Discussion
9.1 MORPHOLOGICAL EVALUATION OF HepG2 CELLS AFTER INFECTION WITH *Salmonella enterica* serovar Typhimurium.

*Salmonella enterica* serovar Typhimurium induces morphological changes at higher multiplicity of infection (1:200) in HepG2 cells (in present study), whereas MOI of 1:10, 1:50, 1:100 did not produce visible changes (Fig. 8). During natural infections the MOI can range between 1:10 to 1:500 depending on the invasiveness of bacteria and nature of host cells. Phagocytic cells like macrophages, dendritic cells require lower MOI whereas non-phagocytic cells like epithelium cells, liver cells, usually show higher MOI. Moreover, bacteria themselves negotiate their entry into non-phagocytic cells this phenomenon commonly termed as macro-pinocytosis. *Salmonella enterica* serovar Typhimurium follows normal course of infection in HepG2 which include adherence, internalization and replication. Phase contrast microscopic analysis of infected HepG2 cells shows disrupted and disfigured morphology along with adhered bacteria. Adherence of *Salmonella enterica* serovar Typhimurium was more pronounced in scanning electron micrograph where usually 3 to 4 bacteria were found attached to a single HepG2 cell (Fig.10). Internalization is accompanied with the gross cytoskeleton changes where actin is accumulated due to local cytoskeletal rearrangement and the uptake of bacteria in that particular area of membrane ruffle has been demonstrated (Finlay et al., 1991). It has been reported that *S. Typhimurium* induces macro-pinocytosis in the area of membrane ruffle which contains bacteria and also in the extracellular fluids in the immediate vicinity of internalized bacteria (Garcia-del Portillo and Finlay, 1994; Alpuche-Aranda et al., 1994) which produce the characteristic membrane ruffling. This characteristic ruffling was evident in infected HepG2 cell micrograph at 36 hr of post-infection (Fig.11).

Membrane ruffling following *Salmonella enterica* serovar Typhimurium invasion has been reported both *in vivo* and *in vitro*. When *S. enterica* serovar Typhimurium is administered into GIT of mice, the bacteria invade the mucosa of the small intestine and display a tropism for the M cells of Peyer's patches (Carter and Collins, 1974; Hohmann et al., 1978; Clark et al., 1994; Jones et al., 1994). Entry of *S. enterica* serovar Typhimurium M cells is accompanied by the formation of membrane ruffle in a manner similar to that seen in the invasion of epithelial cells in tissue culture (Francis et al., 1993; Ginocchio et al., 1992), and M cell internalization of *S. enterica* serovar Typhimurium causes a
destruction of the cytoskeletal architecture (Jones and Falkow, 1996). After intestinal penetration, *S. enterica* serovar Typhimurium causes a transient bacteraemia and disseminates to systemic sites of infection, most importantly the liver and spleen, where the bacteria multiply.

The *Salmonella enterica* serovar Typhimurium pathogenicity island 1 (SPI-1) type three secretion system (TTSS) is essential for Salmonella invasion of host cells through its triggering of actin-dependent membrane ruffles. The SPI-1 effectors SipA, SopE, SopE2 and SopB all have actin regulating activities and contribute to invasion. SipA plays a more prominent role in induction of invasion-competent membrane ruffles by *Salmonella enterica* serovar Typhimurium lacking a full complement of SPI-1 effectors (Perrett and Japson, 2009). Following host cell contact, *Salmonella enterica* serovar Typhimurium employs a specialized organelle termed the type III secretion system to inject multiple bacterial effector proteins directly into the host cell cytosol. A specific subset of these effector proteins taps into the eukaryotic signaling networks responsible for regulating actin dynamics and together they orchestrate profuse remodeling of the cytoskeleton at the site of entry (Patel and Galan, 2005). As key modulators of cytoskeletal remodeling in eukaryotes, the Rho family of small GTPases presents ideal targets for bacterial effector proteins to promote internalization into non-phagocytic cells (Bustelo et al., 2007). Rho-family GTPases, which have been implicated in mediating actin-dependent *Salmonella* entry by interacting with N-WASP or WAVE-complex, well-established activators of the actin nucleation machine Arp2/3-complex. *Salmonella enterica* serovar Typhimurium invasion is not always necessary to be accompanied with bacteria-induced membrane ruffling as an Arp2/3-complex-independent actin assembly activity was shown in *Salmonella enterica* serovar Typhimurium invasion (Hanisch et al, 2010).

Invasion of cultured polarised epithelial cells, such as MDCK and Hep-2 cells, by *S. enterica* serovar Typhimurium shows that the pathogen can efficiently enter the host cell from the apical surface, where membrane ruffling and subsequent macro-pinocytosis are evoked on the epithelial cell surface to engulf the attached bacteria. The bacteria are surrounded by a membrane vacuole, multiply in the vacuole, and are then transcytosed toward the outside of the host cell. Unlike *Yersinia*, *Salmonella* invasion involves the triggering of host cellular signaling events that lead to the membrane ruffling and macro-
pinocytosis around the site of bacterial entry. Through this mode of bacterial entry, other non-invasive bacteria can be entrapped and can co-infect together with the pathogen (Francis et al., 1993). Such cellular responses are triggered upon delivery of a subset of Salmonella effector proteins through the type III secretion machinery after contact with the host cell. Internalized Salmonellae remain within a membrane vacuole which is surrounded by large dense structures composed of various cytoskeletal components including F-actin, α-actinin, ezrin, talin and tropomyosin (Garcia-del Portillo et al., 1994; Garcia-del Portillo and Finlay, 1995). Many actin-binding proteins use a set of structural modules to achieve diverse activities and generate distinct topologies of F-actin bundles. F-actin-bundling proteins generally exert their actin-bundling activities by encoding two discrete actin binding domains within their sequence or by forming a multimeric complex that bridges two F-actin filaments together into a bundle. For example, plastin (Fimbrin) has two actin-binding domains in close proximity to tightly bundle F-actin in microvilli. Furthermore, proteins like α-actinin form homodimers in an anti-parallel manner to produce more loosely ordered F-actin structures such as stress fibres). The C-terminus of SipC bundles Factin promote Salmonella invasion (Myeni and Zhou, 2010).

Replication of Salmonella enterica serovar Typhimurium results into death and disruption (cytotoxic) of infected cells though the number of Salmonella enterica serovar Typhimurium recovered was found to be low. This cytotoxicity was mediated by invasive Salmonella through apoptosis. Mutant S enterica serovar Typhimurium that is incapable of inducing host cell membrane ruffling fails to induce apoptosis in phagocytic cells. It is the entry and not the replication step that triggers the signal transduction pathway which leads to apoptosis (Fig. 12). During invasion, S enterica serovar Typhimurium triggers an increase in several host cell second messengers, such as intracellular calcium levels, phospholipase A2 activity, and leukotriene production, as well as enhanced protein kinase activity (Bliska et al., 1993, Saito et al., 1994). These second messengers may play a role in the activation of programmed cell death.

Oxidative stress mediated by ROS has been implicated as the central pathway in ischemia/reperfusion injury, autophagy, acetaminophen toxicity and alcohol-induced liver injury (Jaeschke et al., 2002). Autophagy contributes to the degradation of cytoplasmic components to recycle nutrients under starvation, and clearance of aberrant organelles,
protein aggregates and even microbes. Autophagy has been linked to many human disease conditions and is an important innate immune defense mechanism. Autophagy can target intracellular bacteria in the cytosol (e.g., Autophagy can target intracellular bacteria in damaged vacuoles (e.g., *Salmonella enterica* serovar Typhimurium) (Birmingham *et al.*, 2006). However the signals involved in these targeting processes remain largely unclear. Bacterial pattern recognition receptors (PRRs), including TLRs and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), are recently found to be involved in activating autophagy. Autophagy can be induced by a variety of stimuli in different cell types under different conditions, and ROS are one of these autophagy activators. Excessive ROS accumulation in cells can cause oxidative stress and cell death, including autophagic cell death (Azad *et al.*, 2009, Chen *et al.*, 2008). However, the role of oxidative stress in inducing liver damage following *Salmonella enterica* serovar Typhimurium infection, particularly in reference to different environmental conditions encountered by the pathogen, within the host has not been examined. Cell death can be triggered by Toll like Receptor (TLR) ligands such as lipoproteins, dsRNA and LPS. Many bacterial pathogens have developed strategies to exploit TLR signaling and interfere with the immune response of the host hence TLR signalling could also play a role in *Salmonella*-induced killing of macrophages. Hsu *et al.* (2004) showed that Salmonella-induced SipB-independent cell death requires TLR4 stimulation of the dsRNA responsive protein kinase PKR and postulating that this may be Trif dependent (Cook *et al.*, 2007).

### 9.2 PROTEOMIC STUDY OF *Salmonella enterica* serovar Typhimurium INFECTION IN VITRO MODEL

Proteomics is relevant to numerous aspects of microbial disease pathogenesis and treatment. Comprehensive identification of microbial proteins during infection as well as host cell responses in defense can be established through techniques of proteomics (2D-PAGE and mass spectroscopy). Interest in the application of proteomics to microbiology goes back at least a decade, with the pioneering work of Fred Neidhardt (1999) to characterize protein expression patterns in *Escherichia coli* under different growth conditions. The sequencing of complete genome of the malaria parasite *Plasmodium falciparum* has provided a basis for conducting comparative proteomics studies of this
pathogen, leading to the identification of new potential drug and vaccine (Lasonder et al., 2002; Florens et al., 2002).

*Salmonella* which is well studied in terms of pathogenesis causes illness when translocated from the intestine, mainly the distal ileum to bloodstream and finally to liver and spleen. Two distinct tissues in the ileum have been identified as targets for *Salmonella* entry into the host: the ileum mucosa (IM) and the Peyer’s patches (PP) (Darwin et al., 1999 and Wallis et al., 2000). PP are immune tissues that are part of the gut-associated lymphoid tissue. M cells are located in the follicle-associated epithelium overlaying the PP that have a role in sampling of pathogens. Numerous studies are available on the gene transcription response of epithelial cells exposed to various bacterial pathogens *in vitro*, such as *Vibrio cholerae*, *Listeria monocytogenes*, *Shigella flexneri*, and *Salmonella* (Stokes et al., 2004; Baldwin et al., 2003; Pedron et al., 2003; Eckmann et al., 2000) but little is known of the response of deeper tissues system such as liver.

There are confirmed evidences of intracellular survival and growth of *Salmonella* in liver (*in vitro* as well as *in vivo*). The molecular mechanism of invasion is available at gene level but there is a lack of knowledge at protein level. Given that the level of transcript of a gene does not necessary correspond to the absolute amount of protein of that gene, the two molecules may be regulated differently. Moreover, functional changes are produced by post translational modification of proteins and not by genes itself; therefore, further studies are required at protein level to understand the scenario in toto. The present study aims at exploring the molecular cross talk between *Salmonella enterica* serovar Typhimurium and host cells at proteomic level.

Human hepatoma cell line (HepG2) was chosen for the infection studies as representative of liver cells, as the cells belong to human itself, the data might therefore reflect closely the possible *in vivo* mechanism.

In present study total cellular protein from *Salmonella enterica* serovar Typhimurium infected HepG2 cells showed substantial down-regulation in various proteins between 43 kDa to 29 kDa range, whereas marked up-regulation were seen in different proteins between 60 kDa to 43 kDa range on simple SDS-PAGE (Fig.13). When the same protein sample was analyzed on two-dimensional electrophoresis, a total of three hundred and fifty three (353) protein spots were seen (Fig.14). Further analysis of the gel revealed
significant differential expression (on the basis of quantity as calculated by PDQuest software) only in thirty one (31) protein spots when compared to control gel (Table.4). Some of these thirty one spots were discernable only in one gel whereas in other, they seem totally missing. These spots were marked as highly up-regulated or highly down-regulated accordingly. Table.4 shows the details of each (out of 31) individual protein spot along with their SSP number, approximate molecular weight/isoelectric point (Mw/pl) and percentage increase/decrease. Histograms show the quantitative relation of proteins between normal and infected hepatoma cells (Fig. 16).

9.2.1 Identification of protein spots by HCC-M database

On the basis of molecular weight/isoelectric point (Mw/pl), individual spot were compared in hepatocellular carcinoma cell line (HCC-M) database, spots revealed the similarity with proteins (Table.5) such as,

A) SSP-5201 {Capping protein (actin-filament) gelsolin like}
B) SSP-1505 (Vimentin like)
C) SSP-3104 (Rho-GDP-dissociation inhibitor protein like)
D) SSP-6103 (Growth factor receptor-bound protein like)
E) SSP-7106 (Glutathione-S-transferase homologue like)
F) SSP-7201 (BAG-family molecular chaperon regulator like)

A) SSP-5201 {Capping protein (actin filament) gelsolin like}

HCC-M database- MW- 38.51, pI-5.8 (Gel ID-HCM074, A.N-4502561).
PDQuest analysis- MW- 38.30, pI-5.5

Gelsolin, first isolated from rabbit lung macrophages (Yin and Stossel, 1979), is the prototype and founding member of a family of actin binding proteins involved in controlling the organization of the actin cytoskeleton in cells (Sun et al., 1999). Upon activation by calcium, they fragment and cap the fast-growing end of actin filaments. Removal of gelsolin from the (+) end of actin filaments (uncapping) favors actin polymerization and is controlled by phospholipids such as phosphatidylinositol 4,5-bisphosphate (PIP2) (Tolias et al., 2000) or lysophosphatidic acid (Meerschaert et al., 1998). Cells deficient in gelsolin exhibit defective chemotaxis and wound healing, and are
defective in neurite retraction (Lu et al., 1997). Conversely, over-expression of gelsolin increases membrane ruffling and chemotaxis (Cunningham et al., 1991).

Invasive Salmonella enterica serovar Typhimurium delivers two actin binding proteins (ABPs)- SipA and SipC (Zhou et al., 1999; Hayward and Koronakis, 1999), into target mammalian cells to promote bacterial internalization. SipA has several biological activities, including a capacity to nucleate filamentous actin (F-actin) and promote F-actin bundling (Zhou et al., 1999). Moreover, SipA can stabilize F-actin by directly antagonizing the action of depolymerizing factors within the cell, such as ADF/cofilin and gelsolin (McGhie et al., 2004 and Dai et al., 2004). The second actin binding protein from Salmonella, SipC, comprises de novo actin polymerization and F-actin bundling activity, in addition to its distinct role in mediating the translocation of effector proteins (Chang et al., 2005). Together, SipA and perhaps SipC are believed to promote efficient bacterial uptake by ensuring the spatial localization of actin foci beneath invading Salmonella.

When overexpressed in Jurkat cells, gelsolin inhibits apoptosis. A similar anti-apoptotic activity has been observed in mouse fibroblast NIH 3T3 cells and other human tumor cell lines with several different apoptotic stimuli, indicating that this is not restricted to Jurkat cells. During apoptosis, gelsolin is proteolyzed by caspase-3 to a form that no longer needs Ca$^{2+}$ for activity. This active fragment of gelsolin contributes to progression of apoptosis, presumably through severing actin filaments (Kwiatkowski, 1999).

B) SSP-1505 (Vimentin like)

HCC-M database - MW- 53.68, pI-5.06 (Gel ID-HCM074, A.N-P08670).
PQQuest analysis - MW- 54.00, pI-5.00

Vimentin is a major type of intermediate filament (IF) protein. Intermediate filaments (IF) are main cytoskeletal components of mammalian cells that appear to be relatively stable structures because of their insolubility in vitro and the lack of biochemical evidence for the existence of significant pools of soluble subunits in vivo (Soellner et al., 1985). Intermediate filament proteins have a common basic subunit monomer structure consisting of three domains: the N terminus variable region, the central highly conserved 38 kDa region, and the C terminus variable region (Renner, 1981). IF proteins can be classified in to five groups namely- I) Acidic keratins, II) Neutral/basic keratins, III) Vimentin, desmin, and glial fibrillary acidic protein, IV) Neurofilaments, V) Lamins.
Vimentin besides other functions also facilitates infection of intracellular pathogens (bacteria as well as virus). Vimentin serves as potential target for tyrosine phosphatase activity of secreted effector-SptP during \textit{Salmonella} internalization (Murli \textit{et al.}, 2001). SptP is a bifunctional protein, with its GAP domain at the amino terminus, and a protein tyrosine phosphatase domain at the carboxy terminus (Zhou and Galán, 2001). Other studies have also identified another intermediate filament protein involved in \textit{Salmonella} entry: SipC binds cytokeratins and expression of dominant negative cytokeratin-18 inhibits \textit{Salmonella} entry into HEP2 cells (Carlson \textit{et al.}, 2002). As virtually nothing is known about the disruption of intermediate filaments by pathogens, this emerging area of investigation shows promise for future advances.

It has been reported that many viruses also interact with cytoskeletal elements, which is considered to be critical at each step along the replication cycle. Vaccinia virus requires MTs and cytoplasmic dynein for efficient transport in HeLa cells. Microtubule (MT), as depolymerizing agent, significantly reduces viral capsid accumulation at perinuclear sites (Ploubidou \textit{et al.}, 2000). Respiratory syncytial virus replication requires actin (Kallewaarda \textit{et al.}, 2005), and intermediate filament integrity is required for Junin virus replication (Cordo \textit{et al.}, 2003). In a latest study, conducted on ECV304 cells, dengue virus serotype 2 (DV2) infection induced microtubules and vimentin reorganization (Chen \textit{et al.}, 2008).

C) SSP-3104 (Rho-GDP-dissociation inhibitor protein like)

| HCC-M database | MW-32.56, pI-5.11 (Gel ID-HCM074, A.N-P52565). |
| PDQuest analysis | MW-32.70, pI-5.20 |

RhoGDI (Rho GDP-dissociation inhibitor) protein was identified as a down-regulator of Rho family GTPases typified by its ability to prevent nucleotide exchange and membrane association. RhoGDI proteins constitutes a family with three mammalian members: RhoGDIα, the ubiquitously expressed member of the family; RhoGDIβ (Ly/D4-GDI), which has haematopoietic tissue-specific expression, particularly in B- and T-lymphocytes; and RhoGDI-γ, which is preferentially expressed in brain, pancreas, lung, kidney and testis (Sasaki and Takal, 1998). RhoGDIs have a long evolutionary history and
are present in yeast (Masuda et al., 1994), Caenorhabditis elegans (Yap et al., 1999), Dictyostelium (Imai et al., 2002), Arabidopsis (Bischoff et al., 2000).

Rho family GTPases (namely RhoA, Rac1 and Cdc42) play important roles in a variety of cellular functions (like cell cycle progression, cytokinesis, phagocytosis and vesicular traffic) including the changes in the filamentous actin system involving the formation of stress fibres, membrane ruffles/lamellipodia and filopodia respectively (Nobes and Hall, 1995). Salmonella directly activate Rho GTPases using secreted effectors and a TTSS encoded within the Salmonella pathogenicity island 1 (SPI-1) locus. The SPI-1-secreted effectors SopE and SopE2 act as guanine-nucleotide-exchange factors (GEFs) for the small GTPases Cdc42 and Rac (Zhou and Galan, 2001).

One SPI2-dependent morphological alteration observed during Salmonella infection is the formation of tubular membranous extensions of the phagosome, which are microtubule-dependent and have been termed Sif (Salmonella-induced filaments). SifA is a SPI2 TTSS effector, which localizes to the phagosome and is required for its tubulation. Salmonella ΔsifA mutants are attenuated for virulence in mice and for intracellular replication in cultured macrophages, indicating that phagosome tubulation is likely an important pathogenic mechanism that promotes intracellular replication. Other SPI2 effectors that localize to the phagosome, including SseF, SseG, SopD2, and PipB2, have been shown to modulate phagosome tubulation, however, only SifA seems to be absolutely required (Knodler and Steele-Mortimer, 2005). SifA interacts with GDP-bound RhoA family GTPases as a mechanism to manipulate host cell processes. In addition, it is possible that SifA binds and/or activates RhoA as a mechanism to modulate the activity of SseJ, since it also binds RhoA (Ohlson et al., 2008). SifA binds to a host protein termed SifA kinesin interacting protein (SKIP) that also binds the plus-end directed microtubule motor kinesin (Boucrot et al., 2005). SifA has been shown to regulate the stability of the Salmonella phagosome with the SPI2 effector SseJ. SseJ has homology to glycerophospholipid-cholesterol acyl transferase enzymes of the lipase superfamily and localizes to the phagosome membrane during infection (Nawabi et al., 2008).

Rho family GTPases act as molecular switches cycling between inactive (GDP-bound) and active (GTP-bound) forms. Post-translational modification with a C-terminal prenyl moiety allows them to associate with membranes where they can interact with, and
activate, their effectors (Bishop and Hall, 2000). Because of their crucial roles, several levels of regulation tightly control their activation state and accessibility. Activation through exchange of GDP for GTP is catalysed by GEFs (guanine nucleotide-exchange factors) (Schmidt and Hall, 2002) and promotes downstream signalling; GAPs (GTPase activating proteins) (Moon and Zheng, 2003) accelerate the intrinsic GTPase activity to inactivate the protein and terminate the signal. A third level of regulation also exists: RhoGDIs (Rho GDPdissociation inhibitors). These function by extracting Rho family GTPases from membranes and solubilizing them in the cytosol. Moreover, both in vitro and in vivo they interact only with prenylated Rho proteins. They also inhibit nucleotide exchange and GTP hydrolysing activities on Rho proteins by interacting with their switch regions and probably restricting accessibility to GEFs and GAPs.

D) SSP-6103 (Growth factor receptor-bound protein like)

HCC-M database-MW-25.20, pI-5.89 (Gel ID-HCM074, A.N-P29354).
PDEquest analysis-MW-25.60, pI-5.70

The growth factor receptor-bound protein 2 (Grb2) is an adapter protein that consists of the Src homology 2 (SH2) flanked by two SH3 domains. The function of Grb2 SH2 domains is to specifically recognize the phosphorylated state of tyrosine residues, thereby allowing SH2 domain-containing proteins to localize to tyrosine-phosphorylated sites. Tyrosine phosphorylation leads to activation of a cascade of protein-protein interactions whereby SH2 domain-containing proteins are recruited to tyrosine-phosphorylated sites. This process initiates a series of events which eventually result in altered patterns of gene expression or other cellular responses (Lowenstein et al., 1992). Thus where Grb2 SH2 domain binds to phosphotyrosine on several receptor-type tyrosine kinase, the Grb2 SH3 domain on the other hand binds to the proline-rich region of Son-of-Sevenless (SOS), a guanine nucleotide exchange factor for Ras thus providing a critical link between cell surface growth factor receptors and the Ras signaling pathway (Chardin et al., 1993). In mammalian cells, especially in nonhemopoietic lineage cells, Grb2 binds to a receptor-type tyrosine kinase and mediates the Ras signal pathway (Egan et al., 1993). Grb2 may play an essential role in mediating oncogenic signaling by TGF-β through the
docking of Grb2 protein with the transforming growth factor- (TGF-β) type II receptor (TβR-II) upon its phosphorylation on Tyr284 by Src.

The human growth factor receptor–bound protein 7 (Grb7) is the founding member of a family of adaptor molecules (Janes et al., 1997) including Grb10 and Grb14. The family members have a highly conserved structure. Grb7 has been shown to be amplified and over-expressed in breast cancer (Stein et al., 1994) and esophageal cancer (Tanaka et al., 1997). Grb7 and Grb7 variant, a novel variant of human Grb7 lacking the Src homology 2 domain, were associated with cell invasion and metastatic progression of esophageal cancer (Tanaka et al., 1998).

E) SSP-7106 (Glutathione-S-transferase homologue like)

Glutathione-S-transferases (GSTs), a family of cytosolic multifunctional enzymes, are detoxifying enzymes that are present in all aerobic organisms. Three major families of proteins that are widely distributed in nature exhibit glutathione transferase activity. Two of these, the cytosolic and mitochondrial GST, comprise soluble enzymes that are only distantly related (Lardner et al., 2004). The third family comprises microsomal GST and is now referred to as membrane-associated proteins in eicosanoid and glutathione (MAPEG) metabolism (Jakobsson et al., 1999). They catalyze the conjugation of glutathione with a variety of reactive electrophilic compounds, thereby neutralizing their active electrophilic sites and subsequently making the parent compound more water soluble. 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. In addition to catalytic functions, the GSTs can also bind covalently/non-covalently to a wide number of hydrophobic compounds, such as haem, drugs and carcinogens. The cloned Arabidopsis thaliana GST was found to function as a
glutathione peroxidase, and may be involved in the removal of reactive organic hydroperoxides, such as the products of lipid peroxidation (Bartling et al., 1993).

*Salmonella* infection can trigger the oxidative stress mediated through the reactive oxygen and reactive nitrogen species. Overall redox status of infected cells fast deteriorates through the electrophilic compounds which are bound to glutathione with the help of glutathione S transferase and subsequently eliminated.

Glutathione transferases are of interest to pharmacologists and toxicologists because they provide targets for antiasthmatic and antitumor drug therapies (Matsushita et al., 1998), and they metabolize cancer chemotherapeutic agents, insecticides, herbicides, carcinogens, and by-products of oxidative stress. Overexpression of GST in mammalian tumor cells has been implicated with resistance to various anticancer agents and chemical carcinogens (Hayes and Pulford, 1995).

F) **SSP-7201 (BAG-family molecular chaperon regulator like)**

<table>
<thead>
<tr>
<th>HCC-M database</th>
<th>MW- 30.00, pI-6.04 (Gel ID-HCM074, A.N-4757834).</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDQuest nanalysis-</td>
<td>MW- 30.90, pI-6.10</td>
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Bcl-2-associated athanogene (BAG)-family proteins were originally identified by their ability to associate with the anti-apoptotic protein, Bcl-2. BAG-family proteins were also found to interact with heat shock proteins 70 (Hsc70/Hsp70) and can modulate, either positively or negatively, the functions of these chaperone proteins. Therefore, BAG-family proteins are characterized as co-chaperones (Takayama, et al., 1997). Currently, six human BAG proteins have been reported. However, only four of them (BAG-1, -3, -4 and -6) have been confirmed in vivo and shown to interact with Hsc70/Hsp70. BAG-family proteins contain a single BAG domain, except for BAG-5 which has four BAG repeats.

The BAG domain is a conserved region located at the C-terminus of the BAG-family proteins that binds the ATPase domain of Hsc70/Hsp70 (Takayama et al., 1999). The BAG domain is evolutionarily conserved region at the C-terminus of the BAG-1 protein, and BAG domain containing 110–124 amino acids (Briknarova et al., 2001).

BAG-1 has been demonstrated to enhance cell resistance to apoptotic stimuli (Takayama et al., 1995). BAG-1 promotes cell growth by binding to and stimulating Raf-1 activity. The activated Raf-1 turns on its downstream extracellular signal-related kinase (ERK) activities and stimulates cell proliferation. When cells are under stress, such as heat
shock, Hsp70 levels increase. The increased quantity of Hsp70 may compete with Raf-1 for BAG-1 binding. The binding of Hsp70 to BAG-1 diminishes Raf-1 signaling and inhibits subsequent events, such as DNA synthesis, as well as arrests the cell cycle (Wang et al., 1996).

BAG-1 has been suggested to function as a molecular switch that encourages cells to proliferate in normal conditions. The anti-apoptotic effects of the BAG-family proteins may be caused by the interaction of the BAG domain with Hsc70/Hsp70. BAG-family proteins act as co-chaperones and indirectly regulate the Hsc70/Hsp70-mediated protein refolding or protein degradation process. BAG-1, in the presence of ATP and Hsp40, has been shown to bind the ATPase domain of Hsp70 inducing conformational changes, thereby reducing Hsp70 nucleotide binding affinity and inhibits Hsp70-mediated chaperone activity. All BAG-1 isoforms contain a UBL domain, suggesting involvement with proteosome-mediated protein degradation. The ubiquitin/proteosome system (UPS) functions as a regulator of targeted protein turnover by directing degradation of proteins via the proteosome (Luders et al., 2000).

The BAG-1 protein is also a binding partner to a wide range of signaling molecules including Siah (Matsuzawa et al., 1998), steroid hormone receptors (Bardelli et al., 1996) and the Raf-1 protein kinase (Song et al., 2001). Siah is the vertebrate homolog of the Drosophila sina gene-encoded protein that is required for fly eye development. BAG-2 has been shown to interact with the carboxyl terminus of HSP70- interacting protein (CHIP) (Arndt et al., 2005; Dai et al., 2005), an HSP70-associated ubiquitin ligase that participates in the ubiquitin–proteasome system by ubiquitylating misfolded proteins associated with cytoplasmic chaperones. BAG2 inhibits ubiquitylation of misfolded proteins by CHIP by a co-chaperone-dependent regulatory mechanism (Arndt et al., 2005; Dai et al., 2005), suggesting that BAG2 plays an endogenous role in regulating proteasome-mediated degradation of chaperone substrates.

9.3 Identification of protein spots by MALDI/LCMS

Protein spots (SSP-5206) and (SSP-3204) (Fig. 18) were identified to be TRIP-1(Transforming growth factor-β interacting protein-1) (Fig. 19, 20, 21, 22) and Ubiquitin ligase specificity factor (Fig. 23, 24, 25, 26) respectively.
9.3.1 TGF-β (Transforming growth factor-β) interacting protein (TRIP-1)

Transforming growth factor beta (TGF-β) receptor interacting protein 1 (TRIP-1), a modulatory molecule in the TGF-β signaling pathway and designated as TRIP-1. The TGF-β family regulates proliferation, differentiation, and death of cells and plays multiple roles in the development and homeostasis of organisms from yeast to human (Massague 1998). TGF-β polypeptides are perceived by transmembrane Ser/Thr kinase receptors that form a heterotetrameric complex of type I and type II or a cognate dimer as the ligand binding. To identify intracellular binding partners of the type II TGF-β receptor kinase, the cytoplasmic domain of the type II receptor was used as a bait in a yeast two-hybrid screen, and a WD-domain protein was isolated and named TRIP-1 (Chen et al., 1995). TRIP-1 binds to the cytoplasmic domain of the TGF-β type II receptor in a kinase-dependent manner and is phosphorylated on Ser and Thr residues by the receptor kinase. Subsequent experiments showed that TRIP-1 functioned as a modulator of TGF-β receptor signaling in vivo and inhibited the expression of specific TGF-β responsive genes (Choy and Derynck, 1998), suggesting that TRIP-1 plays a role in the TGF-β signaling pathway. Surprisingly, TRIP-1 also functions as a subunit of the eukaryotic translation initiation factor 3 (eIF3; Asano et al., 1997). eIF3 is the largest of the elFs and is composed of ten or more subunits, with TRIP-1 as the p36 subunit. The eIF3 promotes dissociation of 80S ribosome and is required for binding of mRNA to the ribosomal 40S subunit, thus playing an essential role in the initiation of eukaryotic protein synthesis. Therefore, characterization of the structure of CsTRIP-1 is significant to understand its role.

Transforming growth factor-β (TGF-β) is the prototype member of a superfamily of growth factors, which have many roles in growth regulation, wound healing, immunity, and development. Most notably, TGF-β is a potent growth inhibitor for many cell types and induces a variety of extracellular matrix proteins and adhesion receptors (Derynck et al., 1998). The current model for TGF-β signaling invokes the binding of ligand to the type II TGF-β receptor, which results in the recruitment, phosphorylation, and concomitant activation of type I TGF-β receptors. The activated type I receptor then phosphorylates Smad2 or Smad3 which is then released from the receptor and forms a complex with
Smad4. This complex then translocates to the nucleus where it interacts with transcription factors and regulates gene expression (Heldin et al., 1997).

Although Smads clearly act as effectors of TGF-β receptor signaling, several lines of evidence indicate that other factors are probably involved in TGF-β signal transduction. The ability of a dominant negative type II receptor to inhibit TGF-β-mediated growth inhibition but not extracellular matrix production suggests that the pathways leading to these effects of TGF-β are distinct and separable (Chen et al., 1993) and may be differentially regulated via quantitatively different thresholds of signaling (Feng et al., 1996). Consistent with this notion, specific residues on the type I (Saitoh et al., 1996) or type II (Wieser et al., 1993) receptor have been identified that are dispensable for TGF-β stimulation of extracellular matrix production but required for the antimitogenic effect. The Smads have been found to mediate both the extracellular matrix and the anti-mitogenic signaling pathways (Lagna et al., 1996), and no evidence yet presented has indicated a role for Smads in differentially modulating distinct TGF-β signaling pathways. Thus, although the basis for distinction between these pathways is not well understood, it is possible that other proteins interacting directly with the receptors are involved in this differential regulation.

In addition to Smads, other proteins have been identified that interact with the TGF-β receptors. Two-hybrid screening approaches resulted in the cloning of FKBP-12 (Wang et al., 1994) and a subunit of farnesyltransferase (Kawabata et al., 1995), which interact with the type I receptor, and TRIP-1, a WD40 repeat-containing protein which interacts with the type II receptor. However, farnesyltransferase activity has since been shown to be dispensable for TGF-β signaling, and TGF-β does not affect the activity of this enzyme (Ventura et al., 1996); therefore the functional significance of the interaction of a subunit of farnesyltransferase with TGF-β receptors is unknown. FKBP-12 has been found to have an inhibitory effect on signaling through TGF-β family type I receptors and can inhibit phosphorylation of the type I receptor by the type II TGF-β receptor (Chen et al., 1997). No function for TRIP-1 has as yet been reported. Unlike a subunit of farnesyltransferase and FKBP-12, TRIP-1 does not appear to have any enzymatic function, a common phenomenon among WD40 repeat-containing proteins (Neer et al., 1994).
WD40 repeats, first recognized in the β-subunit of heterotrimeric G proteins (Fong et al., 1986), have been identified in many proteins involved in diverse functions, such as transcriptional regulation, RNA processing, signal transduction, cell cycle progression, and vesicular trafficking. These proteins are often found in multi-subunit protein complexes, and it has been proposed that the WD40 repeats are important for protein-protein interactions. The crystal structure of Gβγ has revealed the three-dimensional structure of the WD40 repeat units in Gβ as forming a β-propeller structure, with each propeller blade being formed from a four-strand β-sheet (Sondek et al., 1996). Subsequent studies suggest that other WD40 repeat proteins are also likely to form similar propeller structures (Garcia-Higuera et al., 1996). TRIP-1 interacts with the type II TGF-β receptor both in vivo and in vitro but does not interact with the closely related type II activin receptor. The kinase activity, and therefore, presumably, auto-phosphorylation of the receptor greatly enhance this interaction, and TRIP-1 is a substrate of the receptor kinase. TRIP-1 can be found in the ligand-bound complex of type I and II receptors but does not interact directly with the type I receptors (Chen et al., 1995). Recently, another WD40 repeat-containing protein has been identified that interacts with TGF-β receptors. Ba, a subunit of protein phosphatase 2A, interacts with type I TGF-β receptors and potentiates the antimitogenic signaling pathway in a receptor-dependent manner. However, Ba has little or no effect on PAI-1 expression induced by the activated receptor complex, suggesting that Ba preferentially regulates the antimitogenic signaling found that TRIP-1 acts to inhibit signal transduction by the activated TGF-β receptor complex. Furthermore, TRIP-1, like Ba, exhibits selectivity with respect to its effects on signaling pathway and does not modulate all TGF-β effects equally.

9.3.1.1 TGFβ and bacterial infection

The three mammalian isoforms of TGF-β (TGF-β1, -β2, and -β3) mediate a wide array of physiological processes. In addition to their functions in development, cell cycle control, and wound healing, the TGF-β regulates the function of cells involved in the immune response to antigens, mitogens, and pathogens (McCarmey et al., 1994). TGF-β is a potent immunosuppressive cytokine that has been implicated in the susceptibility to infection with intracellular pathogens such as Trypanosoma cruzi and Leishmania (Barral-
Netto et al., 1992; Silva et al., 1991). Macrophages infected with these microorganisms produce active TGF-β, which in turn suppresses normal cytokine-stimulated macrophage cytotoxic activity and allows for the permissive growth of these pathogens. The role of TGF-β in the regulation of events within 24 h after infection with encapsulated extracellular bacterial pathogens, revealed that active TGF-β given at the time of infection can convert non-susceptible murine strains into permissive environments for the growth of the microorganism and that giving anti-TGF-β at the time of infection provides protection to susceptible murine strains. In fact, when given either TGF-β or anti-TGF-β 24 h before infection, no effect was seen on the infectious process in the appropriate murine strain. In total, these studies suggest that active TGF-β is involved in early events during infection with both intracellular parasites and extracellular bacteria. Salmonella colonization of the cecum and colon followed by edema, mucosal ulcerations, and severe inflammation, was also accompanied by significantly elevated expression of transforming growth factor-beta1 (TGF-β), connective tissue growth factor, and insulin-like growth factor-I along with extensive type I collagen deposition in the cecal mucosa, submucosa, and muscularis mucosa of infected mice (Grassl et al., 2008).

The mechanism by which TGF-β inhibits host defense at the site of infection cannot be explained currently. Because previous studies demonstrated TGF-β induced defects in PMN phagocytic function, one possibility to explain the decreased clearance of the pathogen from sites of infection is decreased phagocytic uptake of the organisms by PMN. However, these organisms are not readily ingested by either normal PMN or macrophages, consistent with the presence of the capsule. In addition, neither normal PMN nor macrophages have bactericidal activity in vitro for the encapsulated bacteria used in experiments, even in the presence of complement. Therefore, it is believed that the effect of TGF-β observed on host defense is mediated by the suppression of bacteriostatic activity in vivo. This is in contrast to the effect of TGF-β on the cytotoxic activity of macrophages against intracellular pathogens. Thus, TGF-β may adversely affect both bacteriostatic and bactericidal host defense mechanisms. The precise bacteriostatic mechanisms inhibited by TGF-β remain to be elucidated.

Among the other immunomodulatory effects attributed to the TGF-β is the suppression of the production of nitrogen free radicals by macrophages in vitro (Ding et al.,
Nitric oxide (NO) and its derived nitrogen free radical have been the subject of much investigation, owing to their pleiotropic effects. An enzyme inducible by cytokines and microbial products present in many cell types (iNOS; NOS type II) produces large quantities of NO. The cytotoxic or cytostatic effects of high levels of NO are beneficial during infection but can damage host tissue when unchecked (Nathan, 1992). Accordingly, suppression of NO production by exogenously administered TGF-β can have both deleterious and beneficial effects in different contexts (Vodovotz, 1994).

9.3.1.2 Ubiquitin ligase specificity factor

The identified ubiquitin ligase specificity factor is an F-box and WD-40 domain-containing protein. More than 47 F-box proteins are identified in mammals, which are divided into three subclass depending on the types of substrate interaction domains in addition to the F-box motif. The two largest classes of interaction domains are WD40 repeats (Smith et al., 1999) and leucine-rich repeats (LRRs) (Kobe and Kajava 2001). A third generic class of F-box proteins contained various other types of protein interaction domains or no recognizable domains. These classes of F-box proteins were designated FBWs, FBLs, and FBXs, respectively, followed by a numerical identifier (Cenciarelli et al., 1999). F-box proteins are used as specificity factors by SCF. SCF (Skp1 – Cullin – F-box protein) is a cullin based E3 ubiquitin ligase which mediates ubiquitinylation of many target proteins in mammalian cells for subsequent degradation by proteosomes.

The ubiquitin–proteasome pathway is a major pathway for the targeted degradation of proteins and involves multistep enzymatic reactions catalyzed by a cascade of enzymes, including ubiquitin-activating enzyme E1, ubiquitin conjugating enzyme E2, and ubiquitin ligase E3. Ubiquitin is first activated by binding to E1 through a thioester bond between a cysteine residue at the active site of E1 and the C-terminus glycine (G76) of ubiquitin.

Activated ubiquitin in an E1–ubiquitin complex is then transferred to E2, which also forms a thioester bond between its active-site cysteine residue and the G76 of ubiquitin. Finally, ubiquitin is covalently attached to the target protein through an isopeptide bond between the G76 of ubiquitin and the q amino group of an internal lysine residue of the target protein, catalyzed by E3 ubiquitin ligase. Through multiple runs of
reactions, ubiquitin is covalently attached to substrates to form K48-linked polyubiquitinated conjugates that are rapidly recognized and degraded by the 26S proteasome (Ciechanover, 1998).

Up-regulation of ubiquitin ligase specificity factor in *Salmonella enterica* serovar Typhimurium infected HepG2 cells can be explained as the defense mechanism employed by host cell to degrade TTSS encoded effector proteins such as SopE and SptP (Kubori and Galan, 2003). A small, reduction/oxidation-regulatory protein, thioredoxin was found to be the mammalian binding partner of the Salmonella effector SirP. *In vitro*, SirP was able to mediate ubiquitination of ubiquitin and thioredoxin. A Cys residue conserved in other effectors of the same family that also possess E3 ubiquitin ligase activity was essential for this catalytic function. Stable expression of SirP in HeLa cells resulted in a significant decrease of thioredoxin activity and in an increase of cell death (Bernal and Ramos, 2009). SopE acts as a GTP–GDP (guanosine 5’-triphosphate–guanosine 5’-diphosphate) exchange factor (GEF) that activates the signaling molecules Rac-1 and Cdc42, two proteins of the Rho GTPase family, thus provoking cytoskeleton reorganization, which results in bacterial internalization. In contrast, the SptP effector functions as a GTPase-activating protein that deactivates Rac and Cdc42, allowing the recovery of the actin cytoskeleton’s normal appearance a few hours after infection. For successful colonization, the activity of these two TTSS effector proteins has to be temporally regulated within the host cell.

The mechanism of this regulation was shown to be due to their differential degradation by the host proteasome. SopE and SptP are delivered in equal amounts during infection, but SopE undergoes poly-ubiquitination and rapid proteasome-dependent degradation following translocation, whereas SptP is degraded at a much slower rate (Kubori and Galan, 2003). Two other *Salmonella* SPI-1 T3SS effectors, SopA and SopB, are functionally regulated by host ubiquitination. SopB is a phosphoinositide phosphatase that modulates vesicle trafficking by altering the phosphoinositide metabolism. It was shown to be monoubiquitinated and degraded, although probably not via the proteasome (Marcus et al., 2002). SopA, a protein required for the elicitation of intestinal inflammation, has been shown to be ubiquitinated within the host cell by the membrane-anchored RING-type E3 Ub ligase HsMRA1, and degraded by the proteasome in an HsMRA1-dependent manner (Zhang et al., 2005).
9.4 NITRIC OXIDE AND *Salmonella enterica* serovar Typhimurium INFECTION

9.4.1 Effect of NO donors on nitric oxide production in *Salmonella enterica* serovar-Typhimurium infected HepG2 cells

*Salmonella enterica* serovar Typhimurium infection causes a rise in nitric oxide production which enhances further with exogenous supplementation of L-arginine (Fig.27). This increase was found highest with 4mM concentration of L-arginine, whereas a decline was observed with 8mM and 16mM. This downfall in NO production was caused by some unknown pathway.

Nitric oxide production through iNOS induction following *Salmonella* infection has been reported not only *in vivo* but also *in vitro* (Witthaft *et al.*, 1998). As L-arginine is the only substrate for nitric oxide synthases (for all isoforms), intracellular production of NO can be augmented by L-arginine supplementation *in vivo* (Reynolds *et al.*, 1988; Norris *et al.*, 1995). L-citrullin which is the product of nitric oxide synthases along with nitric oxide can be recycled back in to L-arginine by citrullin-L-arginine cycle. There are enough accumulated evidences which indicate that highly reactive and short-lived NO is involved in host defense against intracellular pathogens such as *Leishmania*, *Mycobacteria* and *Salmonella* (Fang, 1997). With these considerations in mind we tested the earlier (*in vivo*) reports of intracellular NO production *in vitro* using HepG2 cells after *Salmonella enterica* serovar Typhimurium infection. As the main source of nitric oxide is iNOS induction during infection, therefore a selective inhibitor of iNOS; aminoguanidine (AG) is used. A concentration of 1mM (AG) was found sufficient for iNOS inhibition.

HepG2 cells are permeable to L-arginine and normally have cationic transport system $y^+$ (Sodium independent L-arginine uptake) (Goenner *et al.*, 1992). The usual concentration of L-arginine provided in DMEM (Dulbecco’s Modified Eagle’s Medium) culture medium is 84mg/L (approx. 0.48mM). A variable concentration of L-arginine ranging from 2mM, 4mM, 8mM and 16mM were used with infection whereas in uninfected control only lowest concentration (2mM) was found to be sufficient (approx. four-fold of optimized amount i.e. 0.48mM).
The possible reason for decrease in nitric oxide production with higher L-arginine concentration (i.e. 8mM, 16mM) may be attributed to declining activity of enzyme (iNOS). Autoinhibition of constitutive nitric oxide synthases (cNOS) have been reported by the nitric oxide generated by iNOS after LPS administration in rats (Schwartz et al., 1997). Moreover, it has already been reported that iNOS and arginase-I (hepatic isoform), not arginase-II (extrahepatic isoform), are coinduced by LPS in cultured rat peritoneal macrophages and in the lung and spleen in vivo (Sonoki et al., 1997). As L-arginine is a common substrate for NOS and arginase, arginase may compete with NOS for L-arginine, and downregulate production of NO.

The induction of arginase-I, is slower than that of iNOS. Therefore it can be concluded that iNOS is first induced, NO is produced, and then arginase is induced and the sustained overproduction of NO that is toxic to surrounding cells is prevented. NOS inhibitors usually inhibit all types of NOS and inhibition of constitutive types of NOS may cause side effects. On the other hand, the demand of iNOS for its substrate L-arginine is much higher than those of constitutive NOSs because of its very high activity. The induction of arginase may lead to selective inhibition of the high output NO production by iNOS.

9.4.2 Effect of L-arginine on bacterial load in HepG2

Bacterial load on HepG2 cells decreased sharply with rising amount of L-arginine with lowest (58.73% of control; C+B) in 8mM, therefore optimum concentration of NO donor which acts against the bacterial load was found to be 8mM. Moreover, 16mM indicates a lower (9.44%) rise in bacterial load as compared to 8mM (49.29%) (Fig.28). How does higher amount of L-arginine favor bacterial growth? The reason for this discrepancy is not known presently and requires further work in this area. If we presume nitric oxide to be the main antimicrobial agent against the internalized bacteria then the lowest bacterial load should have been observed with 4mM of L-arginine where the production of NO was highest (Fig.27). Lowest bacterial load with 8mM of L-arginine suggests that NO is not acting alone as an antimicrobial agent, excess of NO might react with superoxide radicals to produce highly potent oxidant peroxynitrite (ONOO⁻). Peroxynitrite is reported to have bactericidal properties (Brunelli et al., 1995). Apart from
peroxynitrite other NO derivatives such as nitrosothiol, that is sulfhydryl adduct of nitrosonium, have potent bacteriostatic activity against serovar Typhimurium (De Groote et al., 1995).

9.4.3 Protection assay (Effect of nitric oxide donors on cell viability)

*Salmonella enterica* serovar Typhimurium infected HepG2 cells show sharp decrease (61.73%) in viability (Fig. 29). This decline in viability is attributed to cell death caused by invading bacteria. Kim et al. (1998) found that apoptosis in human intestinal epithelium cells is a delayed event during bacterial invasion. This is validated in our study where cell fragmentation was seen 48 hr after infection (Fig. 12) in SEM (Scanning Electron Microscopy).

As depicted in (Fig. 29), cells viability gradually raises with increase in L-arginine concentration, highest viability was recorded at 4mM concentration. This data is consistent with earlier observation showing highest NO production in the same group (i.e 4mM of L-arginine), thus a direct relationship can be inferred between cells viability and nitric oxide production.

Nitric oxide may improve cell viability through its strong anti-apoptotic activity, Derivatives of NO such as nitrosothiols inhibit the caspases via S-nitrosylation (Mannick et al., 1997). *Salmonella enterica* serovar Typhimurium- derived SipB protein activates caspases-1 and -2 inside the host cell and triggers the cell death (Hersh et al., 1999). Thus, it is evident that NO is a potent inhibitor of caspase activity both *in vitro* and *in vivo* (Kim et al., 1998). This observation has significant implication for the regulation of apoptosis by NO. Under an oxidizing intracellular environment, the caspases get inactivated, and the cell must therefore, maintain a reducing environment for the execution phase of apoptosis to occur.

9.5 Effect of NO donors on *Salmonella enterica* serovar Typhimurium induced oxidative stress

Nitric oxide production in *Salmonella enterica* serovar Typhimurium infected cells though seems beneficial initially but excess amount of NO can change the redox status of the host cell enormously. NO itself is capable of combining with other reactive species
which can initiate the cell damage. The overall effect of nitric oxide on host, therefore requires the measurement of oxidative stress markers.

9.5.1 Reduced glutathione

Reduced glutathione declines significantly in (B+Arg8) whereas (C+B) and (Arg) groups showed a nonsignificant fall in glutathione content as compared to control (Fig. 30). This decrease in glutathione amount may results from induced as well as constitutive nitric oxide (in C+B group and Arg respectively) which react with free thiols to form S-nitrosothiole. Such S-nitrosothiol compounds, which also include S-nitrosocysteine and S-nitrosogluthathione, assume bioactivity through their capacity to donate NO and may therefore serve as stable intermediaries. It has been speculated that this bioactive extracellular pool of S-nitroso proteins serves as a source of NO, buffering its free concentration (Stamler et al., 1992). These observations have suggested that NO also exerts effects within cells by reacting with intracellular thiols.

Glutathione- the most abundant non-protein thiol, maintains the redox state of critical protein sulphhydrals in cytosol and nucleus. Oxidized glutathione is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism. Too high a concentration of GSSG (glutathione di-sulphide) may damage many enzymes oxidatively. Apart from being a cofactor of many enzymes GSH is necessary for maximum activity of iNOS. Reduced glutathione also upregulates NO synthesis by increasing iNOS mRNA levels and iNOS activity in hepatocytes (Harbrecht et al., 1997). A higher concentration of L-arginine (B+Arg16) and inhibitor (B+AG) treatment with infection, however, registered a higher amount of GSH in HepG2 cells. Since GSH amount reflects a healthy status of cells, this data is consistent with the earlier observation in present study (Fig. 29) where cells viability (infected group) was higher at L-arginine concentration (16mM) than at concentration (8mM). AG (Aminoguanidine) which specifically inhibits iNOS enhanced the level of GSH after infection (B+AG) through unknown pathway.
9.5.2 Glutathione peroxidase

Glutathione peroxidase activity declines significantly in all *Salmonella enterica* serovar Typhimurium infected groups (C+B, B+Arg16, B+Arg8, B+AG) except (Arg) and AG groups (Fig.31). Further, infection along with exogenous supply of L-arginine causes more inhibition. Lower inhibition was observed with 8mM, which was reversed by the higher dose of 16mM, this data is consistent with the earlier reports that intracellular NO inhibits GPx (Asahi *et al.*, 1997). Moreover, L-arginine alone and iNOS inhibitor in both cell groups (Infected and uninfected) also registered a decline in activity, suggesting a role of constitutive nitric oxide.

Glutathione peroxidase (GPx) is an antioxidative enzyme that scavenges various peroxides including \( \text{H}_2\text{O}_2 \). Three isozymes, cellular GPx, extracellular GPx, and phospholipid hydroperoxide GPx, are known, and each contains a seleno-cysteine in its catalytic center. Cellular GPx, the most characterized form, can react with hydrogen peroxide and organic peroxides but not lipid hydroperoxide. Nitric oxide inactivates GPx through the binding with the seleno-cysteine catalytic centre. A significant decrease in intracellular GPx activity was also observed in LPS-treated RAW 264.7 cells (Asahi, *et al.*, 1995), presumably due to induction of NO synthase by LPS because GPx activity was protected in the presence of \( N^\omega \)-methyl-L-L-arginine. Inactivation of GPx was likely a consequence in certain cells that produce NO (through iNOS), thereby; increasing accumulation of peroxides within cells after treatment with an NO donor. Reports showed that, in addition to having a cytostatic effect, NO induced apoptotic cell death in several types of cells (Albina, *et al.*, 1993). Because redox regulation of cells and GPx activity are closely tied to apoptosis (Sandstrom *et al.*, 1994), inactivation of GPx by NO may be one of the causes of apoptotic cell death in these cells. Recent reports suggested that *salmonella* induces prominent gene expression in the rat colon, among other protein, GPx-2 level was found increased significantly during oxidative stress (Rodenburg *et al.*, 2007).

9.5.3 Catalase

*Salmonella enterica* serovar Typhimurium infection generated the production of reactive species which enhanced the catalase activity in HepG2 cells (C+B) Fig.32.
Discussion

Whereas the catalase activity declined with the L-arginine treatment, this fall in activity may be caused by nitric oxide (NO) generated by iNOS.

Catalase has strong NO binding due to electron donating properties of the proximal tyrosinate ligand (Brown, 1995). Catalase being a heme protein is often 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). Inhibition of Catalase by induced NO, disrupts the intracellular balance of $H_2O_2$ thereby enhancing the possibility of pathogen killing. This was found to be true where NO / $H_2O_2$ is capable of killing $E. coli$ in presence of iron (Pacelli et al., 1995). Apart from catalase NO can deactivate other iron-sulfur protein such aconitase.

Administration of iNOS inhibitor (AG) in infected cells, limited the available NO for catalase inhibition therefore high activity was observed but the exact cause of this highest rise is unclear.

9.5.4 Xanthine oxidase

In present study XO activity was increased following Salmonella enterica serovar Typhimurium infection whereas a gradual decline in activity was observed in treated (Arg, B+Arg16, B+Arg8) groups after post-infection (Fig.33). Major reason for reduced XO activity, especially in treated and Salmonella enterica serovar Typhimurium infected group (B+Arg16, B+Arg8), is attributed to the generation of peroxynitrite (ONOO) through superoxide (from XO) and NO (from iNOS). ONOO$^-$ inhibited XO function primarily by oxidative disruption of the molybdenum catalytic site. Taken together, ONOO$^-$ in biological systems can feedback and down-regulate XO activity that in turn may serve to limit further ONOO$^-$ formation and oxidant-derived injury. As reported earlier, XO catalyzes the conversion of xanthine into uric acid with the concomitant reduction of oxygen into superoxide ($O_2^-$) and hydrogen peroxide. Superoxide anion ($O_2^-$) was reported to be an important antimicrobial molecular species in S. enterica serovar Typhimurium-infected mice (Umezawa et al., 1995) thus XO was responsible for the host-defense mechanism.
Moreover, excessive production of NO by iNOS expressed in mouse liver was also critically involved in the antimicrobial mechanism against *S. enterica* serovar Typhimurium. It is already reported that both O$_2^-$ and NO$^-$ are major effector molecules in the host defense against *S. enterica* serovar Typhimurium *in vivo*. There is evidence that XO activity is expressed in a variety of cells, including endothelial cells (Dupont *et al.*, 1992), hepatocytes (Suematsu *et al.*, 1992), fibroblasts (Falciani *et al.*, 1992), and neutrophils and macrophages (Tubaro *et al.*, 1980). Also, it has been reported that XO is upregulated by stimulation with lipopolysaccharide and proinflammatory cytokines, e.g., gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α), which are induced in infections with various viruses and bacteria, such as influenza virus, cytomegalovirus, and *S. enterica* serovar Typhimurium (Takahashi *et al.*, 1988).

NO produced excessively, in particular by activated macrophages, has been shown to function as a cytotoxic or cyostatic molecule and inhibits the growth of a diverse array of infectious agents. Moreover, overproduction of NO has been implicated in the pathogenesis of sepsis; of carcinogenesis induced by parasites, viruses, or bacteria (*Helicobacter pylori*); and of cerebral malaria. Identification of the role of NO in protection against microbial infections will therefore provide insight into the delicate balance of host-microbe interaction. The present result for *S. enterica* serovar Typhimurium infection in HepG2 cells clearly illustrates the protective effect of NO produced during salmonellosis.

It is now well documented that NO *per se* is not a potent bactericidal molecular species. The most effective cytotoxic effect of NO appears to be via reaction with superoxide (Miles *et al.*, 1996). NO reacts with O$_2^-$ to yield peroxynitrite (ONOO$^-$). Peroxynitrite, mediate recruitment of inflammatory cells such as PMN cells and monocyte-derived macrophages to the infectious foci, resulting in containment of the intruding bacteria in the restricted area with formation of localized microabscesses and granulomatous lesions in tissues like lung. This is supported by results obtained in present study where inhibition of NO production by the administration of iNOS inhibitor (Aminoguanidine) (B+AG) group significantly raised the XO activity. These results may suggest that NO and its oxidized metabolites such as peroxynitrite function as inflammatory mediators in microbial infections.
9.5.5 Lipid peroxidation

Salmonella enterica serovar Typhimurium infection causes significant increase in MDA (Malondialdehyde) level reflecting cell damage in (C+B) group (Fig.34). Increasing L-arginine supplementation (8mM, 16mM) in infected HepG2 cells (Arg8+Arg16), shows corresponding decrease in MDA level. This trend clearly shows a correlation of nitric oxide with lipid peroxidation. Moreover, L-arginine treatment in uninfected cells shows lower MDA level, signifying the role of NO found in cells group, where specific inhibitor (AG) was used but administration of inhibitor along with infection (B+AG) shows higher level of MDA, indicating the involvement of some other oxidants. Main reactive oxygen species (ROS) such as superoxide (O$_2^-$), hydroxyl radical (OH$^-$), peroxynitrite (ONOO$^-$) induce lipid peroxidation per se.

Lipid peroxidation is a chain reaction and a single oxidative event can oxidize many lipid molecules. In presence of iron or copper ions, the chain reaction can become a cascade and the oxidation process can rapidly become unstoppable (Halliwell and Gutteridge, 1984). Eukaryotic cells are equipped with a variety of primary and secondary defenses against lipid peroxidation and other deleterious effects of oxidative stress (Fridovich, 1978). Primary defenses are mainly preventative, whereas secondary defenses have a “back-up” protective role, which might typically involve excision/repair of any lesions that do develop. Primary cytoprotection relies on the scavenging/inactivation of ROS or redox metal ions before lipid peroxidation takes place.

Enzymes involved in primary cytoprotection include; the copper/zinc-dependent (cytosolic) and manganese-dependent (mitochondrial) superoxide dismutases (SODs); cytosolic and mitochondrial glutathione peroxidase (GPX), which scavenges H$_2$O$_2$ efficiently at relatively low concentrations (low $K_m$); and peroxisomal catalase (CAT), which scavenges H$_2$O$_2$ efficiently at relatively high concentrations (high $K_m$). (GPX may also be effective at the secondary stage, e.g., by detoxifying fatty acid hydroperoxides). Hydroxyl radical (HO') species can be intercepted (often indiscriminately) by a variety of low molecular weight antioxidant compounds (Buettner, 1993). Chain-breaking antioxidants such as α-tocopherol (α-TOH) and butylated hydroxytoluene (BHT) can afford primary as well as secondary stage protection; by competing with LHs for peroxyl radicals,
they also produce LOOHs, but in much lower overall yield than without competition, and this basically accounts for their efficacy.

Recent studies have revealed that NO• can also function as a chain-breaking antioxidant by reacting with peroxyl radicals, and appears kinetically to be more effective in this than a-TOH, at least in model systems (O’Donnell et al., 1997). In the process, lipid nitrites and nitrates appear to be generated via rearrangement of unstable nitroso-peroxyl intermediates. Thus, NO• by itself can act as a lipid antioxidant, but upon reacting with superoxide it becomes ONOO'/ ONOOH, a powerful lipid pro-oxidant. The expression and regulation of this dual activity in vivo, e.g., in the vascular system, is clearly a matter of great importance. In addition to these various agents there are iron-sequestering proteins, lactoferrin and ferritin being prime examples, which play an important role in limiting peroxidation potency, both at the primary and secondary level.

Thus the present study tried to explore, not only the morphological consequences of Salmonella enterica serovar Typhimurium infection in HepG2 cells, but also explains the underlying molecular events at protein level. Most of the proteins, which showed differential expression pattern as compared to control cells, either belong to cell cytoskeleton or a part of cell signaling pathway. All evidences, which indicate the involvement of cell cytoskeleton in bacterial infection, are consistent with the previous reports in vivo as well as in vitro. Moreover, the role of nitric oxide against bacterial infection was also verified by the present study. Nitric oxide production, under infection conditions, can be modulated by the NO donors (Arginine in present study) supplementation so as to contain infection.
Summary and Conclusions
SUMMARY AND CONCLUSION

Typhoid fever is a febrile and often serious systemic illness caused by *Salmonella enterica* serovar Typhi. It remains a serious health threat in developing countries and in Asia, it represents the most common cause of community acquired bacteremia. The genus *Salmonella* is extremely heterogeneous comprising almost 2600 serotypes. The etiologic agents of murine and human typhoid fever are *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Typhi respectively. In humans, there are three major diseases caused by *Salmonella*: typhoid fever (caused by *S enterica* serovar Typhi and related strains), gastroenteritis (caused by most *Salmonella* strains such as *S. enterica* serovar Typhimurium and *S enterica* serovar Enteritidis), and an invasive disease (caused by *S enterica* serovar. Choleraesuis).

The pathogenesis of this disease depends on the ingested inoculum size of *S. enterica* serovar Typhi, the virulence of the strain, the host’s immune response and previous exposure, and local protective factors. The pathological hallmark of enteric fever is mononuclear cell infiltration and hypertrophy of the reticuloendothelial system, including the intestinal Peyer’s patches, mesenteric lymph nodes, spleen, liver and bone marrow. During systemic infection, *Salmonella* invades spleen and liver predominantly. *Salmonella* evades humoral immunity by multiplying intracellularly with in hepatocytes.

Microscopic studies of liver carcinoma cell line (HepG2 in present study) post infection revealed that *Salmonellae enterica* serovar Typhimurium invade epithelial cells by a morphologically distinct process termed bacteria-mediated endocytosis. Shortly after bacteria (usually 3-4) adhere to the HepG2 cell surface (Fig.10), profound cytoskeletal rearrangements occur in the host cell, disrupting the normal cell structure and subsequent formation of membrane ruffles that reach out and enclose adherent bacteria in large vesicles. This process resembles the membrane ruffling and macropinocytosis induced in many cells by growth factors, and it is morphologically and functionally distinct from receptor-mediated endocytosis, the mechanism by which many other pathogens enter nonphagocytic cells. *S enterica* serovar Typhimurium triggers caspase signaling pathways causing cell fragmentation through apoptosis in later period of infection (Fig.12).
Proteomic studies were undertaken to gain better insight of molecular nature of *Salmonella enterica* serovar Typhimurium invasion of HepG2 cells. SDS-PAGE revealed sharp downregulation of different protein bands between 43 kDa to 29 kDa range whereas overexpression among different proteins were observed between 66 kDa to 43 kDa range at 24 hrs of post infection. Proteins from the infected HepG2 cells were further subjected to two-dimensional electrophoresis (Iso-electric focusing and SDS-PAGE) for better resolution.

PDQuest analysis of gels produced a total of 353 (Three hundred and fifty three) protein spots out of which only 31 (Thirty one) spots showed significant differential expression. Out of 31 protein spots 4 spots (SSP-0101, 1202, 2702, 7601) were present only in control gel whereas 14 spots (SSP-2203, 3103, 3104, 3204, 5206, 6103, 6705, 7105, 7106, 7108, 7201, 7202, 7303, 8406) were found only in infected HepG2 gel, rest of 13 (SSP-1505, 2003, 2102, 2501, 3402, 4301, 4402, 5201, 6301, 6303, 7002, 7302, 8007) spots were present in control as well as infected gel. A list of spots quantity and molecular weight and iso-electric point (Mr/pl) is provided on page 68-69 in results (Table.4).

On the basis of calculated experimental molecular weight and Iso-electric point (Mr/pl), similarity search in hepatocellular carcinoma (HCC) database identified protein spots (SSP-5201, 1505, 3104, 6103, 7106, 7201) as Capping protein (actin filament) gelsolin like, Vimentin like, Rho-GDP-dissociation inhibitor protein like, Growth factor receptor-bound protein like, Glutathione-S-transferase homologue like, BAG-family molecular chaperone regulator like proteins respectively.

Capping protein gelsolin like, is an actin binding protein involved in controlling and organization of the actin cytoskeleton in cells. Gelsolin over-expression increases membrane ruffling by depolymerizing filamentous actin (F-actin). Apart from role in cell cytoskeleton gelsolin also has a role in apoptosis inhibition. Gelsolin was down regulated in *Salmonella* infected HepG2 cells as a defensive mechanism against membrane ruffling and apoptosis.

Vimentin is an intermediate filament (IF) protein which forms the component of mammalian cell cytoskeleton. Bacteria and many viruses interact with IF during invasion. *Salmonella* secretes different effector proteins into host cell cytoplasm during internalization, some of these effector proteins (SptP, SipC), having tyrosine phosphatase
activity, target vimentin thereby causing the disruption of cell cytoskeleton to facilitate the pathogen entry. Vimentin like protein was found upregulated in infected HepG2 cells possibly to counter the degrading intermediate filament during bacterial invasion.

RhoGDI (Rho GDP-dissociation inhibitor) protein down-regulates Rho family GTPases by its ability to prevent nucleotide exchange and membrane association. Rho family GTPases (namely RhoA, Rac1 and Cdc42) play important roles in a variety of cellular functions (like cell cycle progression, cytokinesis, phagocytosis and vesicular traffic) including the changes in the filamentous actin system involving the formation of stress fibres, membrane ruffles/lamellipodia and filopodia during Salmonella enterica serovar Typhimurium invasion respectively.

The growth factor receptor-bound protein 2 (Grb2) is an adapter protein that consists of the Src homology 2 (SH2) flanked by two SH3 domains. Grb2 SH2 domain binds to phosphotyrosine on several receptor-type tyrosine kinase, whereas the Grb2 SH3 domain binds to a guanine nucleotide exchange factor for Ras thus providing a critical link between cell surface growth factor receptors and the Ras signaling pathway. Salmonella enterica serovar Typhimurium invasion of mammalian cells (such as HepG2) triggers the signaling cascade which stimulates responses during Salmonella enterica serovar Typhimurium invasion.

Glutathione-S-transferase (GST) is a major detoxifying enzyme in aerobic organisms. This protein is highly up-regulated in infected HepG2 cells. Salmonella enterica serovar Typhimurium invasion triggers the generation of reactive species and depletion of glutathione. GST activity reduces GSSG to provide and maintain GSH level in the cells. GST can also bind to different hydrophobic compounds (such as haem, drugs and carcinogens).

Bcl-2-associated athanogene (BAG)-family proteins enhance cell resistance to apoptotic stimuli. The anti-apoptotic effects of the BAG-family proteins mediated by the interaction of the BAG domain with Hsc70/Hsp70. Salmonella enterica serovar Typhimurium invasion, as evident from the morphological studies, causes apoptosis in late phase of infection. Infected host cell shows high up-regulation of BAG like protein as a mechanism to resist the cell death.
MALDI/LCMS analysis of protein spots (SSP-5206) and (SSP-3204) produced similarity with TRIP-1 (Transforming growth factor β interacting protein-1) and Ubiquitin ligase specificity factor respectively.

TRIP-1, a modulatory molecule in the TGF-β signaling pathway, mediates cell proliferation, cell differentiation and cell death. TRIP-1 binds to the cytoplasmic domain of the TGF-β type II receptor in a kinase-dependent manner and is phosphorylated on Ser and Thr residues by the receptor kinase. Moreover, TRIP-1 also functions as a modulator of TGF-β receptor signaling in vivo and inhibits the expression of specific TGF-β responsive genes. TGF-β regulates the function of cells involved in the immune response to antigens, mitogens, and pathogens. TGF-β is a potent immunosuppressive cytokine that has been implicated in the susceptibility to infection with intracellular pathogens such as *Trypanosoma cruzi* and *Leishmania*. Immunomodulatory effects of TGF-β are mediated by the suppression of the production of nitrogen free radicals by phagocytic cells (such as macrophages).

Ubiquitin ligase specificity factor is an F-box and WD-40 domain-containing protein. F-box proteins are used as a specificity factors by SCF. SCF (Skp1– Cullin–F-box protein) is a cullin based E3 ubiquitin ligase which mediates ubiquitinylation of many target proteins in mammalian cells for subsequent degradation by proteosomes. One of likely functions of Ubiquitin ligase specificity factor in bacteria-infected host cell is to degrade the TTSS encoded effector proteins (such as SopE and Spt). These bacterial effector proteins facilitate the pathogen internalization and intracellular replication. Most of the effector proteins undergo poly-ubiquitination and rapid proteasome-dependent degradation following translocation in to host cells.

*Salmonella enterica* serovar Typhimurium infection in HepG2 cells causes significant increase in NO production as confirmed by the nitric oxide oxidation products (nitrite and nitrate) in cell cytosol. Nitric oxide is produced by the induction of iNOS which utilizes the only substrate (L-arginine). Effect of substrate availability on NO production established a correlation under infection condition. The most effective L-arginine concentration *in vitro* was found to be four (4) millimolar in present study.
Summary & conclusions

NO-related antimicrobial activity has been demonstrated against a number of pathogenic microorganisms including viruses, bacteria, fungi, and parasites. Simultaneous production of reactive nitrogen and reactive oxygen intermediates may lead to the formation of a variety of antimicrobial molecules including nitric oxide (NO), peroxynitrite (OONO'), S-nitrosothiols (RSNO), nitrogen dioxide (NO₂), dinitrogen trioxide (N₂O₃), dinitrosyl-iron complexes (DNIC), nitrous acid (HNO₂). Administration of nitric oxide donors significantly reduces the bacterial load in HepG2 cells. Effective concentration which reduced the bacterial burden in vitro was found to be eight (8) millimolar.

NO has been implicated in host defense against intracellular pathogens such as Leishmania, Mycobacteria and Salmonella which induce host cell death by apoptosis. NO is reported to has protective effects against apoptosis in a variety of cell types including lymphocytes, hepatocytes, endothelial Cells, neurons and eosinophils. Nitric oxide inhibition of caspases provides a mechanism to abort the apoptotic cascade. Caspases contain a highly conserved cysteine residue within their active site and therefore are a target for S-nitrosylation. NO donor supplementation considerably improves the HepG2 cells viability (highest with 4mM of L-arginine) against the Salmonella enterica serovar Typhimurium infection in present study.

Nitric oxide has a dual nature, if it can be protective in one circumstance, in other it can be quite detrimental, especially when expressed beyond a threshold value. It has enormous potential to change cell redox status, which might culminate in to inflammation and even cell death in some instances. NO at high concentrations readily combines with other oxidants to form reactive nitrogenous species, which can damage a variety of cellular targets such as DNA and proteins. This can ultimately lead to apoptosis, mutagenesis or carcinogenesis. Therefore to assess the effect of NO on oxidative stress, biochemical estimation of oxidative markers were undertaken in Salmonella enterica serovar Typhimurium infected and L-arginine treated HepG2 cells.

Reduced glutathione content was found increased with L-arginine treated (16 mM) HepG2 cells (B+Arg16), whereas untreated Salmonella enterica serovar Typhimurium infected cells (C+B) showed decreased glutathione amount. Glutathione decrease in (C+B) group was 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.
Summary & conclusions

thiols. Inhibitor treated and infected group (B+AG) showed higher GSH amount because of the inhibition of induced iNOS thereby reducing the peroxynitrite formation which actually reduce GSH amount.

Glutathione peroxidase activity declines significantly in all *Salmonella enterica* serovar Typhimurium infected groups (C+B, B+Arg16, B+Arg8, B+AG) except (Arg) and AG groups (Fig.31). Further, infection along with exogenous supply of L-arginine causes more inhibition. Nitric oxide inactivates GPx through the binding with the seleno-cysteine catalytic centre. In addition to having a cytostatic effect, NO induced apoptotic cell death in different types of cells. As redox regulation of cells and GPx activity are closely tied to apoptosis, inactivation of GPx by NO may be one of the causes of apoptotic cell death in these cells.

CAT activity is reduced in L-arg treated infected and uninfected groups. However, aminoguanidine treated groups enhanced CAT activity. CAT activity is also diminished in group Arg. 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$_2$O$_2$ production which will combine with NO to produce bactericidal activity.

XO catalyzes the conversion of xanthine into uric acid with the concomitant reduction of oxygen into superoxide (O$_2^-$) and hydrogen peroxide. Superoxide anion (O$_2^-$) is reported to be an important antimicrobial molecular species in *S. enterica* serovar Typhimurium-infected host cells. XO activity is significantly inhibited in infected and NO donors treated group (B+Arg16, B+Arg8). This inhibition in XO activity is resulted from generation of highly oxidant peroxynitrite (ONOO$^-$) from superoxide (O$_2^-$) and NO (from iNOS). Inhibitors of iNOS curtails excess NO production and thereby increasing XO activity (B+AG).

Lipid peroxidation is a chain reaction and a single oxidative event can oxidize many lipid molecules. In presence of iron or copper ions, the chain reaction can become a cascade and the oxidation process can rapidly become unstoppable. Lipid peroxidation is mediated by reactive oxygen species (ROS) such as superoxide (O$_2^-$), hydroxyl radical (OH$^-$), peroxynitrite (ONOO$^-$). *Salmonella enterica* serovar Typhimurium infection showed high amount of malondialdehyde (MDA) indicative of lipid peroxidation whereas NO
Summary & conclusions

donor treated groups (Arg, B+Arg16, B+Arg8) reduced MDA formation. Low level of lipid peroxidation resulted from NO' which can function as a chain-breaking antioxidant by reacting with peroxyl radicals. In the process, lipid nitrites and nitrates appear to be generated via rearrangement of unstable nitroso-peroxyl intermediates.

Based on the results of our studies following conclusion have been drawn

- *Salmonella enterica* serovar Typhimurium infects the HepG2 cells *in vitro*.
- Multiplicity of infection of *Salmonella enterica* serovar Typhimurium which induces morphological changes in HepG2 cells, was found to be (1:200).
- *Salmonella enterica* serovar Typhimurium invasion of HepG2 cells resulted in gross changes in HepG2 cell morphology at 24 hr post infection (PI) under phase contrast microscope.
- The number of *Salmonella enterica* serovar Typhimurium adhered to HepG2 cells at 24 hr PI, Was found to be 3-4 in scanning electron microscopy (SEM).
- Infected HepG2 cells undergo ruffling at 24-36 hr PI as verified in scanning electron microscopy.
- *Salmonella enterica* serovar Typhimurium infection initiates HepG2 cell fragmentation at 48 hr PI.
- Total protein profile from infected HepG2 cells showed differential expression of protein bands (Compared to control) on SDS-PAGE at 24 hr of PI. Major downregulation of proteins were found between 43 kDa to 29 kDa range whereas upregulation were observed between 66 kDa to 43 kDa range.
- 2-DE of total cellular protein from infected HepG2 cells yielded a total of 353 (Three hundred and fifty three spots).
- PDQuest analysis of gels (control and infected HepG2 protein) revealed significant variable expression only in 31 protein spots out of total 353. Quantity and Mr/pl of each of 31 spots were calculated with the help of standards run along with protein sample.
Out of 31 protein spots, 4 spots (SSP-0101, 1202, 2702, 7601) were present only in control gel, whereas 14 spots (SSP-2203, 3103, 3104, 3204, 5206, 6103, 6705, 7105, 7106, 7108, 7201, 7202, 7303, 8406) were found only in infected HepG2 gel, rest of 13 (SSP-1505, 2003, 2102, 2501, 3402, 4301, 4402, 5201, 6301, 6303, 7002, 7302, 8007) spots were present in control as well as infected gel.

Similarity of each of 31 protein spots (on the basis of experimentally calculated Mr/pl) in HCCM database (Hepatocellular carcinoma database), identified protein spot SSP-5201 (as Capping protein (actin filament) gelsolin like), SSP-1505 (as Vimentin like), SSP-3104 (as Rho-GDP-dissociation inhibitor protein like), SSP-6103 (as Growth factor receptor- bound protein like), SSP-7106 (as Glutathione S transferase homologue like), SSP-7201 (as BAG-family molecular chaperone regulator like).

LCMS analysis of protein spots SSP-5206 and SSP-3204 identified as TRIP-1 (Transforming growth factor-β interacting protein-1) and Ubiquitin ligase specificity factor respectively.

NO donor supplementation to the infected HepG2 cells upregulates the nitric oxide production in vitro. L-arginine concentration (4mM) produced highest amount of NO in present study.

NO donor treatment significantly reduces the bacterial burden from Salmonella enterica serovar Typhimurium infected HepG2 cells. The Most effective concentrations of L-arginine were found to be 8mM and 16mM respectively.

NO donor significantly improves infected cells viability. Highest cell viability was found at 4mM concentration of arginine. Thus there seems to be a direct correlation between highest NO generation (at 4mM of arginine) and host cell protection (at 4mM of arginine).

NO has a dual tendency, if it can protect against intracellular pathogens, it can also damage the host cells by changing intracellular redox status. Salmonella enterica serovar Typhimurium infection significantly reduces the glutathione content in HepG2 cells whereas NO donor treatment (16mM of arginine concentration) increases glutathione amount.
• GPx activity is inhibited in treated (lowest in 8mM of arginine) HepG2 cells following *Salmonella enterica* serovar Typhimurium infection. Moreover inhibitor (Aminoguanidine) treated cell group also observed decrease in GPx activity.

• Catalase (CAT) activity is inhibited in arginine treated HepG2 cells, whereas untreated cells register an increase. Aminoguanidine treatment following *Salmonella enterica* serovar Typhimurium infection enhances CAT activity *in vitro*.

• NO donor supplementation decreased xanthine oxidase activity which in turn blocks the formation of peroxynitrite. Whereas aminoguanidine treatment increases xanthine oxidase activity.

• LPO activity is decreased in the HepG2 cells treated with NO donor whereas aminoguanidine infected (B+AG) and bacterial infected groups (B+AG) enhanced LPO activity.
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