International Research Journal of Microbiology (IRJM) (ISSN: 2141-5463) Vol. 2(6) pp. 259-264, July 2011
Available online http://www.interesjournals.org/IRJM


*Full Length Research Paper*

# Effect of nitric oxide donors and antibiotic against typhoid

**S.S.Haque**

Department of clinical Biochemistry, Indira Gandhi Institute of Medical Sciences, Patna-14
Email: sshaq2002@yahoo.co.in

Accepted 15 June, 2011

**Typhoid fever is an important communicable disease in many developing countries. Salmonella, gram negative bacilli, and can survive during certain stages of host parasite interaction. 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 maxim NO production. Taking these facts in to consideration, it was thought pertinent to see the effect of oral administration of NO donors i.e. L-Arginine. In this study we have evaluated NO donor and low doses of ciprofloxacin in connection with oxidative stress generated in Swiss albino mice by Salmonella typhimurium.**

**Key words:** Typhoid, Xanthine oxidase, Catalase

## INTRODUCTION

Typhoid fever (TF) remains a major health problem in India and other developing countries. TF is an important cause of morbidity and mortality in many regions of the world, with an estimated 12 – 33 million cases leading to 216,000 - 600,000 deaths annually (Pang et al., 1995; DeRoeck, 2007).The treatment against this disease was antibotics and vaccination. A major impediment to the effective chemotherapy of typhoid is the ever increasing numbers of resistant strains of S.typhi (Goldstein et al., 1986; Eykyn and Williams, 1987; Atoba and Charzt ,2001).

Oxidative stress engendered by the sustained synthesis of NO mediates cytotoxicity against a variety of eukaryotic and prokaryotic cells (Hibbs et al., 1988; DeGroote et al., 1995; MacMicking et al., 1995). Alternatively, reactive nitrogen species (RNS) generated through the interaction of NO with O2 and superoxide (O2-) indirectly mediate cytotoxicity of this diatomic radical. The autooxidation of NO with O2 gives rise to RNS such asNO2- and N2O3 with potent oxidative and nitrosative activity. Independently, NO reacts with O2 to generate ONOO-, a species capable of oxidizing amino acids, [Fe-S] clusters, and DNA (Pryoo and Squadrito, 1995; Keyes and Imalay, 1997). NO has lessen oxidative stress endured by mammalian host cells exposed to a variety of inorganic or organic peroxides (Johson et al., 1991, Wink et al., 1993, Gorbunov et al., 1997; Joshi et al.,1999). Despite its well-documented pro-oxidant functions, low concentrations of NO can paradoxically be cytoprotective. The majorities of the disease occur mainly due to the imbalance between the pro-oxidant and antioxidant homeostatic phenomenon in the body. The condition of proxidant dominates either due to the enhanced generation of free radicals and/or their poor quenching/scavenging into the body. The ability of salmonellae to replicate within the macrophages allows this enteric pathogen to cause this disseminated disease. The bacterial entrance causes the production of superoxide and nitric oxide. Superoxide and nitric oxide react together to form peroxynitrite a strong biological oxidant. Consequently, pathological conditions characterized by oxidative stress can greatly elevate the production of peroxynitrite (Rastaldo et al., 2007). The exposure of isolated rat enterocytes to Salmonella typhimurium enterotoxin resulted in an increased Xanthine oxidase (XO) activity (Mehta et al., 1999). To minimize ROS (Reactive oxygen species) toxicity, prokaryotic and eukaryotic organisms express a battery of low-molecular-weight thiol scavengers, a legion of detoxifying catalases, peroxidases, and superoxide dis-

| Groups | Treatments |
|---|---|
| Group1 | Negative control (Normal Saline) |
| Group2 | Positive control (*S. typhimurium*($0.6xLD_{50}$)+Saline |
| Group3 | *S. typhimurium* ($0.6xLD_{50}$)+Ciprofloxacin (400mgper kg b. wt) |
| Group4 | *S. typhimurium* ($0.6xLD_{50}$) +Arginine (1000mg perKg 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(250mgper kg b. wt) +Ciprofloxacin(200 mg per kg b. wt) |

mutases, as well as a variety of repair systems.

## MATERIAL 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 *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. 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.

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* ($0.6xLD_{50}$) 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 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 14th days of post treatment, liver was removed aseptically in sterile condition, homogenate was made and post mitochondrial supernatant was prepared for NO estimation.

### Catalase activity

Catalase activity was measured by the method of Claiborne et al, (1985). Briefly, the reaction mixture consisted of 1.95 ml of phosphate buffer (0.1M, pH 7.4), 1ml $H_2O_2$ (0.09M) and 0.05ml PMS (10%w/v) in final volume of 3ml. Change in absorbance after 3 minutes was recorded at 240nm. Catalase activity was calculated in term of nmol $H_2O_2$ consumed/min/mg protein.

### Xanthine oxidase activity

Xanthine oxidase (XO) catalyses the conversion of xanthine to uric acid. The spectrophotometric method for XO estimation is based on the procedure of Stripe & Corte, (1969) as modified by Ali et al, (2000). Briefly, 0.2 ml of post mitochondrial supernatant was diluted to 1 ml with Tris-buffer (0.5 M, pH 8.1) and incubated for 5 min at 37°C. Reaction was started by adding 0.1ml of 1mM xanthine. The reaction was kept at 37°C for 20 min. The reaction was terminated by the addition of 0.5 ml ice-cold perchloric acid (10%, v/v in distilled water). After 10 min, 2.5 ml of distilled water was added to the precipitated mixture, which was centrifuged at 1,200xg for 10 min. The clear supernatant was decanted and the O.D was read at 290 nm. The results were expressed as μmoles of uric acid formed/mg protein. The activity of xanthine oxidase has been calculated by using a 2mM stock

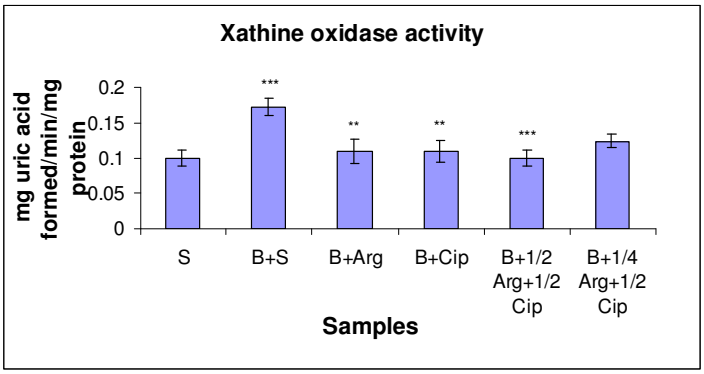


**Figure 1.** Hepatic XO activities were measured in mice: drugs were given and study was made at day 14. S=Saline, B+S=S.typhimurium+Saline, B+Arg=S.typhimurium+1000mg per kg b.wt L-Arginine, B+Cip=S. typhimurium+400mg per kg b. wt Ciprofloxacin, B+1/2Arg +1/2Cip=S. typhimurium+500mg per kg b. wt Arginine+200 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<0.01 and ***p<0.001

solution of uric acid to prepare standard curve.

### Protein estimation

Protein content was determined by using Lowry et al, (1951) method using bovine serum albumin as a standard.

## RESUTLS

### Xanthine oxidase (XO) activity

To analyze the effect of L-arginine, Ciprofloxacin and their combination on liver damage caused by *S. typhimurium*, XO activity was measured. The mice were challenged with sublethal dose ($0.6xLD_{50}$) of *S. typhimurium* and then treated with above drugs. The results have been summarized in Figure 1. Infection with bacteria resulted in an increase in XO activity by 11.68%, at day 14, as compared with normal control. The treatment of mice with drugs L-arginine, Ciprofloxacin and their combination, at day 14 of infection, caused an increase in the XO activity was reduced to 17.2%, 36.04%, 41.86% and 47.67% respectively as compared with only *S. typhimurium* infected control mice.

It is hypothesized that, selected drugs were able to minimize the damage of liver by reducing the enhancement of XO activity induced in bacterial infected mice, and maximum reduction was observed particularly in Ciprofloxacin and this combination B+1/2 Arg+1/2 Cip. So it can be speculated that combination of drug (B+1/2 Arg+1/2 Cip) might have the capacity to prevent the salmonellosis in murine model.

### Catalase (CAT) activity

To assess the effect of L-arginine, Ciprofloxacin and their combination on liver cell damage, catalase activity was measured. The mice were infected with sublethal dose of *S. typhimurium* ($0.6xLD_{50}$) and then treated with above drugs. Results have been summarized in Figure 2. The infection with bacteria to control mice resulted in an increase in CAT activity by 27.27% at day 14.
Although, at day 14, treatment of mice with L-arginine, Ciprofloxacin and their combination partially protected the liver, significant enhancement was observed in the infection-induced reduction of the enzyme and the activity of CAT was 15.6%, 21.87%, 15.6% and 0.00% in *Salmonella*-infected mice as compared with control. The drugs were able to confer partial enhancement in CAT activity in bacterial infected mice.

### Glutathione-S-transferase (GST) activity

To assess the effect L-arginine, ciprofloxacin and their combination, on liver function. The mice were challenged with sublethal dose of *S. typhimurium* $0.6xLD_{50}$, and then the mice were treated with above drugs. GST activity was assessed and the results have been summarized in Figure 3. Infection of mice with bacteria resulted in decrease in the GST activity by 21.51% day 14 as compared to saline treated control. Treatment with above drugs, the GST activity was slightly decreased by 15.0%, 20.0%, 20.0% and 16.66% as compared with control.

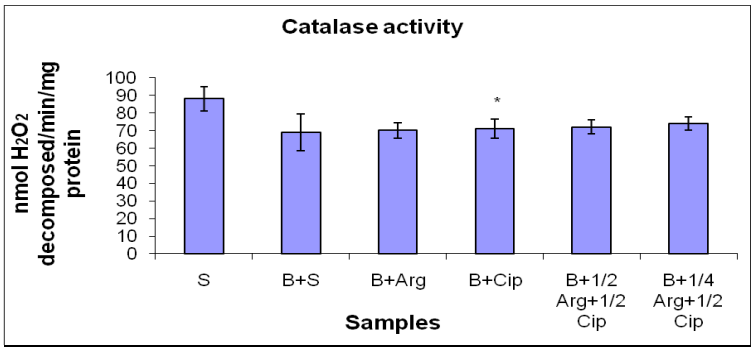


**Figure 2.** Hepatic catalase activities were measured in mice: drugs were given and study was made at day 14. S=Saline, B+S=*S. typhimurium*+Saline, B+Arg=*S. typhimurium*+ 1000mg per kg b. wt L-Arginine, B+Cip=*S. typhimurium*+400mg per kg b. wt Ciprofloxacin, B+1/2Arg +1/2Cip=*S. typhimurium*+500mg 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

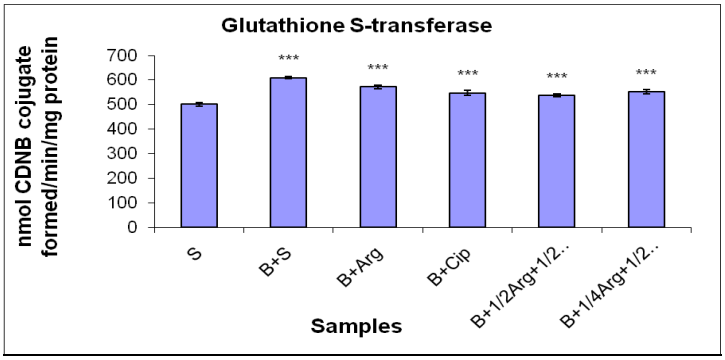


**Figure 3.** Glutathione-S-transferase activity in mice of day 14 treated with arginine, ciprofloxacin and their combination. S=Saline, B+S=*S. typhimurium*+Saline, B+Arg=*S. typhimurium*+ 1000mg per kg b. wt L-Arginine, B+Cip=*S. typhimurium*+400mg per kg b. wt Ciprofloxacin, B+1/2Arg +1/2Cip=*S. typhimurium*+500mg 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<0.001

At day 14, the treatment of mice with above drugs, the GST activity was increased by 18.03%, 31.14%, 26.22% and 19.67% as compared with control.

## Discussion

### Oxidative stress

Oxidative stress takes place when flux of partially reduced form of oxygen is greater than the ability of biological system to cope with its production. Under stressed conditions biological molecules are exposed to pro-oxidant species resulting in irreversible oxidation reactions. The redox state of a cell, which refers to the ratio of the reduced and the oxidized forms of certain cellular components (such as glutathione), is an important signaling molecule in cellular homeostasis. Any additional oxidative insult can change the redox-status of the cell. The main objective of our study was to evaluate the redox status of the cells after treatment with NO donor and its combination with antibiotic followed by bacterial infection. For the purpose of investigation of the role of these agents, we have used liver as the organs of the primary sites for bacterial replication.

### Xanthine oxidase

In the present studies, results showed that Uric acid

production was significantly decreased in animal treated with drugs (Figure 1). The XO activity increased in *S. typhimurium* infected mice, which in turn enhanced $O_2^-$ production in liver. Similar increase in XO activity in salmonella infected animals was also observed by Umezawa et al, (1997). Earlier it was thought that peroxynitrite is the main bacteriocidal species in case of *S. typhimurium* (Umezawa et al., 1997). Careful examination reveals that nitrotyrosine, a molecular signature that can be associated with peroxynitrite synthesis, does not co-localize with internalized bacteria (Ischiropoulos et al, 1992; Crow and Ischiropoulas, 1996).

**Catalase**

Catalase has been shown to have strong NO binding due to electron donating properties of the proximal tyrosinate ligand (Brown, 1995). It is not surprising that catalase, a heme protein, is inhibited by NO (Fang, 1997). Nitric oxide is known to have light affinity for iron in heme proteins (Hoshino et al, 1993). It can reversibly bind to ferric iron. This reaction is responsible for inhibition of catalase by NO (Cooper, 1999). Nitric oxide can inhibit antioxidant metalloenzymes such as catalase (Kim et al., 1995; Fang, 1997) thereby limiting $H_2O_2$ disproportionation.

In the cells that utilize catalase as their primary pathway method for $H_2O_2$, production NO may be expected to raise intracellular $H_2O_2$ levels (Farias-Eisner et al, 1996). Mice infected with bacteria showed increase in NO production that results in inactivation of CAT activity but the effect was less pronounced than groups (B+Arg) and (B+Cip). Mice were treated with L-arginine and ciprofloxacin in a combination (B+1/2Arg+1/2Cip), affect CAT activity (Figure 2). The treatment withL-arginine and ciprofloxacin partially restored the catalase activity especially after 14 days of treatment. however increase in CAT activity was not significant in these case except (B+Cip)

The *S. typhimurium* infected mice also showed inhibition in catalase activity that have some bacteriocidal role but may cause the host toxicity due to generation of strong oxidants such as hydroxyl radicals. Our study suggests that there is depletion of other antioxidants in liver of infected animals. Iron-sulfur proteins such as aconitase are particularly sensitive to damage by RNI (Hausladen et al., 1994; Castro et al., 1994), which would then result not only in enzymatic deactivation but also in an increased cytoplasmic low molecular weight redox-active iron pool, causing bacterial DNA damage. In mammalian cells, the enzymes such as aconitase are not in close proximity to DNA. The differences in compartmentalization and higher amount of non-sulphur proteins in bacteria in comparison to mammalian cells appeared to explain the differential response of mammalian cells (Wink et al., 1993; Farias-Esner et al., 1996) and bacteria to NO/ $H_2O_2$ (Pacelli et al., 1995). It may accord a unique means to combat the invading microorganisms with minimal damage to host cells.

**Glutathione-S-Transferase**

Cells have evolved a GSSG clearance mechanism to avoid potentially harmful disulfide interchange reaction. There are components that in presence of glutathione-S-transferase (GST) can react with -SH to yield conjugates such as mercapturic acid, leukotrienes and other components. The GST activity reduces GSSG to provide or to maintain GSH level in the cells. In other words, GST provides another way to enhance the reduced glutathione or GPx activity in the cells. However, the GR/G6PD system is completely inhibited in the animals infected with $0.6xLD_{50}$ of *S. typhimurium*, suggesting that these enzymes constitute a prooxidant effect on cells. Though these will kill bacteria, however, the overall damage to the host will be more in disease model (Figure 3).

The XO activity increased in group S+B, which in turn enhanced $O_2^-$ production in the liver. The $O_2^-$ radical has been of profound interest owing to its increased dominance *in vivo* in different disease conditions. Oxidation of hypoxanthine to uric acid with simultaneous generation of $O2^-$ and $H_2O_2$ has been observed to play a crucial role during an inflammatory condition and cancer. Thus it helps in reducing the production of uric acid by inhibiting XO. Decrease in uric acid production indicated the liver to be in good rather than in damaged condition. The infection of mice with *S. typhimurium* caused the damaged to liver as reported by (Khan et al., 2008; Khan and Jain, 2009). Infection of the mice with *S. typhimurium* caused an Thus it helps in reducing the production of uric acid by inhibiting XO. Decrease in uric acid production indicated the liver to be in good rather than in damaged condition. The infection of mice with *S. typhimurium* caused the damaged to liver as reported by (Khan et al., 2008; Khan and Jain, 2009). Infection of the mice with *S. typhimurium* caused an elevated level of uric acid thereby causing the increase of XO. In the present study we have shown a decrease in the level of uric acid in animals, because of the damaging effects of ROS, all cells maintain antioxidant defenses. Three levels of protection have been considered: 1) prevention of ROS formation, 2) termination of the ROS using free radical scavengers or antioxidant enzymes, and 3) repair of damaged cellular components. An important aspect of prevention is the segregation or chelation of metals that can catalyze ·OH formation, such as by iron binding to ferritin (Balla et al., 1992). Nonenzymatic antioxidants include glutathione, alpha-tocopherol (vitamin E), ascorbic acid, beta-carotene, and uric acid; these are mostly considered to be chain-breaking antioxidants in that they interrupt the autocatalytic spread of radical reactions (Cadenas,

1989). Enzymes involved in antioxidant defenses exist as a coordinated system and include superoxide dismutase (SOD) which catabolizes superoxide radicals and catalase (CAT), Se-independent peroxidase activity is a function of one class of glutathione S-transferase isozymes;

**REFERENCES**


Atoba MA, Charzt FA (2001). In vivo drug resistance during treatment for typhoid fever: a case report. Central Afr. J.Med. 47: 20-21.

Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW, Vercellotti

GM (1992). Ferritin: a cytoprotective antioxidant strategem of endothelium. *J.*

*Biol.Chem*. 267: 18148- 18153.

Brown GC (1995). Reversible binding and inhibition of catalase by nitric oxide. *Eur. J. Biochem*. 232: 188-191

Cadenas E (1989). Biochemistry of oxygen toxicity. *Annual Rev. Biochem*.,58: 79-110.

Castro L, Rodriguez M, Radi R (1994). Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. *J. Biol. Chem*. 269: 29404-29415.

Cooper CE (1999). Nitric oxide and iron proteins. *Biochem. Biophys. Acta*. 1411: 290-309.

Crow JP, Ischiropoulos H (1996). Detection and quantitation of nitrotyrosine residues in proteins *in vivo* marker of peroxynitrite. *Methods Enzymol*. 269: 185-184.

De Groote MA, Granger D, Xu Y, Campbell G, Prince R, Fang FC (1995). *Proc. Natl. Acad. Sci. USA.* 92: 6399–6403

De Roeck D, Jodar L, Clemens J (2007). Putting typhoid vaccination on the global health agenda. N. Engl. J. Med. 357: 1069-1071.

Eykyn SJ, Williams H (1987). Treatment of multiresistant Salmonella typhi with oral ciprofloxacin. *Lancet*. 12 (2): 1407-408.

Fang FC (1997). Perspectives series, host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *J. Clin. Invest*. 99: 2818-2825.

Farias-Esner R, Choudhuri G, Aeberhard E, Fukuto JM (1996). The chemistry and tumoricidal activity of nitric oxide/hydrogen peroxide and the implications to cell resistance/susceptibility. *J. Biol. Chem*. 271: 6144-6151

Goldstein FW, Chumpitaz JC, Guevara JM, Papadopoulous B, Acar JF (1986). Plasmid-mediated resistance to multiple antibiotics in *Salmonella typhi*. J.Infectious Dis. 153: 261- 266.

Gorbunov NV, Yalowich JC, Gaddam A, Thampatty P, Ritov VB, Kisin ER, Elsayed NM, Kagan VE (1997). *J. Biol. Chem.* 272: 12328–12341

Hausladen A, Fridovich I (1994). Superoxide and peroxynitrite inactivate aconitase, but nitric oxide does not. *J. Biol. Chem*. 269: 29405-29408.

Hibbs JB, Jr Taintor, RR, Vavrin Z, Rachlin EM (1988) *Biochem. Biophys. Res. Commun.* 157: 87–94

Hoshino M, Ozawa K, Seki H, Ford PC (1993). Photochemistry of nitric oxide adducts of water soluble iron (iii) porphyrin and Ferrihemoproteins studied by nanosecond laser photolysis. *J. Am. Chem. Soc*. 115: 9568-9575.

Ischiropoulos H, Zhu L, Beckman JS (1992). Peroxynitrite formation from macrophage-derived nitric oxide. *Arch. Biochem. Biophys*. 298: 446-451

Johnson G, 3rd Tsao PS, Lefer AM (1991). *Crit. Care Med.* 19: 244–252

Joshi MS, Ponthier JL, Lancaster JR (1999). *Free Radic. Biol. Med.* 27: 1357–1366 Keyer K, Imlay JA (1997). *J. Biol. Chem.* 272: 27652–27659

Khan KH, Ganjewala D, Rao KVB (2008). Recent advancement in typhoid research-a review. Advanced Biotech. 7 (4): 35-40.

Khan KH, Jain SK (2009). Regular intake of *Terminalia chebula* can reduce the risk of getting typhoid fever. Advanced Biotech. 8 (9): 10-15.

Kim YM, Bergonia HA, Muller C, Pitt BR, Watkins WD, Lancaster Jr JR (1995). Nitric oxide and intracellular heme. *Adv. Pharmacol*. 34: 277-291.

MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, Xie QW, Sokol K, Hutchinson N, Chen H, Mudgett JS (1995). *Cell* 81: 641–650

Mehta A, Singh S, Ganguly NK (1999). Effect of *Salmonella typhimurium* enterotoxin (S-LT) on lipid peroxidation and cell viability levels of isolated rat enterocytes. Molecular and Cellular Biochem. 196: 175-181.

Mitchell JB (1993). *Proc. Natl. Acad. Sci. USA.* 90: 9813–9817

Pacelli R, Wink DA, Cook JA, Krishna MC, DeGraff W, Friedman N, Tsokos M, Samuni A, Mitchell JB (1995). Nitric oxide potentiates hydrogen peroxide-induced killing of *Escherichia coli*. *J. Exp. Med*. 182: 1469-1471

Pang TZ, Bhutta A, Finlay BB, Altwegg M (1995). Typhoid fever and other salmonellosis: a continuing challenge. Trends Microbiol. 3: 253-255.

Pryor WA, Squadrito GL (1995) *Am. J. Physiol.* 268: L699–L722

Rastaldo R, Pagliaro P, Cappello S, Penna C, Mancardi D, Westerhof N, Losano G (2007). Nitric oxide and cardiac function. Life Sci. 81: 779-793.

Umezawa K, Akaike T, Fujii S, Suga M, Setoguchi K, Ozawa A, Maedia H (1997). Induction of nitric oxide synthesis and xanthine oxidase and their roles in the antimicrobial mechanism against *Salmonella typhimurium* infection in mice. *Infect. Immun*. 65: 2932-2940.

Wink DA, Hanbauer I, Krishna MC, DeGraff W, Gamson J, Mitchell JB (1993). *Proc. Natl. Acad. Sci. USA*. 90: 9813–9817