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Original Article

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A new method for studying cryptococcosis in a murine model using ^{99m}Tc-*Cryptococcus gattii*

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Abstract

Cryptococcus gattii is one of the etiologic agents of cryptococcosis, a systemic mycosis that occurs in healthy and immunosuppressed humans and animals worldwide. Primary pulmonary infection caused by *C. gattii* is usually followed by fungal dissemination to the central nervous system, resulting in high mortality rates. In this context, animal models of cryptococcosis are useful in the study of fungal pathogenesis and host response against the pathogen, and for testing novel therapeutic options. The most frequently applied method to study fungal dissemination from the lungs to other organs is by culturing tissues, which is not accurate for the detection and quantification of fungal load at early stages of the infection. To overcome this problem, the purpose of this study was to develop a new method for the quantification of *Cryptococcus* dissemination. One *C. gattii* strain was efficiently radiolabeled with technetium-99m (^{99m}Tc), without affecting viability of the cells. Further, the ^{99m}Tc-*C. gattii* (111 MBq) strain was used to infect mice by intratracheal and intravenous route for biodistribution studies. ^{99m}Tc-*C. gattii* was successfully used in detection of the yeast in the brain of mice 6 hours postinoculation, while the detection using colony forming units was possible only 24 hours postinfection. Our results provided an alternative method that could be applied in further investigations regarding the efficacy of antifungals, fungal virulence, and host-pathogen interactions.

Key words: *Cryptococcus gattii*, technetium-99m, radiolabeling, fungal dissemination, animal model.

Introduction

Cryptococcus gattii is one of the etiologic agents of cryptococcosis, a neglected systemic mycosis that affects approximately 1 million people worldwide each year. It is the most common fungal infection of the central nervous system (CNS) in both healthy and immunocompromised individuals. Only 40% of these patients are alive after one year of active infection despite undergoing antifungal chemotherapy.[1,2] Cryptococcal infections are acquired by inhalation of environmental spores or desiccated yeasts.[3,4] During infection, the cells colonize the alveoli and further disseminate through the blood until they cross the blood-brain barrier (BBB), leading to cryptococcal meningoencephalitis, i.e. the most severe and lethal form of the disease.[5]

Murine models have been useful in the study of cryptococcal infection, since, mice infected with *Cryptococcus* develop clinical symptoms similar to those observed in humans.[6–9] In this context, studies focused on fungal dissemination to the CNS are important for better understanding of host-fungi interactions, including host defense mechanisms, fungal virulence, and studies on drug efficacy. The most frequently applied method to quantify fungal burden in mice tissues is based on plating and counting colonies on sabouraud dextrose agar (SDA).[9–11] Unfortunately, this method is not useful for the accurate detection and quantification of fungal load at early stages of the infection, probably because of problems associated with dilution in the plating technique.

In a previous study, researchers labeled *Escherichia coli* with technetium-99m (^{99m}Tc) to investigate bacterial translocation in an experimental model of obstructive jaundice.[12] In addition, ischemia followed by reperfusion of the small intestine as well as the role of arginine[13] and glutamine supplementation[14] in bacterial translocation using ^{99m}Tc-*E. coli* were evaluated. ^{99m}Tc is the most widely used radiotracer in nuclear medicine because of its availability and ideal physical characteristics. In daily medical practice, compounds radiolabeled with ^{99m}Tc are used to obtain scintigraphic images of the thyroid gland, gastric mucosa, salivary glands and choroids plexus of the brain.[15] Normally the procedures employed in nuclear medicine allow early diagnosis of several diseases. Other radioactive isotopes have also been used for radiolabeling, among which indium-111 has been used in *E. coli, Staphylococcus aureus, S. epidermidis,* and *Pseudomonas aeruginosa*.[16,17] However, there is a scarcity of studies focused on fungal labeling with radioactive isotopes.

In light of these facts, the purpose of this study was to develop and evaluate a new method based on the radiolabeling of *C. gattii* with ^{99m}Tc atoms to quantify fungal dissemination to the CNS at the early stages of infection in murine models.

Methods

Cryptococcus culture

Cryptococcus gattii strain L27/01 was used in this study. This strain is deposited in the Culture Collection of Microorganisms and Cells of the Universidade Federal of Minas Gerais (code UFMG-CM-Y6141) (Belo Horizonte, MG, Brazil). The yeast was grown on SDA at 37°C for 48 h prior to the tests. For all experiments, yeast cells were suspended in sterile phosphate buffered saline (PBS) with pH 7.4, and the inoculum was adjusted in Neubauer chamber at 3.3×10^7 viable cells per ml.

Radiolabeling

Sodium pertechnetate ($Na^{99m}TcO_4$) was obtained by elution from a sterile ^{99}Mo/^{99m}Tc generator with sterile saline solution. For the $^{99m}TcO_4^-$ ion to be reactive, it was reduced to lower oxidation states by stannous chloride ($SnCl_2.2H_2O$).[18,19] To verify the best experimental conditions for radiolabeling of *C. gattii,* parameters such as optimal concentration of $SnCl_2.2H_2O$ and its incubation time with the radioisotope were investigated. Triplicate yeast suspensions of 3.3×10^7/ml were incubated with variable concentrations (1, 2, and 3 mg/0.5 ml 0.25 N HCl) of $SnCl_2.2H_2O$ for 30 min. After incubation, 111 MBq (3mCi) of $Na^{99m}TcO_4$ were added, followed by incubation at 37°C for 30 min and centrifugation at 3,000*g* for 25 min. Further, 30 μl of the supernatant were collected and the rest was discarded. To the pellet, 1 ml of PBS was added, homogenized, and 30 μl were collected. The supernatant and precipitate radioactivity was determined by Wizard Gama Counter (Perkin-Elmer, Turku, Finland) and the procedure was repeated twice. Regarding incubation time, the same procedure was performed using variable durations (10, 30, and 60 min) of incubation with $Na^{99m}TcO_4$. The optimized concentration of $SnCl_2.2H_2O$ and its incubation time with the radioisotope was used in the next procedures. The percentage of ^{99m}Tc incorporated into the fungal cells (precipitate) was determined using the equation below and the results were expressed in cpm (counts per minute).

$$\%\ \text{labelled}\ C.\ gattii = \frac{\text{CPM (precipitate)}}{\text{CPM (precipitate + supernatant)}} \times 100$$

Stability of radiolabeled *C. gattii*

The ^{99m}Tc-*C. gattii* complex stability was investigated by *in vitro* studies in order to determine if the ^{99m}Tc atoms remained bound to the fungal cells. For this purpose, the radiolabeling was performed as described above, using 3 mg $SnCl_2.2H_2O$/0.5 mL 0.25 N HCl and 111 MBq (3mCi) of $Na^{99m}TcO_4$ for 30 min of incubation. After two washing cycles, the ^{99m}Tc-*C. gattii* was incubated at 37°C with PBS for 6 and 24 h. Aliquots (30 μl) of supernatant and resuspended precipitate were collected for radioactivity determination.

Fungal viability after radiolabeling

To investigate the impact of radiolabeling on the viability of *C. gattii* cells, viability of unlabeled *C. gattii*, *C. gattii* plus labeling reagents without ^{99m}Tc, and ^{99m}Tc-*C. gattii* was determined by plating yeast cells on SDA. The plates were incubated at 37°C for 48 h, the colonies were counted, and the results were expressed as colony forming units per ml (cfu/ml).

Ethics statement for the animal protocols

The animal protocol was approved by the Comitê de Ética no Uso de Animais (CEUA) from Universidade Federal de Minas Gerais (Protocol 34/2016) and all animal experiments were performed according to the guidelines of Brazilian Federal Law 11,794. All mice were housed in clean bedding (six mice per cage) with food and water *ad libitum* in a controlled environment with a 12 h light/dark cycle at 23°C. For intratracheal (i.t.) and intravenous (i.v.) inoculation, mice were anesthetized by intraperitoneal (i.p.) injection of ketamine hydrochloride (80 mg/kg) and xylazine (15 mg/kg) in sterile saline. All efforts were made to minimize suffering. Mice were killed under anesthesia by cervical dislocation done by experienced animal handlers.

Intravenous and Intratracheal infection

C57BL/6 male mice (6 to 8 weeks old) were used in all experiments. Initially, the time-points to quantify the fungal burden in the brain of mice were determined. Groups of mice ($n = 6$) were infected by one route (i.v. or i.t.) with nonlabeled *C. gattii* (1×10^6cfu) and killed 6, 24, 48, 72, or 96 h after inoculation for cfu determination in the brain and lungs.

Further, a biodistribution assay was performed to test the dynamics of distribution of the ^{99m}Tc-*C. gattii* complex in mice. Briefly, groups of mice ($n = 6$) were infected by i.v. route in the lateral caudal vein of the mice with labeled *C. gattii* (1×10^6 CFU). In sum, 1 and 6 hours postinoculation, blood, brain, lungs, liver, spleen, heart, and stomach were collected, weighed and the radioactivity percentage per gram of the tissue was measured (irradiated dose [%ID/g]) in a Wizard Gama Counter. To compare the two methods, mice ($n = 6$) were anesthetized and then inoculated with 30 μl of a suspension containing 1×10^6 cfu of ^{99m}Tc-*C. gattii* by i.t. (in a midline neck incision) or i.v. routes. Further, animals were killed to determine radioactivity and fungal burden in the brain and lungs 6 h postinoculation.

Table 1. Effect of the reducing agent $SnCl_2.2H_2O$ on the radiolabeling of *C. gattii* with ^{99m}Tc.

$SnCl_2.2H_2O$ (mg/0.5 ml HCl 0.25 N)	% of radioactivity in *C. gattii*		
	Before washing	After washing	
		1	2
1 mg	26.0 ± 0.15	60.0 ± 8.35	84.5 ± 0.90
2 mg	50.0 ± 0.31	83.3 ± 0.46	92.1 ± 1.10
3 mg	57.0 ± 0.51	85.7 ± 0.24	94.5 ± 1.07*

In sum, 3.3×10^7 viable cells of *C. gattii* were incubated with distinct concentrations of $SnCl_2.2H_2O$ for 30 min and then with 111 MBq of ^{99m}Tc for 30 min. The percent of radiation was measured before and after two washes with PBS. The values represent the mean ± SD for each concentration ($n = 3$). *$P < .05$: were significantly higher compared to the other concentrations after two washes.

Fungal burden and radioactivity dosage

Organs were removed, weighed, and used for determination of radioactivity percentage per gram of tissue (irradiated dose [%ID/g]) in a Wizard Gama Counter. Brain and lungs were homogenized in 1 ml of PBS, plated on SDA, and incubated for 48 h at 37°C for determination of cfu/g of tissue.

Statistical analysis

All data were expressed as mean ± SEM (standard error of the mean). The results were evaluated using the Mann–Whitney test. Differences were considered significant at $P < .05$.

Results

Radiolabeling

As Table 1 shows, post second wash, the samples incubated for 30 min with 3 mg of $SnCl_2.2H_2O$ per 0.5 ml 0.25 N HCl presented a higher percentage of radioactivity linked to *C. gattii* than those incubated with 1 mg. No difference was observed in radioactivity ($P > .05$) with $SnCl_2.2H_2O$

Table 2. Influence of incubation time on radiolabeling of *C. gattii* with ^{99m}Tc.

	% of radioactivity in *C. gattii*		
		After washing	
Time (min)	Before washing	1	2
10	71.50 ± 0.81	92.99 ± 0.62	93.47 ± 1.26
30	83.75 ± 0.17	94.16 ± 0.69	98.45 ± 0.55*
60	56.85 ± 1.74	91.92 ± 4.36	93.06 ± 0.32

In sum, 3.3 × 10^7 viable cells of *C. gattii* were incubated with 3 mg of $SnCl_2.2H_2O$ for 10, 30, or 60 min, and then with 111 MBq of ^{99m}Tc for 30 min. The percentage of radiation was measured before and after two washes with PBS. The values represent the mean ± S.D. for each concentration (n = 3).
*$P < .05$: were significantly higher compared to the other times after two washes.

Table 3. ^{99m}Tc-*C. gattii* stability.

Time (hour)	% of radioactivity in *C. gattii*
6	90.43 ± 3.49
24	94.43 ± 0.76

After radiolabeling, the ^{99m}Tc-*C. gattii* was incubated at 37°C for 6 and 24 h and the percentage of radiation was measured. The values represent the mean ± SD for each concentration ($n = 3$).

concentrations of 2 and 3 mg. Therefore, 3 mg $SnCl_2.2H_2O$ per 0.5 ml 0.25 N HCl was used in the further experiments. Table 2 demonstrates that binding of ^{99m}Tc with yeast cells was almost immediate, as evidenced by the radioactivity in the yeast pellet at 10 min. Interestingly, the time-point of 30 min demonstrated an increased efficiency and was used for subsequent analyses.

Stability of ^{99m}Tc-*C. gattii*

Table 3 shows that even 6 and 24 h after the radiolabeling procedure, the radiolabeled *C. gattii* were stable.

Viability

The *in vitro* viability study did not show significant differences between the investigated groups, revealing that both the reducing agent and the ^{99m}Tc atoms had no effect on the fungal growth (Table 4).

Biodistribution

As demonstrated in Table 5, the biodistribution assay reveals rapid extravascular distribution, with smaller quantities in blood (around 0.1% ID/g) and high penetration in lungs (3–12% ID/g) in the first hour. Slightly lower levels of the ^{99m}Tc-*C. gattii* complex were detected in brain, heart, and stomach, in which concentrations ranged from 0.1 to 3.0 %ID/g. These results were not altered after 6 h.

Table 4. *C. gattii* viability.

Group	cfu/ml
PBS	2.35 × 10^7 ± 1.72 × 10^6
$SnCl_2$	2.26 × 10^7 ± 7.9 × 10^6
$SnCl_2$ + ^{99m}Tc	2.22 × 10^7 ± 6.14 × 10^6

After the radiolabeling process, yeast cells were plated on SDA to determine cfu per ml. The values represent the mean ± SD for each treatment ($n = 3$).

Table 5. ^{99m}Tc-*C. gattii* biodistribution.

	%ID/g	
Organ	1 hour	6 hours
Brain	0.01 ± 0.00	0.01 ± 0.00
Lungs	8.24 ± 5.39	6.28 ± 3.17
Liver	2.02 ± 1.03	2.00 ± 1.00
Spleen	1.07 ± 0.62	1.32 ± 0.63
Heart	0.08 ± 0.03	0.19 ± 0.38
Blood	0.10 ± 0.07	0.07 ± 0.01
Stomach	0.07 ± 0.03	0.05 ± 0.05

Six animals per group were inoculated by i.v. route with 1 × 10^6 ^{99m}Tc-*C. gattii* cells. Blood and organs were removed and weighed for measurement of radioactivity percentage per gram (ID/g%) 1 and 6 h postinoculation. The values represent the mean ± SD for each organ.

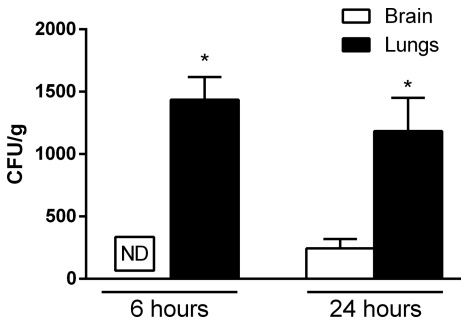

Figure 1. *C. gattii* CFU/g of brain and lungs determined in intravenous murine model of cryptococcosis. n = 6 animals per group were inoculated by the intravenous route with 1 × 10^6 non-labeled *C. gattii*. After 6 and 24 hours, mice were sacrificed to obtain brain and lungs. The organs were homogenized in PBS and the CFU number was determined by plating on SDA. *p: were significantly different ($p < 0.05$). ND: Not detected.

cfu and radioactivity dosage

The earliest time for cfu recovery from the brain was 24 h (Fig. 1). Shorter durations (6 h postinfection) did not allow the recovery of colonies from the brain. Because of this result, the time duration of 6 h postinfection was chosen for subsequent experiments. The cfu in the lungs were easily quantified after 6 and 24 h of infection. The other

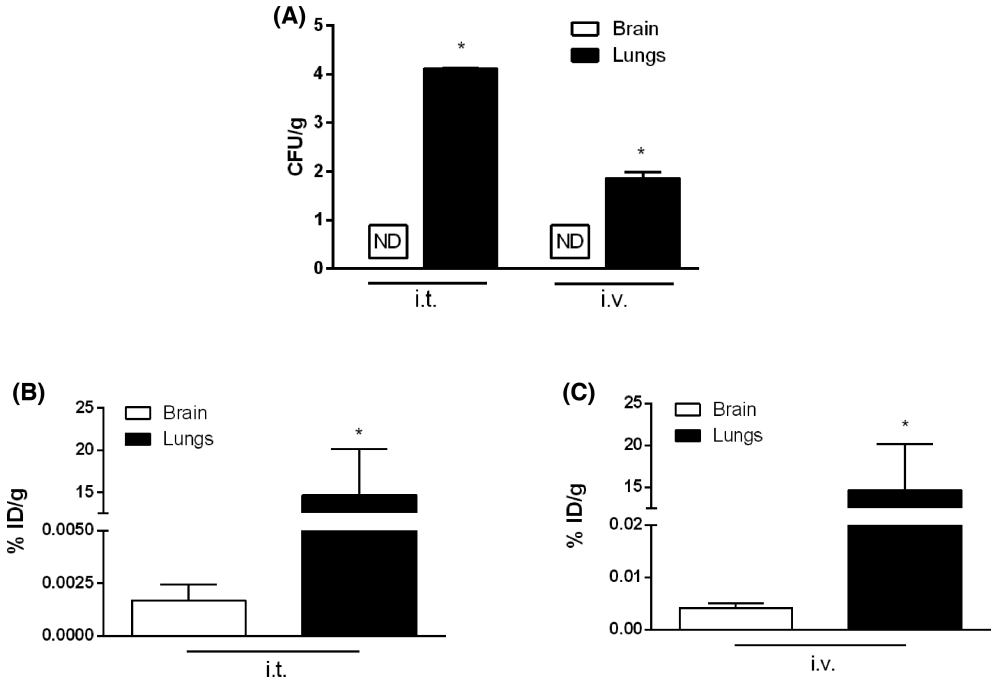

Figure 2. Fungal burden and radioactivity in the brain an lungs of mice infected with *C. gattii*. n = 6 animals per group were inoculated by intratracheal (i.t.) or intravenous (i.v.) route with 1×10^6 ^{99m}Tc-*C. gattii* cells, then 6 hours post inoculation, brain and lungs were removed and weighted for determination of CFU/g (A) and for measurement of radioactivity percentage per gram (ID/g%) (B-C). *p: were significantly different ($p < 0.05$). ND: Not detected.

time points included (48, 72, and 96 h postinfection), revealed an increase in the fungal burden with time (data not shown).

As shown in Figure 2A, no fungal burden was detected in the brain of mice infected with ^{99m}Tc-*C. gattii*. Interestingly, the results demonstrated by Figure 2B and 2C reflect that the ^{99m}Tc-*C. gattii* were detected in the brain 6 h postinoculation by radioactivity counting (%ID/g), in both intratracheal and intravenous murine models.

Discussion

Technetium-99m is an artificial element obtained by the radioactive decay of molybdenum (Mo). ^{99m}Tc presents ideal nuclear properties such as physical half-life time ($t_{1/2}$ = 6.01 h), gamma emission, and energy (140 keV) suitable for *in vivo* studies. Thus, it is the most widely used radioisotope in nuclear medicine.[15,19] A variety of ^{99m}Tc based radiopharmaceuticals have been developed and approved by the US Food and Drug Administration (FDA) for determining organ function or assessing disease status by scintigraphic imaging.[20]

^{99m}Tc is obtained from the ^{99m}Mo/^{99m}Tc generator as $^{99m}NaTcO_4$ (15). In order to obtain a high yield of radiolabeling, reduction of $^{99m}TcO_4^-$ ion is essential, since, there is no effective procedure available for attaching a pertechnetate ion to an organic moiety.[21] Stannous chloride is one of the most powerful reducing agents available, and it is widely used for radiopharmaceutical preparation. The amount of stannous chloride is empirically optimized for each formulation with ^{99m}Tc, maintaining the balance of radioactivity added.[18,22] We tested various amounts of $SnCl_2.2H_2O$ and optimized labeling was achieved when the reactions were carried out with 3 mg of $SnCl_2.2H_2O$. Regarding the incubation time, better radiolabeling yield was obtained at 30 min. The binding of ^{99m}Tc atoms with the yeast remained stable and detectable for at least 24 h, which is in accordance with the findings of Diniz et al. (1999), who demonstrated that ^{99m}Tc-*E. coli* remained stable up to 36 h. These results corroborate the successful radiolabeling of *C. gattii* without affecting cell viability.

Intratracheal inoculation with *C. gattii* provides an excellent model of human pulmonary cryptococcosis, mimicking natural infection.[8,9,11] Inoculation through the tail vein in mice has also been commonly used. Since i.v. inoculation bypasses the respiratory route, it is especially useful in elucidating determinants of pulmonary *versus* CNS infection.[23] The detection of fungal burden in the brain can be limited due to technical limitations,[24] and previous animal studies report conflicts between cfu and histology results.[25]

Using the plating method, *C. gattii* was not detected in the brain homogenates of mice infected by i.t. routes within

early hours of inoculation. Detection of *C. gattii* as determined by cfu was observed in the brain only 24 h postinfection. On the other hand, when the radioactivity counting was employed, the results showed radioactivity uptake by brain at 6 h for both infection routes, suggesting fungal dissemination and indicating that ^{99m}Tc-*C. gattii* was able to cross the blood brain barrier. Even at 1 h, as assessed in the biodistribution assay, the complex was detected in the brain of mice infected by i.v. route. Although absolute uptake was lower in the brain, the %ID/g revealed that sufficient number of the yeast cells were available to cross the blood-brain barrier and establish fatal meningoencephalitis upon i.v. inoculation.[23] This enabled efficient labeling of *C. gattii* for effective detection of fungal dissemination.

The results also showed that the ^{99m}Tc-*C. gattii* constitute a simple and rapid method for studies involving fungal dissemination at early phases of cryptococcosis. It can circumvent the limitations of commonly used methods, that is, detection based on plating and histopathology, and can improve the chances of gaining crucial information regarding yeast virulence, host-pathogen interactions, or the efficacy of antifungals.

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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.

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