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Short Communication

Anti *Salmonella* activity of nitric oxide donor and antibiotic: *In vitro* studies

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Typhoid fever (TF) is an important cause of morbidity and mortality with estimated more than 21 million cases per years. 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 unique molecule produced in a biological system, which regulates a different array of physiological functions and acts as an inter and extra cellular messenger in most mammalian organs. In the present study, antimicrobial tests were done to look for anti-bacterial activity against *S. typhi* and *S.typhimurium*. *In vitro* antimicrobial activity provided the required preliminary observation to select the combination, those have potentially useful properties. As evaluated by the diameter of the inhibition zone, Ciprofloxacin alone, 1/2 L-Arg+1/2 Cip showed potent anti-*Salmonella* activity 18 mm i.e. 100%.

Key words: Typhoid, Arginine, Antibiotic, Nitric oxide.

INTRODUCTION

Typhoid fever (TF) is an underestimated important health problem in India and other developing countries (Haque, 2011). The causative agent *Salmonella enterica* serovar *typhi* is pathogenic both to man and animals with associable inflammatory reaction in the intestinal tract. Like other enteric pathogens, *S. enterica* serovar typhi is transmitted through food or water that has been contaminated with faeces from acutely infected person's persistent excretors (that is constant stooling or diarrhoea) or from chronic asymptomatic carriers (Rajiv et al., 2007). *Salmonellae* are typically members of the enterobactericeae. The enterobactericeae are a large group of facultative anaerobic gram negative, catalase negative rods (Jawetz and Adelbergs, 1995). The bacterial infections are the world's leading killing diseases. In recent years, drug resistance to human pathogenic bacteria has been commonly reported all over the world. The situation is alarming in both developing as well as developed countries due to indiscriminate use of antibiotics. The present scenario of emergence of multiple drug resistance to human pathogenic microorganisms has been necessitated a search for antimicrobial substance from other sources including plants (John et al., 2004). The wide distribution and high prevalence of MDR among *Salmonella* species has led to fluoroquinolones assuming a primary role in the therapy for invasive salmonellosis. Some 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 (Maskey et al, 2008 and Sood et al 1999).Ciprofloxacin continues to be widely used, due to penetrate tissue well. To understand 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 molecule produced in biological system. NO react with the superoxide anion (O_2^-) yielding $ONOO^-$ and peroxynitrous acid (ONOOH). These are strong oxidant molecules that can cause molecular damage to a variety of tissues. The acid ONOOH reacts with the cysteine residues of proteins or glutathione, forming S-nitrosothiols. Peroxynitrite also modifies the tyrosine residues, producing nitrotyrosine (Radi *et al*., 2000). The anti-*Salmonella* activity of RNS (Reactive nitrogen Species) emanates from the modification of redox active thiols and metal prosthetic groups of key molecular targets of the electron transport chain, central metabolic enzymes, transcription factors, and DNA and DNA-associated proteins. Traditionally, RNS have been

recognized as important mediators of host defense against *Salmonella*. The aim of this study was to evaluate the role of NO donors and *ciprofloxacin* are used in different low concentration, which shows better therapeutic results against experimentally induced Salmonellosis.

MATERIALS AND METHODS

Dose and Dosage

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.

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. The drug was administered orally and *S. typhimurium* intraperitoneally.

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* ($0.6xLD_{50}$) +Saline

Group3. *S. typhimurium* ($0.6xLD_{50}$) + Ciprofloxacin (400mg per kg b. wt)

Group4. *S. typhimurium* ($0.6xLD_{50}$) +Arginine (1000mg per kg b.wt)

Group5. *S. typhimurium* ($0.6xLD_{50}$) + Arginine (500mg per kg b . wt) + Ciprofloxacin (200mg per kg b. wt)

Group6. *S. typhimurium* ($0.6xLD_{50}$) + Arginine (250mg 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 given 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* ($0.6xLD_{50}$) along with saline. Third group was administered with sub lethal dose of *S. typhimurium* and given only full dose of ciprofloxacin. Fourth group was administered 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 administered with *S. typhimurium* and then half and one fourth dose of Arginine was administered along with half dose of Ciprofloxacin respectively.

Determination of Anti-*Salmonella* activity

Anti-*Salmonella* activity was determined by agar well diffusion method as described by Perez *et al*., (1996), with minor modifications. The agar plates were prepared by dissolving 2.6 g Nutrient broth in 200 ml of double distilled water, supplemented with 200 mg of glucose and 4 g of agar. The autoclaved media was poured in 55 mm petri plate and allowed to solidify. Culture was prepared by the inoculation of strain of *S. typhi* and *S. typhimurium* in glass tubes containing sterile nutrient broth and to incubate at 37˚C. Aliquot of 100 µl bacterial culture, which corresponded to 10^5 CFU was spread on the agar plate. Wells of 6 mm diameter were then punched into agar plate and test drug (or PBS in case of controls) was applied in this well. The plates were kept at 4˚C for 6 hours and subsequently place at 37˚C for 16 hours. Zone of inhibition and percent activity was calculated.

RESULTS

In vitro susceptibility test for *S. typhi and S. typhimurium*

The in vitro susceptibility activity of the L-Arginine, Ciproflaxacin and their combinations was quantitatively evaluated by the presence or absence of inhibition zone and zone diameter in agar plates. The results obtained at a combination of (1/2 L-Arginine+1/2 Ciprofloxacin) showed better protection and highly active in preventing the growth of bacteria (*S. typhi* and *S. typhimurium*). These results have been summarized in the Table 1.

L-Arginine, ciprofloxacin and their combinations (1/2L-Arg+1/2 Cip, 1/4 Arg+1/2 Cip, 1/8 L-Arg+1/2 Cip and 1/16 Arg+1/2 Cip) exhibited anti-*Salmonella* activity and zone of inhibitions were 17.5, 18, 18, 17, 17, 15 mm respectively i.e. percent activity of the above are 97.22%, 100%, 100%, 94.44%, 94.44% and 83.3% respectively against *S. typhi.* Similar result was observed except in 1/8 Arg+1/2 Cip dose against *S. typhimurium* it was 91.66% where as this combination (1/32 Arg+1/2 Cip) showed 13.0 mm and 12.0 mm and percent activity was less as 72.22% and 66.66% respectively against *S. typhi* and *S. typhimurium* respectively.

DISCUSSION

Antimicrobial agents have been used in clinical practice

Table 1. Anti-*Salmonella* activity of Formulated drugs.

	S. typhi		*S. typhimurium*	
	Zone of inhibition (mm)	**% Activity**	**Zone of inhibition (mm)**	**% Activity**
Arg	17.5 mm	097.22%	17.5 mm	097.22%
Cip	18.0 mm	100.00%	18.0 mm	100.00%
1/2 Arg+1/2 Cip	18.0 mm	100.00%	18.0 mm	100.00%
1/4 Arg+1/2 Cip	17.0 mm	094.44%	17.0 mm	094.44%
1/8 Arg+1/2 Cip	17.0 mm	094.44%	16.5 mm	091.66%
1/16 Arg+1/2 Cip	15.0 mm	083.30%	15.0 mm	083.30%
1/32 Arg+1/2 Cip	13.0 mm	072.22%	12.0 mm	066.66%

Table.1 Arg=Arginine, Cip=Ciprofloxacin, Arg+1/2 Cip= ½ Arginine+1/2Ciprofloxacin, 1/4 Arg+1/2Cip=1/4 Arginine+1/2 Ciprofloxacin, 1/8 Arg+1/2Cip=1/8 Arginine+1/2 Ciprofloxacin, 1/16Arg+1/2Cip=1/16Arginine+1/2 Ciprofloxacin, 1/32 Arg+1/2Cip=1/32 Arginine+1/2 Ciprofloxacin.

for over 40 years (Zhanel et al., 1991). In the present study, in vitro antibacterial activity of nitric oxide donor L Arginine, ciprofloxacin and their combinations against Salmonella was measured by disk diffusion method. In vitro antimicrobial activity provided the required preliminary observation to select the combination; those have potentially useful properties for biochemical, toxicological and immunological studies. In the present study, antimicrobial tests were done to look for anti-bacterial activity against *S. typhi* and *S. typhimurium*. The anti-*Salmonella* activity of the L-Arginine, Ciproflaxacin and their combination and their potency was quantitatively assessed by the presence or absence of inhibition zone and zone diameter in agar plates when the test material was applied in the well. Anti-*Salmonella* activity of nitric oxide donor L-Arginine, ciprofloxacin and their combinations were checked by antimicrobial test. It was found that this combination (1/2 L-Arginine+1/2 Ciprofloxacin) preventing the growth of bacteria (*S. typhi* and *S. typhimurium*). These results have been summarized in the Table 1.

L-Arginine, ciprofloxacin and their combination (L-Arg alone, Cip alone, 1/2 L-Arg+1/2 Cip, 1/4 Arg+1/2 Cip, 1/8 L-Arg+1/2 Cip and 1/16 Arg+1/2 Cip) exhibited anti-*Salmonella* activity and percent activity of the above were 97.22%, 100%, 100%, 94.44%, 94.44% and 83.3% respectively against *S. typhi* and *S. typhimurium*.

This combination (1/32 Arg+1/2 Cip) showed 13.0 mm and 12.0 mm and percent activity was less as 72.22% and 66.66% respectively against *S. typhi* and *S. typhimurium* respectively.

Conclusion

From the above results, it can be inferred that NO donor with combination of antibiotic may be able to provide significant protection against *S. typhimurium* challenge through the enhancement of NO production. This view is further supported by the observation that NO production was higher in control animals. The zone of inhibition incases of Cip alone and 1/2 L-Arg+1/2 Cip showed potent anti-*Salmonella* activity.

Treatment of L-arginine increased the NO production in liver following sub lethal challenge with *S. typhimurium* ($0.6xLD_{50}$). It seems that iNOS is not induced which is necessary to up- regulate NO production. Animals treated with NO donors (L-arginine) and ciprofloxacin had significantly lower incidence of bacterial translocation to liver in comparison to control animals.

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