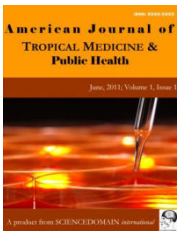

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Evaluation of Biochemical Aspects of Formulated Drugs against Typhoid

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Research Article

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ABSTRACT

Typhoid fever one of the major health problems in many developing countries. *Salmonella* is a gram-negative, rod-shaped facultative anaerobic bacterium. Salmonella has developed resistance to many antibiotics used now a day that has complicated its management, that thus it has necessitated the search of formulated drugs for its treatments. Nitric oxide (NO) is an important signaling molecule that regulates a diverse range of patho-physiological processes in many tissues. Earlier studies have suggested that exogenous administration of Larginine 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, i.e., L-Arginine. Bacterial Clearance Study shows the bacterial burdens in the liver of *S. typhimurium* infected mice were consistently greater as compared to formulated drugs (L-Arginine+Ciprofloxacin) treated mice and ALT and AST decreases by 38.84% and 53.61% in 1/2 L-Arg+1/2 Cip group as compared to bacterial treated groups.

Keywords: Nitric oxide; ciprofloxacin; bacterial clearance study;

1. INTRODUCTION

Typhoid fever is endemic throughout Africa and Asia and persists in the Middle East, and certain part of southern and eastern European countries and central and South America. In

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the US and in most part of Europe typhoid is epidemics, typhoid is predominantly a disease of the returning traveler (Ackers et al., 2000). *Salmonella typhimurium* can access systemic tissue (mainly spleen and liver) via the lymphatic system and the Peyer's patches. In a second pathway, phagocytes are believed to carry intestinal bacteria directly into the bloodstream without passing through the Peyer's patches (Worley et al., 2006). Ciprofloxacin, a new synthetic fluoroquinolone with a broad spectrum of bactericidal activity and effective tissue penetration (Drug ther bull., 1987), are highly susceptible to circulating strains of *S. typhi* (Saha et al., 1992). Ciprofloxacin has also been used successfully in the treatment of typhoid fever in children (Sen et al., 1991).

Nitric oxide (NO) the "molecule of the decade," is a highly reactive gas that's also a biologically relevant molecule. It is used by cells and organisms for a wide range of functions, from relaxing blood vessels to fighting infection. NO has been implicated in host defense against intracellular pathogens such as Leishmania, *Mycobacteria* and *Salmonella* (Sternberg et al., 1994). NO effects are mediated through secondary oxidants, especially peroxynitrite in macrophages, neutrophils and Kupffer cells. Activated macrophages generate superoxide radicals through membrane bound NADPH oxidase along with NO leading to generation of peroxynitrite (ONOO–) (Ischiropoulos et al., 1992). Peroxynitrite is a strong oxidant capable of modifying lipids, amino acids, DNA, and redox active metal centers of dehydratases (Radi, 2004). In analogy to the extensive functional overlap in the enzymatic detoxification of reactive oxygen species (Hebrard et al., 2009), *Salmonella* can antagonize ONOO– through both indirect and direct mechanisms. *Salmonella* periplasmic Cu/Zn superoxide dismutase Sod CI prevents ONOO– formation (De Groote et al., 1997). NO can inhibit several enzymes of central metabolic pathways (Castro et al., 1994; Keyer and Imlay, 1997; Brandes et al., 2007; Hyduke et al., 2007). *Salmonella* likely coordinates a metabolic response to this diatomic radical. In accordance with this idea, glucose-6-phosphate dehydrogenase (Zwf) of the pentose phosphate pathway is important for resistance to RNS *in vitro* and *in vivo* (Lundberg et al., 1999). The gene encoding Zwf is part of the SoxR regulon, an [Fe–S] cluster-containing transcription factor activated by NO in *E. coli* (Ding and Demple, 2000). Zwf shuffles the flow of carbon through the pentose phosphate pathway, producing NADPH reducing equivalents in the process. NADPH could fuel glutathione oxidoreductase or thioredoxin reductase to repair damage caused by RNS. NO produced by iNOS in response to Salmonella infection is involved in a broad range of pathophysiological processes, acting both as a signaling molecule and a potent antimicrobial mediator. RNS inhibit assorted bacterial targets involved in a variety of cellular processes. Given this strong selective pressure, *Salmonella* have developed mechanisms to counteract the cytotoxicity of RNS. It is unclear whether *Salmonella* bacteria produced hepatic dysfunction by direct invasion or by endotoxemia (Khosla et al., 1988). The study was designed to determine the clinical and hepatic biochemical changes in the course of typhoid fever.

2. MATERIALS AND METHODS

2.1 Dose and Dosage

2.1.1 Animals

Swiss albino mice (25-30g) 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 (14/10h). 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.

2.1.2 Bacteria

In this experiment only *Salmonella 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.

Groups	Treatments
Group1	Negative control (Normal Saline)
Group2	Positive control (*S. typhimurium* (0.6xLD50)+Saline
Group3	*S. typhimurium* (0.6xLD50) + Ciprofloxacin (400 mg per kg b. wt)
Group4	*S. typhimurium* (0.6xLD50) +Arginine (1000 mg per kg b.wt)
Group5	*S. typhimurium* (0.6xLD50) + Arginine (500 mg per kg b. wt) +Ciprofloxacin (200mg per kg b. wt)
Group6	*S. typhimurium* (0.6xLD50) + Arginine (250 mg per kg b. wt) +Ciprofloxacin (200 mg per kg b. wt)

Effects of above drugs on infected mice by *S. typhimurium* were analyzed. Post-treatment of drugs were done at above dose orally to the experimental animals, first group was considered as control that receive only saline, second group considered as positive control which was challenged with sub lethal dose of *S. typhimurium* (0.6xLD50) along with saline. Third group was challenged with sub lethal dose of *S. typhimurium* and given only full dose of ciprofloxacin. Fourth group was challenged with sub lethal dose of *S. typhimurium* and then mice were treated with full dose of Arginine only. In fifth and sixth group animals were challenged with *S. typhimurium* and then half and one fourth dose of Arginine was administered along with half dose of Ciprofloxacin respectively. On 8th days of post treatment, liver was removed aseptically in sterile condition, homogenate was made and post mitochondrial supernatant was prepared for biochemical estimation.

2.2 Bacterial Clearance Study

The bacterium gains entry to its reticulo-endothelial system, especially liver and spleen as the mice were infected with *S. typhimurium*. The number of bacteria (as seen by CFUs) increases slowly up to 1 week and then attains a constant value. To analyze the effect of Larg, ciprofloxacin, the mice were post treated with above mentioned procedure. Different groups were treated with bacteria and drugs as mentioned above. The mice were sacrificed by cervical dislocation at day 8-post infection (PI). The liver of the animals were aseptically removed and washed with PBS. Livers were weighed and an equal amount of them by weight from each group was homogenized separately in PBS containing 1.15% KCl at room temperature. A small aliquot from each homogenate was cultured on nutrient agar plates. Bacterial colonies were obtained after overnight incubation of the culture plates at 370C, which were screened for *S. typhimurium* by standard dabbing method on triple-sugar-

ironagar (TSI-agar) plates and counted. The *Salmonella* bacteria impart a blackish hue to pink colored plates. The results of the experiments were expressed as a number of viable bacteria (log10 CFUgm-1 tissue).

2.3 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 800xg in a fixed rotor centrifuge at room temperature. After centrifugation serum was collected carefully using a Pasteur pipette. It was kept at -200C till the enzyme analysis.

2.4 Serum Enzymes

The activities of Alanine aminotransferase (ALT) and Aspartate aminotransferase (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 Reitman and Frankel (1956) (Reitman and Frankel, 1956).

3. RESULTS

3.1 Bacterial Clearance Study

Mice were infected with sub-lethal dose (0.6xLD50) of *S. typhimurium* (wild) by an intraperitoneal route. After 7 days of infection, mice were treated with L-Arginine, ciprofloxacin and their combination up to 7 days, and on day 14 liver of animals were remove aseptically, the control group was highly infected with *S. typhimurium* indicating that in absence of any resistance from host, the bacterium reach the liver and rapidly colonize. Treatment with above dose of drugs in pre-infected mice, inhibited the bacterial translocation into the liver and exhibited rapid clearance of bacteria. L-arginine and Ciprofloxacin treated groups had 20.28% and 47.28% decrease in the level of bacterial burden as compared with control, where as in case of 1/2 L-Arg+1/2 Cip, 1/4 L-Arg+1/2 Cip combination, there was 31.15% and 21.01% decrease was observed as compared with control. The maximum decrease was observed in Ciprofloxacin alone and 1/2 L-Arg+1/2 Cip combination of treated mice as compared with control. This lowered bacterial load resulted in the lowering of bacterial infection (Figure 1).

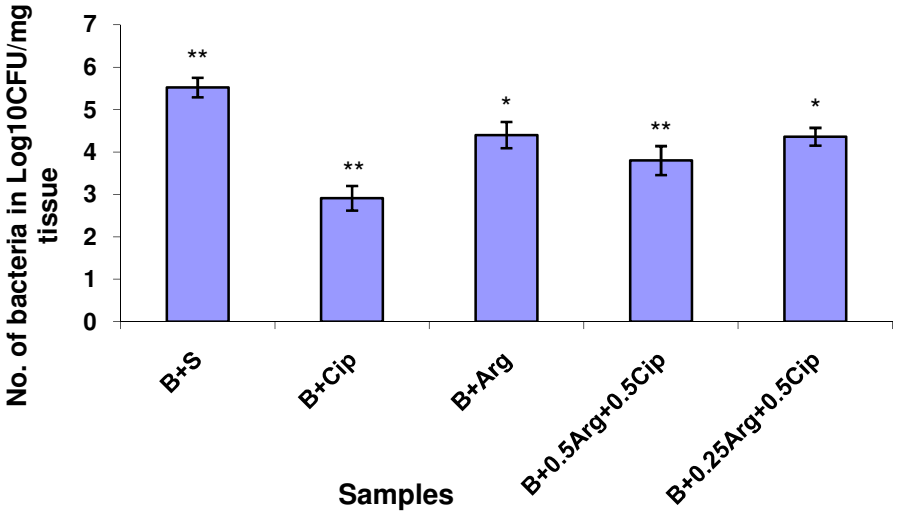

Fig. 1. Bacterial clearance from the reticuloendothelial system

Drugs were treated for seven days after 7 day of infection. B+S=S. typhimurium+Saline,B+Arg=S. typhimurium+ 1000mg per kg b. wt L-Arginine, B+Cip=S. typhimurium+400mg perkg b. wt Ciprofloxacin, B+1/2Arg +1/2Cip=S. typhimurium+500mg per kg b. wt Arginine+200mg per kg b. wt ciprofloxacin, B+1/4Arg+1/2Cip=S. typhimurium+250mg per kg b. wtArginine+200mg per kg b. wt Ciprofloxacin.
*Values are significantly different *p<0.05 and **p<0.01.*

3.2 Serum Enzymes

The mice were challenged with sub-lethal dose of *S. typhimurium* ($0.6xLD_{50}$) and then drugs were treated. The results have been summarized in Table 1.

Table 1. Effect of formulated drugs on serum Liver enzymes

Groups	ALT (Units/L)	AST (Units/L)
S	36.00 ± 01.80	50.00 ± 04.40
B+S	80.66 ± 06.27	123.60 ± 02.41
B+Arg	57.33 ± 07.85	81.75 ± 04.25
B+Cip	46.00 ± 05.24	59.11 ± 09.55
B+1/2Arg+1/2 Cip	39.50 ± 05.31	57.33 ± 04.65
B+1/4 Arg+1/2 Cip	49.33 ± 05.98	59.66 ± 05.76

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 8.
Each value represents mean ±SE (n=5).

The activity of serum enzymes ALT and AST were estimated. Infection with bacteria in control mice resulted in an increase in the activity of ALT and AST by 124.05% and 147.2% respectively on day 8. However, in therapeutic dose of drugs in treated mice with L-Arginine, ciprofloxacin and their combination have shown the significant decrease in ALT & AST by 28.9%, 42.97%, 51.02% and 38.84% & 33.85%, 52.17%, 53.61% and 51.73%.

Thus, the treatment of mice with this combination (B+1/2 Arg+1/2 Cip), 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.

4. DISCUSSION

4.1 Bacteria Clearance Study

Infections begin with the penetration of the intestinal epithelium by *Salmonella* and their subsequent dissemination throughout the reticuloendothelial system, where they multiply, especially in liver (Nnalue, 1992), also liver was chosen as a focus of the present study because this organ plays a pivotal role in the maintenance of metabolic homeostasis. *Salmonella* infection in mice increases the incidence of bacterial translocation into the liver, and causes a major site of salmonellosis. The occurrence of disease is a function of several major variables: the virulence of the microorganism, its mode of transmission, and the host susceptibility. As expected, the bacterial burdens in the liver of *S. typhimurium* infected mice were consistently greater as compared to drugs treated mice. This fact supports the studied results obtained in the Figure 1. The fast clearance of bacteria from reticuloendothelial system by this specific combination (1/2 Arg+1/2 Cip) is due to their high bactericidal property and gradual decrease was seen and maximum clearance was found on day 14.

4.2 Serum Enzymes

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 7 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 (Table 1). Thus drugs are cytoprotective against the toxic effects of bacteria as seen by the present study. This effect suggests probably that serum level of liver enzymes come back to normal with the healing of hepatic parenchyma and regeneration of hepatocytes. 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 alanine aminotransferase (ALT) activities but aspartate aminotransferase (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. Also necrosis of liver causes enhancement of serum marker in the blood (Ashok Shenoy et al., 2002).ALT and AST are considered as a

important marker for liver function test (Tolman and Rej, 1999; Hilaly et al., 2004). ALT is located in the cytosol of the hepatocytes. This enzyme is considered a more sensitive marker of hepatocellular damage than AST.

5. CONCLUSION

Although its pathogenesis remains unclear, hepatic insult in typhoid fever may occur through a variety of mechanisms, including local or systemic effects of endotoxin or non-specific reactive inflammation in response to ulcerations in the intestine or due to the effects of cytotoxin produced by *S. typhi* that have infected Kupffer cells . In hepatocytes, the uptake of extracellular L-arginine is the rate-limiting step for its conversion to urea and ornithine. We obtained better results at lower dose (0.5 gm per kg body wt) of L-Arginine in combination with Ciprofloxacin.

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