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Pioglitazone as an adjuvant of amphotericin B for the treatment of cryptococcosis

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ABSTRACT

Approximately 180,000 people worldwide die from cryptococcosis each year, probably due to the ineffectiveness and toxicity of drugs currently available to treat the disease. Amphotericin B (AMB) is effective for killing the fungus, but has serious adverse effects linked to excessive production of reactive oxygen species which compromise renal function. Pioglitazone (PIO) is a peroxisome proliferator-activated receptor-γ agonist widely repositioned as an adjuvant of various drugs that have toxic effects due to its antioxidant and anti-inflammatory effects. This study evaluated PIO in combination with AMB for the treatment of cryptococcosis. PIO was found to reduce serum creatinine and glutamic-oxalacetic transaminase levels in mice treated with PIO+AMB. *In vitro*, PIO was able to control harmful oxidative bursts induced by AMB without compromising the antifungal effect. *In vivo*, PIO+AMB increased the survival rate compared with AMB alone, and improved the morbidity of the animals. PIO+AMB was more efficient than AMB alone for inhibiting fungal transmigration from the lungs to the brain, and killing yeasts that reached the central nervous system, avoiding the establishment of meningoencephalitis. In a phagocytosis assay, PIO did not influence the engulfment and fungicidal activity of macrophages induced by AMB, but reduced the oxidative bursts after the reduction of fungal burden, pointing to control of the pathogen without leading to excessive stress which can be damaging to the host. In conclusion, PIO+AMB was found to ameliorate cryptococcosis in a murine model, indicating that it is a promising therapeutic adjuvant for combating and controlling this fungal infection.

1. Introduction

Treatment for cryptococcosis is restricted to use of the antifungals fluconazole (FLZ), amphotericin B (AMB) and 5-flucytosine (5-FC) [1]. A combination of AMB+5-FC is recognised as the 'gold standard' therapy [2]. However, there are serious issues related to this small therapeutic arsenal, including fungal resistance to FLZ, toxicity of AMB to the host, and unavailability of 5-FC in many countries. These factors limit the anticryptococcal therapeutic options [1–3], leading to a mortality rate nearing 80% [4].

As such, the search for new effective drugs to control cryptococcosis is crucial. In recent years, drug repositioning has emerged as an interesting alternative to establishing new, fast and affordable therapies [5,6]. To treat fungal infections, a given drug can be used on its own or in combination with other standard antifungal drugs [6–8]. The repurposed drug may target the micro-organism or the host [6,8,9], and reduce the toxicity of AMB.

Pioglitazone (PIO), a peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist, was originally prescribed to control blood sugar [10]. However, some reports have described its use in the treatment of cerebral malaria [9,11]. Due to its immunomodulatory action, PIO prevents exacerbated inflammations [9,10,12], and has been tested as an anti-inflammatory, neuroprotective and neuroregenerative agent in models of brain injury, stroke and ischaemia [9]. Recently, PIO has been used in patients with acute myelogenous leukaemia [13] and brain tumours [14] to reduce

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the toxicity of cancer treatment, to reduce symptoms of autism in children [15], and to reduce the toxicity of gentamicin [16].

The aim of this study was to evaluate the effect of PIO in an experimental model of cryptococcosis. In summary, PIO reduced renal toxicity caused by AMB, and PIO+AMB increased the survival rate, diminished morbidity and inhibited fungal growth in the central nervous system.

2. Methods

2.1. Ethics and effects of PIO on creatinine and glutamic-oxalacetic transaminase levels of mice

The protocol for animal experimentation was approved by the Comissão de Ética no Uso de Animais at Universidade Federal de Minas Gerais, Brazil (Protocol 366/2013). The animal experiments were conducted in strict accordance with Brazilian Federal Law 11,794. All mice were housed in clean bedding (five mice per cage), with food and water *ad libitum*, in a controlled environment at 23°C with a 12-h light/dark cycle. C57BL/6 male mice (aged 6–8 weeks) were used in all procedures.

For quantification of serum creatinine and glutamic-oxalacetic transaminase (GOT) levels, non-infected (NI) mice (six per group) were treated daily for 15 days with PIO (6.2 mg/kg/day, corresponding to 30 mg/day dose in humans [17]), AMB (2.0 mg/kg/day) or PIO+AMB (6.2 and 2.0 mg/kg/day, respectively). Serum was collected for evaluation of creatinine and GOT levels in accordance with the manufacturer's instructions (Bioclin, Belo Horizonte, Brazil).

2.2. In-vitro susceptibility of Cryptococcus gattii strains to PIO

Thirteen *C. gattii* strains were tested (clinical, environmental and reference isolates provided by the Mycology Laboratory, Universidade Federal de Minas Gerais, Brazil). They were used to determine the minimum inhibitory concentration (MIC), according to the method of the Clinical and Laboratory Standards Institute [18]. The inoculum was prepared to obtain 1×10^3 to 5×10^3 colony-forming units (CFU)/mL. The MICs of PIO (Amphora, Belo Horizonte, MG, Brazil) and AMB (Sigma-Aldrich, St. Louis, MO, USA) were determined visually at 100% growth inhibition. The combination of PIO and AMB was evaluated using the chequerboard microdilution method, and assessed quantitatively by determining the fractional inhibitory concentration index (FICI). The association was classified as synergy (FICI $\leq$0.5), antagonism (FICI $>$4.0) or indifferent (FICI$>$0.5 and $\leq$4.0).

2.3. Quantification of reactive oxygen species and peroxynitrite production

The endogenous reactive oxygen species (ROS) and peroxynitrite (PRN) were determined inside yeast cells by fluorometric assay [19]. Cultures with 1.0×10^5 cells/mL of *C. gattii* strain L27/01 (UFMG-M-Y6141) were treated with PIO (32 μg/mL), AMB (0.03 μg/mL), or PIO (32 μg/mL) + AMB (0.03 μg/mL) in an RPMI-1640 medium without phenol red. Next, they were incubated in 2′,7′-dichlorofluorescein diacetate at 10 mM (Invitrogen, Life Technologies, Carlsbad, CA, USA) for ROS quantification, or in dihydrorhodamine 123 at 20 mM (Invitrogen) for PRN quantification.

Fluorescence was measured with a fluorometer (Synergy 2 SL Luminescence Microplate Reader; Biotek, Winooski, VT, USA) at the excitation and emission wavelength of 500 nm. After 3 h and 24 h, 50 μL of each suspension was plated on Saboraud dextrose agar (SDA) for CFU determination. Data were expressed as the quotient of arbitrary fluorescence units (AU) and CFU (AU/CFU).

2.4. Animal protocol

2.4.1. Intratracheal infection

Mice were anaesthetised with an intraperitoneal (i.p.) injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Next, they were inoculated, via an intratracheal (i.t.) injection, with 30 μL *C. gattii* strain L27/01 (UFMGM- Y6141) at 10^4 CFU per animal, or with PBS (NI group).

2.4.2. Survival curve and behavioural analysis

After infection, mice were divided into five groups. The treatments started 24 h after infection, via i.p. injections once daily, as follows: (1) PIO (6.2 mg/kg); (2) AMB (0.5 mg/kg); (3) PIO (6.2 mg/kg) + AMB (0.5 mg/kg); (4) non-treated (NT); and (5) NI. The glycaemic index was determined 1 day before infection, and 7 and 15 days after infection.

The behavioural and functional checking for neurological diseases was conducted according to the SmithKline/Harwell/Imperial College/Royal Hospital/Phenotype Assessment (SHIRPA) protocol [6]. Mice were monitored daily to evaluate five functional categories: neuropsychiatric state, motor behaviour, autonomous function, muscle tone and muscular strength, and reflex and sensorial function. The score of each functional category was calculated as the sum of the parameters evaluated, and was analysed using EpiData 3.1. All groups were compared with the NI group; better results were closer to the results from the NI group. The experiments were performed in triplicate and all results were reproducible.

2.4.3. Determination of fungal burden in mouse lungs and brain, and cell counting in bronchoalveolar lavage fluid

Other groups of mice were infected, treated and killed 15 days post infection (d.p.i.), and lungs and brains were removed. The euthanasia time-point was determined after analysis of the survival curve and the SHIRPA protocol. After 15 days of infection, all of the animals were still alive (which made it possible to establish a comparison between the groups), and the symptoms of cryptococcosis were evidenced by the behaviour analysis. The organ homogenates were plated on SDA for 48 h at 35°C to determine the fungal burden (CFU/g tissue). Fungal recovery kinetics from the brain were performed for mice treated with PIO+AMB at 1, 3, 7 and 15 d.p.i. to verify whether PIO+AMB prevents fungal dissemination from lungs and/or if it acts by killing *C. gattii* in the central nervous system. In addition, bronchoalveolar lavage fluid (BAL) was obtained for CFU quantification and differential counting of neutrophils and mononuclear cells.

2.4.4. Histopathological analysis

Lungs were fixed in a 10% buffered formalin solution, embedded in paraffin, sliced into 4-μm-thick sections and stained with haematoxylin-eosin. The histopathological alterations (inflammation, necrosis and haemorrhage) were then observed.

2.5. Phagocytosis assay and intracellular proliferation rate

Bone-marrow-derived macrophages (BMDMs) were obtained [20] and 2×10^5 BMDMs/mL (in RPMI medium supplemented with 10% fetal bovine serum) were distributed on to 24-well plates and incubated overnight at 37°C (with 5% CO_2). Afterwards, BMDMs were infected with yeast cells (4×10^4 cells/mL) and treated with PIO (32 μg/mL), AMB (0.03 μg/mL), or PIO (32 μg/mL) + AMB (0.03 μg/mL). The phagocytic index (PI) was determined by counting internalised yeasts with at least 100 BMDMs after 24 h. For the intracellular proliferation rate (IPR) assay, the supernatant of the cell culture was removed, and non-internalised and adherent yeast cells were removed from the well plates by washing with 200 μL

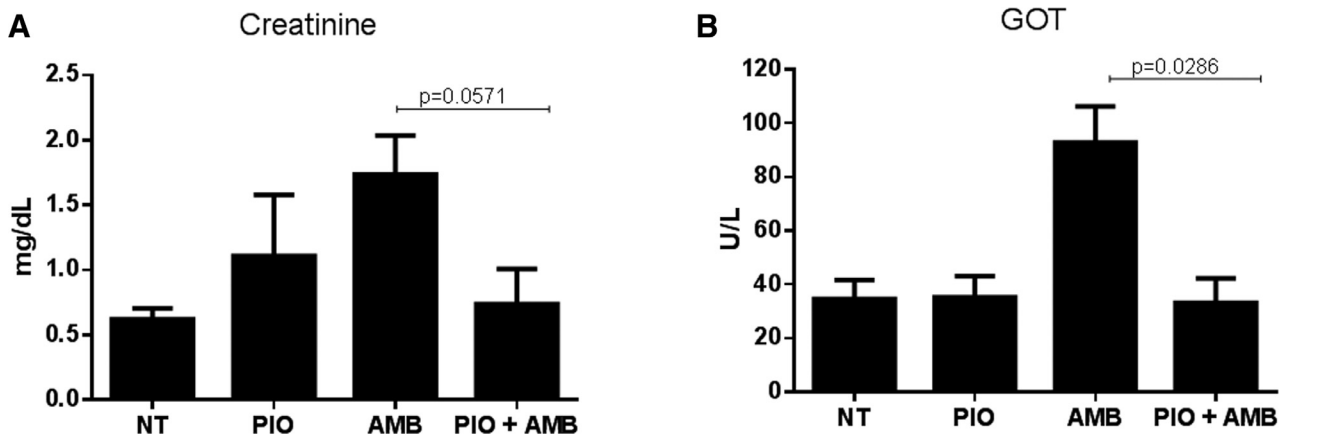

Fig. 1. Levels of serum creatinine (mg/dL) (A) and glutamic-oxalacetic transaminase (GOT) (U/L) (B) in mice treated daily with pioglitazone (PIO), amphotericin B (AMB) or PIO+AMB. NT, untreated.

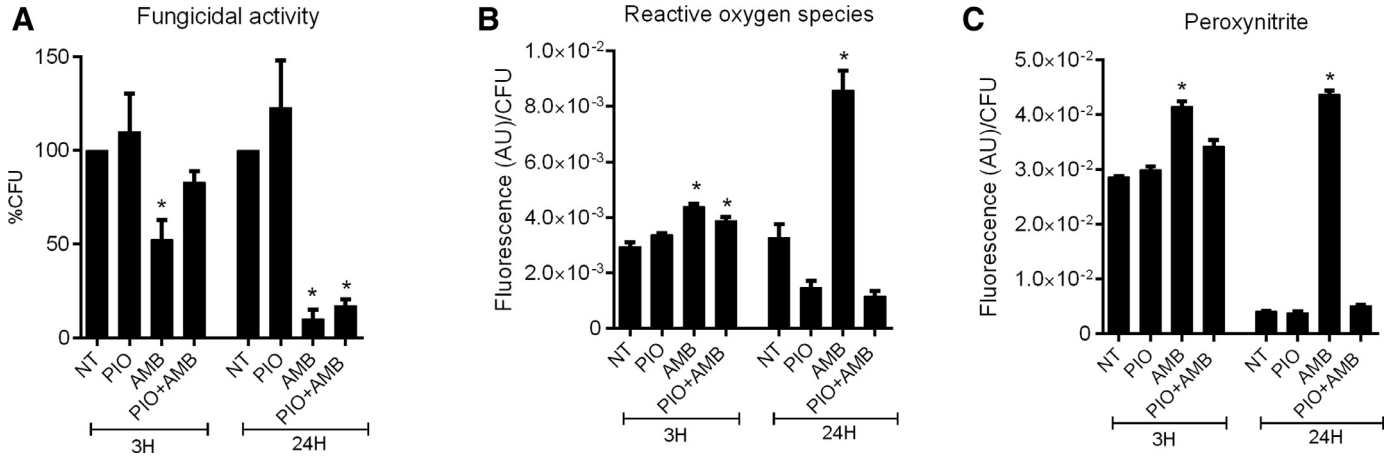

Fig. 2. Pioglitazone (PIO) reduces oxidative and nitrosative bursts caused by amphotericin B (AMB). (A) Fungicidal activity (%). (B) Quotient of reactive oxygen species (ROS) and colony-forming units (CFU) [arbitrary units (AU)/CFU]. (C) Quotient of peroxynintrite and CFU. NT, untreated. $^{*}P<0.05$.

PBS. Next, macrophages were lysed with 200 µL sterile distilled water for 30 min at 37°C. Lastly, 200 µL was collected and plated on SDA for CFU determination. IPR was calculated as the quotient of CFU/PI [6]. Also, the amount of ROS was determined in all groups tested, as mentioned above, and the results were expressed as the quotient between AU and CFU (AU/CFU).

2.6. Statistical analyses

All statistical analyses were undertaken using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA), with $P<0.05$ considered to indicate statistical significance. The survival curve was plotted as indicated by the Kaplan–Meier method, and the outcome was assessed by the log-rank test. Results of ROS, PRN, phagocytosis, IPR and CFU were analysed by analysis of variance (ANOVA) and the non-parametric Friedman test. SHIRPA data were evaluated using area under the curve, ANOVA and Tukey's test.

3. Results

3.1. PIO reduces serum creatinine and GOT levels

AMB alone increased the serum levels of creatinine (Fig. 1A) and GOT (Fig. 1B). The combination of PIO+AMB reduced the levels of these two markers.

3.2. PIO reduces oxidative and nitrosative bursts caused by AMB

PIO did not demonstrate antifungal activity, with MIC values >256 µg/mL (data not shown). Also, the activity of PIO+AMB was classified as indifferent (data not shown). Only AMB was able to inhibit growth of strain L27/01 after 3 h of incubation. On the other hand, AMB and PIO+AMB inhibited growth after 24 h (Fig. 2A), demonstrating that PIO did not compromise the antifungal effect of AMB. The levels of ROS increased after 3 h of treatment, but reduced after 24 h in groups treated with PIO and PIO+AMB (Fig. 2B). The levels of PRN increased after 3 h and 24 h in the groups treated with AMB (Fig. 2C).

3.3. PIO+AMB increases the survival of mice and reduces the morbidity of cryptococcosis

Serum glucose levels were not altered by PIO (data not shown). Survival of the NT and PIO groups was similar, averaging 35 and 34 days, respectively (Fig. 3A). The survival of mice treated with AMB alone was 47 days. It is worth noting that mice treated with PIO+AMB only started to die after 80 days, when all the other groups had already succumbed (Fig. 3A). The protocol finished after 120 days, with an 80% survival rate of mice treated with PIO+AMB.

The SHIRPA protocol was performed to verify behavioural changes which may have been caused by cryptococcosis or by toxic

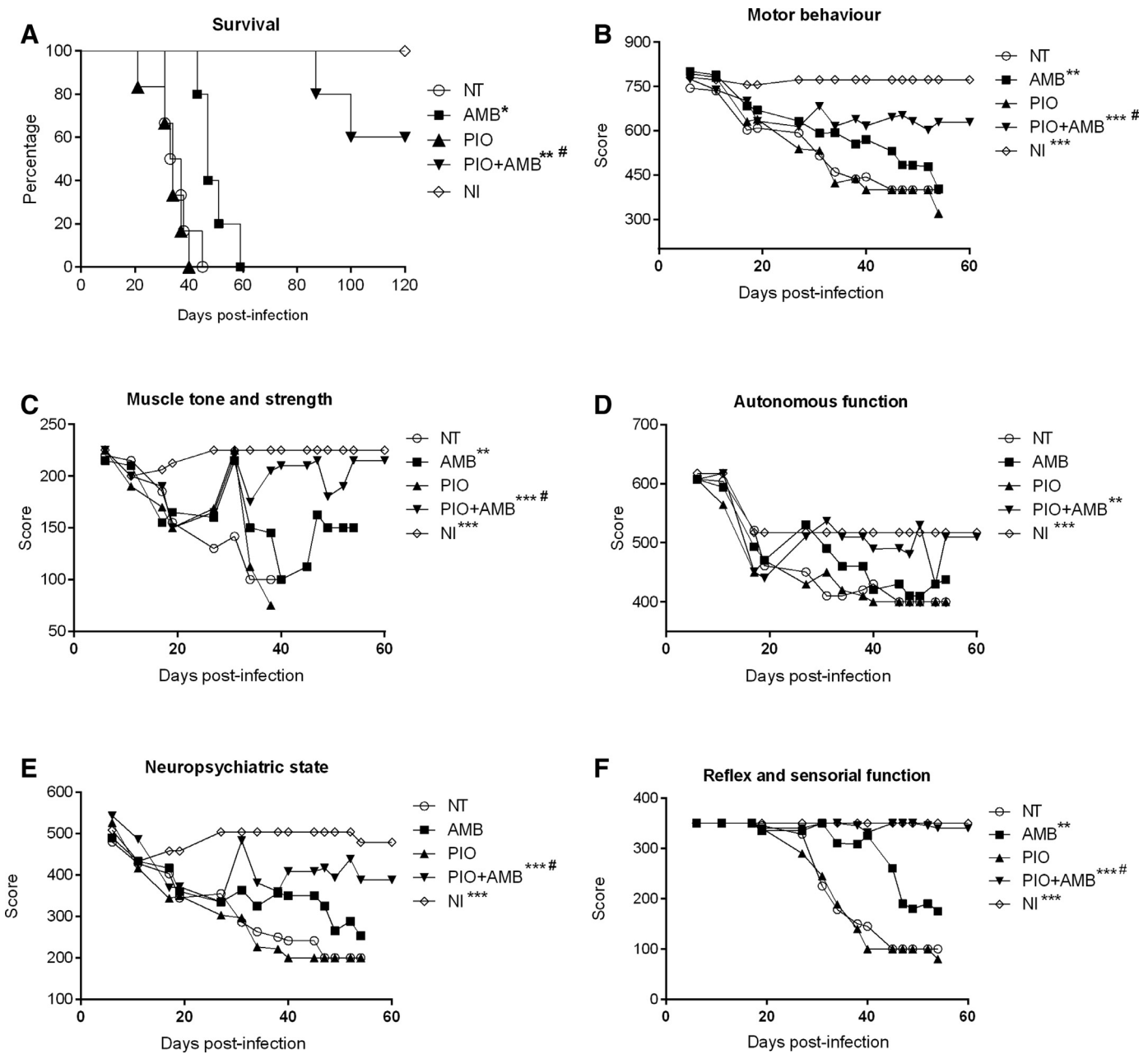

Fig. 3. Pioglitazone (PIO) in combination with amphotericin (AMB) increases survival of mice and reduces morbidity of cryptococcosis. (A) Survival curve of mice inoculated with *Cryptococcus gattii* and treated daily with PIO (6.2 mg/kg), AMB (0.5 mg/kg), or PIO (6.2 mg/kg)+AMB (0.5 mg/kg). The same groups were evaluated for behaviour: (B) motor behavior, (C) muscle tone and strength, (D) autonomous function, (E) neuropsychiatric state, and (F) reflex and sensory function. NI, uninfected; NT, untreated. $^{**}P<0.01$, $^{***}P<0.001$ (difference compared with NT); $^{\#}P<0.05$ (difference between PIO+AMB and AMB alone).

effects of AMB. All groups were analysed in comparison with the NI group (results closer to those of this group indicated better performance in the SHIRPA protocol). The scores are presented in Fig. 3B–F. The values obtained from the PIO+AMB group were close to those of the NI group for all analysed parameters, meaning that the combination reduced behavioural cryptococcosis-related alterations or drug-related symptoms compared with the NT and PIO groups. Compared with the AMB group, PIO+AMB performed considerably better in four categories. Indeed, animals treated with AMB behaved similarly to those in the NT group in the functional categories 'neuropsychiatric state' (Fig. 3D) and 'autonomous function' (Fig. 3E), reflecting the inability to cure cryptococcosis and AMB toxicity.

3.4. PIO+AMB decreases the fungal burden in mouse lungs and brain

All treatments reduced the fungal burden in BAL in comparison with the NT group (Fig. 4A). Indeed, a high number of mononuclear cells was found in the PIO+AMB and AMB groups (being higher in the PIO+AMB group), whereas polymorphonucleated cells increased in both the PIO and NT groups (Fig. 4B). AMB and PIO+AMB reduced the fungal burden in the lungs in comparison with the NT and PIO groups (Fig. 4C). Interestingly, fungal burden was not detected in the brain of mice treated with PIO+AMB 15 d.p.i., which differed from the results for AMB (Fig. 4D). Following these findings, fungal recovery kinetics from the brain were performed for mice treated with PIO+AMB at 1, 3, 7 and 15 d.p.i. to

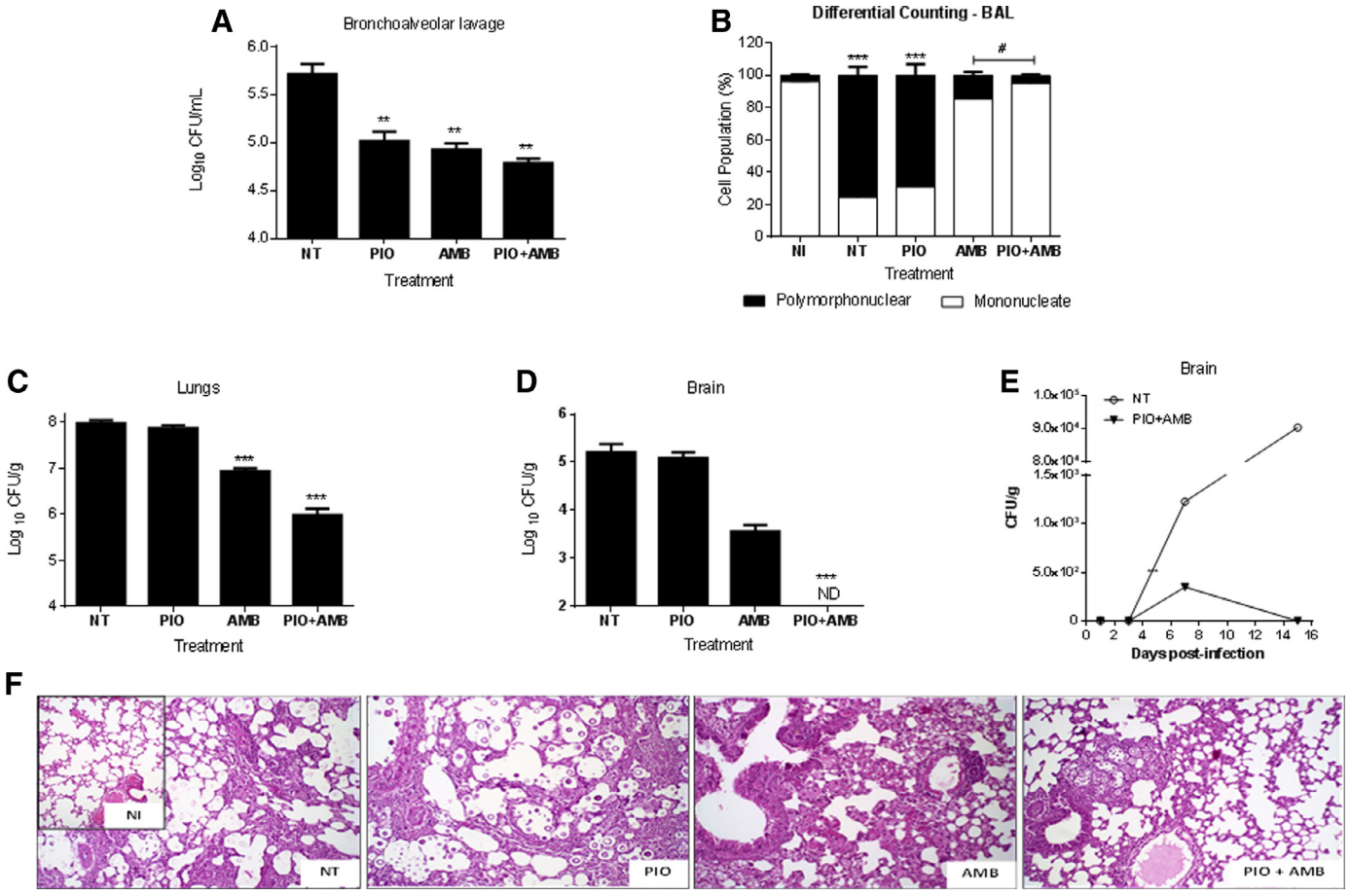

Fig. 4. Pioglitazone (PIO) plus amphotericin (AMB) decreases fungal burden in the lungs and brain, and reduces inflammation in the bronchoalveolar lavage fluid (BAL) and lungs. Mice were inoculated with *Cryptococcus gattii*, treated with AMB, PIO or PIO+AMB and killed 15 days post infection. (A) Colony forming units (CFU) per mL of BAL. (B) Differential cell counting in BAL. (C) CFU per gram of lungs. (D) CFU per gram of brain. (E) Histopathology demonstrating that uninfected (NI) group does not have significant microscopic changes. Untreated (NT) mice showed moderate yeasts and moderate multifocal inflammation. Pulmonary inflammation was more evident in mice infected and treated with PIO than the AMB or PIO+AMB groups. Foam macrophages are well evidenced in the pulmonary parenchyma of mice treated with AMB. $^{**}P<0.01$, $^{***}P<0.001$, $^{\#}P<0.05$ (difference between PIO+AMB and AMB alone).

verify whether PIO+AMB prevents fungal dissemination from the lungs and/or if it acts by killing *C. gattii* in the central nervous system. Yeasts were detected in the brain at 7 d.p.i. but not at 15 d.p.i. (Fig. 4E), revealing that the fungus is able to reach the central nervous system at lower levels but the treatment is able to inactivate *C. gattii* in this organ.

3.5. Histopathology

The histopathological results are presented in Fig. 4F. No change was observed in the lungs of NI mice. On the other hand, mice in the infected group exhibited a moderate multifocal amount of extracellular yeasts in the alveolar and bronchial lumen, associated with moderate peribronchial mixed-inflammatory infiltrate, composed of both macrophages and neutrophils. Infected mice treated with PIO showed a moderate-to-intense multifocal amount of extracellular yeasts in the alveolar and bronchial lumen, associated with moderate perivascular and peribronchial inflammatory infiltrate containing macrophages and neutrophils. Moderate multifocal alveolar thickening due to the mixed-inflammatory infiltrate was also found in the NT and PIO groups.

Mice treated with AMB and PIO+AMB showed a discrete multifocal amount of extracellular yeasts in the alveolar lumen, and little multifocal alveolar and peribronchial mixed-inflammatory infiltrate. Foamy macrophages were observed in a moderate amount in lung parenchyma.

3.6. PIO controls excessive oxidative bursts inside macrophages and did not influence the antifungal activity of AMB

Considering the importance of the trojan horse mechanism for *Cryptococcus* spp. to reach the central nervous system, the increased population of mononuclear cells in BAL and the absence of fungi in the brain at 15 d.p.i. in mice treated with PIO+AMB, the interaction between the fungus and macrophages treated with the different regimens was investigated further. Phagocytosis indices were not influenced by the treatments (Fig. 5A). The fungal proliferation inside the macrophages only reduced in the AMB and PIO+AMB groups, suggesting that AMB is responsible for killing the fungus inside macrophages (Fig. 5B). Interestingly, ROS levels remained high despite the reduction in fungal burden in the AMB group. PIO was able to control the harmful oxidative burst induced by AMB (Fig. 5C) inside the macrophages in the PIO+AMB group. The Fig. 6 summarizes all the results from this study.

4. Discussion

In order to cure cryptococcosis, the treatment must kill the fungus with minimal toxic effects for patients; however, this remains a major challenge as the drugs currently available have high toxicity, high cost and, in some cases, low efficacy [1]. AMB is an effective antifungal; however, it has high nephrotoxicity due to vasoconstriction of afferent arterioles, and reduction of renal blood

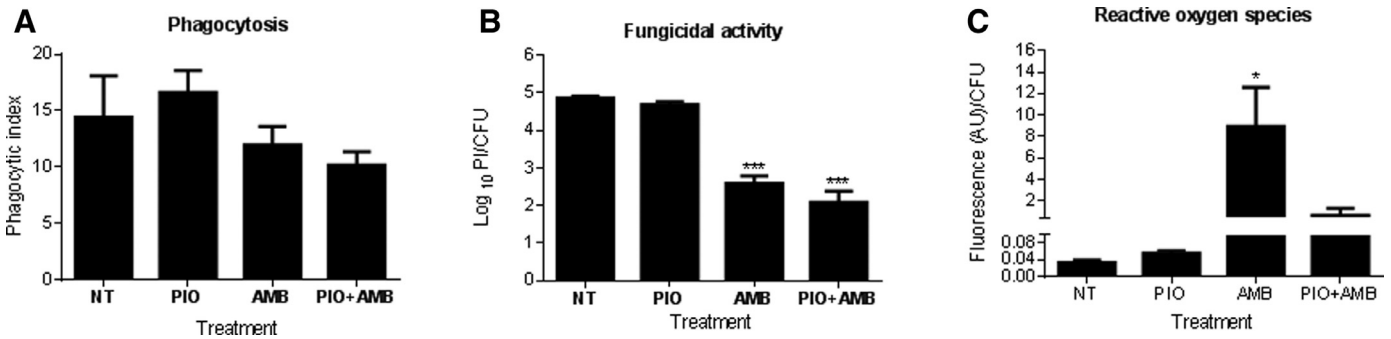

Fig. 5. Phagocytosis assay, intracellular proliferation rate and reactive oxygen species (ROS). (A) Phagocytosis indexes (PI) after 24 h. (B) IPR [log colony-forming units (CFU)/PI]. (C) Quotient of ROS and CFU recovered during the phagocytosis assay (arbitrary units/CFU). NT, untreated; PIO, pioglitazone. $^{**}P<0.05$, $^{***}P<0.001$.

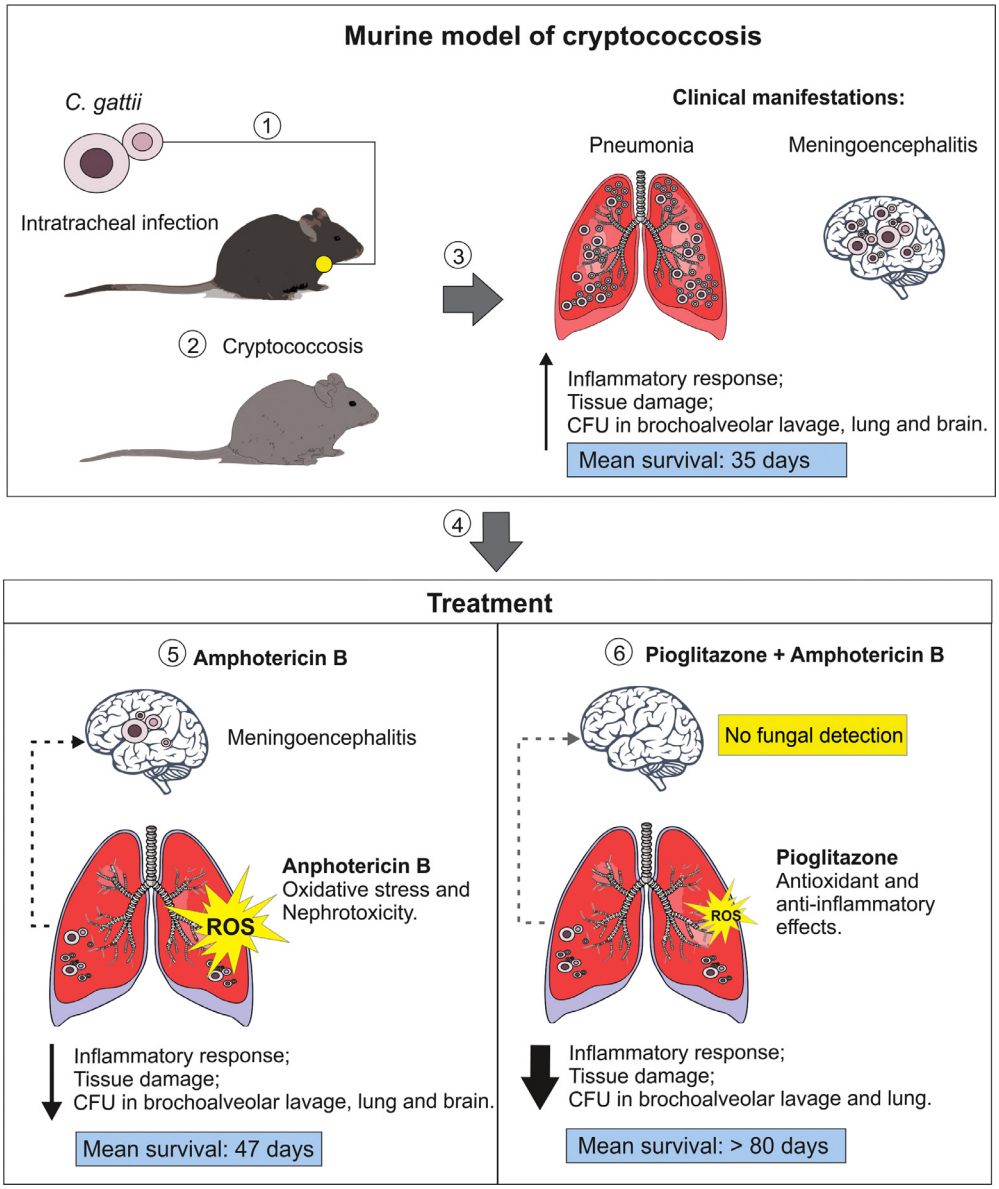

Fig. 6. (1) C57/BL6 mice were infected with *Cryptococcus gattii*. (2) After infection, the mice were evaluated daily through the SHIRPA protocol for analysis of morbidity. (3) Behavioural changes due to pneumonia and meningoencephalitis were observed. (4) Infected mice were treated with amphotericin B (AMB), pioglitazone (PIO)+AMB, or AMB alone. (5) Treatment with AMB alone reduced the inflammatory infiltrate in the lungs and the fungal burden in bronchoalveolar lavage fluid (BAL), lungs and brain. However, treatment with AMB alone is associated with the occurrence of high oxidative stress [reactive oxygen species (ROS)], which causes toxicity and may influence the observed lethality. The black dashed arrow indicates that there is transmigration to the central nervous system (CNS). (6) Treatment with PIO+AMB reduced ROS (induced by AMB), inflammatory infiltrate and fungal burden in BAL and lungs. Interestingly, the yeast was not detected in the CNS after 15 days of treatment. The grey dashed arrow indicates that there is transmigration to the CNS, but PIO+AMB was able to eliminate the fungus.

flow and glomerular filtration. Increased ROS, lipid peroxidation and death of kidney cells occur as a consequence of hypoxia and ischaemia, being an important nephrotoxic mechanism of AMB [21]. In addition, AMB induces an uncontrolled host response called 'immune reconstitution inflammatory syndrome', which has a high death rate [4,22–25]. Otherwise, AMB induces oxidative and nitrosative stresses in fungal cells which are important for fungal killing [19,26].

Therefore, it is important to find: (i) new antifungals to be used alone or in combination with AMB or fluconazole; and (ii) drugs that potentiate AMB activity by stimulating the host response and/or reducing its toxicity. Due to the unreasonable costs and the long time required for the validation of new drugs against *Cryptococcus* spp., repurposing traditional drugs can be an interesting alternative [7,27,28]. Repositioned drugs may have antifungal or immunomodulatory effects when combined with lower AMB, resulting in effective and low-toxicity treatment [1,6].

This study aimed to repurpose PIO (a PPARγ agonist used to control glucose homeostasis and lipid metabolism) as an adjuvant for the treatment of cryptococcosis. PIO has an important role in adaptive immunity, regulates gene expression involved in inflammation [12,29,30], and modulates the redox balance by upregulating the transcription of antioxidant-related genes and inhibiting the generation of ROS [31,32]. The first result (serum creatinine and GOT) shows that PIO is able to reduce toxicity caused by AMB, and the host would probably be more able to fight the fungus. Although the in-vitro MIC results demonstrated that PIO itself and in combination has no antifungal effect against *C. gattii*, it was found to influence the amount of ROS and PRN produced by AMB. ROS and PRN increase within the first few hours, but reduce after 24 h, corroborating that PIO allows AMB to kill the fungus at an early stage, returning ROS and PRN to basal levels after 24 h. These results encouraged the authors to test PIO in combination with AMB to treat murine cryptococcosis.

With a survival rate of 80% at the end of the protocol, PIO+AMB was more successful in increasing the survival of mice than the other treatments. In addition, animals also showed reduced morbidity as they behaved better in the SHIRPA protocol. These results are certainly a consequence of the absence of fungus in the central nervous system, as the signs of meningoencephalitis were more evident in the NT, PIO and AMB groups. PIO+AMB may have acted by reducing fungal translocation from the lungs to the brain and by killing yeasts that had already reached the central nervous system, avoiding the establishment of meningoencephalitis and allowing increased survival. Histological analysis of the lungs and quantification of inflammatory mediators showed similarities between animals treated with AMB and animals treated with PIO+AMB, confirming that PIO does not impair the important antifungal action of AMB.

Further, the difference in the population of mononuclear and polymorphonuclear cells in the BAL of mice should be noted. PIO+AMB led to a greater population of mononuclear cells compared with AMB alone. *Cryptococcus* spp. are able to 'take a ride' within macrophages to reach the central nervous system in a mechanism called a 'trojan horse' [33]. In order to prevent this, macrophages have to be efficient at engulfing, producing ROS and killing the fungus. As mentioned, AMB is able to increase ROS production, which would be a beneficial effect for the macrophage to kill the fungus, but excessive ROS leads to inflammation and toxicity. Although there were no differences in the ability to engulf and kill the fungus between macrophages treated with AMB and PIO+AMB, only PIO+AMB treatment was able to reduce oxidative stress after antifungal action, suggesting that PIO avoided the harmful effects of excessive ROS production, returning to a condition close to homeostasis.

Overall, these data corroborate the findings of other researchers who have demonstrated that PIO may increase the effectiveness of different treatments by reducing the toxicity of radiotherapy or drugs (e.g. capecitabine, temozolomide, cytarabine, daunorubicin and gentamicin) that are known to be toxic to eukaryotic cells [13,14,16].

In conclusion, this study suggests that PIO+AMB has therapeutic potential for controlling and combating cryptococcosis.

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Competing interests

None declared.

Ethical approval

Comissão de Ética no Uso de Animais, Universidade Federal de Minas Gerais, Brazil (Protocol 366/2013).

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.06.020.

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