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Original Article

In vivo investigation of antimicrobial effects of cinnamaldehyde using immunosuppressed ICR mice with invasive pulmonary aspergillosis

Xiao-jun Liu[a], Jing Luo[b], Jie-hua Deng[c], Ji-hong Li[d], Gang-sheng Wang[c,*]

[a] Department of Hematology, The Second Hospital of Hebei Medical University, Shijiazhuang 050000, China
[b] Department of Research, The Second Hospital of Hebei Medical University, Shijiazhuang 050000, China
[c] Department of Dermatology, The Second Hospital of Hebei Medical University, Shijiazhuang 050000, China
[d] Department of Clinical laboratory, The Second Hospital of Hebei Medical University, Shijiazhuang 050000, China

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ABSTRACT

Objective: Invasive pulmonary *Aspergillus* infection has the characteristics of high morbidity, difficult to be treated, poor prognosis and high mortality. This study aims to investigate the effects of cinnamaldehyde on 1,3-β-*D*-glucans in the pulmonary *Aspergillus fumigatus* cell wall to provide a basis for developing novel antifungal drugs.

Methods: Immunosuppressed ICR mice were intranasally inoculated with 50 μL of *A. fumigatus* suspension (1×10^7 CFU/mL) and then separated into two groups, for the experimental group cinnamaldehyde was orally administered at 240 mg/kg/d consecutively for 14 d. While for the control group, voriconazole was used to treat the fungus infection. Pulmonary tissues were then extracted for 1,3-β-*D*-glucans assay and electron microscopy.

Results: The concentration of 1,3-β-*D*-glucans was significantly different between the cinnamaldehyde and voriconazole groups, which was (1160.89 ± 364.96) pg/mL and (3885.94 ± 845.45) pg/mL, respectively ($P < 0.01$). Electron microscopy showed that 2–3 outer layers (1,3-β-*D*-glucan layer) of *A. fumigatus* cell wall were damaged and fell off, resulting in serious defect of the cell wall, but the cell membrane was clear and intact.

Conclusion: Cinnamaldehyde has a significant influence on the integrity of 1,3-β-*D*-glucans in the pulmonary *A. fumigatus* cell wall, but the cell membrane is unaffected, suggesting that cinnamaldehyde has unique antifungal properties depending on its action against the 1,3-β-*D*-glucans on the pulmonary *A. fumigatus* cell wall.

1. Introduction

Invasive pulmonary aspergillosis infection in immunodeficiency patients are difficult to be treated, its prognosis is poor, and the mortality rate is as high as 80%–90% (Balaguer, Lopez-Carballo, Catala, Gavara & Hernandez-Munoz, 2013; Saifudin, Kadota & Tezuka, 2013). At present, the target of antifungal drugs is mainly focus on the fungal cell membrane in treatment (Kocevski et al., 2013), but the side effect is considerable, and drug resistant is easy to be induced. For example, up to 27% of *Aspergillus fumigatus* isolated from patients showed drug resistant (Li et al., 2015; Li et al., 2015). Although antifungal drugs have been used for many years in clinical practice, the mortality rate of fungal infection is still very high, and it has become one of the important causes of death for inpatients, and research interest should be called for in the medical community.

Cinnamaldehyde, chemical name 3-phenyl-2-propenal, is a small-molecule olefin aldehyde organic compound found in cinnamon oil extracted from Lauraceae medicinal plants. Cinnamaldehyde has many pharmacological activities (Masevhe, McGaw & Eloff, 2015; Sharma, Gairola, Sharma & Gaur, 2014; Yang et al., 2015; Zhang et al., 2015), including antifungal activity against a variety of pathogenic fungi (Wang, Xu, Wang, Zhu & Cui, 2012; Wang, Deng, Ma, Shi, & Li, 2012; Wang, Deng, & Jiang, 2010;). It was found that cinnamaldehyde acted on *Aspergillus* cell wall to destroy its structure and blocked the exchange of materials between the intracellular and extracellular compartments, thereby preventing fungal nutrient utilization and biological macromolecules synthesis in order to realize the inhibition of fungi growth and reproduction (Wang et al., 2012; Wang, Deng, et al., 2012). A series of *Saccharomyces cerevisiae* knockout strains (lKS1, GHS020, and GHS003) was constructed by inactivating the cell wall

* Corresponding author.
E-mail address: djh092@163.com (G.-s. Wang).

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synthetase gene, and we found that cinnamaldehyde had distinct influence on the synthesis of 1,3-β-*D*-glucans inside the cell wall (Jia, Zou, Shi & Gong, 2015). However, whether this *in vitro* test results working well *in vivo* remains unclear. In this study, the *in vivo* antimicrobial effects of cinnamaldehyde were investigated using immunosuppressed ICR mice with invasive pulmonary aspergillosis. Our results showed that cinnamaldehyde played an antifungal effect through inhibition of proper 1,3-β-*D*-glucans function in the *A. fumigatus* cell wall without affecting its cell membrane. Therefore, cinnamaldehyde might be a potential antifungal agent in the treatment of invasive *A. fumigatus* infections, particularly invasive pulmonary aspergillosis.

2. Materials and methods

2.1. Strains and animals

Aspergillus fumigatus ATCC3626 was gifted by the Institute of Microbiology, Chinese Academy of Sciences. Male ICR mice aged 6–8 weeks and weighing 22–25 g were bought from Beijing Vital River Laboratory Animal Technology Co., Ltd. The quality certificate of the experimental animal was specific pathogen-free level with the license number of SCXK (Beijing) 2012-0001.

2.2. Drugs and reagents

Cinnamaldehyde (purity > 98%) was provided by Jiangxi Xuesong Natural Medicinal Oil Co., Ltd, China. Cyclophosphamide was provided by Jiangsu Hengrui Medicine Co., Ltd, China. Voriconazole was provided by Ebang Pharmaceutical Co., Ltd. (Zhuhai, China). The 1,3-β-*D*-glucans kit was provided by Zhanjiang A & C Biological Ltd., China, and the dynamic test tube detector was provided by Lad Kinetics Ltd, UK.

2.3. Experimental location

The experiment was done at the Animal Experimental Center of the Second Hospital of Hebei Medical University. Individual ventilated cages (IVC) laminar-flow hood with the humidity of 50–70% at the temperature of 22 °C were arranged for animals feeding. The Animal Care and Use Committee of Hebei Medical University approved the experiment.

2.4. Experimental grouping and model construction

(1) Thirty mice were set for immunosuppression group: in this group all the mice were treated with cyclophosphamide through intraperitoneally injection at a dose of 200 mg/kg/d for continuous 2 d (Chen et al., 2013).
(2) Another 30 mice were set for invasive pulmonary *Aspergillus* group: on the 4th day, the immunosuppressed mice were anesthetized by injecting 10% chloral hydrate at a dose of 3 mL/kg, intraperitoneally, and then was nasally injected with 50 μL of *A. fumigatus* spore (10^7 colony form unit CFU/mL). The pulmonary tissues were harvested on the 14th day to perform microscopy and fungal culture, pathological examination, electron microscopic observation, and 1,3-β-*D*-glucans assay to establish invasive *A. fumigatus* infection models of immunosuppressive animal (Li et al., 2015).
(3) Thirty mice with invasive pulmonary aspergillosis infection were set for cinnamaldehyde treated group: cinnamaldehyde was oral administered to the mice treated with *A. fumigatus* spore after 12 h at a dose of 240 mg/kg/d for consecutive 14 d (the effective dose for the human body × 10 times) (Naveed et al., 2013), through which an immunosuppressed ICR mice model with invasive aspergillosis treated with cinnamaldehyde was established.
(4) Positive control group treated with voriconazole: 30 immunosuppressed ICR mice with *A. fumigatus* infection were treated with voriconazole at a dose of 240 mg/kg/d for consecutive 14 d Then, lung tissue was extracted on the 14th day for fungal culture and microscopic examination, 1,3-β-*D*-glucans assay, histopathological examination, and electron microscopic observation.
(5) Negative control group treated with saline (30 mice): After 50 μL of *A. fumigatus* [$(1 \times 10^7$ CFU/mL)] was nasally injected into the immunosuppressed mice, the mice were orally treated with 0.5 mL of 0.9% saline for consecutive 14 d 24 h after the injection.

Lung tissue specimens were taken in time for those dead mice that died before 15th d after the treatment. For those who lived longer than 14 d, the lung tissue samples were taken under anesthesia. Then the lung tissues were used for fungal microscopy, 1,3-β-*D*-glucans detection, histopathology (PAS), culture, and transmission electron microscopy.

2.5. Preparation of drug and fungal suspension

Tween 80 (2 g) were added to 97.76 mL of 0.9% saline, and then 240 mg of cinnamaldehyde or 240 mg of voriconazole were added to Tween 80 physiological saline.

A. fumigatus spore suspension was made at a concentration of (1×10^7) cfu/mL with 0.1% Tween 80 physiological saline.

2.6. Fungal culture and microscope examination

The lung tissue pieces (1.0 g) were taken under aseptic conditions, placed in a grinder together with 4 mL of physiological saline, and ground for 10 min, and then diluted with 5 mL of 0.9% saline. Taking 0.25 mL tissue fluid, glucose protein agar tube 35°, incubating for 72 h, and judging the results. The 0.1 mL of tissue fluid was taken and dropped on a glass slide, and then read under microscope.

2.7. Detection of 1, 3-β-D-glucans

Speed turbidimetry was used to detect the content of 1,3-β-*D*-glucans in lung tissue. The 500 mg tissue homogenate were centrifuged at a speed of 400 g for 10 min; Supernatant (100 μL) was added into sample dilution bottle and gently mixed on vortex mixer, then samples were placed in the tube thermostat at a temperature of 75 °C for 10 min. The 0.25 mL reagent reconstituted solution was added to the LPS reagent with a pipette, then 100 μL of the prepared sample test solution and 50 μL reagent solution were taken and added into the reaction tube, after mixing the solution gently, each test tube was inserted into the LKM dynamic tube tester, react at 37 °C for 75 min before reading the result.

2.8. Statistical analysis

SPSS21.0 statistical software (SPSS, IL, USA) was used for statistical analysis. Continuous data and categorical data were represented as mean ± standard deviation and chi-square test respectively. Analysis of variance was used for comparative analysis between groups. The least significance difference *t*-test was used for pair-wise comparison. The test level was set to be $\alpha = 0.05$, and a *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Cyclophosphamide-induced immunosuppression

Table 1 showed that after intraperitoneal injection of cyclophosphamide at a dose of 200 mg/kg/d for 2 d, the total leukocyte

Table 1
Effects of cyclophosphamide on leukocytes of ICR mice.

Groups	*n*	Count /(10^9•L^{-1})				
		0 d	2 d	4 d	6 d	8 d
Immunosuppression group	30	7.02 ± 0.10	3.60 ± 0.50	2.58 ± 0.20**	4.60 ± 0.90	7.65 ± 1.60
Normal saline group	10	6.82 ± 0.20	7.02 ± 0.66	7.20 ± 0.60	8.00 ± 0.60	7.80 ± 0.830

Immunosuppression group 2, 4, 6, and 8 d after administration was compared with the normal saline group using SPSS21.0 statistical software ($P < 0.01$).

counts in immunosuppressed mice were decreased, the lowest count [(2.58×10^9/L)] emerged on day 4, which was significant ($P < 0.01$) compared to that of control group treated with saline. The weights of the mice reduced to 18–20 g after immunosuppression compared with those of the control group ($P < 0.05$). All mice in the immunosuppression group had significant reductions in food and water intake and activity level with increased fur loss. These findings were consistent with the clinical symptoms of human immune-deficient patients, including poor appetite, fatigue, and hair loss. Leukocyte count and physical symptoms gradually recovered to normal 8 d after cessation of immunosuppression, and no natural death occurred in the mice population within 28 d. This suggests that intraperitoneal injection of cyclophosphamide at 200 mg/kg/d for 2 d can effectively induce immunosuppression in mice without causing death.

3.2. Models of immunosuppressed ICR mice infected with invasive A. fumigatus

Mice immunosuppressed with 2 d of cyclophosphamide injection at 200 mg/kg/d were nasally inoculated with 50 μL of *A. fumigatus* suspension (1×10^7 CFU/mL). Death happened 2 d after inoculation of *A. fumigatus*, 24 mice died in day 7, and the mortality rate reached 80%. Multiple abscesses were found in pulmonary tissues of the dead mice (Fig. 1A). Direct microscopic examination demonstrated septate hyphae with branching at 45° in pul-

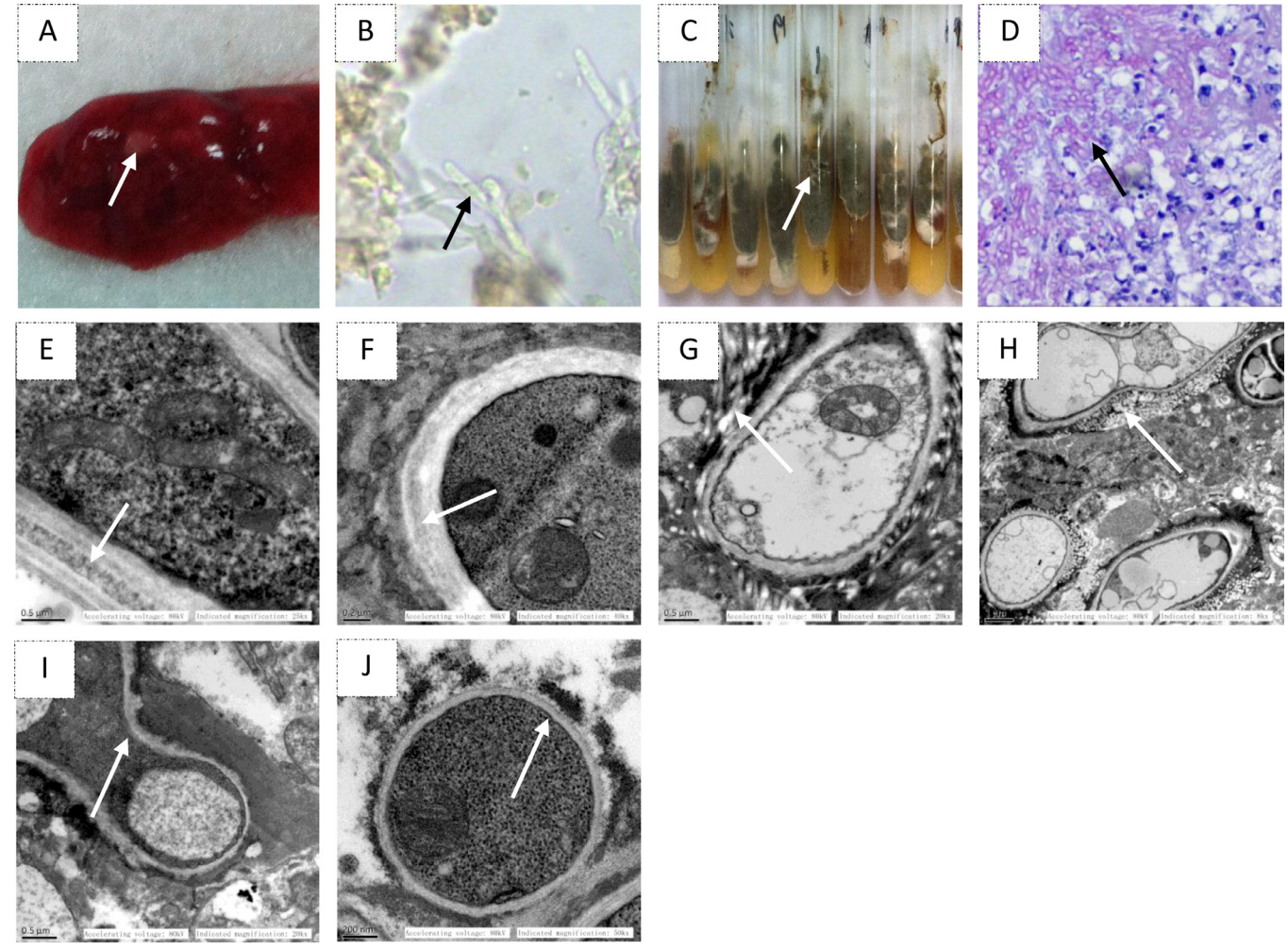

Fig. 1. Electron microscopic observation of cinnamaldehyde for treatment of lung tissue with invasive *A. fumigatus* infection. (A) Pulmonary abscesses; (B) microscopic examination of branched mycelium (10 × 40); (C) lung tissue culture shows the growth of *Aspergillus*; (D) pathological examination shows branch hyphae (PAS); (E) normal mycelial structure under electron microscope (80KV, 25KX); (F) normal spore structure under electron microscope (80KV, 25KX); (G) spore cell wall dissolution after cinnamaldehyde (80KV, 20KX); (H) mycelial cell wall dissolution after cinnamaldehyde (80KV, 20KX); (I) in voriconazole control group, hyphal cell membrane completely fell off and cell wall was dissolved. (80KV, 50KX); (J) in voriconazole control group, spore cell membrane completely fell off and cell wall was dissolved (80KV, 50KX).

monary tissues (Fig. 1B). Growth of *A. fumigatus* was found in the Sabouraud's medium in which the lung tissues were cultured (Fig. 1C). Large numbers of fungal hyphae and spores, inflammatory infiltration and tissue necrosis were found under microscope in pulmonary histopathological examination, (Fig. 1D). Pulmonary tissues were prepared for 1,3-β-*D*-glucans levels detection on day 2 to day 7 after *A. fumigatus* infection. The results were shown in Table 2. Significant differences were found between the infection and saline groups. Electron microscopy examination of pulmonary tissues showed that the layers of the *A. fumigatus* hyphae cell wall were complete. Furthermore, the cellular membrane, nucleus, and content of fungi hyphae were also complete. The study demonstrated that models of immunosuppressed ICR mice with invasive pulmonary aspergillosis were successfully established. Methods we used like pathology examination through microscope, biochemical methods for detection of 1,3-β-*D*-glucans, and electron microscopic are effective in the fungi infection model study.

Table 2
Results of 1, 3-β-*D*-glucan assay in mice that died 2–7 d after *A. fumigatus* infection.

Groups	*n*	1, 3-β-*D*-glucans/ (pg•mL^{-1})	Mean value
Model group	30	4875 ± 19 to 7783 ± 45	5930 ± 36**
Saline group	30	52 ± 49 to 98 ± 80	81 ± 58

Model group 2, 4, and 6 d after administration was compared with saline group using SPSS21.0 statistical software ($P < 0.01$).

**(1, 3)-beta-D-glucans test results of mouse Aspergillus fumigatus infection (pg/ml).

3.3. 1, 3-β-D-glucans detected in tissue

In the cinnamaldehyde group, cinnamaldehyde (240 mg/kg/d) was administered orally for 14 d, and voriconazole was used as control. The efficacy of cinnamaldehyde for 1,3-β-*D*-glucans was 81%, while the efficacy of voriconazole was 62%, suggesting that cinnamaldehyde was superior to voriconazole (Table 3). Our results showed that the concentration of 1,3-β-*D*-glucans in lung tissues of the cinnamaldehyde group was significantly lower than that of the voriconazole group and the model group without treatment (2−14 d), indicating that cinnamaldehyde inhibited the *in vivo* synthesis of 1,3-β-*D*-glucans in lung tissues, compromised the integrity of the fungal cell wall which may cause its antifungal effects by inducing cell lysis.

3.4. Electron microscopic assessment of cinnamaldehyde in treating invasive A. fumigatus infection

Transmission electron microscopy was used to observe the lung tissue of model mice. The *A. fumigatus* hyphae and spores showed a thick-walled structure of the platy layer, and the contents of the nucleus and organelles are clear and complete, as shown in Fig. 1E and F. After oral administration of cinnamaldehyde (240 mg/kg/d) for 14 d, the cell walls of *A. fumigatus* hyphae and spore became thin and the surface was rough. Swelling of fungal cells was also observed. The nucleus and cell contents were completely dissolved and showed under the electron microscope as vacuoles, but the cell membrane was still intact and clear (Fig. 1G and H). In the voriconazole control group, the organelles and content disappeared, the hyphae deformed, and the cell wall was slightly thinned, but the cell membrane completely fell off, as shown in Fig. 1I and J.

These results suggested that cinnamaldehyde acts at the cell wall of *A. fumigatus*, interferes with 1, 3-β-*D*-glucans integrity and damages the skeletal structure of the cell wall, but does not affect the cell membrane. Additionally, cinnamaldehyde is able to cross the cell membrane and causes damage to fungal nucleus and organelles, leading to cell edema, dissolution, and degeneration.

4. Discussion

Cinnamaldehyde is identified as the most active natural antimicrobial agent owing to its low minimum inhibitory concentration (Xing et al., 2014). It has a wide range of pharmacological activities and can impair the integrity of fungal cell wall structure and function. The hydrophilic aldehyde functional group in its structure is attracted to hydrophilic groups on the fungal surface. This interaction causes degradation of the polysaccharide structure and allows drug penetration of the cell wall, disrupting their growth and reproduction (Jiang, Zhou, Sheng & Wang, 2003). The cell wall of *A. fumigatus* has a multilayer plated structure, where 1, 3-β-*D*-glucans are the main components of the middle and outer layers. It acts as the major antigenic component of the cell wall, accounting for 80%–90% of the dry weight of the cell. 1,3-β-*D*-Glucans and chitin form the skeletal structure of the cell wall, maintaining cellular integrity and structural stability. However, mammalian cells do not contain this component and drug-related toxicity can be avoided (Graybill et al., 1998; Jiang et al., 2003). Therefore, 1,3-β-*D*-glucans is an ideal target for research and development of new antifungal drugs with the goal of fungal growth inhibition through targeted hindrance of fungal cell wall synthesis.

This *in vivo* research conducted with immunosuppressed ICR mice reveals that: (1) Cinnamaldehyde acts at the outer layers of the cell wall of *A. fumigatus* against 1,3-β-*D*-glucans, decreasing the integrity and stability of the *A. fumigatus* cell wall and 1,3-β-*D*-glucans content in pulmonary tissues; (2) The outer layer of the cell wall becomes flaky and disintegrates, leading to the dissolution of cytoplasm contents, organelles, and subsequently causes cell death; (3) The fungal cell membrane remains intact, suggesting that cinnamaldehyde acts only at the cell wall and has no effect on the cell membrane. Thus, there is fewer concerns of toxicity against mammalian cells as mammalian cells do not contain this component (Karageorgopoulos, Qu & Korbila, 2013; Metan et al., 2013).

Table 3
Effect of cinnamaldehyde on 1,3-β-*D*-glucans in *A. fumigatus* infected mice.

Groups	*n*	1, 3-β-*D*-glucans/ (pg• mL^{-1})	*F*	*P*
Cinnamaldehyde group	30	1160.89 ± 364.96	618.441	< 0.01
Voriconazole group	30	3885.94 ± 845.45		
Model group	30	5930.36 ± 716.49		
Saline group	30	81.58 ± 11.89		

Cinnamaldehyde and voriconazole groups were compared with saline and model groups using SPSS21.0 statistical software, a significant difference was found between cinnamaldehyde and voriconazole groups ($P < 0.01$).

5. Conclusion

Cinnamaldehyde demonstrated promising results in treating mice with invasive pulmonary aspergillosis. It played an antifungal effect through inhibition of proper 1, 3-β-*D*-glucans function in the *A. fumigatus* cell wall without affecting its cell membrane. Therefore, cinnamaldehyde might be a potential antifungal agent in the treatment of invasive *A. fumigatus* infections, particularly invasive pulmonary aspergillosis. However, the molecular mechanism of action of cinnamaldehyde should be further investigated in future studies. In addition, pharmacological properties of cinnamaldehyde should be explored for greater clinical value.

Declaration of Competing Interest

All authors of this paper confirm that they have no conflicts of interest.

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