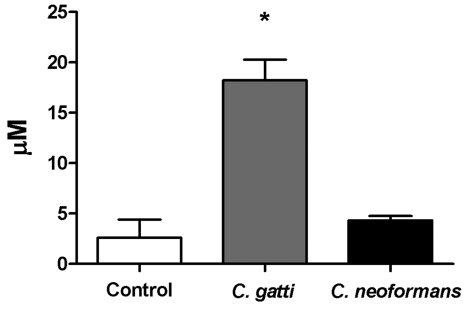


**Figure 2.** NO production by noninfected (control) or *C. neoformans* or *C. gattii* infected PBMCs during 7 days. The results are expressed as mean ± SD of experiments performed with cells obtained from 10 subjects. Statistically significant differences between groups are indicated: $^{*}P < .05$ versus control and *C. neoformans*.

significant DNA damage. However, when oxidative lesions on specific bases (purines and pyrimidines) were analyzed, we concluded that both species are able to induce genotoxicity by oxidative stress. General lesions can occur by several factors, such as radiation, medicines, UV light, and so forth. Damages in purine and pyrimidine bases are specifically due to oxidative stress. Thus, differences detected when general lesions were evaluated can be others than those detected in specific tests. This may explain why only *C. gatti* was shown to induce general lesions, while both species are able to induce specific lesions. To our knowledge, this is the first report detecting this process associated to *Cryptococcus*. Despite all of this, previous studies already showed that this fungus has the ability to cause other types of cell injury mediated by oxidative stress, as impaired mitochondrial function, activation of caspase-1, and altered protein synthesis rate in macrophages. All these cellular damages could promote and potentiate *C. neoformans* survival in macrophages and contribute to its evasion from host immune response.[22] Other studies showed that human alveolar epithelial cells lineage are killed after GXM-mediated interaction with *C. neoformans*.[23]

Our findings allow us to include the two species of *Cryptococcus* among the infectious agents that can cause cells genotoxicity such as *Mycobacterium tuberculosis*,[24] *Trypanossoma cruzi*,[25] *Leishmania chagasi*,[26,27] *Klebsiella pneumoniae*.[28] and others.

The mechanisms involved in the association between carcinogenesis and infection are not totally elucidated. Two main mechanisms are proposed: direct action of the pathogen on DNA or an indirect effect by activating a strong inflammatory response that induces cells to an excessive oxidative metabolism. Oxidative damage to DNA has been recognized as the main factor that promotes carcinogenesis.[29,30] This second mechanism has already been

proven in infections whose resistance requires the generation of an important inflammatory response that culminates with the activation of the oxidative metabolism for the destruction of the pathogen, as in the case of infection with *Leishmania*[26,27] and *T. cruzi*.[25] These data imply that this mechanism can also be suggested by infection with *Cryptococcus*, as host resistance to this fungus is associated with M1 macrophages, which, for activation, require the production of proinflammatory cytokines such as IFN-$\gamma$ and tumor necrosis factor $\alpha$ (TNF-$\alpha$). The main product of activated M1 macrophages is NO that is able to destroy *Cryptoccocus*[7] but that in excess can lead to oxidative stress.

Here, in an attempt to determine the association of DNA damage /high NO production, the levels of this metabolite were evaluated in the supernatants of PBMCs infected with *C. neoformans* and *C. gatti*. Surprisingly, we detected that only *C. gatti* was able to induce high NO production. Thus, an association between DNA damage and high NO levels were only detected in relation to this *Cryptococcus* species. Despite high NO levels not being detected in response to *C. neoformans*, other products of oxidative metabolism not evaluated in the present study, such as $H_2O_2$, peroxide anion, and others can be produced in excess and be involved in DNA damage in response to this strain. Thus, further assays need to be performed in order to confirm the true involvement of NO and other metabolites in the detected lesions induced by two strains, such as those in which the lesions are evaluated before and after neutralization of the possible metabolites involved. Independent of these differences, the results show the potential capacity of both fungus species in inducing cellular damage associated to oxidative stress. These results may indicate a greater susceptibility of these individuals to some diseases that may range from cancer to other metabolic disorders linked to oxidative stress. Future studies should be directed towards confirming these assumptions.

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### Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

## References

1. Kwon-Chung KJ, Fraser JA, Doering TL et al. *Cryptococcus neoformans* and *Cryptococcus gattii*, the etiologic agents of Cryptococcosis. *Cold Spring Harb Perspect Med*. 2014; **4**: 1–27.
2. Sorrell TC. *Cryptococcus neoformans* variety gattii. *Med Mycol*. 2001; **39**: 155–168.
3. Andama AO, Den Boon S, Meya D et al. Prevalence and outcomes of cryptococcal antigenemia in HIV-seropositive patients hospitalized for suspected tuberculosis in Uganda. *J Acquir Immune Defic Syndr*. 2013; **63**: 189–194.
4. Jarvis JN, Harrison TS, Govender N et al. Routine cryptococcal antigen screening for HIV-infected patients with low CD4$^+$ T-lymphocyte counts—time to implement in South Africa? *S Afr Med J*. 2011; **101**: 232–234.
5. Mitchell TG, Perfect JR. Cryptococcosis in the era of AIDS-100 years after the discovery of *Cryptococcus neoformans*. *Clin Microbiol Rev*. 1995; **8**: 515–548.
6. Xia S, Li X, Shi Y et al. A Retrospective Cohort Study of Lesion Distribution of HIV-1 Infection Patients With Cryptococcal Meningoencephalitis on MRI. *Medicine (Baltimore)*. 2016; **95**: e2654.
7. Leopold Wager CM, Hole CR, Wozniak K L et al. *Cryptococcus* and Phagocytes: Complex Interactions that Influence Disease Outcome. *Front Microbiol*. 2016; **7**: 105.
8. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008; **8**: 958–969.
9. Mantovani A, Sica A, Sozzani S et al. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol*. 2004; **25**: 677–686.
10. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. *Ann Rev Immunol*. 2009; **27**: 451–483.
11. Hussell T, Bell TJ. Alveolar macrophages: plasticity in a tissue-specific context. *Nat Rev Immunol*. 2014; **14**: 81–93.
12. Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. *J Immunol*. 1988; **141**: 2407–2412.
13. 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.
14. Arora S, Olszewski MA, Tsang TM et al. Effect of cytokine interplay on macrophage polarization during chronic pulmonary infection with *Cryptococcus neoformans*. *Infect Immun*. 2011; **79**: 1915–1926.
15. Hardison SE, Herrera G, Young ML et al. Protective immunity against pulmonary cryptococcosis is associated with STAT1-mediated classical macrophage activation. *J Immunol*. 2012; **189**: 4060–4068.
16. Davis MJ, Tsang TM, Qiu Y et al. Macrophage M1/M2 polarization dynamically adapts to changes in cytokine microenvironments in *Cryptococcus neoformans* infection. *MBio*. 2013; **4**: 00264–13.
17. Saran M, Beck-Speier I, Fellerhoff B et al. Phagocytic killing of microorganisms by radical processes: consequences of the reaction of hydroxyl radicals with chloride yielding chlorine atoms. *Free Radic Biol Med*. 1999; **26**: 482–490.
18. Valko M, Izakovic M, Mazur M et al. Role of oxygen radicals in DNA damage and cancer incidence. *Mol Cell Biochem*. 2004; **266**: 37–56.
19. Vainio H. Social Responsibility in Cancer Prevention Research: IARC as a 'Global Science Force'. *Asian Pac J Cancer Prev*. 2002; **3**: 267–272.
20. Singh NP, McCoy MT, Tice RR et al. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res*. 1988; **175**: 184–191.
21. Hartmann A, Agurell E, Beevers C et al. Recommendations for conducting the in vivo alkaline Comet assay. *4th International Comet Assay Workshop*. *Mutagenesis*. 2003; **18**: 45–51.
22. Coelho C, Oliveira Souza AC, Derengowski LS et al. Macrophage mitochondrial and stress response to ingestion of *Cryptococcus neoformans*. *J Immunol*. 2015; **194**: 2345–2357.
23. Barbosa FM, Fonseca FL, Holandino C et al. Glucuronoxylomannan-mediated interaction of *Cryptococcus neoformans* with human alveolar cells results in fungal internalization and host cell damage. *Microb and Infect*. 2006; **8**: 493–502.

24. Rao VV, Gupta EV, Thomas IM. Chromosome damage in untreated tuberculosis patients. *Tubercle*. 1990; **71**: 169–172.
25. Ribeiro DA, Calvi SA, Picka MM et al. DNA damage and nitric oxide synthesis in experimentally infected Balb/c mice with *Trypanosoma cruzi*. *Exp Parasitol*. 2007; **116**: 296–301.
26. Kocyigit A, Keles H, Selek S et al. Increased DNA damage and oxidative stress in patients with cutaneous leishmaniasis. *Mutat Res*. 2005; **585**: 71–78.
27. Oliveira LR, Cezário GA, de Lima CR et al. DNA damage and nitric oxide production in mice following infection with *L. chagasi*. *Mutat Res*. 2011; **723**:177–181.
28. Lai YC, Lin AC, Chiang MK et al. Genotoxic *Klebsiella pneumoniae* in Taiwan. *PLoS One*. 2014; **9**: e96292.
29. Ames BN, Gold LS, Willett WC. The causes and prevention of cancer. *Proc Natl Acad Sci U S A*. 1995; **92**: 5258–5265.
30. Lindahl T, Wood RD. Quality control by DNA repair. *Science*. 1999; **286**: 1897–1905.