Effect of Fluconazole on Phagocytic Response of Polymorphonuclear Leukocytes in a Rat Model of Acute Sepsis

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Recently, fluconazole (FLZ) has been shown to improve survival and reduce multiorgan failure in experimental and clinical septic shock. The mechanism by which FLZ affords protection against sepsis remains obscure. This study examines the effect of FLZ on phagocytic activity of polymorphonuclear leukocytes (PMNs) in a rat model of septic shock by inducing fecal peritonitis in male Wistar rats using intraperitoneal instillation (1 mL/kg) of fecal suspension in saline (1 : 1 w/v). Sham control rats received sterile fecal suspension and vehicle treatment. FLZ was administered in the doses of 0, 3, 10, and 30 mg/kg by gavage 30 minutes before fecal instillation. The samples of peritoneal fluid were collected 8 hours following fecal inoculation for the evaluation of phagocytic response of PMNs using zymosan-induced luminol-dependent chemiluminescence (CL). Fecal peritonitis caused massive infiltration of PMNs in the peritoneal cavity (ANOVA $F_{4,45} = 6.322$, $P < .001$). Although FLZ reduced the infiltration of PMNs, this effect was neither significant nor dose dependent. The actual CL response was significantly higher in the peritoneal fluid of rats subjected to peritonitis, which was significantly and dose-dependently attenuated by FLZ treatment (ANOVA $F_{4,45} = 11.048$, $P < .001$). Normalization of CL response for 1000 PMNs revealed that FLZ dose-dependently albeit insignificantly reduced the activity of PMNs. The high dose of FLZ caused 2.29-fold decrement in the area under curve (AUC) pertaining to cumulative CL response. The findings of this study suggest that FLZ protects rats against septic shock by inhibiting PMN-mediated inflammatory cascade without compromising their phagocytic activity.

INTRODUCTION

Sepsis and its sequelae are often lethal and considered to be the leading causes of mortality in intensive care units [1, 2]. The cascade of events initiating from infection to septic shock and multiorgan failure is poorly understood. Polymorphonuclear leukocytes (PMNs) are the first line of defense against foreign antigens. Effective host defense against bacterial infection depends on the recruitment and activation of PMNs that localize, kill, and clear the pathogens [3, 4]. On the other hand, massive inflammatory reaction resulting from uncontrolled sequestration and prolonged activation of PMNs could be potentially deleterious and has largely been implicated in sepsis-mediated multiorgan dysfunction [5, 6, 7, 8]. Thus, PMNs (neutrophils and macrophages) could be regarded as dual-edged weapons, their controlled migration and optimal activity being essential for beneficial effects [4, 9, 10]. Therapeutic strategies to attenuate excessive acute inflammatory responses without compromising essential host defense mechanisms would logically benefit in preventing neutrophil-mediated organ damage in septic shock [11, 12].

The immunomodulating feature of azole antifungal drugs is a known fact [13, 14]. Fluconazole (FLZ) is a well-tolerated antifungal drug with a demonstrated ability to reversibly penetrate into human PMNs [15]. Recently, FLZ has been shown to improve survival and reduce multiorgan damage in experimental [16] and clinical septic shock [17]. Since FLZ has no inherent antibacterial properties, its beneficial effects in bacteremia have been attributed to its action on the modulation of neutrophils sequestration and activation [17]. However, the findings of earlier studies regarding the effect of FLZ on the phagocytic response of PMNs are controversial. Only few studies have reported enhanced bactericidal activity of PMNs in presence of FLZ [18, 19]. Even FLZ-induced increase in the phagocytosis and intracellular killing of *candida* have been linked to its direct effect on yeast rather than on phagocytes [19]. On the other hand, many investigators observed that FLZ did not affect the phagocytic activity of PMNs [13, 20, 21, 22, 23, 24, 25]. It is therefore intriguing to better understand the nature of interaction between FLZ and PMNs in sepsis, and the present investigation is an attempt in this direction.

Table 1. Effect of fluconazole (FLZ) on luminol-dependent chemiluminescence response of phagocyting leukocytes in the peritoneal fluid of rats with septic shock.

Treatment group	Leukocytes count in peritoneal fluid per mm^3	RLU (integral value)		RLU (peak value)	
		Per 500 nL peritoneal fluid	Per 1000 leukocytes	Per 500 nL peritoneal fluid	Per 1000 leukocytes
Control (sham)	9440 ± 780.2	6.67 ± 3.64	1.16 ± .57	8.40 ± 3.10	1.61 ± 0.47
FP	27590 ± 3007.2#	276.94 ± 39.99#	21.21 ± 2.97#	188.30 ± 25.19#	14.42 ± 1.81#
FP + FLZ 3	23730 ± 3397.6	208.60 ± 30.85	18.23 ± 2.33	140.40 ± 19.33	12.28 ± 1.42
FP + FLZ 10	19580 ± 2948.7	164.86 ± 28.24*	17.85 ± 2.39	110.80 ± 19.28*	12.00 ± 1.61
FP + FLZ 30	21630 ± 2589.3	144.78 ± 33.78**	13.06 ± 2.94	97.70 ± 23.25**	8.87 ± 2.05

# denotes $P < .001$ versus control group, $*P < .05$, and $**P < .01$ versus FP group using Dunnett's multiple comparison test. RLU (relative light unit) is an arbitrary unit of chemiluminescence measurement.

MATERIALS AND METHODS

Animals

Adult male Wistar rats were housed in a temperature-controlled room maintained on 12- hour light/dark cycles. The standard laboratory food and water were freely available *ad libitum* except an overnight fasting before induction of experimental sepsis.

Drug and dosing

Animals were divided into 5 groups of 10 animals each. FLZ was administered orally in the doses of 0, 3, 10, and 30 mg/kg body weight, 30 minutes before fecal inoculation. This dose regimen was selected on the basis of our earlier study [16]. Control rats received sham surgery and vehicle treatment.

Induction of sepsis in rats

A fecal peritonitis (FP) model was used to induce acute sepsis in rats [26]. Homogeneous fecal suspension was prepared by dissolving fresh feces (1 : 1 w/v in normal saline) obtained surgically from the caecum of nonfasted healthy rats, and used within 2 hours. A small incision was made in the abdomen of ether-anesthetized rats for intraperitoneal instillation of fecal suspension at a dosage of 1 mL/kg body weight of animals. Control animals received preautoclaved fecal suspension. The wound was closed aseptically and animals returned to home cages. The specimens of peritoneal fluid were collected 8 hours following fecal inoculation. The samples were diluted (100 folds) in Hank's balanced salt solution (HBSS) containing 0.1% gelatin and promptly analyzed.

Measurement of phagocytosis

A sensitive procedure based on zymosan-induced luminol-enhanced chemiluminescence (CL) was used to measure phagocytic response of leukocytes [27]. Fifty microliters of prediluted peritoneal fluid samples were mixed with 150 μL of reaction mixture (4×10^{-4} M luminol + 50 μg opsonized zymosan + 0.1% gelatin in HBSS) in the well of an opaque cliniplate (Labsystems, Finland).

The CL signal produced by phagocytosing leukocytes was measured at 37°C in a luminometer (Model Luminoskan RT, Labsystems). Thirty cycles of measurements using 5- second counting time and 70- second interval time were performed for each sample. The CL intensity was expressed as relative light unit (RLU). Leukocytes were counted in all the samples using a haemocytometer.

The CL response (actual or normalized) has been reported as peak value (highest signal among 30 measurements) and integral value (integration of all the signals for 30- minute measurement time). The actual CL response corresponds to 50 μL of 100-fold diluted peritoneal fluid sample (equivalent to 500 nL of peritoneal fluid), irrespective of the number of leukocytes within the sample. The normalized CL (per 1000 leukocytes) was used to compare phagocytic activity of the leukocytes in different treatment groups. The area under curve (AUC) was measured (in triplicate) using the public domain image processing and analysis program developed at the National Institute of Health, USA. The PC version of this program, known as Scion Image, is available on http://www.scioncorp.com for free download from Scion, Md, USA.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Pearson's test was used for parametric correlations. The statistical significance was defined by $P < .05$.

RESULTS

Leukocyte count

The leukocyte count was significantly increased in the peritoneal fluid of rats subjected to FP (27590 ± 3007 mm^3) as compared to sham control rats (9440 ± 780 mm^3) (ANOVA $F_{4,45} = 6.322$, $P < .001$). Pretreatment with FLZ attenuated leukocyte count in the peritoneal fluid, however this effect was neither dose dependent nor significant (Table 1).
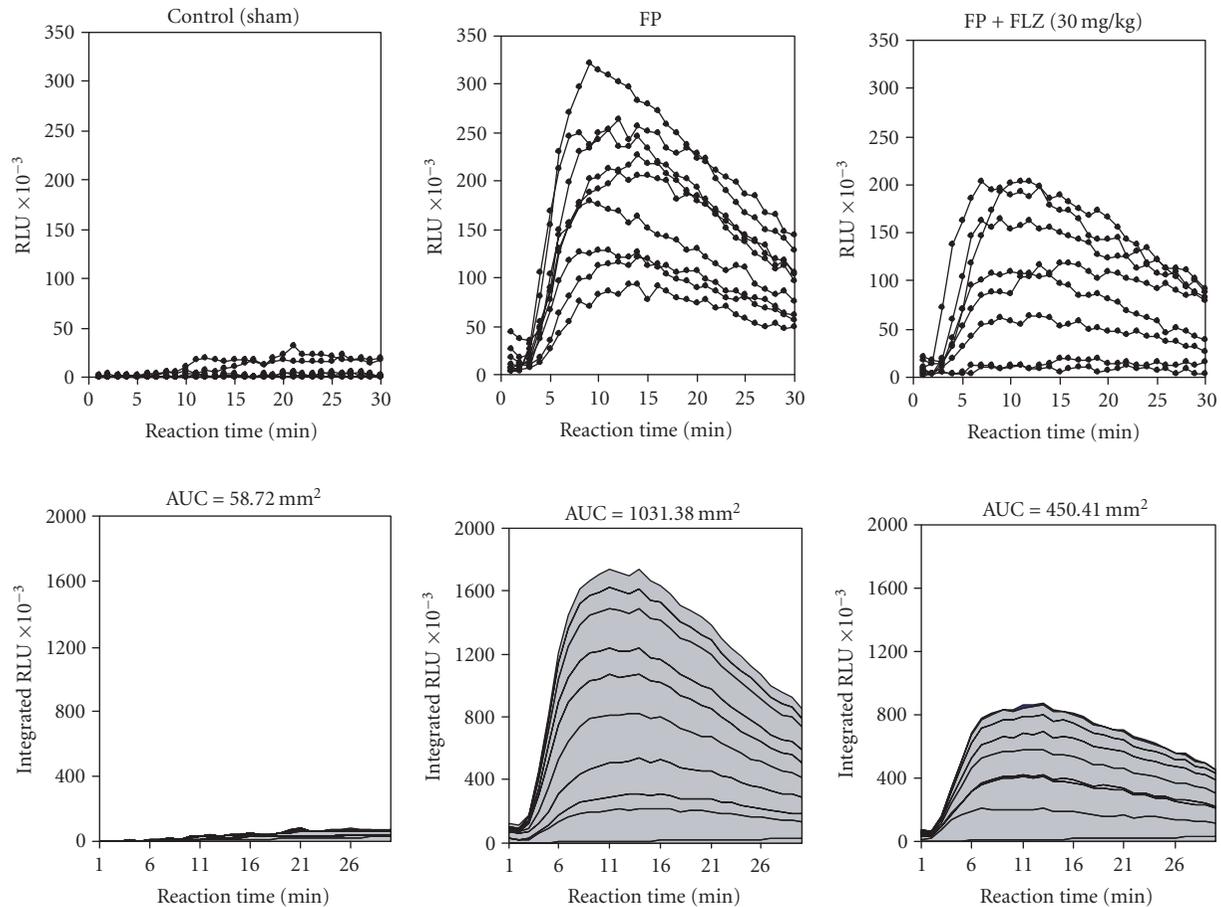
FIGURE 1. Real-time chemiluminescence (CL) signals from individual samples showing the effect of fluconazole (FLZ) on phagocytic activity of leukocytes in the peritoneal fluid (500 nL) of rats with fecal peritonitis (upper-panel line graphs). Areas under curves were used to display cumulative representation of all the samples and to measure area under curve (AUC) (lower panel). x-axes show the measurement time of each sample in the luminometer.

Actual CL response (effective phagocytosis)

Both integral and peak CL responses (RLUs) were significantly higher in peritonitis group as compared to sham control (Table 1). Administration of FLZ significantly and dose-dependently attenuated the integral (ANOVA $F_{4,45} = 11.048$, $P < .001$) and peak (ANOVA $F_{4,45} = 11.345$, $P < .001$) CL responses (Table 1). The representative real-time CL signals for control and FP with or without FLZ (30 mg/kg) groups are shown in Figure 1. The AUC of peritonitis group (1031.38 ± 16.31 mm^2) was 17.56-fold greater than sham control (58.72 ± 3.12 mm^2). Treatments of rats with FLZ (30 mg/kg) markedly reduced (2.29-fold) the AUC as compared to peritonitis alone group (Figure 1, lower panel).

Normalized CL response (leukocyte activity)

The normalization of CL to 1000 leukocytes showed significantly high phagocytic response in peritonitis group as compared to sham control (Table 1). Both inte-gral (ANOVA $F_{4,45} = 10.754$, $P < .001$) and peak (ANOVA $F_{4,45} = 10.164$, $P < .001$) normalized RLUs were significantly higher in the peritonitis alone group. Pretreatment with FLZ dose-dependently reduced the normalized CL responses; however, these effects failed to reach the significance level (Table 1).

Correlation between integral and peak RLUs

A highly significant correlation (correlation coefficient, R = 0.995, $P < .001$) was observed between integral and peak CL responses, irrespective of the treatments administered (Figure 2).

Correlation between leukocyte count and integral RLUs

The plot of leukocyte count versus integral RLUs is shown in Figure 3. The correlation was statistically significant (R = 0.693, $P < .001$); however, the data points from various treatment groups appeared staggered around the trend line (Figure 3).
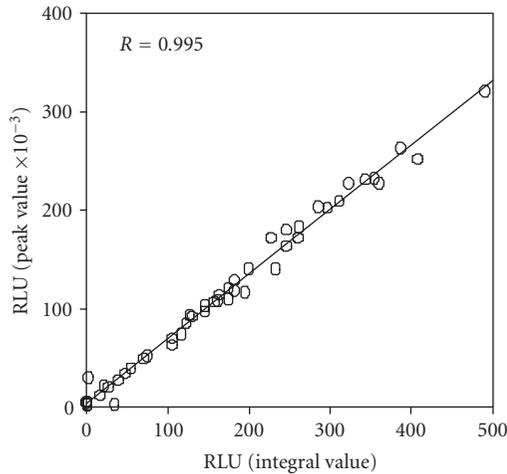
FIGURE 2. Correlation between the two parameters used to measure chemiluminescence (CL) response of phagocyting leukocytes. Peak value represents the maximal signal intensity among the 30 measurement points, whereas the integral value is the total integration of CL signals for a 30-minute period.
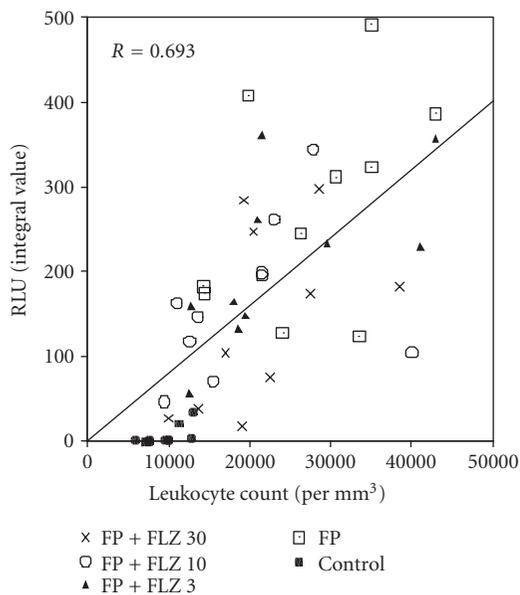

FIGURE 3. Correlation between chemiluminescence signal and leukocyte count in the peritoneal fluid samples.

DISCUSSION

Fecal peritonitis caused massive infiltration of PMNs in peritoneum (Table 1), which is in accordance with our earlier study [28]. Administration of FLZ insignificantly reduced the sequestration of PMNs to the infectious focus. PMNs migration into the peritoneal cavity in response to FP is an important mechanism of host defense against bacterial invasion as the exudative

PMNs localize and contain infection by phagocytizing and killing bacteria [29, 30, 31]. The migrated PMNs in the peritonitis group showed a highly significant upregulation of their phagocytic activity as compared to sham control (Table 1, Figure 1). Although resting PMNs (neutrophils) consume little O_2, their activation involves a marked increase in O_2 uptake, often known as "respiratory burst" that results in the production of potentially toxic reactive oxygen species (ROS) [32, 33]. The upsurge in O_2 uptake is associated with the activation of an enzymatic process that oxidizes NADPH to NADP$^+$, the electron transfer being used to reduce O_2 into superoxide radical ($O_2^{\cdot-}$) that further reacts with H_2O_2 to produce hydroxyl radical (OH$^{\cdot}$) [34]. An enzyme, myeloperoxidase (MPO) is released from activated neutrophils that form hypochlorite (HOCl) after reaction with H_2O_2 and Cl$^-$. Activated neutrophils and macrophages are also able to release nitric oxide that combines with $O_2^{\cdot-}$ to produce peroxynitrile (ONOO$^-$) [35]. All these ROS have a tendency to produce CL in presence of luminol [36, 37, 38]. Therefore, a high CL signal from migrated PMNs during sepsis indicates excessive generation of ROS that might trigger pathways for host damage [39, 40, 41]. The deleterious effects of ROS are limited by antioxidant defense systems. Increased production of ROS and decreased antioxidant status have been reported in septic shock patients [42, 43, 44, 45].

It is widely accepted that the multiple organ dysfunction is a consequence of generalized inflammatory reaction whereas infection *per se* triggers these pathways [6, 46]. The natural resolution of acute inflammation involves bulk clearance of extravasated inflammatory cells in an ordered manner [47]. However, an imbalance between the clearance and infiltration of PMNs and/or disruption of equilibrium between bacterial load and extent of phagocytosing PMNs could be deleterious to host cells. Recent studies have shown that agents with antiinflammatory and/or antioxidant properties significantly protect against experimental [48, 49, 50, 51, 52] and clinical septic shock [53, 54].

Administration of FLZ significantly and dose-dependently reduced the CL response of PMNs in the rats with septic shock (Table 1). On the other hand, the normalized CL values (per 1000 leukocytes) showed that the activity of PMNs is only insignificantly affected by FLZ, which is supported by earlier findings [13, 20, 21, 22, 23, 24, 25]. Taken together, these observations suggest that FLZ effectively counteracts excessive ROS generation by PMNs without significantly altering their functionality. The effect of FLZ on controlling the ROS formation by activated PMNs is further evidenced by more than 50% reduction of AUC in FLZ (30 mg/kg) group as compared to vehicle-treated rats (Figure 1). The correlation between integral and peak CL responses was independent of treatment groups, suggesting a direct relation between the activity of PMN and sustained production of ROS (Figure 2). Although the correlation analysis between PMNs count and CL response showed

a positive trend, data points from various treatment groups appeared staggered (Figure 3). This within-group variation may be attributed to different magnitudes of immunity in animals. However, this finding points towards the usefulness of individual assessment of septic shock patients with regard to PMNs counts and activity for selecting the effective dose regimen of FLZ.

In conclusion, the protective effects of FLZ in experimental sepsis may be attributed to the inhibition of ROS-mediated proinflammatory cascade without compromising effective pathogen clearance. However, the relevance of these findings with human septic shock remains unclear and warrants further clinical study.

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