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Full Length Research Paper

Biochemical role of nitric oxide precursor and antibiotic against typhoid

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Typhoid fever remains an underestimated important health problem in many developing countries. It continues to be a global problem with an annual estimate of 1.6 million cases and 600,000 deaths. *Salmonella*, gram negative *bacilli* can survive during certain stages of host parasites interaction. There are number of drugs being used for the treatment of typhoid, but increasing occurrence of multidrug resistant (MDR) strain of *Salmonella typhi* has complicated its management, that thus, it has necessitated the search of formulated drugs for its treatments. Nitric oxide (NO) is a versatile molecule produced in a biological system. Previous studies have suggested that exogenous administration of L-arginine results in increased NO production, indicating that endogenous substrate is insufficient for maximal NO production. Taking these facts in to consideration, it was thought pertinent to see the effect of oral administration of NO precursor that is, L-arginine. Formulation of nitric oxide precursor and antibiotics shows decreases in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by 54.24% and 53.91% in 1/2 LArg+1/2 Cip group as compared to bacterial treated groups.

Key words: *Salmonella typhi*, L-arginine, ciprofloxacin, alanine aminotransferase (ALT), aspartate aminotransferase (AST).

INTRODUCTION

Typhoid fever is an important health concern in India. Presently available means are not adequate for protection against typhoid; hence there is a need for the development of new methods. *Salmonella* sp. are gram-negative, motile, facultative, intracellular bacilli and their invasion and multiplication within mononuclear phagocytic cells in the liver, spleen, lymph nodes, and Peyer's patches are the hallmark events of typhoid fever (Buchmeier and Heffron, 1989; Hsu, 1989). *Salmonella enterica serovar Typhimurium* is a leading cause of bacterial gastroenteritis, and it can also produce salmonellosis, which is characterized by progressive multiplemicroabscess formations and septicemia (Umezawa et al., 1997; Umezawa et al., 1995). In India more than 2200 strains/serotypes of *Salmonella* are known, most of which are pathogenic to human. Earlier, all the known *Salmonella* were sensitive to commonly administered chemotherapeutic drugs, however; recently there has been a gradual and progressive rise in the multi drug resistant (MDR) strains of *Salmonella*, both in percentage isolates as well as in number of drugs to which these organisms have developed resistance.

Resistance to the traditional first-line antimicrobial agent's ampicillin chloramphenicol, and trimethoprim-sulfamethoxazole defines MDR in *S. enterica.* The MDR phenotype has been shown to be widespread among S. typhi for many years (Umezawa et al., 1995) and is present, albeit at lower rates, among *Salmonella paratyphi* (Rowe et al., 1997; Gupta et al., 2008). Surveillance studies demonstrate considerable geographic variation in the proportion of *S. typhi* isolates that are MDR in the same region, with sites in India, Pakistan, and Vietnam having higher rates of MDR isolates than sites in China and Indonesia (Parry and Threlfall, 2008). The wide distribution and high prevalence of MDR among *Salmonella* sp. has led to fluoroquinolones assuming a primary role in the therapy for invasive salmonellosis. Some

Abrreviations: NO, Nitric oxide; **MDR**, multidrug resistant; **ALT**, alanine aminotransferase.

investigators have noted increases in the prevalence of *S. typhi* and *S. paratyphi* strains susceptible to traditional first-line antimicrobials, coinciding with a switch to fluoroquinolones for the management of enteric fever (Ochiai et al., 2008; Maskey et al., 2008). Ciprofloxacin continues to be widely used, but clinicians need to be aware that patients infected with Salmonella with decreased ciprofloxacin susceptibility may not respond adequately (Sood et al., 1999). To obtain better understanding of the pathogenesis of typhoid fever, it seems crucial to elucidate the host defense function of nitric oxide (NO) against Salmonella. NO is a gaseous, inorganic free radical, and produced in biological system. It regulates a diverse array of physiological functions and acts as inter and extra-cellular messenger in most mammalian organs (Crump et al., 2008). Many types of cells, such as leukocytes, hepatocytes, vascular smooth muscle cells and endothelial cells can produce NO during enzymatic conversion of L-arginine to L-citrulline by NO synthase (NOS). A large amount of NO generated by inducible isoform of the enzyme (iNOS) has been demonstrated. NO functions in biological system in two very important ways. First, it has been found to be a messenger by whom cells communicate with one another (signal transduction). Secondly, it plays critical role in host response to infection. In this second function, it appears that the toxic properties of NO have been harnessed by the immune system to kill or at least slow the growth of invading organisms. However, excess of NO can exert cytotoxic effects (Misko et al., 1993). This may involve both (i) direct toxicity, for example, the reaction of NO with iron-containing enzymes of the respiratory cycle and of the DNA synthetic pathway and (ii) the interaction of NO with free radicals like superoxide ion (O2-) to form peroxynitrite (ONOO), which is a potent oxidizing molecule capable of eliciting lipid peroxidation and cellular damage (Stefenovic-Racic et al., 1993). Hepatic complications of typhoid fever were first reported by Osler (1899). It is uncertain whether Salmonella bacteria produced hepatic dysfunction by direct invasion or by endotoxemia (Khosla et al., 1988). The recent demonstration of intact *S. typhi* in liver tissue of patients with typhoid fever suggests that organisms are phagocytosed by reticuloendothelial system but overcome the cell's killing action and produce hepatic injury by liberating cytotoxic substances in situ (Gitlin, 1999). The present study was designed to determine the clinical and hepatic biochemical alterations in the course of typhoid fever.

MATERIALS AND METHODS

Animals

Swiss albino mice (25-30 g) 6-8 weeks old were obtained from the central animal house of Hamdard University, New Delhi, India. The animals were kept in poly-propylene cages in an air-conditioned room at 22 - 25°C and maintained on a standard laboratory feed (Amrut Laboratory, rat and mice feed, Navmaharashtra Chakan Oil Mills Ltd, Pune) and water *ad libitum*. Animals were allowed to acclimatize for one week before the experiments under controlled light/dark cycle (14/10 h). The studies were conducted according to ethical guidelines of the "Committee for the purpose of control and supervision of Experiments on Animals (CPCSEA)" on the use of animals for scientific research.

Bacteria

In this experiment only *S. typhimurium* (wild) was used. The standard strain of this pathogen was obtained from the National Salmonella Phage Typing Centre, Lady Harding Medical College, New Delhi, India. This bacterial strain was further confirmed by the Department of Microbiology, Majeedia Hospital, New Delhi, India. Animals were divided into six groups. Each group comprised of six animals. The study comprised of following treatment schedules.

The effects of drugs in Table 1 on infected mice by *S. typhimurium* were analyzed. Post-treatment of drugs were done at above dose orally to the experimental animals. On 14th days of post treatment, liver was removed aseptically in sterile condition, homogenate was made and post mitochondrial supernatant was prepared for biochemical estimation.

Serum preparation

Serum was prepared according to the routine method. Briefly, bloods were taken out from retro-orbital sinus using non-heparinised capillary tubes. Blood was collected in dried centrifuge tubes and clot formation was allowed. Serum was separated from the clot by centrifugation for five minutes at 800 x g in a fixed rotor centrifuge at room temperature. After centrifugation serum was collected carefully using a Pasteur pipette. It was kept at -20°C till the enzyme analysis.

Serum enzymes

The activities of ALT and AST were estimated by using the kit supplied by Span Diagnostic Ltd, New Delhi. The procedure of estimation was based on the method described by Reitmann and Frankel (1956). The enzyme activity was expressed in U/ml. Here one unit is defined as one µmole of the pyruvate formed under defined condition per ml of serum. The assay consisted of 0.1 ml of serum diluted to 1.0 ml with α-ketoglutarate-alanine buffer substrate (pH 7.4) for AST determination.

Statistical analysis

All data are expressed as means ± standard errors of the means (SEM). The statistical difference was determined by the two-tailed unpaired t test. A P of <0.05 was considered statistically significant.

RESULTS

Serum enzymes

For analyzing the effect of L-arginine, ciprofloxacin and their combination on liver function. The mice were challenged with sub-lethal dose of *S. typhimurium* (0.6xLD50) and then drugs were given for 3 and 7 days. The results have been summarized in Tables 2 and 3.

The activity of serum enzymes ALT and AST were

Table 1. The six groups and their treatments.

Groups	Treatments
Group 1	Negative control (Normal Saline)
Group 2	Positive control [*S. typhimurium*($0.6xLD_{50}$)]+Saline
Group 3	*S. typhimurium* ($0.6xLD_{50}$) + Ciprofloxacin (400 mg per kg b. wt)
Group 4	*S. typhimurium* ($0.6xLD_{50}$) + L-Arginine (1000 mg per kg b. wt)
Group 5	*S. typhimurium* ($0.6xLD_{50}$) + L- Arginine (500 mg per kg b. wt) + Ciprofloxacin (200 mg per kg b. wt)
Group 6	*S. typhimurium* ($0.6xLD_{50}$) + L- Arginine(250 mgper kg b. wt) + Ciprofloxacin(200 mg per kg b. wt)

Table 2. Serum enzymes: the mice were infected with $0.6xLD_{50}$ of *S. typhimurium*, after 7 days of infection, drugs (L-arginine, ciprofloxacin and their combination) were given and study was made on day 11. Each value represents mean ±SE (n=6).

Groups	ALT (units/L)	AST (units/L)
S	36.00 ± 02.60	50.00 ± 05.36
B+S	83.66 ± 07.29	110.6 ± 04.43
B+Arg	59.33 ± 05.80	79.75 ± 05.38
B+Cip	48.00 ± 06.28	58.85 ± 08.42
B+1/2Arg+1/2 Cip	38.50 ± 06.15	56.31 ± 05.52
B+1/4 Arg+1/2 Cip	47.33 ± 03.89	56.96 ± 06.68

Table 3. Serum enzymes: the mice were infected with $0.6xLD_{50}$ of *S. typhimurium*, after 7 days of infection, drugs (L-Arginine, Ciprofloxacin and their combination) were given and study was made on day 14. Each value represents mean ±SE (n=6).

Groups	ALT (units/L)	AST (units/L)
S	36.00 ± 03.10	50.00 ± 08.30
B+S	82.36 ± 05.29	115.6 ± 04.45
B+Arg	58.33 ± 06.50	82.75 ± 05.11
B+Cip	47.08 ± 04.12	58.25 ± 06.58
B+1/2Arg+1/2 Cip	37.68 ± 03.28	53.27 ± 05.60
B+1/4 Arg+1/2 Cip	48.45 ± 06.55	57.47 ± 03.22

estimated.

Infection with bacteria in control mice resulted in an increase in the activity of ALT and AST by 132.38 and 121.2%, respectively on day 11. However, in therapeutic dose of drugs in treated mice with L-arginine, ciprofloxacin and their combination have shown significant decrease in ALT and AST by 28.29, 42.62, 53.9, 43.42% and 27.89, 46.79, 49.08 48.49%, respectively.

However, infection with bacteria in control mice resulted in an increase in the activity of ALT and AST by 128.77 and 131.2% respectively on day 14. Therefore, in therapeutic dose of drugs in treated mice with L-arginine, ciprofloxacin and their combination have shown significant decrease in ALT and AST by 29.17, 42.83, 54.24 41.17% and 28.40, 49.61, 53.91 50.28%, respectively.

Thus, the treatment of mice with this combination (B+1/2 Arg+1/2 Cip) for 7 days, protected and normalized the liver. Therefore, it is concluded that this dose (B+1/2 Arg+1/2 Cip) was able to minimize the damage of cell caused by bacterial infection and reclaims the effectiveness of drugs against Salmonellosis.

DISCUSSION

Serum enzymes

Endotoxin/LPS derived from gram-negative bacteria play an important role in the pathogenesis of liver injury associated with sepsis (Liang-Takasaki et al., 1983). Liver damage is associated with the estimation of serum transaminases (ALT and AST). Our results suggested

that the activity of serum aminotransferases (transaminases) in mice infected with 0.6xLD50 of *S. typhimurium* showed significant increase in both ALT and AST activity after 14 days of infection. The rise in AST and ALT levels induced by *S. typhimurium* was significantly recouped by the treatment with following drugs combination (B+1/2 Arg+1/2 Cip), suggesting that more recovery effect of cellular leakage and loss of functional integrity of the cell membrane of hepatocytes (Tables 2 and 3). Thus drugs are cytoprotective against the toxic effects of bacteria as seen by the present study. These results indicate that NO has significant host defense functions in Salmonella infections not only because of its direct antimicrobial effect but also via cytoprotective actions for infected host cells.

These results are also supported by the study of serum enzymes. The treatment with L-arginine, ciprofloxacin and their combination showed decline in the serum ALT activities but AST activities is significantly higher in infected groups as compared to respective control groups. These results suggested that L-arginine, ciprofloxacin and their combination are able to protect bacteria induced liver damages. Salmonella infection caused much more extensive liver damage (microabscess formation and induction of apoptosis) in NOS-deficient mice than in wild-type mice. The enhancement of apoptotic change was clearly demonstrated in the Salmonella-infected iNOS-/-mice, even when they were similarly affected by the bacteria in terms of bacterial growth in the liver. These results suggest that, NO potentially mediates its cytoprotective effect through its antiapoptotic activity. It is intriguing that mice lacking iNOS undergo impaired liver regeneration (Rudra et al., 1998), suggesting that hepatic iNOS expression is involved in an adaptive response for minimizing inflammatory injury. Thus, NO formed during salmonellosis may play an important role in hepatocellular regeneration or healing from damage caused by the bacteria, thus helping infected cells and organs maintain an effective defense against and recovery from salmonellosis.

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