6. Discussion

In recent years, hepatic disorders have increased exponentially at global scale. In terminal unresponsive cases of hepatocellular carcinoma, colorectal metastases to liver, porto-pulmonary hypertension, primary sclerosing cholangitis, etc., treatment through surgical intervention or transplantation is the last available option (Ananya et al., 2011; Goldberg et al., 2013). During surgical procedures, the hepatic ischemia reperfusion injury results due to clamping of portal vein. The hypoxic state of hepatocytes during ischemia leads to impaired mitochondrial respiration. Reduction in cellular energy reserve initiates the breakdown of metabolic pathways and impairs membrane ion transport which in turn leads to ionic imbalance thereby causing SECs swelling and activation of Kupffer cell, which are further aggravated during reperfusion (Clavien et al., 1992; Rosser and Gores, 1995). The activated Kupffer cells releases the inflammatory mediators which in turn stimulates the sequestration of neutrophils at later stages, resulting in excessive release of ROS at the sinusoidal interfaces further aggravating the injury (Jaeschke and Farhood 1991; Xu et al., 2008). Since oxidative stress is the major contributor for IR induced liver injury, therapeutic strategies employing antioxidants has gained interest.

Though there are numerous studies regarding the effect of pretreatment of antioxidants on the hepatic IR injury in adult rats, only meager information is available on the comparison of the liver of young and aged rats. There has been no adequate insight into the differences between molecular as well as biochemical changes that occur in young and aged rat liver during IR injury. Since, aged rat liver has reduced antioxidant defense system it may be more susceptible to IR injury, and hence a detailed comparison on the differential outcome of antioxidant pre-treatment in young and aged rats becomes essential.

Young and aged male Wistar rats were subjected to 90 min ischemia and 2 h reperfusion. Schauer et al. (2004) found that 90 min hepatic ischemia induces more liver injury than 60 min hepatic ischemia and no further changes occur after 120 min of ischemia. Later, other studies have employed 90 min as optimum period to induce hepatic ischemia (Xu et al., 2008; Wu et al., 2011; Lin et al., 2012). Previous studies have shown that two distinct phases exist in hepatic reperfusion injury. The early phase (during the first 2h) during which the Kupffer cells are activated leading to
ROS release and expression of TNF-α and interleukin-1, while the late phase (12 h after reperfusion) is characterized by activation of neutrophils and progressive hepatic damage leading to fibrosis (Glantzounis et al., 2005). Reperfusion induced stress stimulus triggers NFκB, which transcriptionally activates TNF-α. Takahashi et al. (2002) observed biphasic activation of NFκB level during 1-3 h (early phase) and 12 h (late phase) reperfusion of liver grafts transplanted in rats, which depicts the occurrence of multiple peaks in inflammatory signal.

Though multiple peaks of inflammatory mediators are observed during hepatic IR injury, the observations from earlier studies suggest that intervention during the initial phase of reperfusion injury (during first 2 h) result in significant recovery of hepatic tissue rather than intervention in later phases (Crockett et al., 2006). Moreover the changes that occur during the initial phase are stronger and determine the ultimate fate of the organ. Hence the current study focused on the changes that occur during 90 min ischemia and 2 h reperfusion (initial phase) induced injury in rat liver, in order to determine the effect of GSH pre-treatment in young and aged rats.

IR injury in liver involves complex network of pathways and mediators. Several pathways such as, Kupffer cell-induced NFκB-mediated proinflammatory pathway, nitric oxide synthase (NOS) pathway and HO-1 system are activated in parallel during the IR stimulus (Vardanian et al., 2008). The proinflammatory pathway leads to deleterious changes in liver post IR injury. On the other hand, the NOS pathway and HO-1 system counteract the IR-mediated injury and proved as beneficial for the improvement of liver function following ischemic stimulus (Hines et al., 2005; Tsuchihashi et al., 2007). Kaizu et al. (2006) had shown that inducible nitric oxide synthase (iNOS) gene therapy reduced the IR injury in rat liver. They have further shown that increased production of NO induces HO-1 which inturn counteracts the inflammatory response. Involvement of such multitude of factors makes it difficult to treat IR injury by targeting a single intervening factor. Since ROS are central mediators of IR injury, better clinical outcome can be achieved through blocking ROS-mediated injury by employing antioxidant treatment prior to surgery.

We have compared the effect of GSH treatment prior to surgery on hepatic recovery from early phases of IR induced injury in young and aged rats. The
outcomes were analyzed by various biochemical markers and histopathological analysis. The elevation of serum transaminases is a reliable indicator for the extent of hepatic damage (Schauer et al., 2004). The elevation of ALT and AST activities in serum of young and aged IR group rats portrays the extent of liver IR injury. The elevation of AST and ALT suggests the increased susceptibility of aged rat liver to IR injury compared with the young and the restoration of transaminase activities explains the reduction in liver damage in both age groups with GSH pre-treatment.

The reduced serum and hepatic GSH level in IR groups (groups II & V) and their resurgence in GSH pre-treated groups reveals the effective absorption and restoration of GSH:GSSG ratio upon extraneous treatment in young and aged rats alike. Lang et al. (1992) had reported that GSH level in young individuals was 17% higher on average than that of elderly subjects. The significant decline in mitochondrial GSH:GSSG ratio in IR groups substantiates the oxidative damage at mitochondrial level. The observed decline in GSH:GSSG ratio of mitochondrial fraction of aged IR groups (group VI) and its recovery with GSH treatment in aged groups are comparable to the similar outcome in young rats thereby suggesting the mitochondrial GSH replenishing efficiency of extraneous GSH administration prior to hepatic surgery across age barrier.

Lipid peroxidation is the key mechanism by which ROS execute the oxidative damage in liver (Knight et al., 2003; Glantzounis et al., 2005; Zhang et al., 2007). Moreover, mitochondrial lipid peroxidation due to oxidative injury has been described as the major culprit in the hepatic IR injury (Mukhopadhay et al., 2012). Ageing has been shown to be associated with increased hepatic lipid peroxidation in aged subjects (Farahmand et al., 2013). In our study, TBARS level was elevated in the aged rats subjected to hepatic IR, but were subdued in the rats treated with GSH prior to surgery. Further we observed that, though the increase in lipid peroxidation was higher in aged rats subjected to hepatic IR as compared to young rats subjected to IR, pretreatment with GSH subdued lipid peroxidation to produced similar outcomes in both young and aged rats.

In the current study, we have observed significantly high expression of TNF-α, active-caspase-3 and PARP-1 in rats subjected to hepatic IR injury without GSH treatment. TNF-α has been observed to play a major role in the onset of IR-mediated
oxidative hepatic injury. Previous studies have elucidated the mechanism of TNF-α-induced activation of apoptosis and its role in hepatic injury (Okajima et al., 2002; Mari et al., 2009; Birk et al., 2013). Circulating neutrophils have been shown to elicit TNF-α induced caspase-3 activation (Geering et al., 2011). PARP-1 cleavage fragments are a signature of apoptotic progression (Chaitanya et al., 2010). Our observation correlated the increase in TNF-α, caspase-3 and PARP-1 and confirms a positive loop of apoptotic signaling involved in hepatic IR injury. The rise in serum and liver tissue TNF-α level upon hepatic IR injury and its reduction in GSH pre-treated rats, as evident by ELISA observations further supports the inflammatory process augmented by IR liver injury.

The increase in TUNEL positive nuclei in young and aged rats of IR group elucidates the DNA damage inflicted by IR-mediated oxidative damage. Earlier studies have reported similar increase in TUNEL positive cells upon reperfusion followed by ischemia (Schmitt-Graff et al., 1991; Serbetci et al., 2012). The effectiveness of GSH pre-treatment prior to surgery in reducing oxidative DNA damage is demonstrated by reduction in number of TUNEL positive cells in GSH treated young and aged groups.

Histological observation of aged IR groups showed vascular endothelial degradation and patches of hepatocyte necrosis with neutrophil intrusion, while hepatocytes ballooning and pyknotic nucleus was observed in young rats subjected to hepatic IR injury. The liver damage in IR injury has been proven earlier to be extensively due to the triggering of inflammatory mediators released by Kupffer cell stimulation and the resulting release of inflammatory mediators like TNF-α, IL1-α and IL6 (Kim et al., 2013c; Menger et al., 1999). The elevated expression of TNF-α has been shown to be the culprit for P-selectin upregulation and neutrophil recruitment (Peralta et al., 2001). Suppression of TNF-α-mediated apoptotic signaling by GSH pre-treatment can be explained by attenuation of Kupffer cell simulation by lowering the oxidative stress stimulus.

These observations showed that the administration of GSH prior to hepatic IR surgery protects both the young and aged rats from early stages of IR-induced oxidative damage. The pre-treatment with GSH significantly reduced the apoptosis and TNF-α by restoration of GSH:GSSG ratio at mitochondrial level, in young and
aged rats. These findings suggest that GSH supplementation prior to surgery would be an efficient therapeutic strategy and can be used synergistically with other treatments to yield better post-operative outcomes, thus irrespective of age factor.