Short Report: Beneficial Effects of Benznidazole during an Infectious-based Situation of Systemic Inflammatory Response: Cecal Ligation and Puncture

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Abstract. We have shown that benznidazole (BZL), a drug used to treat Chagas disease, markedly reduced the production of pro-inflammatory cytokines and NO-derived metabolites in experimentally Trypanosoma cruzi–infected rats. Treatment with BZL exerted beneficial effects in a model of inflammation-based pathology like murine experimental endotoxemia. Based on these findings, we wished to ascertain the effect of BZL in a closer situation to sepsis: the cecal ligation and puncture (CLP) model in C57BL/6 mice. We analyzed clinical course, survival, circulating levels of inflammation-related compounds (NO, tumor necrosis factor [TNF]-α, and bacteremia). Recipients of BZL, 25 mg/kg, had an increased survival rate at 24 hours after CLP, showing a better clinical situation and a significant reduction of TNF-α levels and bacteremia, with respect to the other groups. BZL failed to inhibit in vitro bacterial growth, suggesting that these effects may be partly caused by the immunomodulatory effects of BZL.

Benznidazole (BZL) has been used for the treatment of acute Chagas’ disease for more than three decades. In addition to its trypanocidal activity, our studies indicate that BZL also affects the synthesis of important biological response modifiers with potential implications in inflammatory processes. By using different experimental approaches, it was shown that BZL downregulated NO and cytokine synthesis by lipopolysaccharide (LPS) and/or interferon (IFN)-γ-stimulated murine macrophages. Systemic treatment with BZL in Trypanosoma cruzi–infected rats inhibited the production of pro-inflammatory cytokines and NO-derived metabolites. Beneficial effects of BZL were also seen in murine experimental endotoxemia. C57BL/6 mice given BZL orally and challenged with LPS intraperitoneally had decreased mortality, reduced serum levels of pro-inflammatory cytokines, diminished number of interleukin (IL)-6–producing peritoneal macrophages, and lowered IL-12 and inducible NO synthase (iNOS) mRNA expression in liver samples. These findings provided a stimulating background for analyzing the usefulness of BZL during infections, accompanied by a strong and ultimately detrimental inflammatory reaction. Ineffective clearance of pathogens or failure of regulatory mechanisms may result in systemic inflammation, further progressing to an aggravated situation such as septic shock. Septic shock seems to have a multifactorial basis, with tissue damage being not only caused by the presence of pathogens but also to mediators released in response to infection. Tumor necrosis factor (TNF) is a major inflammatory cytokine in this regard. Factors involved in anti-microbial mechanisms also include NO, which plays diverse biological roles (i.e., vascular tone, neurotransmission, and immune response). NO overproduction may be relevant for the development of some pathological events occurring during septic shock.

Given this background, we analyzed the effect of BZL in a closer situation to sepsis, the cecal ligation and puncture (CLP) model. The CLP murine model mirrors more closely the clinical course of abdominal sepsis in humans, because it is triggered by an endogenous septic focus progressing to a polymicrobial infection with systemic inflammatory response syndrome.

Male C57BL/6 mice, 8–10 weeks of age, were provided by the School of Veterinary Sciences, National University of La Plata. Experiments were conducted according to internationally accepted guidelines for animal handling. Mice were divided into eight experimental groups: 1) sham control plus vehicle (V); 2) sham control plus treatment with BZL 10 mg/kg body weight; 3) sham control plus BZL 25 mg/kg body weight; 4) sham control plus BZL 200 mg/kg body weight; 5) CLP + V; 6) CLP + BZL 10 mg/kg body weight; 7) CLP + BZL 25 mg/kg body weight; and 8) CLP + BZL 200 mg/kg body weight.

The CLP studies were performed according to Baker and others, with slight modifications. Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylacine (10 mg/kg). Under aseptic conditions, the abdomen was opened to extract and ligate the cecum directly under the ileum–cecum valve. A double puncture using a 21-gauge needle was done to spread the cecal content into the peritoneal cavity. The ligated and perforated cecum was placed in the peritoneal cavity, and the surgical incision was closed with 4-0 silk sutures. All mice received 0.1 mL of normal saline subcutaneously for fluid resuscitation and were placed on a heating pad until they recovered from anesthesia. Sham controls underwent the same surgical procedures, but the cecum was neither ligated nor punctured.

BZL (Roche Laboratories, Buenos Aires, Argentina) was dissolved in carboxymethylcellulose (CMC; Sigma-Aldrich, Buenos Aires, Argentina). Mice were given BZL suspended in 1% CMC through the oral route with an intubation syringe for animal feeding in a 0.1-mL volume. Therapy with BZL was given 2 hours before CLP and every 12 hours after surgery. Control animals received vehicle alone. Preventive therapy was applied to allow drug absorption to further reach pharmacologically active concentrations because it was administered by the oral route. The amount of mice surviving at different time points after the surgical procedure is shown in Table 1. Most animals were dead by the end of the experiment (96 hours after CLP) regardless of the group under analysis. However, comparisons at 24 hours after CLP
showed an increased survival rate in mice given BZL 25 mg/kg body weight ($P < 0.05$). These animals showed a better clinical situation, as judged by their response to external stimuli, ability to maintain upright position, and normal breathing. Because recipients of 10- and 25-mg/kg body weight doses were in better condition, further studies concentrated on these two mouse groups.

Parallel mice groups were killed at 90 minutes and 4 and 24 hours after being subjected to CLP to assess changes in serum levels of TNF-$\alpha$ and NO-derived metabolites. Values of NO-derived metabolites were within baseline levels (16–29 $\mu$mol/L) at 90 minutes and 4 hours after CLP, irrespective of the group under analysis. The same was true for sham control mice at 24 hours after CLP. In contrast, increased amounts of nitrate were observed when analyzing samples from the CLP group taken at 24 hours after CLP compared with values seen in sham operated controls. As shown in Figure 1, we found no significant differences in levels of this mediator between animals with CLP treated with BZL and those who were not.

Analysis of TNF-$\alpha$ serum levels at 24 hours after CLP showed that mice given BZL 25 mg/kg body weight had the lowest cytokine values, which were significantly different from the CLP + V group ($P = 0.04$; Figure 2). No measurable amounts of TNF-$\alpha$ were found when analyzing samples taken 90 minutes and 4 hour after CLP or in sera from sham control mice.

For bacteriemia studies, blood samples taken 24 hours after surgery were serially diluted and cultured on agar Columbia-blood plates at 37°C for 16 hours. No bacteriemia was detected in non-manipulated mice or sham operated controls. As shown in Figure 3, mice from the CLP + V and CLP + BZL 10 mg/kg groups displayed increased levels of bacteremia. In contrast, CLP mice given BZL at 25 mg/kg body weight had significantly decreased bacteremia with respect to the CLP + V group ($P < 0.05$). To test whether BZL exerted a direct bactericidal effect, the drug was further added to cultures. Cecal content obtained under sterile conditions was cultured on Petri plates containing different culture media: chocolate-agar, blood-agar, cysteine-lactose electrolyte deficient (CLDE), and Salmonella-Shigella, in an attempt to recover habitual bacteria from intestinal flora. Five-millimeter-diameter disks were embedded with dimethyl sulfoxide (DMSO; Sigma) or BZL dissolved in DMSO at concentrations known to mediate in vitro immunomodulatory effects (0.1 and 1 mmol/L) or above that (10 mmol/L). Petri plates were incubated overnight at 37°C under aerobic or anaerobic conditions. In no case was BZL able to inhibit bacterial growth (data not shown).

In contrast to experimental endotoxemia that is characterized by a hyperacute reaction to LPS, CLP represents a much more serious condition because, in addition to the release of bacterial endotoxins, the presence of bacteremia favors septic colonization and the ensuing life-threatening affection. Despite this, recipients of BZL at 25 mg/kg body weight presented an increased survival rate 24 hours after CLP. The infectious nature of this process may have precluded further effects of BZL to be achieved.

NO overproduction is known to be toxic for different organs and systems; this is related to septic shock pathophysiology. In line with this, NO-derived metabolites were found to be increased in CLP mice 24 hours after surgery. The fact that serum nitrate represents the total amount of NO produced by the host may account for the inability of BZL to

![Figure 1. NO-derived metabolite concentrations in serum samples taken 24 hours after CLP. Endogenously synthesized NO in serum was detected as nitrate. Serum nitrate was assessed by reducing nitrate to nitrite and further measured by the Griess reaction. As shown here, there is no statistically significant differences in perceived levels of this mediator between animals with CLP treated with BZL or V. The results are expressed in box-plot format. The box stretches from the lower hinge (defined as the 25th percentile) to the upper hinge (the 75th percentile) and therefore contains the middle half of the scores in the distribution. The median is shown as a line across the box (3–5 mice/group). Two rounds of experiments, yielding similar results, were performed. Non-parametric analysis was performed by the Kruskal-Wallis test.](image-url)
decrease its levels, because this compound may only inhibit the iNOS-driven production.

TNF-α is involved in the early inflammatory response, and studies in human sepsis showed that increased TNF-α concentrations are correlated with a lethal outcome. In this study, TNF-α was clearly noticeable in circulation 24 hours after surgery, with mice receiving BZL at 25 mg/kg body weight showing significantly decreased levels of this cytokine. Mice given the 25 mg/kg dose also showed a better clinical condition at the same time point evaluation; this was not the case for those treated with 10 mg/kg body weight, for which it may be a suboptimal dose. The fact that mice subjected to BZL 25 mg/kg body weight had a lower bacteriemia by 24 hours after CLP may imply a direct or indirect anti-infectious effect of BZL, resulting in a decreased bacterial load and overall reduced levels of TNF-α. Nevertheless, BZL failed to inhibit bacterial growth, for which beneficial effects may be explained by an immunomodulatory effect favoring microbial clearance. Some preliminary studies in peritoneal adherent cells showed fewer yeast-containing cells on treatment with BZL (data not shown). Future studies are needed to ascertain the mechanisms by which this effect is achieved. The fact that bacteriemia levels at an early point were not predictive of the final outcome in this sepsis model suggests that factors other than the pathogen "per se" may be implied in disease outcome (i.e., inflammatory compounds released in response to infection).

Although BZL has been used for > 30 years for treatment of Chagas disease, the mechanisms accounting for its beneficial effects are not yet fully understood. Results from this initial report provide an additional clue for such protective action and show the need for a more in-depth analysis of the mechanisms involved.

FIGURE 2. TNF-α concentrations in sera obtained 24 hours after CLP. Measurement of serum TNF-α levels was made by using a commercially available ELISA kit according to manufacturer’s recommendations (mouse TNF ELISA Set, BD OptEIA; BD Biosciences, San Diego, CA). Analysis of TNF-α serum levels showed that mice treated with BZL 25 mg/kg body weight had significantly lower values than the treated with vehicle (CLP + V; P = 0.04). No amounts of TNF-α were detected in sham controls mice. Data are the mean ± SD of two different experiments. Multiple groups (5–7 mice/group) were analyzed by analysis of variance (ANOVA) followed by a Tukey multiple comparisons test. A value of P < 0.05 was considered statistically significant. *Statistically different from CLP + V.

FIGURE 3. Bacteriemia 24 hours after CLP. Bacterial counts were performed on aseptically harvested blood by cardiac puncture. CFUs per milliliter was determined from serial dilutions of blood in sterile saline, further plated on blood agar plates, and incubated for 16 hours at 37°C. Mice from CLP + V and CLP + BZL 10 mg/kg groups displayed increased levels of bacteriemia. Of note was the fact that CLP mice given BZL at 25 mg/kg body weight had a significantly decreased bacteriemia with respect to the CLP + V group (P < 0.05), suggesting that such a BZL dose may facilitate bacterial clearance. No bacteriemia was detected in mice without manipulation or sham controls. The results are expressed in box-plot format. The box stretches from the lower hinge (defined as the 25th percentile) to the upper hinge (the 75th percentile) and therefore contains the middle half of the scores in the distribution. The median is shown as a line across the box (3–5 mice/group). Two rounds of experiments, yielding similar results, were performed. Non-parametric analysis was performed by the Kruskal-Wallis test followed by a Dunn multiple-comparisons test. A value of P < 0.05 was considered statistically significant. *Statistically different from CLP + V.
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