Short Communication

INFLUENCE OF MEDIA AND TEMPERATURE ON GLIOTOXIN PRODUCTION IN *ASPERGILLUS FUMIGATUS* STRAINS*

Ivan KOSALEC[1], Stjepan PEPELJNJAK[1], and Marija JANDRLIĆ[2]

Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb[1], Department of Microbiology, Zagreb University Hospital[2], Zagreb, Croatia

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Gliotoxin is a secondary metabolite of the epipolythiodioxopiperazine family with biologically active internal disulfide bridge. It is produced by many fungal species, including *Aspergillus fumigatus* and *A. terreus*. *A. fumigatus*, which produces gliotoxin and more than twenty other secondary metabolites, is the leading cause of invasive aspergillosis. Gliotoxin production *in situ* influence the development of aspergillosis. This study investigated the *in vitro* production of gliotoxin in nine *A. fumigatus* isolates from the upper respiratory tract of immunocompromised patients. The effects of media composition and incubation temperature were studied. Gliotoxin was extracted from biomass and its concentration was semi-quantitatively analysed using thin-layer chromatography. Gliotoxin production was higher in the yeast-extract liquid medium (YES) than in the synthetic Czapek-Dox liquid medium (CZA). Incubation at 37 °C resulted in higher gliotoxin production than at 25 °C, probably because higher temperatures favour expansive growth of the mycelium. Gliotoxin could be detected after three days of incubation at concentrations 4.06 mg mL^{-1} (in YES at 37 °C) and 1.07 mg mL^{-1} (in CZA at 25 °C). YES broth as a medium containing 4 % sucrose and 2 % of yeast extract is a very rich substrate for the production of gliotoxin *in vitro*.

KEY WORDS: *aspergilosis, Czapek-Dox liquid medium, yeast-extracted liquid medium, toxicogenicity*

Mycotoxin gliotoxin (Figure 1) is a secondary metabolite of many moulds: *Aspergillus fumigatus*, *A. terreus*, *A. chevalieri*, *Penicillium terlikowskii*, *P. obscurum*, *P. cinerascens*, *Thermoascus crustaceus*, *Dichotomomyces cejpii*, *Trichoderma viride*, *Gliocladium fimbriatum*, *G. deliquescens* and yeast *Candida albicans* (1-3). It is a member of epipolythiodioxopiperazine family with a biologically active disulfide bridge. There are some other toxins with active (poly)sulfide bridges such as sporidesmins, chetomins, and scabrosins, which also have biological effects on animals (4).

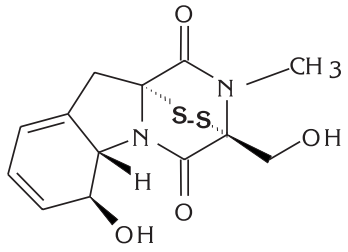

Figure 1 *Structure of gliotoxin (M=326.4)*

Because of very high incidence of invasive aspergillosis with high mortality among immunocompromised patients caused by saprophytic and thermophilic ubiquitous mould *Aspergillus fumigatus Fresenius*, the role of gliotoxin in the pathogenesis of aspergillosis has been a subject of many researchers (5, 6).

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Gliotoxin has received attention over recent years because of its various biological effects, immunosuppression being the most studied. Gliotoxin also shows antimicrobial activity and leads to a drop in ciliary beating frequency, which is associated with epithelial damage. These effects help the spores of *A. fumigatus* to colonise the respiratory system (7). Gliotoxin inhibits phagocytosis and induces apoptosis in macrophages (8-10), thymocytes, spleen cells and cells of the mesenteric lymph nodes (11, 12). Gliotoxin inhibits T- and B-cell activation and proliferation, and inhibits the activity of alcohol dehydrogenase, reverse transcriptase, farnesyltransferase, and the transcription factor NFκB (13). It has been found in tissues infected with *A. fumigatus*: bovine udder (14), turkey lungs (15), mice lungs (16), in serum of patients with invasive aspergillosis (16), and in vaginal fluid of women with vaginitis caused by *Candida albicans* (3).

The ability to produce secondary metabolites *in vitro* is used to test the toxicity of fungal isolates. This can help to predict mycotoxin production in various substrates such as cereals, smoked meat or even in infected tissues or organs. Mycotoxin production depends on genetic properties of fungal strains, and on the substrate as a potential precursor in the biosynthesis of mycotoxins. The aim of this study was to compare *in vitro* gliotoxin production in two media formulations at two different temperatures.

MATERIAL AND METHODS

Fungi tested

Nine isolates of gliotoxin-producing strains of *Aspergillus fumigatus* were used to investigate the influence of medium and temperature on gliotoxin production. The strains were isolated from sputum (four), nose swab (two) or from tracheal aspirate (three) of immunocompromised patients from a haematology ward with the following underlying diseases: myeloid or lymphatic leukaemia (six), Hodgkin's lymphoma (one), non-Hodgkin's syndrome (one). One strain was isolated in a heart operation. Isolates were stored on 2 % Sabouraud glucose agar (Merck, Germany) slants at +4 °C before analysis. At the time of analysis, nine isolates of *A. fumigatus* were grown on Sabouraud 2 % (w/V) glucose agar slants for 3 days at (25±2) °C and the conidia were harvested with sterile saline with 0.1 % polysorbate 80 (Tween® 80). The concentration of conidia was approximately 107 mL^{-1}, estimated by inoculation of 100 µL of conidial suspension on the surface of 2 % Sabouraud glucose agar. One mL of conidial suspension was used for inoculation of 50 mL of media for biosynthesis.

Biosynthesis

Two liquid media were used for biosynthesis: yeast-extract-sucrose broth (YES, composition: yeast extract 20 g, sucrose 40 g, distilled water 1000 mL, pH 5.8±0.2) and Czapek-Dox broth (CZA, composition: $MgSO_4x7H_2O$ 0.5 g, $FeSO_4$ 0.01 g, KCl 0.5 g, KH_2PO_4 1 g, $NaNO_3$ 3 g, sucrose 30 g, distilled water 1000 mL, pH 7.3±0.2). Erlenmayer flasks (250 mL) were filled with 50 mL of media for biosynthesis and autoclaved for 15 minutes at 121 °C. After cooling at room temperature, broths were inoculated with conidial suspension and incubated at two temperatures: (25±2) °C and (37±1) °C. The incubation time was 3, 6, 9, and 12 days. During the incubation, flasks were shaken manually at least twice a day.

All microbiological media and chemicals were purchased from Merck, Germany. All chemicals were of analytical grade.

Extraction and quantification of gliotoxin

After incubation time, the biomass was extracted with 50 mL of chloroform (Kemika, Croatia) and cut up in small pieces with electric homogeniser at 3.500 rpm for 10 min. The biomass was then filtered through a Whatman No.1 filter paper and extracted using 2x25 mL of chloroform, and filtered through anhydrous Na_2SO_4 (Merck, Germany). Chloroform fraction was pooled and evaporated to dryness on rotary evaporator under reduced pressure at 60 °C. Dried extracts were dissolved in 500 μL chloroform and stored at +4 °C until gliotoxin was analysed. The detection and semi-quantitative analysis was performed using a method described by *Bauer et al*. (14). The concentration of gliotoxin in extracts was determined by comparing the fluorescence of gliotoxin from extracts and from standard solutions of known concentration (from 0.01 $\mu g\ mL^{-1}$ to 0.5 $\mu g\ mL^{-1}$) on thin-layer chromatography plates (TLC, silica gel GF254 with fluorescent indicator, thickness 0.25 mm, 20x20 cm, Merck, Germany) under 366 nm. Before that, the plates were developed in mobile-phase toluene:ethyl acetate:formic acid (5:4:1), sprayed with 20 % $AgNO_3$ in ethanol (96 %) and heated for 10 minutes at 110 °C.

The calibration curve for gliotoxin production was constructed in two different media. All chemicals and solvents were of analytical grade.

Statistical analysis

The concentration of gliotoxin obtained from nine *A. fumigatus* isolates cultured for biosynthesis in the same medium and at the same temperature is expressed as mean ±SE. The concentration of gliotoxin from the YES broth was compared with the concentration from the CZA broth using the *t*-test non-parametric Mann-Whitney U post-test (GraphPad Software, USA). The level of significance was set as $p<0.05$.

RESULTS AND DISCUSSION

The effect of temperature and two liquid media formulations on the gliotoxin production of nine clinical isolates of *A. fumigatus* was tested *in vitro*. The results are shown in Figures 2 and 3.

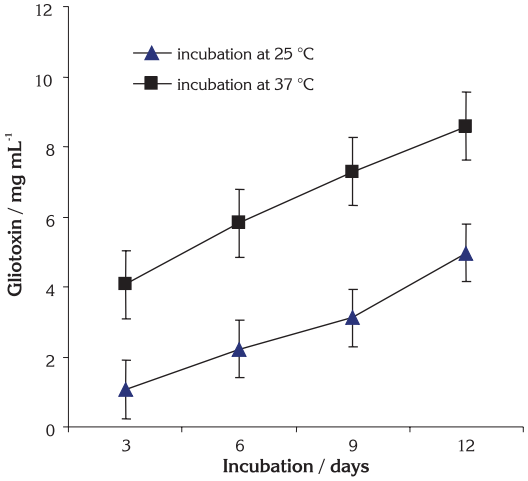

Figure 2 *Gliotoxin production in the YES broth at different incubation temperatures*

Gliotoxin was detected after 3 days of incubation in the YES broth at both temperatures of (25±2) °C and (37±1) °C. Higher production was observed at (37±1) °C than at (25±2) °C, and higher temperature stimulated gliotoxin production during the experiment. After 12 days of incubation at (37±1) °C, the concentration of gliotoxin doubled compared to the concentration incubated at (25±2) °C. The concentration of gliotoxin in the same media

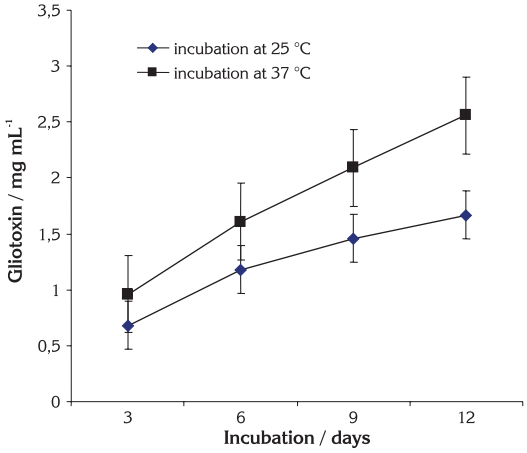

Figure 3 *Gliotoxin production in the CZA broth at different incubation temperatures*

increased progressively during incubation, the average being 4.06 mg mL^{-1} after 3 days, and 8.59 mg mL^{-1} after 12 days. At (25±2) °C, the concentration of gliotoxin in the YES broth doubled more quickly than at (37±1) °C. The average concentration after 3 days was 1.07 mg mL^{-1} and after 6 days 2.22 mg mL^{-1}. The concentration of gliotoxin was five times higher after 12 days than after 3 days at the same temperature and in the same medium. As a medium containing 4 % sucrose (source of carbon) and 2 % yeast extract (source of nitrogen), YES is a very rich substrate for *in vitro* production of gliotoxion, a lipophilic secondary metabolite of *A. fumigatus*.

The influence of a synthetic media Czapek-Dox broth, containing 3 % sucrose as a source of carbon and 0.3 % $NaNO_3$ as a source of nitrogen, on gliotoxin production was also compared between two temperatures. After 3 days, gliotoxin production was higher at (37±1) °C than at (25±2) °C, and the concentrations increased after 6, 9 and 12 days. After 12 days of incubation at (37±1) °C, the concentration of gliotoxin doubled with respect to the concentration at (25±2) °C, indicating that higher temperature better stimulated gliotoxin production in the synthetic Czapek-Dox broth.

The concentration of gliotoxin, produced in different media formulations (YES and CZA), was compared with regard to the incubation temperature. Statistically significant differences in gliotoxin production were noted after 3, 6, 9, and 12 days in six strains of *A. fumigatus* on the YES and CZA broth at both temperatures, with a higher production in the YES broth ($p<0.05$). Our results indicate that *in vitro*

production of gliotoxin is higher in YES broth than in CZA broth, and that gliotoxin could be detected after only 3 days of incubation.

Our results were confirmed by an earlier study by *Belkacemi et al*. (17), who found that incubation at higher temperatures (37 °C vs. 10 °C, 20 °C, and 30 °C) increased gliotoxin production.

As a thermophilic mould, *A. fumigatus* shows faster growth at higher temperatures, which is associated with greater gliotoxin production. We tested gliotoxin production in liquid media containing 4 % or 3 % sucrose. The differences between the two media suggest that the yeast extract (2 %) with a greater amount of sucrose (4 %) better stimulates *in vitro* production of gliotoxin.

CONCLUSIONS

Gliotoxin is one of the mycotoxins produced by *Aspergillus fumigatus*, the most common mould isolate in the life-threatening invasive aspergillosis. It has many biological effects, immunosuppression being the most studied. *In vitro* production of mycotoxins is mediated by substrates as source of its precursors.

Our study shows that liquid media with 4 % sucrose and 2 % yeast extract (YES) at the incubation temperature of 37 °C are both suitable for rapid screening of *A. fumigatus* for gliotoxin production, because it takes no longer than three days to detect gliotoxin in these cultures. YES is a very rich medium for gliotoxin production, and after three days, the concentrations in the YES broth were 4.06 mg mL^{-1} and 1.07 mg mL^{-1} at 37 °C and 25 °C, respectively. Gliotoxin is produced during mycelar growth of *A. fumigatus*.

REFERENCES

1. Shah DT, Larsen B. Clinical isolates of yeast produce a gliotoxin-like substance. Mycopathologia 1991;116:203-8.
2. Richard JL, DeBey MC, Chermette R, Pier AC, Hasegawa A, Lund A, Bratberg AM, Padhye AA, Connole MD. Advances in veterinary mycology. J Med Vet Mycol 1994;32 Suppl 1:169-87.
3. Shah DT, Glover DD, Larsen B. In situ mycotoxin production by *Candida albicans* in women with vaginitis. Gynecol Obstet Invest 1995;39:67-9.
4. Waring P, Eichner RD, Müllbacher A. The chemistry and biology of the immunomodulating agent gliotoxin and related epipolythiodioxopiperazines. Med Res Rev 1988; 8:499-524.
5. Latge J.-P. *Apsegillus fumigatus* and aspergillosis. Clin Microbiol Rev 1999;12:310-50.
6. Bondy GS, Pestka JJ. Immunomodulation by fungal toxins. J Toxicol Environ Health B 2000;3:109-43.
7. Amitani R, Taylor G, Elezis EN, Llewellyn-Jones C, Mitchell J, Kuze F, Cole PJ, Wilson R. Purification and characterization of factors produced by *Aspergillus fumigatus* which affect human ciliated respiratory epithelium. Infect Immun 1995;63:3266-71.
8. Müllbacher A, Waring P, Eichner RD. Identification of an agent in cultures of *Aspergillus fumigatus* displaying anti-phagocytic and immunomodulating activity in vitro. J Gen Microbiol 1985;131:1251-8.
9. Sutton P, Beaver J, Waring P. Evidence that gliotoxin enhances lymphocyte activation and induces apoptosis by effects on cyclic AMP levels. Biochem Pharmacol 1995;50:2009-14.
10. Hogan LH, Klein BS, Levitz SM. Virulence factors of medically important fungi. Clin Microbiol Rev 1996;9:469-88.
11. Waring P, Khan T, Sjaarda A. Apoptosis induced by gliotoxin is preceded by phosphorylation of histone H3 and enhanced sensitivity of chromatin to nuclease digestion. J Biol Chem 1997;272:17929-36.
12. Kroll M, Arenzana-Seisdedos F, Bachelerie F, Thomas D, Friguet B, Conconi M. The secondary fungal metabolite gliotoxin targets proteolytic activities of the proteasome. Chem Biol 1999;6:689-98.
13. Tomee JFCh, Kauffman HF. Putative virulence factors of *Aspergillus fumigatus*. Clin Exp Allergy 2000;30:476-84.
14. Bauer J, Gereis M, Bott A, Gedek B. Isolation of a mycotoxin (gliotoxin) from a bovine udder infected with *Aspergillus fumigatus*. J Med Vet Mycol 1989;27:45-50.
15. Richard JL, Dvorak TJ, Ross PF. Natural occurrence of gliotoxin in turkeys infected with *Aspergillus fumigatus*. Fresenius. Mycopathol 1996;134:167-70.
16. Lewis RE, Chi J, Wiederhold NP, Kontoyiannis DP, Han X, Prince RA. Detection of gliotoxin, an immunonogically-active metabolite secreted by *Aspergillus fumigatus*, in animals and humans with invasive aspergillosis (IA). 43rd Annual Meeting of the Interscience Conference on Antimicrobial Agents and Chemotherapy (43rd ICAAC). Chicago, USA 2003. Abstracts p. 459.
17. Belkacemi L, Barton RC, Hopwood V, Evans EG. Determination of optimum growth conditions for gliotoxin production by *Aspergillus fumigatus* and development of a novel method for gliotoxin detection. Med Mycol 1999;37:227-33.

Sažetak

UTJECAJ MEDIJA I TEMPERATURE NA TVORBU GLIOTOKSINA U SOJEVA *ASPERGILLUS FUMIGATUS*

Gliotoksin je sekundarni metabolit iz skupine epipolitiodioksipiperazina s biološki aktivnim internim disulfidnim mostom u molekuli, koji tvore razne plijesni i gljivica *Candida albicans*.
Plijesan *Aspergillus fumigatus* vodeći je uzročnik invazivnih aspergiloza i također može tvoriti gliotoksin. Pretpostavlja se da *in situ* tvorba gliotoksina utječe na patogenezu aspergiloze.
U ovom radu ispitali smo *in vitro* tvorbu gliotoksina u devet sojeva *A. fumigatus* vrste, izoliranih iz imunokompromitiranih pacijenata. Praćen je utjecaj medija i temperature inkubacije na tvorbu gliotoksina. Gliotoksin je ekstrahiran iz biomase i koncentracija mu je utvrđena polukvantitativno tankoslojnom kromatografijom. Tvorba gliotoksina uočena je već nakon trodnevne inkubacije u koncentracijama 4,06 mg mL^{-1} (u bujonu s kvaščevim ekstraktom – YES na 37 °C) i 1,07 mg mL^{-1} (u sintetskom Czapek-Dox bujonu – CZA na 25 °C). Tvorba gliotoksina bila je veća u YES bujonu, za razliku od sintetskog CZA bujona. Viša temperatura inkubacije (37 °C) također utječe na jaču tvorbu gliotoksina nego niža temperatura (25 °C).
YES bujon s dodatkom 4 % saharoze i 2 % kvaščeva ekstrakta vrlo je bogat supstrat za *in vitro* tvorbu gliotoksina, kao jednog od lipofilnih sekundardnih metabolita plijesni vrste *A. fumigatus*.

KLJUČNE RIJEČI: *aspergiloza, Aspergillus sp.***,** *bujon kvaščevog ekstrakta, Czapek-Dox bujon, toksikogenost*

REQUESTS FOR REPRINTS:

Ivan Kosalec
Faculty of Pharmacy and Biochemistry, University of Zagreb
Department of Microbiology
Schrottova 39/I, HR-10000 Zagreb, Croatia
E-mail: *ikosalec@pharma.hr*