7. Review of literature

In the past decade, there has been an increase in number of clinical cases involving end stage hepatic disorders (Haddad et al., 2015). Under most circumstances, surgical intervention remains as the last available choice for the physicians to prevent mortality (Saidi, 2013). However, the outcomes of surgical procedure vary depending upon the antioxidant and immunological status of the hepatic tissue (Stutchfield et al., 2015). Most patients admitted for hepatic surgery, are moderately steatotic (30%-60%) with history of multiple medications for a chronic period, leading to culminated oxidative stress negatively altering the hepatic function (Anastacio et al., 2013). The post-operative mortality following liver surgery remains high (~70%) despite the advances in clinical field (Paugam-Burtz et al., 2012). Hence, it is essential to investigate the underlying mechanisms involving surgical hepatic injury to improve the post operative hepatic survival.

7.1. Events during late phase hepatic IR injury

In case of end stage liver diseases, the treatment requires extensive surgical measures and the reperfusion period is prolonged. In the later stages of reperfusion, the oxidative stress is overwhelmingly high and amplifies the inflammatory cytokines and chemokines. The excessive release of cytokines such as TNF-α, interleukin-1β (IL-1β) and chemokines such as macrophage inflammatory protein-2 (MIP-2), & cytokine induced neutrophil chemoattractant – 1 (CINC-1) induce the recruitment of neutrophils into the hepatic parenchyma (Jaeschke and Smith, 1997). These changes are parallel with increased expression of β-2 integrins and NOX in the neutrophils (Jaeschke, 2000). The increased oxidative burst encourages the neutrophil extravasation by initial recognition of neutrophil surface receptors and P-selectin on the luminal side of SECs (Peralta et al., 2001). Moreover, the interaction of β-2 integrins on neutrophil surface and intercellular adhesion molecules (ICAMs) on the SECs leads to firm adhesion and rolling of neutrophils – resulting in their parenchymal infiltration (Campbell et al., 1998). Hence, neutrophils are the major contributors of liver injury when liver subjected to prolonged reperfusion. However, a precise understanding of the events involved in the neutrophil activation, chemotaxis and mode of injury is essential to design a therapeutic strategy.
7.2. Neutrophils

Neutrophils constitute ~70% of all granulocytes in blood and are the first line of defense in the innate immune system. The neutrophils are drawn into the hepatic parenchyma, by the stress signals and chemotactic gradient established across the sinusoid – parenchyma intersection (Zhang et al., 2016). The stimulated neutrophils express NOX on their surface, leading to ROS release upon activation (El-Benna et al., 2016). Once in the parenchyma, the neutrophils are exposed to a cocktail of ROS and inflammatory cytokines leading to the release of the azurophilic granules into the parenchyma milieu. The chief contents of the azurophilic granules are myeloperoxidase, neutrophil elastase, neutrophil proteinase 3 and cathepsin G (Faurschou and Borregaard, 2003).

Activation of myeloperoxidase releases hypochlorous acid and chloramines, which are highly detrimental to the hepatocytes (Nusse and Lindau, 1988). Neutrophil proteinase-3, processes the precursors of inflammatory proteins such as TNF-α, IL-1 and IL-8 into their active forms (Wiedow and Meyer-Hoffert, 2005). Neutrophil elastase causes the degradation of the extracellular matrix components such as collagen-IV and elastin (Papayannopoulos et al., 2010). Cumulatively the release of azurophilic granules causes drastic deterioration of the hepatic parenchyma.

7.3. Neutrophil extracellular traps

Based on the extent of inflammatory stimulus the neutrophils usually release their contents in the form of neutrophil extracellular traps (NET). Upon inflammatory stimulus and excess of ROS levels, the neutrophils undergo dramatic morphological changes (Fuchs et al., 2007). The azurophilic granules are translocated into the nucleus, aided by permeation of the nucleopore complex (Papayannopoulos et al., 2010). The proteolytic enzymes in the granules, orchestrate the cleavage of linker histone H1. Moreover, the increased production of superoxide radical (O$_2^\cdot$-) by NOX promotes citrullination of the arginine residues in three of the four core histones by enzyme peptidylarginine deaminase -4 (PAD4). Subsequently, the chromatin structure unfolds and integrates with the cytoplasm to form a homogenous mixture. This mixture of chromatin and proteolytic content is released upon neutrophil rupture in the form of NET (Papayannopoulos and Zychlinsky, 2009). Studies have shown
that excess ROS also activates MAPK-ERK signaling which plays a major role in NET formation (Hakkim \textit{et al.}, 2011). The infiltrated neutrophils in liver, when triggered by inflammatory and oxidative stimulus have shown to release NET, which results in aggravated hepatic injury during late phase reperfusion. Hence, NOX2 and p38MAPK are the key targets in attenuation of NET release and for the suppression of NET associated hepatic IR injury.

The patients undergoing prolonged medication for hepatic ailment would have significant accumulation of oxidative end products in liver parenchyma on par with suppressed hepatic antioxidant status (Amin \textit{et al.}, 2005; Chen \textit{et al.}, 2015). We suspected that these oxidant end products might be a trigger for neutrophil recruitment in to hepatic parenchyma and NET release upon IR injury and might contribute to the observed post surgical necrotic changes in liver. Hence, in the current study we chose acrolein, as stimuli to trigger NET (Arai \textit{et al.}, 2014).

\textbf{7.4. Acrolein the activator of hepatic IR injury though NETosis}

Acrolein, an $\alpha$, $\beta$-unsaturated aldehyde is a common end product of lipid peroxidation within the cells (Fig. 3.1). Acrolein has also been classified as an environmental toxicant, which is released during combustion of fossil fuels and is also the chief content of cigarette smoke attributed to acrolein induced pulmonary injury (Logue \textit{et al.}, 2012; Kassem \textit{et al.}, 2014). The tolerable daily intake for acrolein is 7.5$\mu$g/kg body weight (Abraham \textit{et al.}, 2011).

\textbf{Figure 3.1. Structure of acrolein}

Endogenously acrolein is produced by: i) Lipid peroxidation of polyunsaturated fatty acids (PUFA), ii) Polyamine catabolism, iii) Cyclophosphamide metabolism, and iv) Theonine degradation by neutrophil myeloperoxidase (MPO).
Acrolein being a small water soluble molecule can easily bypass the semi permeable membrane and being a strong electrophilic, can react with divergent biomolecules. Acrolein induces DNA damage by alkylation and protein damage by forming adducts with sulphhydryl group of cysteine, imidazole of histidine and amino group of lysine to significantly alter the protein function. Glutathione is essential for acrolein detoxification, hence excessive acrolein formation leads to depletion of cellular glutathione reserve and weakening the antioxidant capacity.

Acrolein is considered as both the product and initiator of lipid peroxidation (Uchida, 1999). Hence, it has been related to pathophysiology of various chronic diseases mediated by oxidative stress (Adams and Klaidman, 1993). Increased level of acrolein-protein adducts have been reported in Alzheimer’s disease and diabetic nephropathy associated with excessive lipid peroxidation (Suzuki and Miyata, 1999; Calingasan et al., 1999). Acrolein causes oxidative stress, contributing to membrane, DNA and mitochondrial damage. Acrolein induced oxidative stress accompanied by mitochondrial damage and endoplasmic reticulum stress has been shown in liver cells (Mohammad et al., 2012). Also acrolein inhibits mitochondrial complex I and II, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase over doses ranging from 10μM to 1000 μM in rat liver mitochondria (Sun et al., 2006). Acrolein has been implicated in neutrophil induced damage in chronic obstructive pulmonary diseases and in smoking (Yeager et al., 2016). But the exact mechanisms of acrolein induced neutrophil activation and toxicity are still unclear.

7.5. Molecular players in neutrophil activation and liver injury

7.5.1. NADPH oxidase-2

The term “respiratory burst” denotes sudden explosive production of ROS by phagocytes, in response to pathogenic or inflammatory stimulus (Babior, 1984). At the wake of 20th century, respiratory burst has been observed in sea urchin eggs (Warburg, 1908), phagocytes (Baldridge and Gerard, 1933) and spermatocytes (Macleod, 1943). Sbarra and Karnovsky (1959), showed that respiratory burst was endothermic reaction which is dependent upon glucose metabolism. Iyer et al., (1961), showed that H₂O₂ was the product of respiratory burst from phagocytes. Rossi and Zatti (1964), correctly showed that NOX was the complex responsible for
respiratory burst. Babior et al., (1973) reported that the initial product of respiratory burst is $O_2^\bullet^-$ rather than $H_2O_2$.

NOX belong to enzyme family which generates ROS (in the form of superoxide or hydrogen peroxide) from oxygen metabolism as a response to a range of signaling functions and immune defense (Crosas-Molist and Fabregat, 2015). All the isoforms of NOX are membrane bound with cytosolic, transmembrane and extracellular domains. All NOX forms rely upon NADPH, ROS is generated when electron is transferred from cytosolic NADPH to molecular oxygen to produce $O_2^\bullet^-$ or $H_2O_2$. (Lambeth, 2004; Holmstrom and Finkel, 2014). Different isoforms of NOX are expressed both in heptic and stellate cells such as NOX1, NOX2, NOX4, dual oxidase-1 (DUOX1) and dual oxidase-2 (DUOX2). SECs express NOX1, NOX2 and NOX4. However, Kupffer cells mainly express NOX2, which is phagocyte dominant (Guichard et al., 2008; Paik et al., 2013).

NOX2 is the first identified isoform in phagocytes and was considered as the only source of ROS before identification of other isoforms. NOX2 - also labeled as gp91phox, is most studied of all NOX forms and its biochemistry has been well documented (Cross and Segal, 2004; Nauseef, 2004; Moloney et al., 2017). In phagocytes, NOX2 is localized in both intracellular and plasma membrane regions and is in close proximity to p22$^{phox}$ (Borregaard and Heiple, 1983; Huang et al., 1995). In quiescent neutrophils, NOX2 is mostly localized to intracellular compartments, especially in secondary and tertiary (gelatinase-containing) granules. NOX2 is translocated to the surface, when phagocytes are activated resulting in granule fusion with plasma membrane or phagosomes (Garcia and Segal, 1984; Goldblatt and Thasher, 2000). The resultant ROS formation is considered to be responsible for the signaling functions of NOX2.
Figure 3.2. Structure of fully functional – membrane assembled form of NOX2.

NOX2 activation is brought about by a complicated series of protein-protein interactions (Fig. 3.2) (Nauseef, 2004; Sumimoto et al., 2005). NOX2 (gp91phox) is constitutively associated with p22phox, which is highly essential for its stability and activity (Parkos et al., 1989; Dinauer et al., 1990). Phosphorylation of p47phox – the organizer subunit, induces conformational changes which recruits and organizes other cytosolic subunits (Sumimoto et al., 1996). It allows interaction of p67phox (activator subunit) with the gp91phox unit and the smaller gp40phox is also recruited into the complex (Han et al., 1998). Finally GTPase Rac interacts with NOX2 and then with gp67phox, as a result of which electron from NADPH on the intracellular side is transferred to oxygen on the extracellular side to generate O$_2^-$ (Lapouge et al., 2000; Diebold and Bokoch, 2001). NOX2 gene is located on the X chromosome. Its expression has been shown to be induced in pathogenic and inflammatory cases (Chen et al., 2016; El-Benna et al., 2016).
7.5.2. \textit{p38 Mitogen activated activated protein kinases (p38MAPK)}

p38 Family of proteins are termed the mitogen activated protein kinases which are the major players in inflammatory initiation, especially in macrophages and phagocytes (Yang \textit{et al.}, 2013). It was first isolated in 1994 and is the mammalian orthologs to yeast Hog1p MAPK (O’Rourke and Herskowitz, 1998). It is a 38KDa protein which is activated by phosphorylation at a tyrosine residue in response to stimulation with bacterial lipopolysaccharide (LPS) (Lee \textit{et al.}, 1994). Its expression is shown to be upregulated by various stimuli such as, stress, inflammatory cytokines, heat shock, osmotic shock etc., p38 is shown to play an important role in autophagy, apoptosis, and cell differentiation (Clark and Dean 2012; Huh \textit{et al.}, 2004; Kim \textit{et al.}, 2013a). Several studies have stated its role in arthritis, hepatitis, renal and brain inflammation, mediated by macrophages (Ren \textit{et al.}, 2012; Lim and Tesch, 2012; Ko \textit{et al.}, 2013).

\textbf{Figure 3.3. Structure of p38 mitogen activated protein kinases}

![Structure of p38 MAPK](image)

Adapted and modified from Lindin \textit{et al.}, 2014

The structure of p38 MAPK consists of two domains: N-terminal with 135 amino acids and C-terminal with 225 amino acids. The N-terminal is composed of β-sheets as secondary structure, while the C-terminal is predominantly made of α helices. The area linking these two domains comprises the catalytic site for p38 MAPK (Fig. 3.3). The active site termed as the phosphorylation lip, consists of 13 residues: Leu-171 to Val-183. The activation occurs upon phosphorylation of Thr-180.
and Tyr-182 in the phosphorylation lip (Wang et al., 2012). Various isoforms of p38 show variations in the three dimensional orientations N-and C-terminal residues, resulting in various sizes of ATP binding pockets (Patel et al., 2009). The upregulation of p38 has shown to occur in response to various inflammatory stimuli. Studies have shown increased expression of p38 MAPK in macrophages treated with LPS, endothelial cells exposed to TNF-α and human neutrophils subjected to phorbol 12-myristate 13-acetate (PMA), LPS and TNF-α (Nick et al., 1996; Shapiro et al., 1998). Studies have reported that, upon neutrophil stimulation p38 MAPK expression is upregulated (Funamoto et al., 2002; Cuadrado and Nebreda, 2010). Also, specific inhibitors of p38 MAPK have shown to inhibit chemotaxis in stimulated neutrophils (Heit et al., 2002; Xu et al., 2013).

7.6. Therapeutic targeting of NOX2 and p38 MAPK

7.6.1. F-apocynin as a NOX2 inhibitor

Apocynin (4-hydroxy-3-methoxyacetophenone) also known as acetovanillone was first isolated from the roots of Apocynum cannabinum (Canadian hemp). Traditionally, the extracts have been used for remedies from dropsy and heart conditions (Stefanska and Pawliczak, 2008). In 1971, apocynin was identified from extracts of Picrorhiza kurroa (Scrophulariaceae), native to Indian subcontinent and has been used in traditional medicine (ayurveda). Apocynin has a molecular mass of 166.17 and possesses a faint vanilla odor. It forms needle shaped crystals upon crystallization from water and has a melting point of 115°C (Hougee et al., 2006; Lutchtefeld et al., 2008).

Figure 3.4. Structure of apocynin and f-apocynin
Apocynin has been popularly used as an effective inhibitor of NADPH-oxidase multienzyme complex, in several experiments (Lafeber et al., 1999; Stolk et al., 1994; Zhang et al., 2005). Though the mechanism of action of apocynin has not been known fully, it involves impaired translocation of p47phox component of NOX2 to the membrane (Peters et al., 2001; Barbieri et al., 2004). Apocynin selectively inhibits NADPH oxidase and the resultant ROS production in human neutrophils (IC50- 10 µM) (Simons et al., 1990). Following are the reported effect of apocynin: i) reduction of neutrophil respiratory burst, ii) suppression of chemotaxis in polymorphonuclear cells (PMNs,) iii) suppression of neutrophil mediated oxidative injury and iv) inhibition of peroxynitrite. Apocynin has shown to posses radical scavenging properties and has been proven to be protective against respiratory and neuronal diseases (Leferber et al., 1999; Muller et al., 1999; Muijsers et al., 2000). Studies have also demonstrated anticancer effect of apocynin, where it has shown to inhibit migration in MDAMB-435 cells at subtoxic levels (Ljungman et al., 1991).

F-apocynin (4-fluro – 2- methoxyphenol) is a structural analog of apocynin, with higher lipophilicity (Fig 3.4). F-apocynin is more efficient in blocking the extracellular release of $O_2^\cdot$ as well as intracellular formation of ROS in PMNs, as compared to apocynin (de Almeida et al., 2011). Further, f-apocynin also had shown a suppressive effect on myeloperoxidase activity better than apocynin. On the other hand, TNF-α level was shown to be similar in both the cases (de Almeida et al., 2011). Considering these enhanced effects of f-apocynin, when compared to apocynin, we have chosen f-apocynin for pretreatment against NET formation in neutrophils stimulated by acrolein. Further we evaluated the effect of f-apocynin in in vivo model.

7.6.2. Naringin as an inhibitor of p38 MAPK activation

Naringin is a flavanone present in citrus fruits, tomatoes and grapefruits. Chemically, naringin is a 4’, 5, 7-trihydroxy flavanone -7-rhamnoglucoside ($C_{22}H_{32}O_{14}$), with two rhamnose units attached to its aglycon unit at the 7th position (Alam et al., 2014) (Fig 3.5). The word naringin arises from the Sanskrit word “narangi” – denoting orange. Removal of the rhamnose units by gut bacteria yields naringenin – an aglycone. Naringenin is undergoes extensive metabolism in liver and is converted into glucuronide metabolites (Ishii et al., 1997; Lee and Reidenberg,
Major metabolites of naringin include, 4-hydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, phloroglucinol, 4-hydrophenyl-propionic acid and 4-hydroxyphenylacetic acid (Liu et al., 2012a).

Figure 3.5. Structure of naringin and its metabolite naringenin

![Structure of naringin and its metabolite naringenin](image)

Studies have described various pharmacological activities of naringin in various models. Naringin has excellent free radical scavenging properties, which owes its antioxidant properties (Maridonneau-Parini et al., 1986). Naringin was shown to reduce high fat diet induced cardiovascular fibrosis in obese rats (Panchal et al., 2011). Also, naringin has been shown to protect myocardial tissue and liver against isoproterenol and doxorubicin induced oxidative stress and inflammatory damage (Rajadurai et al., 2007; Kwarta et al., 2016). Naringin demonstrated anti-inflammatory effects by suppression of NO, iNOS, cyclic oxygenase-2 (COX-2), TNF-α and IL-6 production in LPS stimulated RAW 264.7– macrophages (Kanno et al., 2006).

Naringin at a concentration of 50-200 µM has shown to inhibit inflammatory cytokine production in RAW 264.7– macrophages, by blocking the activation of NFκB and MAPK signals (Liu et al., 2012b). Naringin has been elucidated to protect against hyperglycemia mediated injury in H9c2 cells (cardiac cells), by inhibiting p38MAPK mediated activation of leptin (a product of ob gene) (Chen et al., 2013). By virtue of inhibitory property against NFκB and p38MAPK activation, naringin has been demonstrated to posses, antidiabetic, neuroprotective, hepatoprotective, antiulcerative, antiallergic and anticancer potentials (Galati et al., 1998; Jung et al., 2006; Olivia et al., 2008; Kumar and Kumar, 2010).
7.7. Pretreatment strategy plan to counteract acrolein augmented liver IR damage

To protect against the effects of acrolein induced NET release, we targeted 2 major markers - NOX2 which is the major contributor of ROS in neutrophils and p38MAPK which plays a major role in activation of neutrophil enzymes and chromatin modification (Fig 3.6). F-apocynin, a more potent analog of apocynin was used for the inhibition of NOX2, while naringin was chosen for its reported p38MAPK inhibition.

Figure 3.6. Representation of acrolein induced NET release coupled with IR hepatic damage

We observed the effects of inhibition of NOX2 or p38MAPK separately and in combination on the outcomes of acrolein induced NET release in neutrophils and HepG2 cocultured cells. The results were correlated with the in vivo experiments involving male albino Wistar rats subjected to acrolein augmented hepatic IR injury. The novel findings in the present study would shed light on the mechanisms involved in liver IR injury aggravated by infiltrating neutrophils and a possible therapeutic approach to improve post surgical survival.