CHAPTER 6

SUMMARY AND CONCLUSIONS
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Typhoid fever is an important health concern in India. Presently available means for protection against typhoid, its proper diagnosis and treatment are not adequate, hence there is a need for the development of new methods. In India more than 2200 strains/serotypes of *Salmonella* are known most of which are pathogenic to human. Most common Indian species are *S. typhi* and *S. typhimurium* both of which produce mild to severe illness. *S. typhi* is the primary causative agent for human typhoid while *S. typhimurium* causes experimental salmonellosis in rodents and murines. 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 of isolates as well as in number of drugs to which these organisms have developed resistance.

*Salmonellae*, Gram negative bacilli, are facultative intracellular bacteria and can survive during certain stages of host-parasite interactions. The organism gains entry into host by oral route following the ingestion of contaminated food, water, and vegetables. The onset of typhoid fever is characterized by a long incubation period ranging from one week to one month. After its entry, the bacterium invades and multiplies in liver and spleen and then migrates to gall bladder and ultimately to intestine.

Pathogenic *Salmonella* initiate infection of a host by inducing their own uptake into epithelial cells. The bacteria display a very sophisticated means to interact with host cells, resulting in the stimulation of a variety of host cellular responses. The bacteria possess a set of genes that encode a type III
secretion system (TTSS) which enables the bacteria to penetrate the membrane of host cells.

Despite the complications in the pathogenesis processes there is continuous need to understand the pathogenesis of typhoid fever. It seems crucial to elucidate the host responses against *Salmonella* with focus on defense oriented molecules such as oxygen radicals and nitric oxide. NO is the smallest molecule of all the known bioreactive mammalian cell secretory products. High chemical activity of NO results in its short half-life inside the cell and the specificity of its interaction is minimal. In iNOS expressing cells both the synthesis of L-cit from L-arg along with the formation of NO and conversion of L-cit to L-arg takes place via arginine-citrulline cycle. The activity of this pathway is markedly enhanced in the activated cells. Animals were pretreated with NO donors and NO inhibitor compounds for 7 days and thereafter exposed to two different challenge doses of *S. typhimurium* wild (1 x LD$_{50}$ and 2 x LD$_{50}$). The L-arg and L-cit pretreated mice exhibited dose dependent protection whereas aminoguanidine treatment resulted in higher mortality.

Pretreatment of L-arg and L-cit increased the NO production in liver and spleen following sublethal challenge with *S. typhimurium* (0.4 x LD$_{50}$) where as NO production was not significant in uninfected animal groups. It seems that iNOS is not induced which is necessary to upregulate the NO production. In both uninfected and infected groups, the animals pretreated with aminoguanidine blocked the NO production significantly in liver as well as in spleen.

Animals pretreated with NO donors (L-arg and L-cit) had significantly lower incidence of bacterial translocation to liver and spleen in comparison to the control animals. Aminoguanidine pretreatment resulted in increased bacterial growth both in liver and in spleen of mice.

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. The redox state of a cell, which refers to the ratio of the reduced and oxidized forms of certain cellular components such as glutathione, is an important signaling device in cellular homeostasis. Any additional oxidative insult can change the redox status of the cell after treating with NO donor and NO inhibitor compounds followed by bacterial infection.

Our results showed that superoxide production was significantly decreased in animals pretreated with L-arg and L-cit in infected and uninfected groups. However, bacteria infected group SB showed increase in XO activity which in turn enhanced $O_2^-$ production in liver and spleen. Simultaneous production of these two activities results in peroxynitrite production which causes damage to host tissue. Aminoguanidine treated infected and uninfected groups showed increased superoxide production.

Lipid peroxidation is a chain reaction and a single oxidative event can oxidize many lipid molecules. Our studies suggested that L-arg and L-cit treated, infected and uninfected groups showed decrease in lipid peroxidation. However, infected group (SB) enhanced the LPO activity. Aminoguanidine treated animal groups also enhanced LPO. From these results it can be inferred that nitric oxide is able to inactivate lipid peroxidation whereas peroxynitrite and superoxide enhanced LPO.

CAT activity is reduced in L-arg and L-cit treated infected and uninfected groups. However, aminoguanidine treated groups enhanced CAT activity. CAT activity is also diminished in group SB. These results suggest that nitric oxide is involved in the inactivation of CAT enzyme. It may be speculated that decreased CAT activity might enhance $H_2O_2$ production which will combine with NO to produce bactericidal activity.

Reduced glutathione is necessary for maximum NOS activity which is reflected by our results. Increased NO production in animals pretreated with L-arg and L-cit was also supported by an increase in GSH levels in liver and spleen. Whereas bacteria infected group (SB) had decreased GSH levels due to production of peroxynitrite, which can oxidize GSH to GSSG, which
suggesting that this reaction could affect the redox status of intracellular and extracellular thiols. In aminoguanidine treated infected and uninfected groups GSH level decreased significantly. Our results also suggest that increased levels of NO and GSH in L-arg and L-cit treated group may lead to form S-nitrosoglutathione which is bacteriocidal against Salmonella typhimurium.

Our results indicated that L-arg and L-cit pretreated infected and uninfected groups showed enhanced GPX, GR and G6PD activities whereas groups SB, I, and IB has declined activities of these enzymes. These results suggested that NO donor compounds on one hand could protect the host cells by providing them reduced equivalents and on the other hand, they generate bacteriocidal compounds from both reduced equivalents such as N-nitrosoglutathione or S-nitrosothiols and NO. Due to decline in the activities of these enzymes, peroxynitrite is not converted to less toxic radicals and it causes the damage to the host. Aminoguanidine treated and infected groups had declined GPX, GR and G6PD activities which would not be able to generate NADPH for the support of NO production. However, peroxynitrite production does not take place in this case due to blockage of NO production.

Our results also suggested that glutathione S-transferase activity (GST) is depleted in L-arg and L-cit pretreated infect and uninfected groups which suggests that these compounds are able to increase GSH in the cells via reducing GST activity. However, group SB and aminoguanidine treated infected and uninfected groups showed increase in GST activity, which suggests that, the rate of depletion of GSH is increased in treated animals in comparison to control groups.

Above biochemical results are supported by the findings of histopathological studies on liver and spleen. Although the control animals showed some granuloma formation, however, necrosis is also observed in these tissues. Animals pretreated with L-arg and L-cit showed higher granuloma formation which is the sign of protection against infection.
necrosis was visible in these animals. Aminoguanidine treated animals showed necrotic tissues in both liver and spleen.

These results are also supported by the study of serum enzymes. The treatment with L-arg and L-cit showed decline in the serum ALT and AST activities in both infected and uninfected groups as compared to their respective control groups. Group SB and aminoguanidine treated animals showed increase in serum ALT and AST activity. These results suggested that L-arg and L-cit are able to protect bacteria induced liver damages.

Inducible nitric oxide synthase is positively modulated by LPS, IFN-γ and TNF-α in the murine typhoid. Our results suggested that L-arg and L-cit treated animal groups are able to enhance NO production in macrophages. Whereas, aminoguanidine blocked the NO production in peritoneal macrophages. Macrophages are the first niche of bacteria in the host. Nitric oxide production increased between days 3 and 7 of PI and thereafter decrease.

Phagocytic capacity and phagocytic index of peritoneal macrophages are increase in animals challenged with 0.4 x LD₅₀ S. typhimurium. NO donors pretreatment further modulated these responses. This increasing trend was observed upto 3 days thereafter phagocytosis declined towards basal levels. However, aminoguanidine pretreatment blocked the response, which suggested that nitric oxide is involved in the process of phagocytosis.

L-arginine is a versatile amino acid that plays a central role in the normal function of several organs systems including the immune system. Dietary supplementation of L-arg has gained approval to enhance cellular immune responses. In our study we found that L-arg and L-cit both are able to enhance cell mediated immune responses such as DTH and T-cell proliferation. A frequent consequence of infectious diseases is the development of delayed type hypersensitivity to one or more specific microbial antigens. Pretreatment of animals with NO donors like L-arg and L-cit increased foot pad swelling significantly as compared to saline treated control
groups. Contrary to NO donors, aminoguanidine reduced foot pad swelling significantly as compared to control group at 48 hours by using S. typhimurium cell lysate as an antigen.

*S. typhimurium* infection depletes the T cell proliferation whereas NO donors treated groups increase T-cell proliferation induced by Con A. Pretreatment of aminoguanidine depletes Con A induced proliferation of PBMC, however, the effect was less pronounced as compared to infected control group (SB).

Based on the results of our studies following conclusions have been drawn.

- Nitric oxide plays an important role in host response against *S. typhimurium* infection.
- L-arg (1.0 g kg⁻¹ body wt) and L-cit (0.7 g kg⁻¹ body wt) provides 100% protection against 1 x LD₅₀ of *S. typhimurium* and conferred 80% and 70% protection, respectively against 2 x LD₅₀ challenge of *S. typhimurium*. Aminoguanidine (1.0 g kg⁻¹ body wt) treatment increased the mortality of animals.
- The nitric oxide production increased in liver, spleen and peritoneal macrophages following bacterial challenge. This response is further enhanced by pretreatment with NO donors whereas aminoguanidine pretreatment blocked the response.
- NO donor compounds decreased xanthine oxidase activity which in turn blocks the formation of peroxynitrite. Whereas groups SB, I and IB increase xanthine oxidase activity.
- LPO activity is decreased in the animals pretreated with NO donor compounds whereas aminoguanidine infected (IB) and bacterial infected groups (SB) enhanced LPO activity.
- CAT activity is decreased in groups AB, CB, and SB. However, their uninfected groups have no significant effect on CAT activity.
Aminoguanidine treated infected and uninfected groups increased the CAT activity.

- The level of GSH enhanced in animals pretreated with L-arg and L-cit whereas depleted in groups I, IB and SB.
- GPX, GR and G6PD activities were enhanced in NO donor treated, infected as well as uninfected groups whereas depleted in groups I, IB and SB.
- Glutathione-S-transferase (GST) activity is decreased in animals pretreated with NO donors whereas increases in the groups I, IB and SB.
- Serum ALT and AST activities are depleted in the animals pretreated with NO donors while enhanced in groups I, IB and SB.
- Histopathological examinations in liver and spleen suggested that treatment with NO donors showed no pathological lesions whereas SB group animals showed necrotic lesions. Aminoguanidine pretreated group showed prominent areas of necrosis in liver and spleen.
- Phagocytosis increased by NO donors and by bacteria itself whereas aminoguanidine pretreatment decreased the response.
- Animals pretreated with NO donors showed increased cell mediated immune responses as evident by DTH response and T-cell proliferation whereas groups SB and IB had depleted CMI responses.