Synergetic action of atorvastatin and fluconazole against fluconazole-resistant *Candida albicans in vitro* and in a murine model for intra-abdominal Candidiasis

Ação sinérgica da atorvastatina e fluconazol contra *Candida albicans* resistente ao fluconazol *in vitro* e em um modelo murino contra Candidíase intra-abdominal

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ABSTRACT
Introduction: *Candida albican*s is the most common causative agent of Intra-abdominal Candidiasis (IAC) and it is resistant to most antifungal drugs currently available. Here we investigated atorvastatin *in vitro* and *in vivo* antifungal activities against a fluconazole-resistant *C. albicans* strain as a potential repurposed drug. The following tests were carried out: antifungal susceptibility tests to determine minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC), determination of time-kill curve, biofilm assays, *Candida albicans* yeast-hyphae transition inhibition assay, murine model

of Intra-abdominal candidiasis, survival curve, fungal load quantification, histopathology analysis, quantification of TNF-α and IL-17 cytokines, quantification of N-acetyl-β-D-glucosaminidase. *In vitro* assays showed the synergetic action of atorvastatin and fluconazole against *C. albicans* growth and biofilm maturation while the time-kill curve assay revealed their fungicidal effect after 24 h of treatment. When yeast-to-hyphae transition was assessed, the synergetic effect of atorvastatin and fluconazole reduced *C. albicans* filamentation significantly. *In vivo* tests showed that one of the most noticeable signs of IAC is the intense systemic inflammation. However, our survival curve test showed that despite being ill, animals exhibited little to no clinical signs of systemic inflammation when treatment included a combination of atorvastatin and fluconazole. Altogether, these findings suggest that atorvastatin could be feasibly used in the treatment fluconazole-resistant *C. albicans* strains, showing that drug repurposing is an important strategy when considering the limited number of antifungal drugs available for treatment in addition to financial hardship experienced in research and development of new antifungal drugs.

Keywords: atorvastatin, *Candida albicans*, drug repurposing, fluconazole, resistance, synergism.

RESUMO

Introdução: A Candida albicans é o agente causador mais comum da Candidiase Intra-abdominal (IAC) e é resistente à maioria dos medicamentos antifúngicos actualmente disponíveis. Aqui investigámos as actividades de atorvastatina in vitro e antifúngicas in vivo contra uma estirpe de C. albicans resistente ao fluconazol como potencial medicamento repursivo. Foram efectuados os seguintes testes: testes de susceptibilidade a antifúngicos para determinar a concentração inibitória mínima (MIC) e a concentração fungicida mínima (MFC), determinação da curva tempo-queda, ensaios de biofilme, ensaio de inibição da transição de Candida albicans-hipfae, modelo murino de candidíase intra-abdominal, curva de sobrevivência, quantificação da carga fúngica, análise histopatológica, quantificação de citocinas TNF-α e IL-17, quantificação de N-acetil-β-D-glucosaminidase. Os ensaios in vitro mostraram a acção sinergética da atorvastatina e fluconazol contra o crescimento de C. albicans e a maturação do biofilme, enquanto que o ensaio da curva tempo-queda revelou o seu efeito fungicida após 24 h de tratamento. Quando a transição de levedura para hifa foi avaliada, o efeito sinergético da atorvastatina e do fluconazol reduziu significativamente a filamentação de C. albicans. Os testes in vivo mostraram que um dos sinais mais notáveis da IAC é a intensa inflamação sistémica. Contudo, o nosso teste de curva de sobrevivência mostrou que, apesar de estarem doentes, os animais apresentavam pouco ou nenhum sinal clínico de inflamação sistémica quando o tratamento incluía uma combinação de atorvastatina e fluconazol. No conjunto, estes resultados sugerem que a atorvastatina poderia ser utilizada no tratamento das estirpes de C. albicans resistentes ao fluconazol, demonstrando que a repreensão de medicamentos é uma estratégia importante quando se considera o número limitado de antifúngicos disponíveis para tratamento, para além das dificuldades financeiras experimentadas na investigação e desenvolvimento de novos antifúngicos.

Palavras-chave: atorvastatina, *Candida albicans*, repurposição de fármacos, fluconazol, resistência, sinergismo.

1 INTRODUCTION

In the systemic forms of fungal infections, the intra-abdominal candidiasis (IAC) is the most common followed by candidemia [1–3]. Patients with gastrointestinal perforations and acute necrotizing pancreatitis also experience IAC in 40% of cases [4]. However, different from other invasive candidiasis, IAC is still poorly understood, making diagnosis, treatment, and clinical management of this infection a challenge to clinicians worldwide [5].

IAC is mainly caused by *C. albicans* since it has been isolated and identified in 70% of cases [6,7]. *C. albican*s is a dimorphic fungus that grows both as yeast and filamentous cells [8]. *C. albicans* polymorphism is not only essential for its pathogenicity but it is also the foremost virulence factor. As a filamentous form, called hyphae, *C. albicans* is described as being more invasive and resistant to the immune response than its yeast form [9,10]. Furthermore, hyphae formed by *C. albicans* is known to have the ability to form biofilms, which is usually resistant to chemical and physical antimicrobial methods and antifungal drugs [11].

Another characteristic that facilitates *C. albicans* adaptation to invasive fungal infections is its ability to acquire resistance to virtually all antifungal drugs currently available. According to a recent CDC study, about 7% of all *Candida* blood samples tested in US were resistant to the antifungal drug fluconazole [12]. Moreover, less than 1% of pharmaceutical companies investments is focused on the development and/or discovery of new antifungal agents [13]. Therefore, repurposing of drugs is the new pharmacological strategy to overcome these concerns [14]. Drug repurposing or repositioning is defined as a strategy to identify new uses for approved/investigational drugs that are outside of the scope of the original medical indication [15]. Nowadays, this strategy has gained more importance against infectious diseases due to reduction of the overall research/development costs, reduction of the speeding process by regulatory drug agencies, and also reduction of drugs price [15]. In fact, many non-antifungal drugs, such as statins which are hypolipidemic agents, have been further studied for their potential antifungal activity [3]. Studies have shown that yeasts of the genera *Candida* and *Cryptococcus* as well as the filamentous fungi *Aspergillus* spp as sensitive to statins *in vitro* and *in vivo*. Furthermore, simvastatin was shown to break *C. albicans* mature biofilm and to have a synergetic action when combined with one of the antifungals drugs fluconazole, ketoconazole, or itraconazole [16]. However, it is still unknown whether fluconazole-resistant *C. albicans* would be sensitive to the synergic action of statins and

azoles *in vivo*. Therefore, this study aimed to investigate atorvastatin potential *in vitro* and *in vivo* antifungal activity against a fluconazole-resistant *C. albicans* strain.

2 MATERIALS AND METHODS

2.1 REAGENTS AND MICROORGANISMS

The following drugs were used in this study: Atorvastatin (Fagron, Brazil), Nystatin (Pharma Nostra, Brazil), Fluconazole (Fagron, Brazil) and Dexamethasone (Teuto, Brazil). *Candida albicans* ATCC 1023, which is resistant to not only fluconazole but also anidulafungin, voriconazole, itraconazole, and ketoconazole, was kindly provided by the Reference Microorganisms Laboratory at the Oswaldo Cruz Foundation (FIOCRUZ; Rio de Janeiro, Brazil) and it was originally obtained from the American Type Culture Collection (ATCC).

2.2 *IN VITRO* ASSAYS

2.2.1 Antifungal susceptibility tests to determine MIC and MFC

Antifungal activity was evaluated by determining the minimum inhibitory concentration (MIC) using the broth microdilution method according to M27-A3 document of the Clinical and Laboratory Standard Institute (CLSI) [17,18], with minor modifications. Briefly, 100 µL of *Candida* inoculums at 10^3 colony forming units (CFU) mL^{-1} were transferred to sterile microplates containing two-fold serial dilutions (1 to 512 $\mu g.mL^{-1}$) of either atorvastatin or fluconazole in Sabouraud-dextrose broth (SDB) (Kasvi, Italy). Nystatin was used as a positive control in both MIC and MFC assays.

2.2.2 Determination of time-kill curve

An anti-*C. albicans* time-kill curve was performed according to [19] in presence or absence of atorvastatin, or in combination with fluconazole. In brief, tubes containing 10 mL of *C. albicans* ATCC 10231 SDB cultures (10^6 CFU/mL^{-1}) were left untreated (negative control) or were treated with atorvastatin either at 32 $\mu g.mL^{-1}$ (MIC) or 128 $\mu g.mL^{-1}$ (MFC). Atorvastatin in combination with fluconazole were tested at their synergetic MFC concentration of 0.25 and 0.01 $\mu g.mL^{-1}$, respectively. Nystatin at 0.25 $\mu g.mL^{-1}$ was used as a positive control. All tubes were incubated at 35±2ºC, and at different time points (0, 2, 4, 6, 10, 12, 24, 36 and 48 h), 0.1 mL aliquots were removed and streaked on SDA plates. After 48 h of incubation at 35±2ºC, SDA plates were evaluated, and yeast colony formation was measured in $CFU.mL^{-1}$.

2.2.3 Biofilm assays

The antifungal action of atorvastatin upon mature biofilm formation of *C. albicans* ATCC 10231 was evaluated either in combination or not with fluconazole using the crystal violet method [20]. Negative controls were performed by leaving biofilms untreated. In the biofilm formation inhibition assay, microplates were incubated at 35±2°C for 48 h in presence of atorvastatin at concentrations ranging from 0.25 to 16 $\mu g.mL^{-1}$, or atorvastatin in combination with fluconazole at the concentrations ranging from 0.125 to 7.81 $\mu g.mL^{-1}$ of atorvastatin and from 0.005 to 0.312 $\mu g.mL^{-1}$ of fluconazole. On the other hand, the mature biofilm reduction assay was performed by growing biofilms in microplates for 48 h at 35±2°C followed by treatment with either atorvastatin ranging from 4 to 512 $\mu g.mL^{-1}$, or atorvastatin in combination with fluconazole at the following concentrations that ranged from 0.25 to 128 $\mu g.mL^{-1}$ of atorvastatin and from 0.01 to 5.12 $\mu g.mL^{-1}$ of fluconazole. Next, microplates were reincubated at 35±2°C for another additional 48 h. Using a microplate reader (Bio-Tek instruments, Winooski, VT) at the optical density (OD) of 550 nm, percentages of biofilm reduction in treated versus untreated groups were calculated by comparing absorbance values. Eight wells were used for each concentration tested.

2.2.4 *Candida albicans* yeast-hyphae transition inhibition assay

A yeast-hyphae transition inhibition assay was performed according to [17] in order to test the antifungal action against *C. albicans* ATCC 10231 of either atorvastatin at 32 $\mu g.mL^{-1}$ (MIC), or fluconazole at 32 $\mu g.mL^{-1}$, or a combination of atorvastatin at 0.25 $\mu g.mL^{-1}$ plus fluconazole at 0.01 $\mu g.mL^{-1}$. For that, hyphal induction was done by culturing *C. albicans* ATCC 10231 (10^3 $CFU.mL^{-1}$) in microplates containing fetal bovine serum (FBS) in addition to either atorvastatin, or fluconazole, or both. Sets of microplates were incubated for 24, 48, and 72 h at 35±2°C and hyphal formation was observed through microscopy (EVOS™ FL Auto Imaging System by Thermo Fisher Scientific). Positive control included cells grown in the presence of FBS and nystatin at 0.25 $\mu g.mL^{-1}$ while negative control had cells induced with FBS but left untreated.

2.3 *IN VIVO* STUDIES

Six 8-week-old female Swiss mice (Biotério Central da UFSJ, São João Del-Rei, MG, Brazil) were used for this study. Animals were kept in 30x19x13 cm polypropylene

boxes under controlled temperature of 25 ± 2°C, 40% humidity, and a 12/12 h light/dark cycle. All experimental procedures strictly followed the international protocols for laboratory animal management, and methods performed in this study were approved by the Laboratory Animal Research Ethics Committee of the Universidade Federal de São João del-Rei (CEUA-UFSJ: 036/2017).

2.3.1 Murine model of Intra-abdominal candidiasis

In order to study drugs' effectiveness in intra-abdominal candidiasis (IAC), a murine model that included immunosuppressed and non-immunosuppressed mice was created according to [21], with modifications. To induce immunosuppression mice received an intraperitoneal (i.p.) injection of dexamethasone (Teuto, Brazil) at a single daily dose of 10 $mg.Kg^{-1}$ for nine consecutive days. On the seventh day, both immunosuppressed and non-immunosuppressed mice were infected intraperitoneally (i.p.) with 0.2 mL of 10^7 cells. mL^{-1} of *C. albicans* ATCC 1023 in saline.

2.3.2 Treatment of animals

Animals were randomly divided into six experimental groups (n = 8) as it follows: NIC - Non-immunosuppressed infected control mice treated with 0.9% saline i.p.; IIC, F4, ATV40, ATV20-F4, and ATV40-F4 - Immunosuppressed with dexamethasone and infected, but IIC group was treated with 0.9% saline i.p.; F-4 was treated with fluconazole at 4 $mg.Kg^{-1}$ i.p.; ATV was treated with atorvastatin at 40 $mg.Kg^{-1}$ i.p.; ATV20-F4 was treated with atorvastatin at 20 $mg.Kg^{-1}$ in combination with fluconazole at 4 $mg.Kg^{-1}$ i.p.; and ATV40-F4 was treated with atorvastatin 40 $mg.Kg^{-1}$ in combination with fluconazole at 4 $mg.Kg^{-1}$ i.p. Treatments were conducted at 2, 24, and 48 h post-infection, and at the appropriate times, mice were then anesthetized and euthanized via cervical dislocation.

2.3.3 Survival curve

To evaluate animals' survival curve, IAC was induced as previously described and mice survival rate was followed for 15 days after the last treatment.

2.3.4 Fungal Load Quantification

Animals' livers were removed aseptically and used to determine fungal load as described. [21], and results were represented as $Log_{10}CFU.g^{-1}$ of liver.

2.4 HISTOPATHOLOGY ANALYSIS

Hematoxylin and eosin (H&E), and examined using light microscopy (EVOS™ FL Auto Imaging System by Thermo Fisher Scientific) [3].

2.5 QUANTIFICATION OF TNF-A AND IL-17 CYTOKINES

TNF-α and IL-17 levels were measured in the liver tissue using a commercially available enzyme-linked immunosorbent assay (ELISA), and following the instructions provided by the manufacturer (DuoSet kits; R&D Systems, Minneapolis, MN). Cytokine levels were represented in picograms (pg) per100 mg of tissue [22].

2.6 QUANTIFICATION OF N-ACETYL-B-D-GLUCOSAMINIDASE

Liver macrophages were quantified according to [23] by measuring the level of the lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAG). Results were obtained using a microplate reader at OD of 405 nm and results were represented as relative number of macrophages per 100 mg of tissue.

2.7 STATISTICAL ANALYSIS

All *in vitro* tests were performed in triplicate after at least three independent experiments. Biofilm reduction percentages in treated groups were calculated compared to the results of untreated biofilms. Six wells were used for each concentration. One-way analysis of variance (ANOVA) followed by Tukey's multiple analysis were used to compare differences between treated and untreated biofilms.

Data that exhibited normal distribution in *in vivo* tests were analyzed using unpaired Student's t test to compare two groups or ANOVA for multiple comparisons followed by Tukey's test. Data that did not show a normal distribution were analyzed by the Kruskal-Wallis test for multiple comparisons using Dunn's post test.

All statistical analyses were done using GraphPad Prism 7.0 (GraphPad Software Inc., LaJolla, CA) and p values < 0.05 were considered statistically significant.

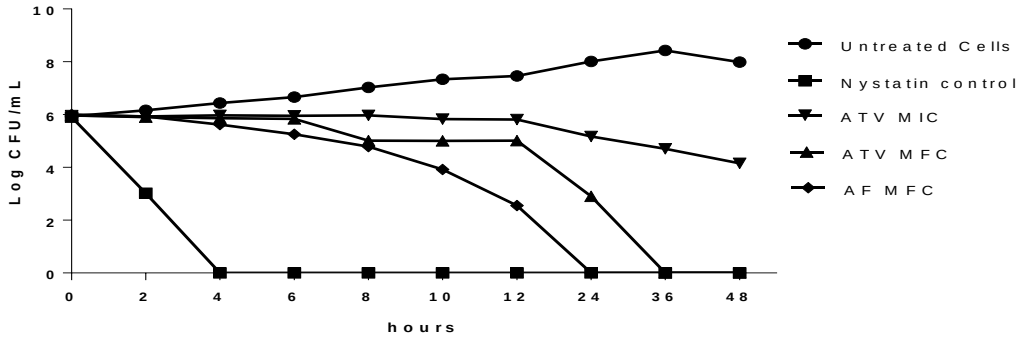
3 RESULTS

At first, the antifungal activity atorvastatin exhibited MIC and MFC concentrations at 32 $\mu g.mL^{-1}$ and 128 $\mu g.mL^{-1}$, respectively, against *C. albicans* ATCC 10231. Nystatin, in turn, was founded to have both fungistatic and fungicidal actions at 0.25 $\mu g\ mL^{-1}$. On the other hand, fluconazole had a MIC > 512 $\mu g.mL^{-1}$, confirming so that C. *albicans* ATCC 1023 is indeed highly resistant to azole drugs. However,

atorvastatin combined with fluconazole at 0.25 and 0.01 μg.mL^{-1} respectively, exhibited a synergetic antifungal action against C. *albicans* ATCC 1023, indicating fluconazole enhanced atorvastatin antifungal action at much lower concentrations than their MICs and MFCs.

Next, the synergetic effect as shown in Figure 1, when *C. albicans* ATCC 10231 was treated with atorvastatin at either 32 μg.mL^{-1} or 128 μg.mL^{-1} (MFC), an approximate 2-3 log reduction in cellular density was observed starting at 24 h of treatment. Furthermore, after 36 hours of treatment with atorvastatin at 128 μg.mL^{-1}, yeast cells were all dead. Our results also revealed that yeast cells treated with both atorvastatin and fluconazole were significantly affected, exhibiting over 3 log reduction in cellular density at 12 h of incubation, and a fungicidal effect after 24 h of treatment when compared to untreated cells. Nystatin at 0.25 μg.mL^{-1} was used as positive control and killed all yeast cells within 4 hours.

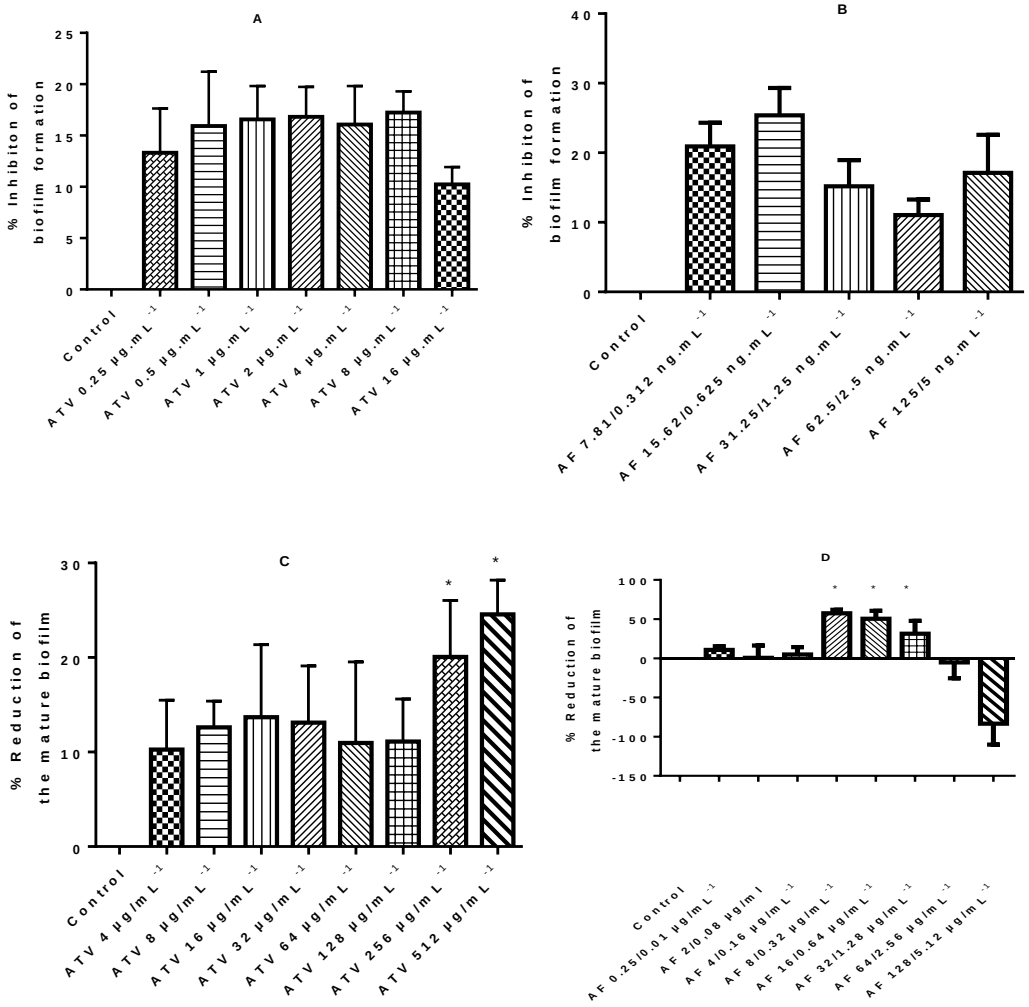
Figure 1: Time-kill curve of atorvastatin or atorvastatin combined with fluconazole against *C. albicans* ATCC 10231. Yeast cells were left either untreated (negative control) or treated with nystatin at 0.25 μg/mL^{-1} (positive control). ATV MIC: atorvastatin at 32 μg.mL^{-1}); ATV MFC: atorvastatin at 128 μg.mL^{-1}; AF MFC: Atorvastatin combined with fluconazole at 0.25 and 0.01 μg.mL^{-1} respectively.



Additionally, we evaluated the antifungal action biofilm formation and maturation. In the biofilm formation assay, we did not observe a significant reduction (Figure 2A) and (Figure 2B). In contrast, maturation of biofilms was significantly affected when biofilms were solely treated with atorvastatin at the concentrations of either 256 μg.mL^{-1} (20% reduction) or 512 μg.mL^{-1} (25% reduction) compared to the untreated control (Figure 2C). Furthermore, biofilm maturation was more affected by the synergistic action of atorvastatin in combination with fluconazole. Our results showed a reduction in biofilm biomass when these drugs were combined at the concentrations of 8 μg.mL^{-1} of atorvastatin and fluconazole at 0.32 μg.mL^{-1} (50% reduction), 16 μg.mL^{-1} of

atorvastatin and fluconazole at 0.64 μg.mL^{-1} (50% reduction), and 32 μg.mL^{-1} of atorvastatin and fluconazole at 1.28 μg.mL^{-1} (25% reduction) (Figure 2D). Surprisingly, when these two drugs were combined at higher concentrations, we observed a dramatic increase in biofilm biomass.

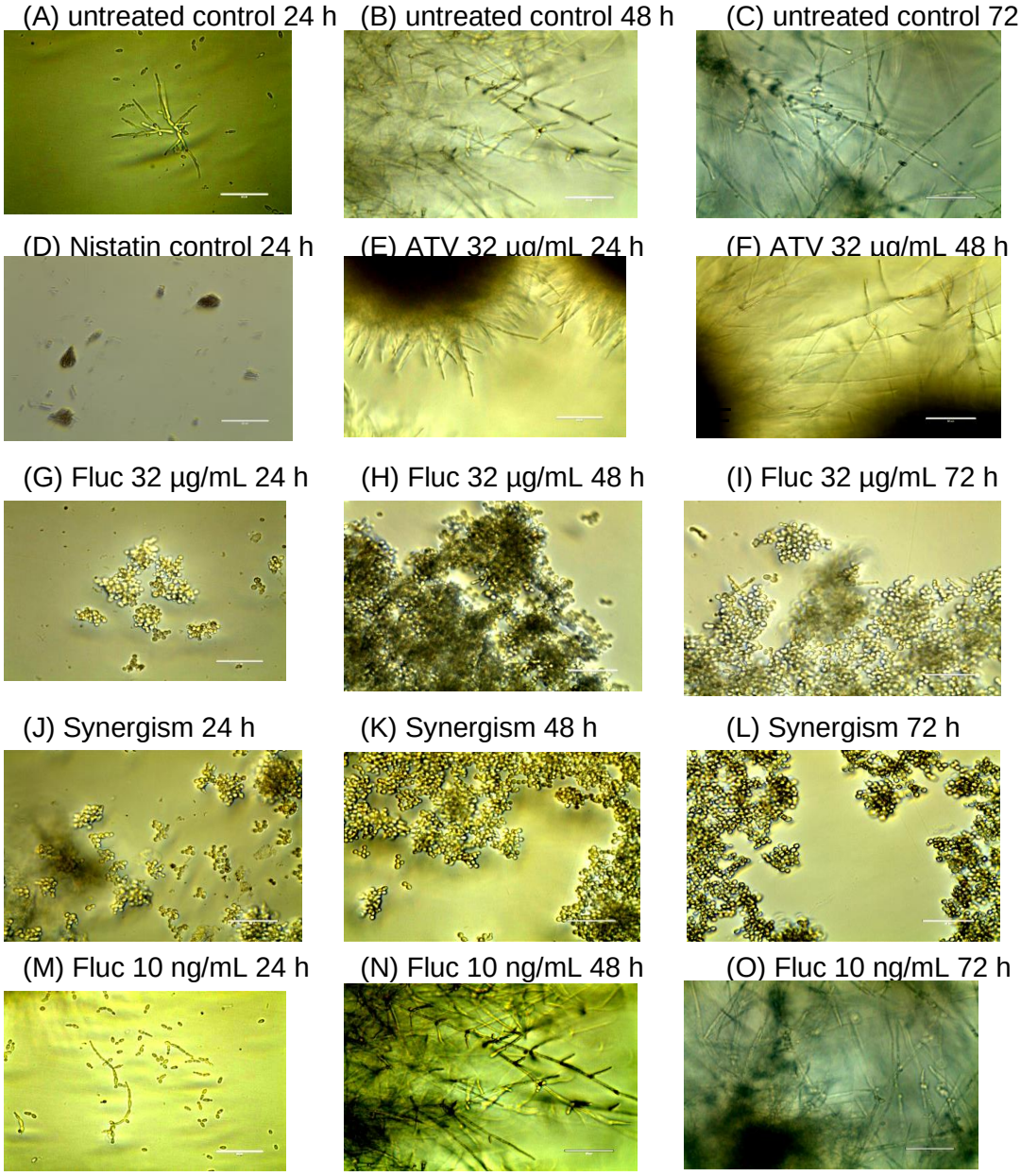
Figure 2: Inhibition of biofilm formation of *C. albicans* ATCC 10231 (A and B); Inhibition of biofilm maturation of *C. albicans* ATCC 10231 (C and D). A and C: Atorvastatin (ATV), (B and D: Atorvastatin in combination with fluconazole (ATV-F). Negative control indicates untreated biofilm, defined as 0% reduction. Each concentration of ATV or AF had six replicates. Data are presented as the mean ± standard deviation of two independent experiments. * p<0.05 compared to the negative control.



We performed a yeast-hyphae transition inhibition assay to investigate the antifungal effect of atorvastatin as well as atorvastatin in combination with fluconazole on *C. albicans* dimorphism was observed in a timely manner (24, 48, and 72 hours). Our results revealed that solely treatment with either atorvastatin at 32 μg.mL^{-1} or fluconazole at 0.01 μg.mL^{-1} were not effective to inhibit yeast-to-hyphae transition. On the other side, our results also showed that when yeast cells were treated with fluconazole at a higher

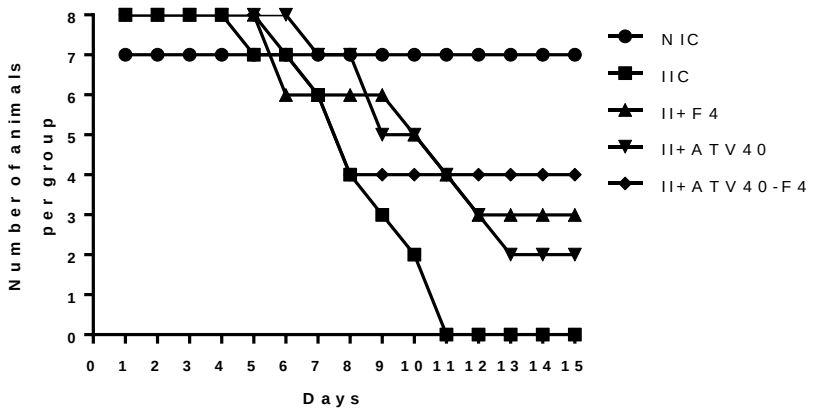
concentration (32 µg.mL^{-1}) (Figure 3G-I), yeast growth was still observed, however, there was a complete inhibition of filamentation. Nevertheless, the combination of atorvastatin with fluconazole at 0.25 and 0.01 µg.mL^{-1}, respectively, exhibited significant inhibition of *C. albicans* filamentation showing how the antifungal action of these drugs were enhanced at low concentrations when combined.

Figure 3: Yeast-hyphae transition of *Candida albicans* ATCC 10231. *C. albicans* was cultured either in the presence of atorvastatin at 32 µg.mL^{-1} (E, F), or fluconazole at 32 µg.mL^{-1} (G, H, I), or with both atorvastatin at 0.25 µg.mL^{-1} and fluconazole at 0.01 µg.mL^{-1} (J, K,L), or fluconazole at 0.01 µg.mL^{-1} (M,N, O) for 24, 48, and 72 h at 37°C. Nystatin was used as a positive control (D) and the negative control included untreated yeast cells (A, B, C). Measurement bars refer to 50 µm.



Due to the promising inhibitory synergetic effect of atorvastatin and fluconazole on the yeast-to-hyphae transition *in vitro* assay, next we investigated their antifungal action against intrabdominal candidiasis using an *in vivo* murine model. Our results revealed that mice in the non-immunosuppressed but infected group showed no clinical signs of infection (data not shown) and therefore, remained alive throughout the entire experiment (Figure 4, IC) while immunosuppressed, infected, and untreated mice (Figure 4, IIC) exhibited a decrease in foraging activity, apathy, piloerection, and irritability. At the 5th day, these immunosuppressed and infected mice started to die, and by the 15th day they were all dead. When immunosuppressed and infected animals were treated with either atorvastatin at 40 mg.kg^{-1} (Figure 4, II+ATV40 group) or fluconazole at 4 mg.kg^{-1} (Figure 4, II+F4 group) lethality rate dropped to 80% and 70%, respectively. Furthermore, the combination of these two drugs was more effective, reducing IAC lethality rate to 60% (Figure 4, II+A40-F4 group).

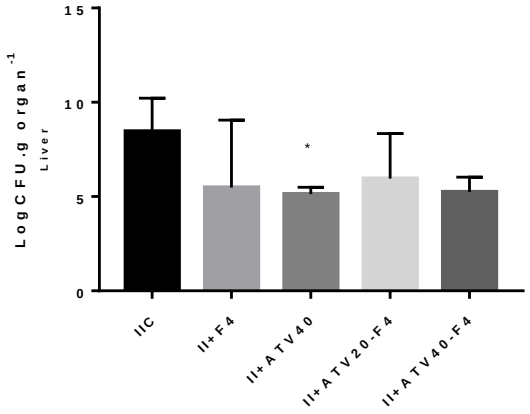
Figure 4: Mice survival rate after induced intra-abdominal candidiasis *C. albicans* ATCC 10231. NIC: negative control, non-immunosuppressed and non-infected; IIC: immunosuppressed and infected control group; II+F4: immunosuppressed and treated with fluconazole at 4 mg.kg^{-1}; II+ATV40: immunosuppressed and treated with atorvastatin at 40 mg.kg^{-1}; II+ATV40-F4: immunosuppressed and treated with both atorvastatin at 40 mg.kg^{-1} and fluconazole at 4 mg.kg^{-1}.



Because the liver is largely affected in IAC, mice livers were collected to investigate the therapeutic effect produced by either atorvastatin, or fluconazole, or both combined. As shown in Figure 5, treatment with atorvastatin at 40 mg.kg^{-1} significantly reduced fungal load in the liver about 40 percent. As expected, treatment with fluconazole at 4 mg.kg^{-1} did not significantly reduce fungal load in the liver, since *C. albicans* ATCC 10231 is highly resistant to this drug. But surprisingly, animals treated with both

atorvastatin (at either 20 or 40 mg.kg^{-1}) and fluconazole (at 4 mg.kg^{-1}) showed no further reduction in fungal load in the liver compared to the infected but untreated mice.

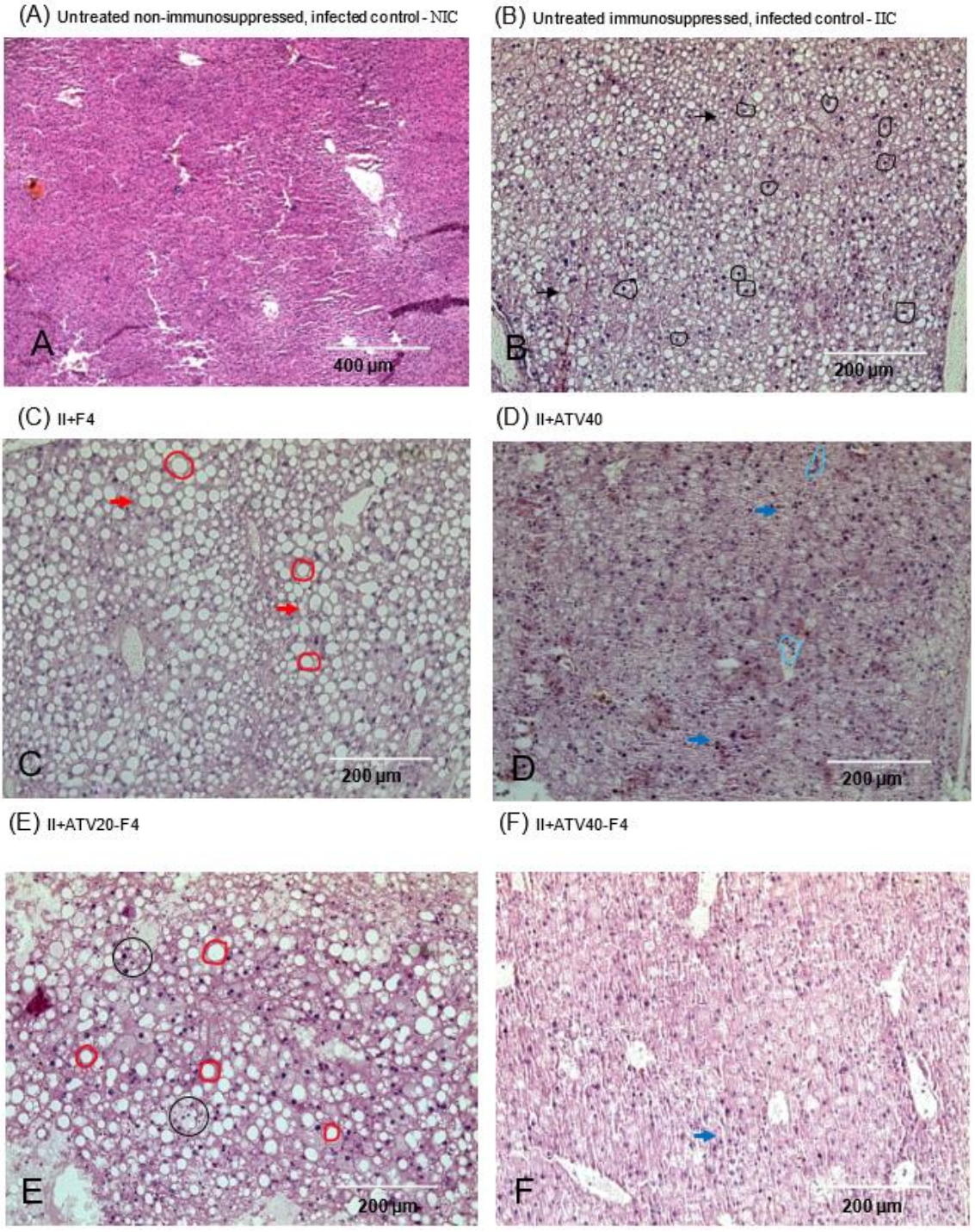
Figure 5: Fungal load in livers from mice infected with *C. albicans* ATCC10231. All groups were immunosuppressed with dexamethasone and then infected with *C. albicans* ATCC10231 followed by treatment. IIC (infected control group was treated with 0.9% saline), II+F4: immunosuppressed and treated with fluconazole at 4 mg.kg^{-1}; II+ATV40: immunosuppressed and treated with atorvastatin at 40 mg.kg^{-1}; II+ATV20-F4: immunosuppressed and treated with both atorvastatin at 20 mg.kg^{-1} and fluconazole at 4 mg.kg^{-1}; II+ATV40-F4: immunosuppressed and treated with both atorvastatin at 40 mg.kg^{-1} and fluconazole at 4 mg.kg^{-1}. Statistical analysis was performed via Man Whitney test (n=8). *p < 0.05 compared to negative control.



A histopathological study of the liver was also performed using hematoxylin-eosin (HE) staining. As shown in Figure 6A, hepatic tissue of uninfected animals (NC group) remained preserved with normal hepatocytes. In contrast, liver of infected but untreated mice (Figure 6B; IC group) had vacuolated hepatocytes associated with cytoplasm degeneration. In addition, apoptotic cells and pyknotic nuclei as well as necrotic areas associated with inflammatory cells were noticed.

Animals treated solely with fluconazole (Figure 6C) had hepatocytes containing vacuoles and peripheric nuclei, indicating steatosis, whereas animals treated solely with atorvastatin (Figure 6D) exhibited moderately preserved tissue and no vacuolated hepatocytes, indicating a hepatoprotective effect of this drug in IAC. However, animals treated with a combination of atorvastatin at 20 mg.Kg^{-1} and fluconazole at 4 mg.Kg^{-1} (Figure 6E) had hepatocytes with multiple vacuoles but when atorvastatin was tested at 40 mg.Kg^{-1} instead (Figure 6F), results were similar to the solely treatment with atorvastatin at 40 mg.Kg^{-1} (Figure 6D), indicating that atorvastatin antifungal action was not enhanced by fluconazole, and therefore, synergism did not occur.

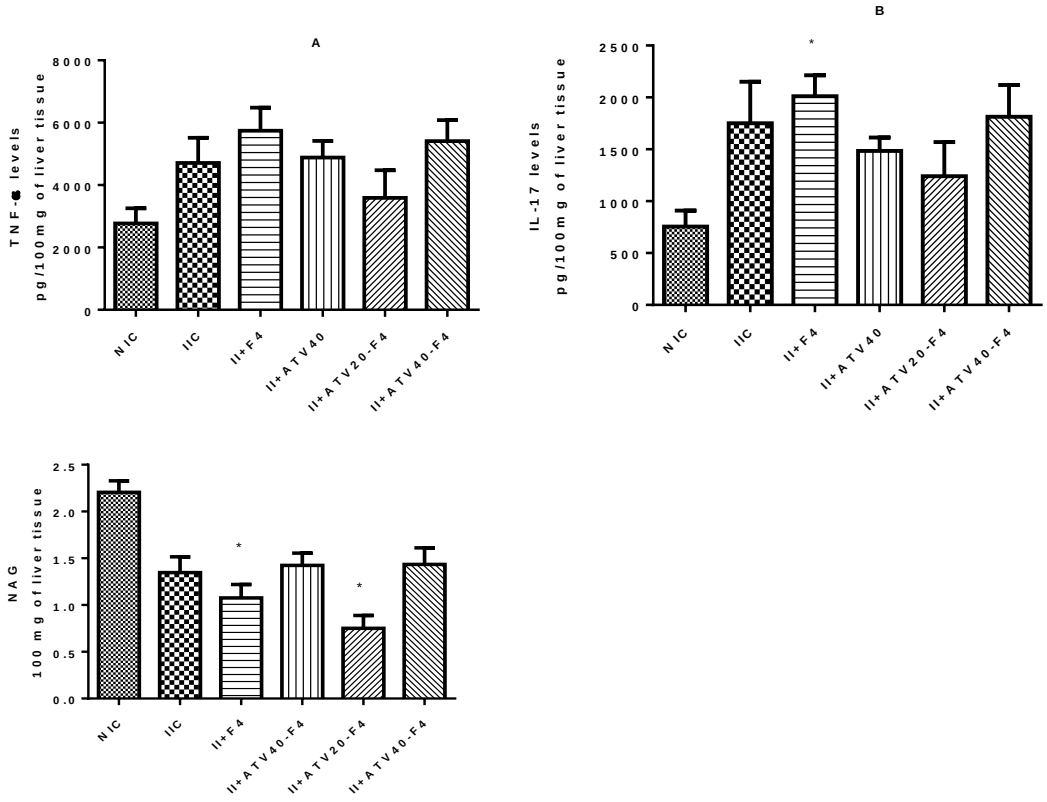
Figure 6: Histological section of the liver of Swiss mice infected intraperitoneally with *Candida albicans* ATCC 10231 at 10^6 CFU/mL^{-1} (n = 2). (A) (NIC) non-immunosuppressed and infected, treated with 0.9% saline; (IIC, II+F4; II+ATV40; II+ATV20-F4; II+ATV40-F4) immunosuppressed with dexamethasone and then infected; (B) (IIC) treated with 0.9% saline; (C) II+F4 treated with fluconazole at 4 mg.kg^{-1}, (D) II+ATV40 treated with atorvastatin at 40 mg.kg^{-1}; (E) II+ATV20-F4 treated with both atorvastatin 20 mg.kg^{-1} and fluconazole at 4 mg.kg^{-1}; (F) II+ATV40-F4 treated with both atorvastatin 40 mg.kg^{-1} and fluconazole at 4 mg.kg^{-1}. Black arrows: hydropic degeneration by accumulation of water in the cytoplasm. Red arrows: hepatic steatosis. Blue circle: inflammatory cells.



Thus, to investigate the effect of atorvastatin as well as atorvastatin in combination with fluconazole on the inflammatory response triggered by *C. albicans* ATCC 10231

infection, we measured the levels of TNF-α (tumor necrosis factor alpha), IL-17 (interleukin 17), and NAG (N-acetyl-β-D-glucosaminidase) in liver samples from treated animals. As shown in Figure 7, treatment with atorvastatin did not reduce the levels of either TNF-α (Figure 7A) or IL-17 (Figure 7B). On the other hand, solely treatment with fluconazole at 4 $mg.kg^{-1}$ increased TNF-α (Figure 7 and IL-17 levels (Figure 7B) while reduced NAG levels (Figure 7C). Furthermore, treatment with both atorvastatin at 20 $mg.kg^{-1}$ and fluconazole at 4 $mg.kg^{-1}$ also reduced NAG levels to, suggesting a decrease in macrophage recruitment in the liver, and consequently favoring the infection. When fluconazole was combined with atorvastatin at higher concentration (40 $mg.kg^{-1}$), NAG levels were similar to the immunosuppressed and infected but untreated group (Figure 7C).

Figure 7: Anti-inflammatory activity of atorvastatin in an intra-abdominal candidiasis murine model for *Candida albicans* ATCC 10231. (A) Liver tissue quantification of TNF-α (tumor necrosis factor alpha), (B) IL-17 (interleukin 17), and (C) NAG (N-acetyl-β-D-glucosaminidase). In (A) and (B), values are shown in picograms (pg) per 100 mg of tissue (n = 8) and in (C), results are represented as relative number of macrophages per 100 mg of tissue. NIC group: non-immunosuppressed and infected, treated with 0.9% saline; Groups IIC, F4, ATV40, ATV20-F4, ATV40-F4: immunosuppressed with dexamethasone and then infected; IIC: treated with 0.9% saline; F4: treated with fluconazole at 4 $mg.kg^{-1}$, ATV40: treated with atorvastatin at 40 $mg.kg^{-1}$; ATV20-F4: treated with both atorvastatin 20 $mg.kg^{-1}$ and fluconazole at 4 $mg.kg^{-1}$; ATV40-F4: treated with both atorvastatin 40 $mg.kg^{-1}$ and fluconazole at 4 $mg.kg^{-1}$. Statistical analysis was performed by ANOVA with Tukey's post-test, Kruskal Wallis with Dunn's post-test (n=8).* $p<0.05$ compared to control.



4 DISCUSSION

In the past years, hypolipidemic agents such as statins have been further investigated for their potential antimicrobial activity [3]. In this study, the time-kill curve showed that atorvastatin in combination with fluconazole at low concentrations (0.25 $\mu g.mL^{-1}$ of atorvastatin and 0.01 $\mu g.mL^{-1}$ of fluconazole) exhibited a synergetic effect against fluconazole-resistant *C. albicans*, which worked more efficiently than the solely treatment with atorvastatin at its minimum inhibitory concentration of 32$\mu g.mL^{-1}$. These results suggest that the combination of statin/azole may be an alternative treatment in the early stages of the infection.

Others have also shown the synergetic effect of statins combined with azoles (i.e. clotrimazole fluconazole, itraconazole, ketoconazole, and miconazole) against *C. albicans*, *C. sutilis*, *S. cerevisiae*, *C. tropicalis*, and *C. neoformans* [16,24–27]. It has been suggested that the antifungal action of statins works by targeting the synthesis of ergosterol and inhibiting the homologous enzyme of HMG-CoA reductase (HMGR) [28]. This enzyme plays a role in the sterol pathway, so it does 14-α-lanosterol demethylase, which can be inhibited by azoles. Consequently, ergosterol production is in yeast is considerably reduced and cell membrane integrity is compromised, therefore suggesting the synergetic acting of statins and azoles.

The combination of atorvastatin and fluconazole exhibited ambiguous effects in biofilm maturation. When atorvastatin was used at 8 $\mu g.mL^{-1}$ in combination with fluconazole at 0.32 $\mu g.mL^{-1}$, biofilm biomass was reduced in 50% whereas atorvastatin at 128 $\mu g.mL^{-1}$ and fluconazole at 5.12 $\mu g.mL^{-1}$ increased biofilm biomass in 100%. This increase in growth may be associated with farnesol which is an important quorum-sensing molecule that negatively regulates biofilm formation and maturation. [16] have shown that simvastatin was able to inhibit formation and maturation of *Candida* and *Cryptococcus* biofilms but others [41, 42,43]. have also shown that statins stimulate biofilm formation in *C. albicans* because farnesol synthesis was inhibited.

The transition from yeast to hypha (Y-H) is a very important virulence factor in the development of IAC [10,31], and it is related to tissue invasion and antifungal resistance among *Candida* species. Our results showed that atorvastatin at 0.25 $\mu g.mL^{-1}$ in combination with fluconazole at 0.01 $\mu g.mL^{-1}$ efficiently inhibited yeast to hypha transition in the first 24 hours of treatment. Yeast cells growth was still observed until 72 hours but filamentation did not occur, suggesting that the combination of these drugs could possibly be considered in the early treatment of patients experiencing IAC. Others

have found opposite results [32], and authors suggested that statins may stimulate Y-H transition by inhibiting HMGR, leading to a reduction of farnesyl pyrophosphate synthesis, which is a precursor of farnesol. Farnesol inhibits Y-H transition, and consequently, biofilm formation and maturation are compromised, [3,33]. Our findings imply otherwise and in fact, azoles have been shown to increase the production of farnesol [34], however further investigation is required to elucidate whether farnesol levels are indeed affected in the presence of atorvastatin and fluconazole at the concentrations we have observed a significant inhibition of Y-H.

Our results showed that immunosuppressed and infected mice that were left untreated showed evident clinical piloerection, lethargy, persistent tremors, low body temperature, lack of appetite, weight loss, aversion to touch, and all animals died withing 15 days. Similarly, [35] also demonstrated that mice with systemic infection by *C. albicans* ATCC 18804 died within 15 days after infection, mainly due to weakness and cachexia. In another study that used a model for candidemia [28], authors showed that animals infected with *C. albicans* intravenously all died within13 days, but treatment with pravastatin reduced mortality to 75% within 21 days. Additionally, they showed that the combination of pravastatin at 50 $mg.Kg^{-1}$ and fluconazole at 4 $mg.Kg^{-1}$ reduced mortality rate to 80% compared to untreated animals. Likewise, our results showed that mortality rate was reduced in our IAC murine model to 80% when treatment involved atorvastatin at 40 $mg.kg^{-1}$ within 15 days.

Next, because the liver is the main organ affected during IAC, we quantified fungal load and also evaluated this tissue histologically from treated and untreated animals. Regarding fungal load in the liver, we determined that was a significant reduction after solely treatment with atorvastatin at 40 $mg.kg^{-1}$ compared to untreated animals. However, when atorvastatin (40 $mg.kg^{-1}$) was combined with fluconazole (4 $mg.kg^{-1}$), fungal load was not greater than solely treatment with atorvastatin (40 $mg.kg^{-1}$). Combination of drugs implies in a higher concentration delivered to the bloodstream, which has to be processed in the liver and affect the final active concentration available in the palsma. For instance, [26] showed that when fifteen seriously ill patients were treated with fluconazole at an average dose of 4.9 $mg.kg^{-1}$, there was a wide variation in the concentrations of the antifungal in the blood, and in 5 patients it was not possible to achieve a sufficient plasmatic concentration needed for this drug to be effective [26].

Atorvastatin has a high hepatic uptake due to its lipophilic characteristic, and induces recruitment of Kupffer cells, which are resident phagocytic cells of the liver

[36,37]. Here, we showed that solely treatment with fluconazole or fluconazole combined with atorvastatin (at 20 μg.mL^{-1}) reduced the levels of N-acetyl-β-D-glucosaminidase, indicating a decrease in macrophage recruitment in the liver. Similar results were still obtained when atorvastatin at 20 μg.mL^{-1} was combined with fluconazole but not with atorvastatin at a higher dose (40 μg.mL^{-1}). When checking TNF-α and IL-17 levels in the liver, our results showed that solely treatment with fluconazole increased the levels of these cytokines whereas the combination of fluconazole and atorvastatin did not. IL-17 expression is modulated by NF-kβ, and it has been shown that statins inhibit this transcription factor, which may have contributed to the downregulation of IL-17 when atorvastatin was combined with fluconazole [38].

Lastly, histological analyses showed that atorvastatin reversed liver damage caused by *C. albicans* infection, reducing areas of suggestive steatosis. Also, when a solely treatment was done with fluconazole, liver damage was still observed but when combined atorvastatin, liver damaged was reversed again. [39] showed that simvastatin has high anti-inflammatory effect in an epithelial cells model, and 50 μM of this agent significantly inhibited TNF-α-induced IL-8 expression. Moreover, [39,40] have found that atorvastatin at 15 mg.kg^{-1} reduced doxorubicin toxicity, a drug used in chemotherapy that can generate reactive oxygen species and cause liver and kidney damage, which was also associated with inhibition of NF-κB pathway. Overall, our results suggest that atorvastatin beneficial effects in the liver of mice with IAC might be associated with its anti-inflammatory properties and antifungal action.

5 CONCLUSION

In summary, atorvastatin combined with fluconazole exhibited very promising results that could be considered in the early treatment of systemic candidiasis caused by fluconazole-resistant *Candida* strains. The synergetic action of atorvastatin and fluconazole dramatically reduced yeast-to-hyphae transition, and also biofilm maturation. Also, our findings demonstrated that mortality rate dropped to 60% in mice with IAC. Atorvastatin seems to have anti-inflammatory properties and antifungal action, ensuring that the body can respond effectively to the infection. Atorvastatin reversed the inflammatory process stimulated by fluconazole, increasing macrophage recruitment in the liver, and therefore, improving mice survival rate.

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CONFLICT INTEREST

All authors declare no conflicts of interest.

REFERENCES

[1] Maseda E, Rodríguez AH, Aguilar G, Pemán J, Zaragoza R, Ferrer R, et al. EPICO 3.0. Recommendations on invasive candidiasis in patients with complicated intra-abdominal infection and surgical patients with ICU extended stay. Rev Iberoam Micol 2016;33:196–205. https://doi.org/10.1016/j.riam.2016.02.003.

[2] G. D, D.K. M, E. R, M. M, M. B. Elderly versus non-elderly patients with intra-abdominal candidiasis in the ICU. Minerva Anestesiol 2017. https://doi.org/10.23736/S0375-9393.17.11528-2.

[3] Lima WG, Alves-Nascimento LA, Andrade JT, Vieira L, de Azambuja Ribeiro RIM, Thomé RG, et al. Are the Statins promising antifungal agents against invasive candidiasis? Biomed Pharmacother 2019;111:270–81. https://doi.org/10.1016/j.biopha.2018.12.076.

[4] de Ruiter J, Weel J, Manusama E, Kingma WP, van der Voort PHJ. The Epidemiology of Intra-Abdominal Flora in Critically Ill Patients with Secondary and Tertiary Abdominal Sepsis. Infection 2009;37:522–7. https://doi.org/10.1007/s15010-009-8249-6.

[5] Guo LN, Yu SY, Xiao M, Yang CX, Bao CM, Yu YH, et al. Species distribution and antifungal susceptibility of invasive candidiasis: A 2016-2017 multicenter surveillance study in beijing, china. Infect Drug Resist 2020;13:2443–52. https://doi.org/10.2147/IDR.S255843.

[6] Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB. Nosocomial Bloodstream Infections in US Hospitals: Analysis of 24,179 Cases from a Prospective Nationwide Surveillance Study. Clin Infect Dis 2004;39:309–17. https://doi.org/10.1086/421946.

[7] Marchetti O, Bille J, Fluckiger U, Eggimann P, Ruef C, Garbino J, et al. Epidemiology of Candidemia in Swiss Tertiary Care Hospitals: Secular Trends, 1991–2000. Clin Infect Dis 2004;38:311–20. https://doi.org/10.1086/380637.

[8] Andrade JT, Lima WG, Sousa JF, Saldanha AA, Nívea Pereira De Sá, Morais FB, et al. Design, synthesis, and biodistribution studies of new analogues of marine alkaloids: Potent in vitro and in vivo fungicidal agents against Candida spp. Eur J Med Chem 2021;210:113048. https://doi.org/10.1016/j.ejmech.2020.113048.

[9] Lo HJ, Köhler JR, Didomenico B, Loebenberg D, Cacciapuoti A, Fink GR. Nonfilamentous C. albicans mutants are avirulent. Cell 1997;90:939–49. https://doi.org/10.1016/S0092-8674(00)80358-X.

[10] Mayer FL, Wilson D, Hube B. Candida albicans pathogenicity mechanisms. Virulence 2013;4:119–28. https://doi.org/10.4161/viru.22913.

[11] Herrera KMS, Silva FK da, Oliveira ME de, Paiva MC de, Soares AC, Siqueira Ferreira JM. First report of polymyxin B activity in Klebsiella pneumoniae biofilm. J Chemother 2019;31:127–31. https://doi.org/10.1080/1120009X.2018.1558751.

[12] CDC. Antifungal Resistance in Candida. Https://WwwCdcGov/Fungal/Diseases/Candidiasis/Antifungal-ResistantHtml 2020.

[13] Talbot GH, Bradley J, Edwards JE, Gilbert D, Scheid M, Bartlett JG. Bad bugs need drugs: An update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. Clin Infect Dis 2006;42:657–68. https://doi.org/10.1086/499819.

[14] Kim K, Zilbermintz L, Martchenko M. Repurposing FDA approved drugs against the human fungal pathogen, Candida albicans. Ann Clin Microbiol Antimicrob 2015;14:1–11. https://doi.org/10.1186/s12941-015-0090-4.

[15] Martin JH, Bowden NA. DRUG REPURPOSING—Overcoming the translational hurdles to clinical use. Pharmacol Res Perspect 2019;7. https://doi.org/10.1002/prp2.548.

[16] Brilhante RSN, Caetano EP, de Oliveira JS, Castelo-Branco D de SCM, Souza ERY, de Alencar LP, et al. Simvastatin inhibits planktonic cells and biofilms of Candida and Cryptococcus species. Brazilian J Infect Dis 2015;19:459–65. https://doi.org/10.1016/j.bjid.2015.06.001.

[17] Andrade JT, Santos FRS, Lima WG, Sousa CDF, Oliveira LSFM, Ribeiro RIMA, et al. Design, synthesis, biological activity and structure-activity relationship studies of chalcone derivatives as potential anti-Candida agents. J Antibiot (Tokyo) 2018;71:702–12. https://doi.org/10.1038/s41429-018-0048-9.

[18] CLSI. Reference Method for Broth Dilution Antifungal Suscetibility Testing of Yeasts. CLSI Ref Method Broth Dilution Antifung Suscetibility Test Yeasts 2017;37:13.

[19] Zore GB, Thakre AD, Jadhav S, Karuppayil SM. Terpenoids inhibit Candida albicans growth by affecting membrane integrity and arrest of cell cycle. Phytomedicine 2011;18:1181–90. https://doi.org/10.1016/j.phymed.2011.03.008.

[20] Merritt JH, Kadouri DE, O'Toole GA. Growing and Analyzing Static Biofilms. Curr. Protoc. Microbiol., Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2005. https://doi.org/10.1002/9780471729259.mc01b01s00.

[21] do Couto FMM, do Nascimento SC, Júnior SFP, da Silva VKA, Leal AFG, Neves RP. Antifungal activity of the piroctone olamine in experimental intra-abdominal candidiasis. Springerplus 2016;5:4–7. https://doi.org/10.1186/s40064-016-2130-8.

[22] Coelho FM, Pinho V, Amaral FA, Sachs D, Costa V V., Rodrigues DH, et al. The chemokine receptors CXCR1/CXCR2 modulate antigen-induced arthritis by regulating adhesion of neutrophils to the synovial microvasculature. Arthritis Rheum 2008;58:2329–37. https://doi.org/10.1002/art.23622.

[23] de Paula TP, Santos PC, do Nascimento Arifa RD, Vieira AT, de Matos Baltazar L, Ávila TV, et al. Treatment with atorvastatin provides additional benefits to imipenem in a model of gram-negative pneumonia induced by klebsiella pneumoniae in mice. Antimicrob Agents Chemother 2018;62:1–14. https://doi.org/10.1128/AAC.00764-17.

[24] Chin NX, Weitzman I, Della-Latta P. In vitro activity of fluvastatin, a cholesterol-lowering agent, and synergy with flucanazole and itraconazole against Candida species and Cryptococcus neoformans. Antimicrob Agents Chemother 1997;41:850–2. https://doi.org/10.1128/AAC.41.4.850.

[25] Menezes EA, Cavalcante M de S, Farias RB, Teixeira AB, Pinheiro FG, Bezerra

BP, et al. Freqüência e atividade enzimática de Candida albicans isoladas da mucosa bucal de crianças de uma creche da prefeitura de Fortaleza. J Bras Patol e Med Lab 2005;41:9–13. https://doi.org/10.1590/s1676-24442005000100004.

[26] Bellmann R, Smuszkiewicz P. Pharmacokinetics of antifungal drugs: practical implications for optimized treatment of patients. Infection 2017;45:737–79. https://doi.org/10.1007/s15010-017-1042-z.

[27] Zhou Y, Yang H, Zhou X, Luo H, Tang F, Yang J, et al. Lovastatin synergizes with itraconazole against planktonic cells and biofilms of Candida albicans through the regulation on ergosterol biosynthesis pathway. Appl Microbiol Biotechnol 2018;102:5255–64. https://doi.org/10.1007/s00253-018-8959-8.

[28] Tashiro M, Kimura S, Tateda K, Saga T, Ohno A, Ishii Y, et al. Pravastatin inhibits farnesol production in Candida albicans and improves survival in a mouse model of systemic candidiasis. Med Mycol 2012;50:353–60. https://doi.org/10.3109/13693786.2011.610037.

[29] Lopez D, Vlamakis H, Kolter R. Biofilms. Cold Spring Harb Perspect Biol 2010;2:1–12. https://doi.org/10.1101/cshperspect.a000398.

[30] Hirota K, Yumoto H, Sapaar B, Matsuo T, Ichikawa T, Miyake Y. Pathogenic factors in Candida biofilm-related infectious diseases. J Appl Microbiol 2017;122:321–30. https://doi.org/10.1111/jam.13330.

[31] Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ. Invasive candidiasis. Nat Rev Dis Prim 2018;4:18026. https://doi.org/10.1038/nrdp.2018.26.

[32] Lima WG, Alves-Nascimento LA, Andrade JT, Vieira L, de Azambuja Ribeiro RIM, Thomé RG, et al. Are the Statins promising antifungal agents against invasive candidiasis? Biomed Pharmacother 2019;111:270–81. https://doi.org/10.1016/j.biopha.2018.12.076.

[33] Nyilasi I, Kocsubé S, Krizsán K, Galgóczy L, Pesti M, Papp T, et al. In vitro synergistic interactions of the effects of various statins and azoles against some clinically important fungi. FEMS Microbiol Lett 2010;307:175–84. https://doi.org/10.1111/j.1574-6968.2010.01972.x.

[34] Yu L, Wei X, Ma M, Chen X, Xu S. Possible Inhibitory Molecular Mechanism of Farnesol on the Development of Fluconazole Resistance in Candida albicans Biofilm. Antimicrob Agents Chemother 2012;56:770–5. https://doi.org/10.1128/AAC.05290-11.

[35] Ibrahim F, Sivak O, Wasan EK, Bartlett K, Wasan KM. Efficacy of an oral and tropically stable lipid-based formulation of Amphotericin B (iCo-010) in an experimental mouse model of systemic candidiasis. Lipids Health Dis 2013;12:1. https://doi.org/10.1186/1476-511X-12-158.

[36] Ioannou GN, Van Rooyen DM, Savard C, Haigh WG, Yeh MM, Teoh NC, et al. Cholesterol-lowering drugs cause dissolution of cholesterol crystals and disperse Kupffer cell crown-like structures during resolution of NASH. J Lipid Res 2015;56:277–85. https://doi.org/10.1194/jlr.M053785.

[37] Schwartz YS, Dushkin MI, Komarova NI, Vorontsova E V., Kuznetsova IS. Cholesterol-induced stimulation of postinflammatory liver fibrosis. Bull Exp Biol Med 2008;145:692–5. https://doi.org/10.1007/s10517-008-0175-6.

[38] Ortego M, Bustos C, Hernández-Presa MA, Tuñón J, Díaz C, Hernández G, et al. Atorvastatin reduces NF-κB activation and chemokine expression in vascular smooth muscle cells and mononuclear cells. Atherosclerosis 1999;147:253–61. https://doi.org/10.1016/S0021-9150(99)00193-8.

[39] Lee JY, Kim JS, Kim JM, Kim N, Jung HC, Song IS. Simvastatin inhibits NF-κB signaling in intestinal epithelial cells and ameliorates acute murine colitis. Int Immunopharmacol 2007;7:241–8. https://doi.org/10.1016/j.intimp.2006.10.013.

[40] El-Moselhy MA, El-Sheikh AAK. Protective mechanisms of atorvastatin against doxorubicin-induced hepato-renal toxicity. Biomed Pharmacother 2014;68:101–10. https://doi.org/10.1016/j.biopha.2013.09.001.