Assessment of the Anti-Biofilm Effect of Micafungin in an Animal Model of Catheter-Related Candidemia

*Beatriz Salinas, MSc, PhD[1,4]

*María Guembe, PharmD, PhD[1,2]

Lorena Cussó, MSc, PhD[1,3,4,5]

Martha Kestler, MD, PhD[1]

Jesús Guinea, PharmD, PhD[1,2,6]

Manuel Desco, MD, MSc Eng, PhD[1,3,4,5]

Patricia Muñoz, MD, PhD[1,2,6,7]

Emilio Bouza, MD, PhD[1,2,6,7]

*These authors have contributed equally to this work

[1] Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain

[2] Servicio de Microbiología y Enfermedades Infecciosas, Hospital General Universitario Gregorio Marañón, Madrid, Spain

[3] Departamento de Bioingeniería e Ingeniería Aeroespacial, Universidad Carlos III de Madrid, Madrid, Spain

[4] Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid, Spain

[5] Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM), Spain

[6] Departamento de Medicina, Facultad de Medicina, Universidad Complutense de Madrid, Madrid, Spain

[7] CIBER Enfermedades Respiratorias-CIBERES (CB06/06/0058), Madrid, Spain

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Corresponding author:

Manuel Desco, MD, MSc Eng, PhD

Universidad Carlos III de Madrid

Avenida de la Universidad 30, 28911 Leganés. Madrid. Spain

Tel.: +34 91426 5067, Fax: +34 91 426 5108

e-mail: manuel.desco@uc3m.es

Alternative corresponding author:

María Guembe, PharmD, PhD

Servicio de Microbiología Clínica y Enfermedades Infecciosas

Instituto de Investigación Sanitaria Gregorio Marañón

Hospital General Universitario "Gregorio Marañón"

C/. Dr. Esquerdo, 46

28007 Madrid, Spain

Phone: +34- 91- 586 80 27

Fax: +34- 91- 586 86 67

E-mail: mariaguembe@hotmail.com

ABSTRACT

In cases where catheter-related candidemia (CRC) must be managed without catheter withdrawal, antifungal lock therapy using highly active anti-biofilm (HAAB) agents is combined with systemic treatment. However, the activity of HAAB agents has never been studied in in vivo models using bioluminescence. We assessed the efficacy of micafungin using a bioluminescent *Candida albicans* SKCA23-ACTgLuc strain in an animal model of CRC. We divided 33 female Wistar rats into five groups: sham (A), infected non-treated (B), treated with lock therapy (0.16 mg/ml) (C), systemically treated only (1 mg/kg) (D), and systemically treated+lock (E). Catheters were colonized 24h before insertion into the femoral vein (day 0). Treatment started on day 1 and lasted 7 days, followed by 7 days of surveillance. Bioluminescence assays were carried out on days 1, 3, 5, and 14, together with daily monitoring of clinical variables. Postmortem microbiological cultures from the catheter and several tissue samples were also obtained. Overall, 28 rats (84.8%) completed the study. Group B animals showed significant weight loss at days 2, 4, and 5 compared with groups C and D ($p<0.05$). In group B, no animals survived after day 7, 75% had CRC, and bioluminescence remained constant five days after catheter implantation. Positive catheter culture rates in groups C, D, and E were, respectively, 83.3%, 62.5%, and 25.0% ($p=0.15$). Micafungin proved to be a HAAB agent when administered both systemically and in lock therapy in an animal model of CRC, although the bioluminescence signal persists after treatment. This persistence should be further analyzed.

Keywords: Catheter-related candidemia, micafungin, bioluminescence, lock therapy.

INTRODUCTION

Candida spp. is responsible for approximately 8% of catheter-related infections and is the third cause of CRI after coagulase-negative staphylococci and *Staphylococcus aureus* infection, with high associated morbidity and mortality rates (1, 2).

Many episodes of candidemia have their origin in central venous catheters (3). Catheter-related candidemia (CRC) results mainly from the ability of some *Candida* strains to produce biofilms, especially *C. albicans* and *C. parapsilosis* (4, 5).

In addition to perpetuating infection, biofilms are difficult to eradicate owing to antifungal resistance. In vitro and in vivo models have been used to assess antifungal activity against *Candida* biofilms in order to test the efficacy of catheter lock therapy, and echinocandins, especially micafungin, have proven to be the most efficacious anti-biofilm agents (6-9).

Although recent guidelines for the management of candidemia recommend removing the catheter when there is suspicion of CRC (2), catheter withdrawal is not always possible because of the risks and comorbidities associated with replacement (10-13). Nucci et al. (14) demonstrated that non-neutropenic patients treated with echinocandins or L-AmB did not benefit from early removal of the catheter. Therefore, the possibility of treating an episode of CRC without catheter removal using systemic antifungals in combination with catheter lock therapy is an attractive approach (15, 16).

Several authors have tested biofilm formation in different in vivo models, although all of them were based on ex vivo techniques, such as microscopy (17-22), to assess biofilm properties, thus hampering in vivo study of progression of CRC and the

evaluation of treatment. Additional shortcomings of ex vivo studies are the need to use a large number of animals and the fact that they are not able to monitor biofilm formation and pathogenesis in vivo (21). In this context, molecular imaging techniques, specifically bioluminescence imaging (BLI), have proven able to assess progression of biofilm in vivo and have thus become a new tool in the study of infections (21, 23). Nevertheless, the BLI approach also has drawbacks, such as low spatial resolution, low tissue penetration (24, 25) and the need of using animal models with superficially implanted catheters, which may not resemble real-world clinical practice (20, 26, 27). Hence, the objective of our study was to circumvent these shortcomings and test micafungin treatment in vivo by using an intravascular catheter animal model that better mimics clinical CRC.

MATERIAL AND METHODS

In vitro micafungin activity studies

Microorganism: We used bioluminescent SKCA23-ACTgLuc *Candida albicans*, which was kindly provided by Dr. Patrick Van Dijck (21). For each experiment, the strain was thawed and inoculated in Sabouraud dextrose agar (SDA) (Difco Laboratories, Detroit, Michigan, USA). Biofilm production was tested using the crystal violet assay and absorbance was quantified in a spectrophotometer at 550 nm (28).

Minimal inhibitory concentration (MIC) of micafungin by CLSI and EUCAST: Susceptibility against micafungin (Astellas Pharma, Inc, Tokyo, Japan) was tested using the EUCAST EDef 7.3.1 microdilution procedure and the CLSI M27-A3 procedure. Inoculated plates were incubated for 24 hours at 35°C. MIC values were determined spectrophotometrically at 530 nm (Multiskan FC microplate photometer, Thermo

Scientific, Madrid, Spain) for EUCAST and visually for CLSI and were defined as the lowest concentration of drug that resulted in inhibition of ≥50% of growth in comparison with a drug-free control growth well. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 isolates were used as quality control strains (29, 30).

In vitro micafungin activity against 24-hour SKCA23-ACTgLuc *C. albicans biofilm:* SKCA23-ACTgLuc *C. albicans* was grown on SDA for 24 hours at 37°C. Two to three colonies from each plate were inoculated into 20 ml of yeast peptone dextrose (YPD) medium and incubated at 30°C overnight on a rocker table. They were then centrifuged at 3,500 rpm for 5 minutes, washed twice with 10 ml of phosphate-buffered saline (PBS), and re-suspended in RPMI1640 medium. After being standardized to 1 x 10^6 colony forming units (cfu)/ml in RPMI1640, 100 µl of each suspension was placed in the wells of a 96-well, flat-bottomed microtiter plate and incubated for 24 hours at 37°C. The suspensions were discarded, and the wells were washed 3 times with sterile PBS and filled with 100 µl of micafungin (16 µg/ml) in RPMI1640. Negative-control wells received 100 µl of RPMI1640 without micafungin. Microtiter plates were incubated at 37°C for an additional 72 hours. The media were discarded and the wells washed 3 times with sterile PBS.

Metabolic SKCA23-ACTgLuc *C. albicans* activity was assessed by measuring the absorbance of each well at 492 nm with XTT to determine the reduction in absorbance compared with the level for the control well (percent metabolic activity reduction = [1-X/C] x 100, where X is the absorbance of micafungin-treated wells and C is the absorbance of negative control wells).

The cfu/well count was assessed by completely scratching the biofilm of the wells with 100 µl of PBS using the tip of the pipette to detach it from the well. Serial dilutions of each well suspension were performed, and 100 µl of the dilutions were cultured on SDA plates followed by a 24-hour incubation at 37°C. After incubation, cfu/plate was calculated to determine the reduction in the number of cfu/well compared with the number for the control well (percent cfu reduction = [1-X/C] x 100, where X is the cfu count of the micafungin-treated wells and C is the cfu count of the negative control wells).

Data for metabolic activity were assessed every 24 hours during the 72-hour treatment, and the cfu/well counts were assessed after the 72-hour treatment. Each experiment was tested in triplicate, and the average value was reported.

In vivo virulence assay of bioluminescent C. albicans strain on a Galleria mellonella model: We tested the virulence of the SKCA23-ACTgLuc strain using the *G. mellonella* infection model. Final instar larvae of *G. mellonella* (Bichosa) were stored in wood shavings in the dark until infection. Each experimental group contained 10 randomly chosen larvae of appropriate weight (330±20 mg); as a control, we used a group of 10 larvae inoculated with 10 µl of PBS to monitor trauma, and a group of 10 non-injected larvae.

SKCA23-ACTgLuc strain was grown in yeast extract-peptone-dextrose (YPD) overnight at 30°C with shaking (150 rpm); the centrifuged pellet was washed twice with PBS and the inoculum was adjusted in PBS to 5×10^7 yeasts/ml (MOXI Z Mini Automated Cell Counter Kit, Orflo Technologies). Larvae were inoculated with 10 µl of the cell suspension (5×10^5 yeasts/larva) directly into the hemocoel via the last left proleg. Larval

death was determined by visual inspection daily from the day of infection up to 7 days. Viable counts were performed to confirm the inoculum. We tentatively classified the isolates according to the percentage of mortality of the larvae at day +1 postinfection as low pathogenic (0 - 30 %), moderate pathogenic (31% - 70%), and highly pathogenic (>70%).

In vivo biofilm model

Experimental procedures: The experimental design is presented in **figure 1**. We used 33 female Wistar rats weighing 250 g. The animals were divided into 5 groups as summarized in **table 1**.

Catheters were contaminated (except for the sham group) 24 hours before femoral implantation to allow biofilm to grow on the internal surface. The inoculum (10^4 cfu/ml in RPMI1640) was made by selecting 2-3 colonies from the 24-hour culture of SKCA23-ACTgLuc *C. albicans* in SDA. The suspension was inoculated into 14-cm-long catheter samples followed by incubation at 37°C for 24 hours. Before implantation, in vitro bioluminescence catheter images were acquired to ensure homogeneous SKCA23-ACTgLuc *C. albicans* catheter colonization.

Catheter implantation: Catheters were implanted into the femoral vein. The animals were under anesthesia and complete analgesia for 1 hour (1-2% inhaled sevoflurane, subcutaneous buprenorphine [0.1 mg/kg], intraperitoneal fentanyl [0.5 ml/kg], subcutaneous ceftriaxone [100 mg/kg]. In vivo BLI images were acquired 24 hours after implantation (day 1) and at days 3, 5, and 14 after implantation (last time point only in groups D and E).

Clinical variables such as survival, weight, diarrhea, blood in feces and respiratory difficulty were monitored daily. In addition, we also carried out a daily evaluation of signs of pain based on general appearance (face expression, ears position, curvature of the back and hair curling) and behavior (emission of screams, abnormal aggressiveness)(. Animals were sacrificed after the last BLI, and the catheter and tissue samples (blood, kidneys, urine, lungs) were obtained for microbiological culture.

Antifungal treatment: Systemic micafungin was administered intravenously at a dose of 1 mg/kg every 24 hours (31). Micafungin lock treatment was administered at a dose of 16 µg/ml every 24 hours (32). The sham group was treated with sterile saline. Treatment started at day 1, lasted 7 days, and was followed by 7 days of surveillance with no treatment in the case of animals that were alive at the end of the study.

Ethics Statement: All animal procedures were approved by the Animal Experimentation Ethics Committee of Hospital General Universitario Gregorio Marañón (ES280790000087) Madrid and performed according to European regulations (2010/63/EU) and national regulations (RD 53/2013).

In vivo BL imaging acquisition and analysis

To prevent light absorption by tissues during acquisition, the skin covering the catheter was slightly removed, to expose the vessel with the catheter implanted. Animals were anesthetized throughout the procedure with inhaled anesthetic (3% sevoflurane at 0.8 l/min of O_2).

During image acquisition, animals were centered in the camera's field of view, and the correct position was confirmed by an anatomical black and white image.

Activated bioluminescence images were acquired in darkness with the following parameters: slow-motion configuration, gain = 32-72, and exposure time = 5-20 seconds. BL was activated by filling the catheter with 0.1 ml (6 µM) of coelenterazine (CTZ, Prolume Ltd., Pinetop, Arizona, USA).

The BL signal was measured from each catheter by thresholding using ImageJ software. The BL signal was represented as a percentage of the signal measured on day 1.

Ex vivo (tissues and catheter) analysis

Tissue samples were collected in sterile containers and cultured using the following approach. Blood was drawn through the vena cava, inoculated into an aerobic blood culture bottle (approximately 10 ml), and incubated in the Bactec FX for 5 days or until positivity. Positive bottles were cultured in SDA plates to assess candidemia. Swabs were rubbed over the surface of the kidneys and lungs and were qualitatively cultured on SDA plates to confirm the presence of SKCA23-ACTgLuc *C. albicans*; 50 µl of urine was quantitatively cultured on SDA plates. Plates were incubated for 48 hours at 35°C to assess the presence of SKCA23-ACTgLuc *C. albicans*. The kidneys and lungs were also preserved in formol for macroscopic examination. Two fragments (distal and proximal), each measuring 2 cm, were cut from the catheter and further divided into 2 new fragments, each measuring 1 cm. One segment was longitudinally sliced (2-3 fragments) and rubbed onto SDA plates. Another segment was transversally sliced and sonicated (1 min + vortex) in 0.5 ml brain-heart infusion, and 100 µl of the sonicate was cultured on

SDA plates. Plates were incubated for 48 hours at 35°C and cfu were counted. Cultures were quantified as the number of cfu/1 cm of catheter.

Definitions

Positive catheter culture: Isolation of SKCA23-ACTgLuc *C. albicans* in the proximal and/or distal segment of ≥3 cfu/1 cm of catheter by the roll-plate method and/or ≥20 cfu/1 cm of catheter in sonication. Candidemia: Isolation of SKCA23-ACTgLuc *C. albicans* in the blood culture during or after a 5-day incubation in the Bactec FX system (32).

CRC: Microbiological confirmation of the isolation of SKCA23-ACTgLuc *C. albicans* both in the catheter culture and in the blood cultures (32).

Statistical analysis

For the *Galleria mellonella* model, survival curves for each isolate were constructed using the Kaplan–Meier method. Statistical analyses were performed using GraphPad Prism (Version 5.0). The log-rank test (Mantel-Cox test) was applied to assess differences between the survival curves.

Chi-squared or Fisher exact tests were used to compare categorical variables. The Kruskal-Wallis test was used to compare weight, and the Cox test was used to assess survival. Statistical significance threshold was set at $p<0.05$ for all the tests. The statistical analysis was performed with SPSS 18.0.

RESULTS

In vitro studies

A bioluminescent SKCA23-ACTgLuc *C. albicans* strain was found to be susceptible to micafungin either by CLSI (0.015 mg/l) or by EUCAST (≤0.0015 mg/l) and was a high biofilm-producing strain (absorbance at 550 nm with crystal violet=2.459).

The efficacy of 72 hours of treatment with micafungin was evaluated in RPMI1640. Compared with positive control wells, metabolic SKCA23-ACTgLuc *C. albicans* activity was already eradicated (99.7%) 24 hours after treatment. No metabolic activity was detectable (% reduction of XTT = 100%) 72 hours after treatment. Moreover, cfu counts were also reduced to 99.9%.

Virulence assay

The median survival of the infected larvae was 1 day. Supplementary data file 1 shows a Kaplan-Meier curve with the survival proportions of the infected larvae. According to the percentage of mortality (60%) in the day +1 post-infection, the SKCA23-ACTgLuc isolate was classified as moderate pathogenic (corresponding to mortality rates in *G. mellonella* model of between 31% - 70%).

In vivo studies

Survival after 5 days of catheter implantation was 35% in infected animals (table 1, group B) and 65% in infected animals with micafungin lock therapy (table 1, group C), (**figure 2A**).

Compared with day 0, a decrease in body weight was observed between day 2 and day 6 in all the experimental groups (**Figure 2B**). Weight loss was significantly greater in animals from group B (infected non-treated animals) at days 2, 4, and 5 than in groups

C and D ($p<0.05$), which even seemed to have recovered after day 7. The trend in group B is unknown, as all of the animals had died by day 7.

Figure 3 shows bioluminescence images of 1 animal per group after 1 day of catheter insertion. Quantification of in vivo catheter images using bioluminescence revealed that fungal load remained almost constant over time in all the groups (**Figure 4**).

Animals were sacrificed after the last bioluminescence image and blood, kidneys, urine, lungs, and the catheter were analyzed. Kidneys are the most sensitive organ for assessing candidemia, and visual signs, such as increased size and presence of white spots suggestive of *Candida* colonization, were only observed in infected animals without treatment (Supplementary data file 2).

Table 2 shows the culture results for the presence of SKCA23-ACTgLuc *C. albicans* in postmortem samples (catheter and blood). As for catheter cultures, 5 out of 6 infected animals with lock therapy (83.3%) had positive SKCA23-ACTgLuc *C. albicans* cultures. Similar results were obtained in systemically treated animals (5 out 8 animals, 62.5%), whereas the frequency of positive *C. albicans* catheters decreased to 25% (2/8) when lock treatment was combined with systemic micafungin therapy. Differences did not reach statistical significance ($p=0.15$). Candidemia was only detected in infected non-treated animals (75%). No animals receiving micafungin had candidemia.

DISCUSSION

Similarly to previous results reported in in vitro studies (6-9), our femoral rat model of CRC showed a clear impact of micafungin treatment on animals with CRC

Regarding in vitro studies, our in vitro model confirmed that micafungin was efficacious against *C. albicans* biofilm, tested by XTT and cfu counts, as both methods showed an almost 100% reduction between treated and non-treated wells after 72 hours of treatment. There was also a good correlation between the XTT assay and cfu counts in a static 96-well microtiter plate.

As for clinical progression in our in vivo model, weight loss (the most common prognostic parameter in infected rodent research experiments) was lower in treated animals, and both systemically-treated groups gained weight starting from day 7. Survival was also higher in treated groups than in non-treated infected animals. Moreover, candidemia was not detected at day 14 in any treated animal. We observed a better prognosis (100% survival) for systemically treated animals (groups C and D) than for micafungin-locked animals (65% survival at day 5). Micafungin was stopped at day 7 after implantation in the treated groups (C, D, and E), and animals from both the systemic treatment group and the systemic+lock group did not present external signs of relapse of infection, such as weight loss, at day 14. In these groups, routine techniques, such as catheter culture and blood culture, revealed a decrease or complete absence of SKCA23-ACTgLuc *C. albicans*. This finding could be interpreted as decolonization. In contrast, BL signal was still detectable despite the catheters were decolonized and animals had a good outcome. This could be explained by the presence of a remaining fungal viability, but clinical impact of this phenomena has to be further assessed.

Currently, conservative treatment of CRC remains open to debate. Guidelines recommend removing the catheter as soon as CRC is suspected (2), although in specific situations the risk-benefit of catheter replacement is still under discussion (10-13). Some authors showed that early catheter withdrawal in candidemic patients treated only systemically was associated with reduced mortality (33-36). In contrast, Nucci et al. (14) demonstrated that early removal of the catheter in non-neutropenic patients did not yield benefits when using anti-biofilm antifungal agents, such as echinocandins or L-AmB. This observation was also supported by Tumbarello et al. (5). Therefore, some authors have proposed to treat CRC episodes without catheter withdrawal by combining systemic and lock therapy (15, 16). Clinical prospective studies using a conservative approach to manage CRC are scarce. Only 2 clinical studies have reported successful management of patients with CRC using antifungal lock therapy (11, 37).

Our study had some limitations such as the small number of animals that completed the experiment, due to the candidiasis progression. Moreover, some animals did not finish the experiment because they pulled the catheter out themselves and were thus euthanized. In addition, we did not anticipate the need for frequent catheter infusions (almost daily), bioluminescent substrate administration, or treatment. Constant manipulation of the catheter may lead to the catheter becoming dislodged, thus removing the biofilm and hampering its detection by bioluminescence. Infection may even spread quicker because of this frequent manipulation. Finally, with the aim of maintaining a similar initial bioluminescence point, we measured the catheter

bioluminescence signal before implantation, although this does not guarantee equal numbers of cfu in all of them before starting treatment.

Obviously, as wild *C. albicans* does not express luciferase, bioluminescence imaging cannot be a translational tool, thus being its use limited to experimental non-clinical models.

In summary, our experimental model does not totally disentangle the dilemma about maintaining/removing catheters in candidemic patients, but it sheds light on the underlying mechanisms. Our results support that, although a complete catheter sterilization is not achieved (as suggested by the remaining bioluminescence, indicative of alive *Candida*), micafungin treatment, in particular, when combining systemic+lock therapy, may lead to negative cultures and a positive clinical outcome.

CONCLUSIONS

Total sterilization of the catheter was achieved in 75% of the animals under systemic and/or lock therapy within 7 days after treatment had finished, suggesting that the combination of systemic micafungin with micafungin lock therapy was the best regimen for reducing *C. albicans* biofilm in the catheter lumen and for eradicating candidemia in an animal model. However, bioluminescence was still detectable in the catheter even in cases where candidemia and cultures were negative. Therefore, further research is warranted to evaluate the possible persistence of viable but non-culturable cells, and its clinical meaning. Prospective clinical studies are also needed to confirm that conservative CRC treatment is successful and safe. In addition, future fields of interest include the administration of intermittent-high doses of micafungin through the infected

catheter, correlation of bioluminescence with cfu, and the potential utility of the BL measurement in animal models with other types of *Candida* infection.

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Conflicts of interest. The authors declare that they have no conflicts of interests.

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FIGURE LEGENDS

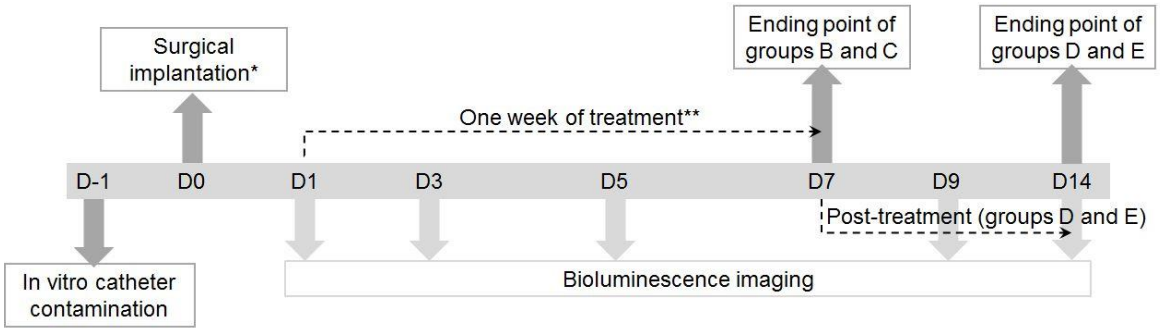

Figure 1. Experimental design. Thirty-three Wistar rats were divided into 5 experimental groups. *Homogeneous bioluminescence signal was confirmed before catheter implantation (Day 0).

**Micafungin treatment was administrated daily. A = sham (non-infected catheter); B = infected non-treated; C = infected + lock; D = infected + systemic treatment; E = infected + lock + systemic treatment.

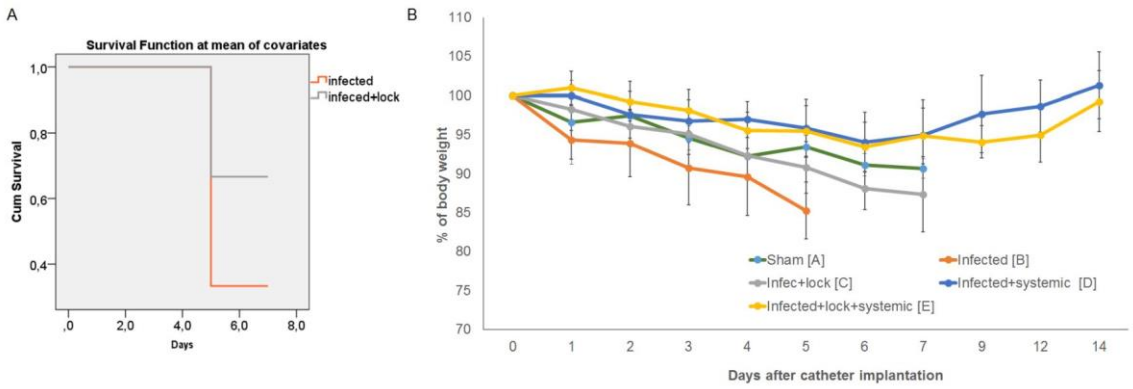

Figure 2. Clinical progression of the study animals

(A) Survival plot of groups B and C. (B) Mean weight loss of each group over time.

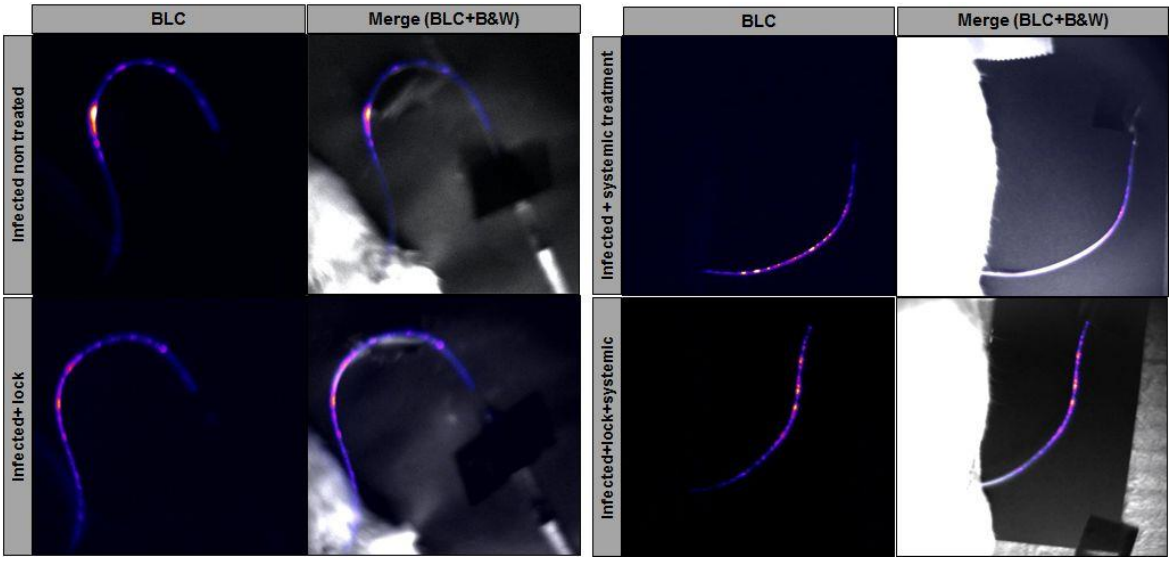

Figure 3. In vivo bioluminescence images. Example of 1 animal per group (B to E) before starting treatment with micafungin (day 1 after femoral catheter implantation). BLC: Bioluminescence catheter; B&W: black and white

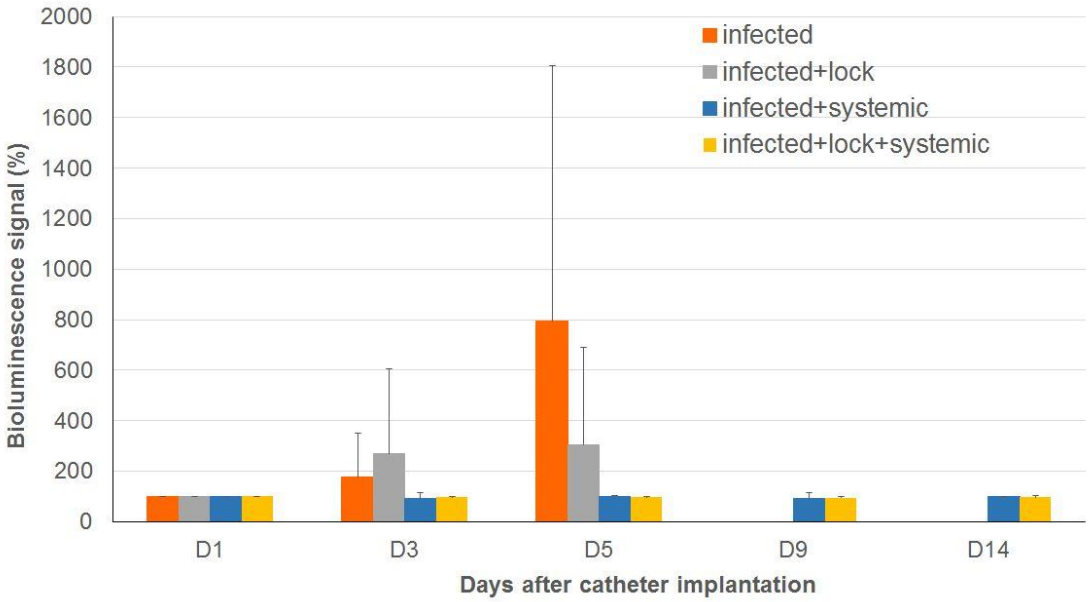

Figure 4. Bioluminescence signal along time as percentage of day 1