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Full Length Research Paper

Formulation of nitric oxide donors and antibiotic against typhoid

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Typhoid fever remains a major health problem in India and other developing countries. It often becomes difficult to precisely estimate the global impact of typhoid, as clinical symptoms resemble many other febrile infections. Salmonella, gram negative bacilli, are facultative intra cellular bacteria and can survive during certain stages of host parasite interaction. There are a 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; thus, it has necessitated the search of formulated drugs for its treatments. Nitric oxide (NO) is a versatile molecule produced in a biological system, it regulates divers array of physiological function and acts as an inter and extra-cellular messenger in most mammalian organ in host's defense functions for many bacterial infections. 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 into consideration, it was thought pertinent to see the effect of oral administration of NO donors that is L-arginine, which is one of the semi essential amino acids used as food supplement. Formulation of NO donors and ciprofloxacin are used in low concentrations to reduce toxicity which shows better therapeutic results against experimentally induced Salmonellosis.

Key words: Typhoid, Nitric Oxide and MDR.

INTRODUCTION

Enteric (typhoid) fever remains a common disease in many parts of the world where access to clean water is limited. In places such as India, Nepal, Pakistan, Indonesia and parts of sub-Saharan Africa, typhoid is still a substantial public health problem (Bhan et al., 2005; Crump et al., 2004; Karkey et al., 2008; Parry et al., 2002). Resistance to the traditional first-line antimicrobial agent's ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole defines multidrug resistance (MDR) in *Salmonella enterica.* The MDR phenotype has been shown to be widespread among *Salmonella typhi* for many years (Rowe et al., 1997) and is present, albeit at lower rates, among *Salmonella paratyphi* (Gupta et al., 2008; Parry and Threlfall, 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 (Ochiai et al., 2008). 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 anti-microbials, coinciding with a switch to fluoroquinolones for the management of enteric fever (Maskey et al., 2008; Sood et al., 1999).

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 (Crump et al., 2008). 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 (Misko et al., 1993). Many types of cells, such as leucocytes, hepatocytes, vascular smooth muscle cells and endothelial cells can produce NO during enzymatic conversion of L-arginine to L-citrulline by NO synthetase (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 (Stefenovic-Racic et al., 1993). This may involve both i) direct toxicity, e.g., 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 (O_2^-) to form peroynitrite ($ONOO^-$), which is a potent oxidizing molecule capable of eliciting lipid peroxidation and cellular damage (Michael and Feron, 1997). NO is produced in mammalian cells by a group of isoenzymes collectively termed NO synthases (NOS). Nitric oxide has been suggested to inhibit the enzymes such as cyclooxygenase, lipo-oxygenase and cytochrome P_{450} reduction of active site heme or non-heme iron to inactive ferrous form (Kanner et al., 1992). NO has been shown to reduce the ferryl heme by which it prevents many oxidative processes (Gorbunov et al., 1995; Wink et al., 2001).

MATERIALS AND METHODS

Dose and dosage

Animals were divided into six groups. Each group comprised of six animals. The study comprised of the following treatment schedules.

Groups	Treatment
Group 1	Negative control (normal saline)
Group 2	Positive control (*Salmonella typhimurium* ($0.6xLD_{50}$) +Saline
Group 3	*typhimurium* ($0.6xLD_{50}$) +Ciprofloxacin (400 mg per kg b.wt)
Group 4	*S. typhimurium* ($0.6xLD_{50}$) +Arginine (1000mg per Kg b.wt)
Group 5	*S. typhimurium* (0.6xLD50) +Arginine (500mg per kg b.wt)+Ciprofloxacin (200mg per kg b. wt)
Group 5	*S. typhimurium* (0.6xLD50)+Arginine(250mgper kg b. wt) +Ciprofloxacin (200 mg per kg b. wt)

Effects of the aforementioned drugs on infected mice by *S. typhimurium* were analyzed. Post-treatment of drugs were done at the stated dose orally to the experimental animals, the first group was considered as control that received 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 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 a full dose of arginine only. The 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 the 14th day of post treatment, liver was removed aseptically in sterile condition, homogenate was made and post mitochondrial supernatant was prepared for biochemical estimation.

Estimation of lipid peroxidation (LPO)

The modified method of Utley et al. (1967) was used for the estimation of lipid peroxidation. Liver homogenate (1.0 ml) was pipetted in a glass vial of 20 ml and incubated at 37±1°C in a water bath shaker for 60 min at 120 strokes up and down. The other 1.0 ml was pipetted in a centrifuge tube and placed at 0°C and marked as 0 h incubation. After 1 h of incubation, 1.0 ml of 5% TCA and 1.0 ml of 0.67% TBA was added in both samples (that is 0°C and 37°C). The reaction mixture from the vial was transferred to the tube and centrifuged at 1500xrpm for 15 min. The supernatant was transferred to another tube and placed in a boiling water bath for 10 min. Thereafter, the test tubes were cooled and the absorbance of the color was read at 535 nm. The formation of lipid peroxidation was expressed as nmol of malondialdehyde (MDA) formed/h/mg protein.

Reduced glutathione (GSH)

Reduced glutathione in the liver was determined by the same modification in the methods of Jollow et al. (1974). 1.0 ml of PMS (10% w/v) was precipitated with 1.0 ml of sulfosalicylic acid (4%). The samples were kept at 4°C for at least 1 h and then subjected to centrifugation at 1200xg for 15 min at 4°C. The assay mixture contained 0.1 ml of PMS (10% w/v), 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml DTNB (40 mg/10 ml of phosphate buffer, 0.1 M, pH 7.4) in a total volume of 3.0 ml. The yellow color developed was read immediately at 412 nm.

Glutathione peroxidase (GPx) activity

Coupled enzyme assay with glutathione reductase (GR) was used for the estimation of GPX activity. The glutathione disulphide produced as a result of GPX activity, which is immediately reduced by GR thereby, maintaining a constant level of reduced glutathione in a reaction system. The assay takes advantage of concomitant oxidation of NADPH by GR, which was measured at 340 nm.

GPX

$$H_2O_2 + 2GSH \xrightarrow{\text{GPX}} 2H_2O + GSSG$$

GR

$$GSSSG + 2NADPH \xrightarrow{\text{GR}} 2GSH + 2NADP^{+} + H^{+}$$

Specific activity of enzyme was measured according to the procedure described by Mohandas et al. (1984). The reaction mixture consisted of 1.44 ml phosphate buffer (0.05 M, pH 7.0), 0.1 ml of EDTA (1 mM), 0.1 ml of sodium azide (1 mM), 0.1 ml of glutathione (1 mM), and 0.1 ml of NADPH (0.2 mM) ,0.01 ml of hydrogen peroxide (0.25 mM) and 0.1 ml of PMS (10% w/v) in a final volume of 1.95 ml. The enzyme activity was calculated as nmole NADPH oxidized/min/mg protein by using molar extinction coefficient of $6.22 \times 103\ M^{-1}\ cm^{-1}$.

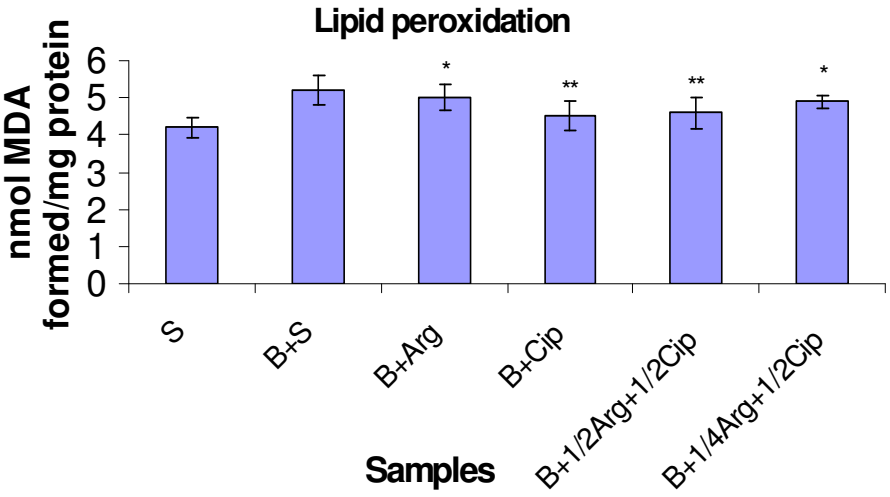

Figure 1. Hepatic malonadialdehyde level measured in mice: drugs were given and study was made at day 14. S = Saline, B + S = *S. typhimurium* + Saline, B + Arg = *S. typhimurium* + 1000mg per kg b. wt L-Arginine, B + Cip = *S. typhimurium* + 400 mg per kg b. wt Ciprofloxacin, B+1/2Arg +1/2Cip=*S. typhimurium* + 500 mg per kg b. wt Arginine + 200 mg per kg b. wt ciprofloxacin, B + ¼ Arg+1/2 Cip = *S. typhimurium* + 250 mg per kg b. wt Arginine + 200 mg per kg b. wt Ciprofloxacin. Values are significantly different *p<0.05 and **p<0.01.

RESULTS

Lipid peroxidation (LPO)

The mice were challenged with a sublethal dose of *S. typhimurium* (0.6xLD_{50}), after seven days, drugs were given up to the next seven days and malonadialdehyde (MDA) production by TBA was measured as an indicator for LPO. The results have been summarized in Figure 1. Infection with *S. typhimurium* significantly induced lipid peroxidation at day 14, as indicated by enhancement of MDA levels compared to the control mice (14.63%). After treatment of L-arginine, Ciprofloxacin and their combination on day 14, it showed 23.40, 25.53, 36.17 and 21.27% decrease in the level of LPO in mice as compared to control. Thus, treatment with L-arginine, Ciprofloxacin and their combination partially protected the liver against the infection-induced damage and some recovery was seen in this dose (B+1/2 Arg+1/2 Cip) in case of lipid peroxidation.

Reduced glutathione (GSH)

To analyze the effect of L-arginine, ciprofloxacin and their combination on liver damage, hepatic GSH levels in mice were measured. The mice were challenged with sublethal dose (0.6xLD_{50}) of *S. typhimurium* and then treated with drugs. The results have been summarized in Figure 2. Infection with bacteria to control mice resulted in significant decrease in the GSH level by 18% at day 14 of infection.

On day 14, the treatment of mice with L-arginine, Ciprofloxacin and their combination, the GSH level was slightly increased by 46.34, 58.53, 53.65 and 46.34% in *S. typhimurium* infected mice as compared with control. Thus, drugs were able to protect the liver from the injury induced by bacterial infection.

Glutathione peroxidase (GPx) activity

To assess the effect of L-arginine, ciprofloxacin and their combination, on liver function. The mice were challenged with a sub lethal dose (0.6xLD_{50}) of *S. typhimurium*, and then treated with the previously stated drugs. GPx activity was assessed and the results have been summarized in Figure 3. Infection of mice with bacteria resulted in a decrease in the GPx activity by 8.63% at day 14 as compared to saline treated control.

On day 14, after the treatment of mice with the previously stated drugs, the GPx activity was significantly increased by 14.54, 15.6, 16.0 and 10.90% as compared with control. However, at day 14, drugs protected the liver, by increasing the decrease of GPx activity and caused to remain near normal to mice.

DISCUSSION

Lipid peroxidation is a chain reaction and a single oxidative event can oxidize many lipid molecules. In the presence of iron or copper ions, the chain reaction can become a cascade and the oxidation process can rapidly

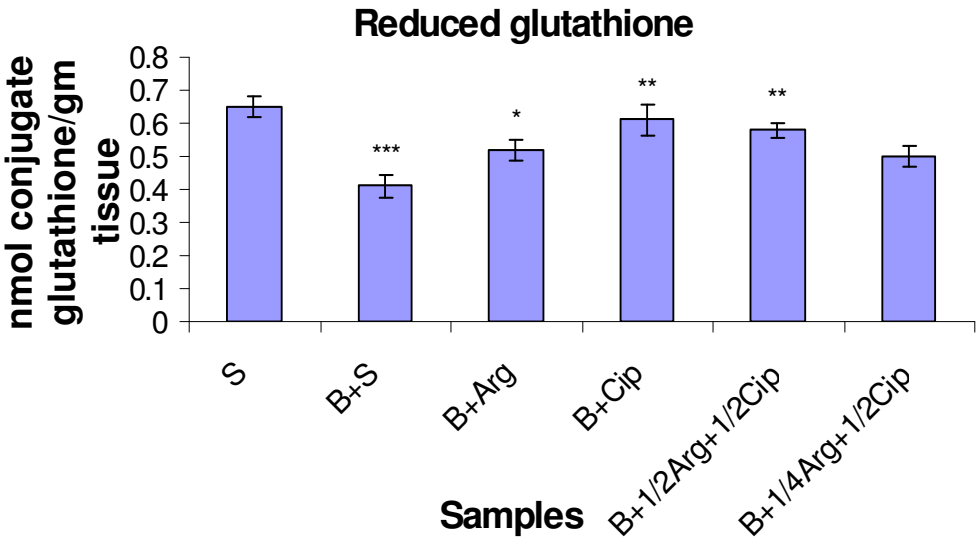

Figure 2. Hepatic reduced glutathione levels in mice: drugs were given and study was made on day 14 with arginine, ciprofloxacin and their combination. S = Saline, B + S = *S. typhimurium* + Saline, B + Arg = *S. typhimurium*+ 1000mg per kg b. wt L-Arginine, B + Cip = *S. typhimurium* + 400 mg per kg b. wt Ciprofloxacin, B + 1/2 Arg + 1/2 Cip = *S. typhimurium* + 500 mg per kg b. wt Arginine + 200 mg per kg b. wt ciprofloxacin, B+1/4Arg+1/2Cip=*S. typhimurium*+250mg per kg b. wt Arginine + 200mg per kg b. wt Ciprofloxacin. Values are significantly different *p<0.05, **p<0.01 and ***p<0.001

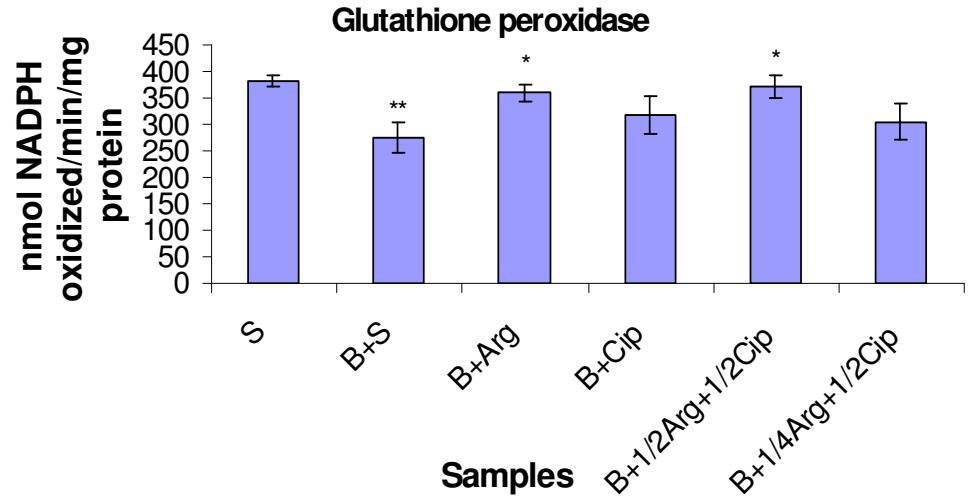

Figure 3. Hepatic GPx activity in mice: drugs were given and study was made on day 14 with arginine, ciprofloxacin and their combination. S = Saline, B + S = *S. typhimurium* + Saline, B + Arg = *S. typhimurium* + 1000mg per kg b. wt L-Arginine, B + Cip = *S. typhimurium* + 400mg per kg b. wt Ciprofloxacin, B + 1/2 Arg + 1/2 Cip = *S. typhimurium* + 500 mg per kg b. wt Arginine + 200 mg per kg b. wt ciprofloxacin, B+1/4Arg+1/2Cip=*S. typhimurium*+250mg per kg b. wt Arginine + 200mg per kg b. wt Ciprofloxacin. Values are significantly different *p<0.05 and **p<0.01.

become unstoppable (Holliwell and Gutteridge, 1984). Nitric oxide reacts with hydroxyl radicals at diffusion-limited rates to generate nitrite, but it clearly cannot exist *in vivo* at a high enough concentration to be an effective scavenger. Nitric oxide has been shown to inhibit the Fenton reaction by binding to ferrous iron and thus, preventing the formation of hydroxyl radicals (Kanner et al., 1991). The most important cellular damage caused by ROS is lipid peroxidation, wherein these reactive oxygen species attack the lipids in the membrane of hepatic cell

and cause peroxidation, resulting in complete destruction of the cell membrane and is associated with Salmonella induced tissue damage. The cell damage was estimated by measuring the changes in the level of lipid peroxidation.

Malondialdehyde (MDA) is a major reactive aldehyde resulting from the peroxidation of biological membranes (Vaca et al., 1988). A secondary product of lipid peroxidation, is used as an indicator of tissue damage involving a series of chain reactions (Ohkawa et al., 1979). Enhancement of the lipid peroxidation in *S. typhimurium* infected mice, as observed in the present study indicates damage to the hepatic cells. It correlates with the change in levels of the serum enzyme also. It has been hypothesized that, one of the principal causes of *S. typhimurium* induced hepatic damage is lipid peroxidation of hepatocytes membrane by generation of free radical derivatives (Recknagel et al., 1991). The observation of elevated levels of hepatic MDA in control mice in the present study is consistent with this hypothesis. In the present study, bacterial infection causes an increase in the LPO levels by 14.63% at day 14 of PI in liver. Drugs showed ability to prevent the *S. typhimurium* induced enhancement of MDA content, suggesting that this combination (B+1/2 Arg+1/2 Cip) inhibit lipid peroxidation more as compared with others (Figure 1).

Reduced glutathione (GSH)

Reduced glutathione (GSH) plays an important role in the detoxification of reactive toxic metabolites of *S. typhimurium*; liver necrosis is initiated when reserves of GSH are markedly depleted (Recknagel et al., 1991; Williams and Burk, 1990). Anand et al. (1996) have suggested that lowered GSH levels may occur due to increased utilization of GSH by antioxidant enzymes. The reduced form of GSH becomes readily oxidized to GSSG on interacting with free radicals. GSH participates in the reductive processes that are essential for the protection of cells through quenching the reactive intermediates and the radicals generated during oxidative toxicity.

Administration of *S. typhimurium* to control mice resulted in reduce levels of GSH. Interestingly, our results showed that L-arginine and ciprofloxacin both were able to enhance GSH levels in infected (B+A and B+C) mice (Figure 2). The maximum increase was found in Ciproflaxacin treated mice at each day 14 of the experiment. These increases in GSH level up regulates NO formation. Infected mice showed a decrease in GSH level, but increase in NO production. The cost for depletion of GSH might be due to the injury mediated by peroxynitrite in host tissues. Peroxynitrite can oxidize GSH to GSSG, which suggests that, this reaction could affect the redox status of intracellular and extracellular thiols (Wink et al., 1997). Thus, in bacterial infected group, the peroxynitrite may deplete GSH by converting it to GSSG. This GSSG may not be recycled back to GSH because there is depletion of glutathione dependent enzymes. The iNOS inhibitor group (IB) showed depletion in GSH level and NO production.

S-nitrosothiols such as *S*- nitrosoglutathione (GSNO) can be formed from NO and reduced thiols (reduced glutahione) in the presence of an electron acceptor (Gow et al., 1997). S-nitrosoglutathione can be bacteriostatic (DeGroote et al., 1995) and bacteriocidal (Shiloh and Nathan, 1997; Chen et al., 2000; Miyamoto et al., 2000). Studies with the Gram-negative bacterium *S. typhimurium* indicate that, S-nitrosoglutathione appears to be recognized as a substrate by the periplasmic enzyme γ-glutamyltranspeptidase, with subsequent conversion to S-nitrosocysteinyl-glycine. This nitrosylated dipeptide in turn is imported into bacterial cytoplasm across the inner membrane by a specialized dipeptide permease (DPP). The presence of dipeptide permease, a member of the ABC (ATP-binding cassette) transporter family, is absolutely essential for GSNO mediated inhibition of *S. tyhphimrium* growth *in vitro*. The ability of GSNO to halt the replication of *S. typhimurium* is markedly reduced by a mutation in DPP, which encodes the dipeptide permease (De Groote et al., 1995).

Glutathione peroxidase

Similarly, our results suggest that enhanced GPx activity was found in liver in combination (B+1/2 Arg+1/2 Cip) of drugs at day 14, maximum increase was seen in L-arginine and combination of drugs (B+1/2 Arg+1/2 Cip) (Figure 3). These results are consistent with report of Farias-Eisner et al. (1996). In contrast to our study, Asahi et al. (1995) on the other hand reported that, GPx could be inhibited by putative NO donor S-nitro-N-acetyl pencillianine (SNAP) in U937 cells. Gluthathione peroxidase can catabolize peroxynitrite *in vitro* (Briviba et al., 1998) and many small biological molecules including glutathione, cysteine, methionine and tyrosine can react with peroxynitrite or its toxic products. Sies et al. (1997) reported a new function for selenoproteins as peroxynitrite reductase. It increased the formation of nitrite from peroxynitrite and was able to defend human fibroblast cells against peroxynitrite mediated oxidation. Peroxynitrite is known to inactivate GPx by the oxidation of essential thiol or selenol (Asahi et al., 1997). Similar trends were observed in case of glutathione reductase activity (Figure 3).

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