4. Materials and methods

4.1. Animals

Male Wistar (approved by the Institutional Animal Ethics Committee, Pondicherry University, Puducherry, India; Approval no: PU/IAEC/12/02) young rats of age 6 weeks weighing 140±20 g and aged rats of age 24 months weighing 300±30 g at the time of surgery were used for the study. Animals were maintained in central animal facilities of Pondicherry University. They were allowed free access to food and water *ad libitum* until 8 h before surgery. Animals were treated and experimented under the guidelines of the Committee (Purpose of Control and Supervision of Experiments on Animals, Government of India).

4.2. Experimental design

The animals were divided into 6 groups (n=6):

Group I - Young sham (without surgical procedure).

Group II - Young IR -subjected to saline pre-treatment via intraperitoneal (i.p.), 30min before IR surgery (90 min ischemia and 2 h reperfusion).

Group III – Young rats pre-treated with GSH (200 mg/kg body wt, i.p.) 30min before IR surgery.

Group IV - Aged sham (without surgical procedure).

Group V - Aged IR – subjected to saline pre-treatment (i.p.) 30 min before IR surgery.

Group VI - Aged rats pre-treated with GSH (200mg/kg body wt, i.p.) 30min before IR surgery.

The rats were anesthetized by ketamine/xylazine (100mg/kg body wt/10mg/kg body wt, i.p.) and then subjected to midline laparotomy (Day *et al.*, 2004). Hepatic IR was achieved by subjecting the rats to hepatic ischemia by partial hepatic occlusion (covers 70% of hepatic parenchyma) for 90 min and released after 2 h for reperfusion.
The hepatic pedicle was identified and the branch left of portal vein which supplies median and left lobe were clamped with microvascular clamp for 90min (ischemia) and released exactly after 2h (reperfusion) (Fig 2.4). This method prevented congestion of mesenteric venous drain by permitting decompression of portal supply through right and caudate lobes (Day et al., 2004). About 2 ml of blood was collected for serum analysis from vena cava caudalis before sacrificing the rats. Liver tissues were washed with saline (0.9% NaCl) and divided into three portions. First portion was wrapped in aluminum foil and stored in -80°C, for biochemical assays. Second portion was fixed in neutral buffered formalin for histopathological studies and the third portion was saved for western blot analysis.

**Figure 2.4. Process of inducing partial hepatic ischemia followed by reperfusion in rat liver**

![Rat liver before clamping](image1)

![Tracing the portal triad](image2)

![Hepatic reperfusion (2 h)](image3)

![Partial hepatic occlusion (90 min)](image4)

**Figure 2.4.** Images representing the procedure for partial hepatic ischemia in rat liver. A. Rat liver exposed by midline incision along the abdominal wall; B. Tracing the left branch of portal triad with forceps; C. Occlusion of the left portal triad branch to occlude median and left lobe with pediatric clamp for 90 min (discolouration of median and left lobe shown in marked area); D. Removal of clamp to allow reperfusion for 2h (restoration of reddish brown color in left and median lobes indicating reperfusion).
The 2 h reperfusion model was chosen for the analysis of reperfusion injury in young and aged rats because Kupffer cell activation and the release of proinflammatory cytokines are initiated at this stage, which later progresses to induce hepatic fibrosis (Ozkan et al., 2010). The molecular changes during the early phase of reperfusion are critical since they determine the ultimate fate of the cells. We assume that the effective suppression of these changes in IR may yield better postoperative outcome.

4.3. Serum transaminases analysis

Hemolysis free serum samples from rats were subjected to alanine transaminase (ALT) and aspartate transaminase (AST) analysis using commercially available kit (Cayman chemical company, MI, USA).

4.3.1. Assay for alanine transaminase

The commercial kit to assay the serum ALT activity has the following kinetic reaction.

\[
\text{L-Alanine} + \alpha\text{-ketoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{L-glutamate}
\]

\[
\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}
\]

Kit contains two reagents labeled as reagent1 and reagent2. The working reagent was prepared by mixing 4 volumes of reagent1 with 1 volume of reagent2. 1ml of working reagent was added to 100 µl of sample and incubated for 1min at 37°C. The change in absorbance per minute at 405 nm (OD/min) was measured for 3min. ALT activity was calculated using following formula.

\[
\text{SGPT (IU/L)} = (\Delta \text{Abs.} \text{ / min}) \times 1745
\]

The enzyme activity was expressed as IU/L serum. One IU is defined as the amount of the enzyme that catalyzes the conversion of 1 µmole of substrate per minute.
4.3.2. Assay for aspartate transaminase

The commercial kit to assay the AST activity has the following kinetic reaction.

\[
\text{L-Aspartate} + \alpha\text{-ketoglutarate} \xrightarrow{\text{AST}} \text{oxaloacetate} + \text{L-glutamate}
\]

\[
\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L-malate} + \text{NAD}^+
\]

Kit contains two reagents labeled as reagent1 and reagent2. The working reagent was prepared by mixing 4 volumes of reagent1 with 1 volume of reagent2. 1 ml of working reagent was added to 100 µl of sample and incubated for 1min at 37°C. The change in absorbance per minute at 405 nm (OD/min) was measured for 3min. AST activity was calculated using following formula:

\[
\text{SGOT (IU/L)} = \left( \Delta \text{Abs. / min} \right) \times 1745.
\]

The enzyme activity was expressed as IU/L serum. One IU is defined as the amount of the enzyme that catalyzes the conversion of 1 µmole of substrate per minute.

4.4. Preparation of tissue homogenate

Following animal sacrifice, the liver tissue was surgically removed and washed thoroughly in PBS for removal of excess blood. A portion of liver tissue was homogenized and used for biochemical analysis. Tissues were homogenized with 0.25 M sucrose-phosphate buffer solution (50 mM, pH 7.4) at 4°C to obtain 10% homogenate (1 g tissue in 10 ml of ice cold buffer) in a motorized homogenizer. Mitochondrial fraction was separated by differential centrifugation method (Sahoo and Chainy, 1997). The fractions were stored on ice until further use.

4.5. Estimation of protein

Protein content of the liver homogenate was estimated by the method of Bradford (1976).
4.5.1 Procedure

For protein estimation 3 different dilutions (undiluted, 1:5 dilution and 1:10 dilution) of tissue homogenate were used. 20 µl of tissue homogenate was mixed with 250 µl of Bradford reagent in a 96 well microtitre plate. A series of standards ranging in concentration from 120 µg/ml to 2000 µg/ml were prepared by dilution from 20 mg/ml bovine serum albumin solution. The blue color developed due to interaction of Coomassie brilliant G-250 with peptide bonds was read at 640 nm. The protein concentration was arrived by comparison with standards and calibration from standard graph. The protein value of tissue homogenate was expressed as mg/g of wet tissue.

4.6. Liver tissue antioxidant enzymes

4.6.1 Superoxide dismutase

SOD activity was assayed by the method of Marklund and Marklund (1974). To 1 ml of sample 0.25 ml of absolute ethanol and 0.15 ml of chloroform were added and kept in mechanical shaker at 2500 rpm for 1 min. Then to the supernatant 2 ml of tris HCl buffer (pH 8.2) was added. To this 0.5 ml of 2 mM pyrogallol and 1.5 ml of distilled water were added and mixed. Then readings were recorded at 420 nm at an interval of 30 seconds for 2 min. The activity of SOD was expressed as µmol of pyrogallol oxidized/min/mg of protein at 37°C.

4.6.2 Catalase

Liver tissue CAT activity was assayed by the method of Claiborne (1985). To 2.94 ml of 0.05 M phosphate buffer pH 7.0, 10 µl of 0.019 M H₂O₂ and 50 µl of tissue homogenate were added and the reaction was monitored for decrease in absorbance at 240 nm at an interval of 30 sec for 2 min. Readings were obtained against phosphate buffer as blank. CAT activity was expressed as nmol of H₂O₂ utilized per min/mg of protein at 37°C.

4.6.3 Glutathione peroxidase

GPx was assayed by using the method of Rotruck et al., (1973). To a mixture of 0.2 ml of 0.4M phosphate buffer, 0.2 ml of 4 mM ethylene diamine tetraacetic acid
(EDTA) and 0.1 ml of 10 mM NaN₃, 0.2 ml tissue homogenate (homogenized in 0.4 M phosphate buffer, pH 7.0) was added. To this, 0.2 ml of GSH, followed by 0.1 ml of H₂O₂ were added. The contents were mixed well and incubated at 37°C for 10 min along with a control tube containing all reagents without enzyme. After 10 min, 0.4 ml of 10% trichloroacetic acid (TCA) was added to arrest the reaction. To determine the residual GSH content, the supernatant was removed by centrifugation and to this 3 ml of 0.4 M Na₂HPO₄ and 1 ml of 0.6 mM 5, 5ʹ-dithiobis-2-nitrobenzoic acid (DTNB) reagent were added. The color developed was read at 412 nm. The solution treated with Na₂HPO₄ and 1 ml of DTNB reagent alone was used as blank to eliminate the reagent color. Suitable aliquots of standards were taken and treated in the same way. The activity of GPx was expressed as nmol of GSH consumed/min/mg protein at 37°C.

4.6.4. Glutathione reductase

The activity of hepatic GR was assayed by employing the method of Carlberg and Mannerick (1975). The reaction mixture contains 1.7 ml of 0.1 M phosphate buffer pH 7.6, 100 µl of 0.2 µM NADPH and 100 µl of 0.01 M EDTA. To this mixture 50 µl of oxidized glutathione (GSSG) and 50 µl of liver homogenate are added to initiate the reaction. The reduction in absorbance of NADPH at 340 nm was recorded at 30 sec intervals for 3 min. The reaction mixture with GSSG alone and without homogenate was used as reagent control to nullify auto reduction. The results were expressed as µmol of NADPH oxidized/min/mg of protein at 37°C.

4.7. Oxidative stress markers

4.7.1. Reduced and oxidized glutathione

The GSH and GSSG glutathione content of liver tissue and serum samples were estimated by using commercially available kit. (HT Glutathione Assay Kit, Trevigen, inc. USA). The kit uses enzymatic recycling method for estimation of glutathione. GR reduces GSSG to GSH which reacts with DTNB to form yellow colored 5-thio-2-nitrobenzoic acid (TNB). The TNB formed, absorbs at 414 nm. The intermediate glutathione thio nitrobenzoic acid (GSTNB) is reduced by GR to form more GSH and TNB. The kit contains the following: Assay buffer, reaction mix
containing GR, 5% metaphosphoric acid, GSSG and 2 M 4-vinylpyridine. The samples were diluted to 1:10 with 1× assay buffer. The standards were prepared and set as described in the kit manual. For total GSH estimation, 50 µl of GSSG and 50 µl of diluted sample were mixed with 150 µl of reaction mix and the reading was obtained at 1min intervals for 10min period. For GSSG estimation, 1 µl of 2 M 4-vinylpyridine was added to 50 µl of sample and 4 µM GSSG and incubated for 1h at room temperature. Following incubation period, 150 µl reaction mix was added and readings were obtained at 414 nm as mentioned for total GSH. GSH value was obtained by subtracting GSSG valued from total GSH. The values of serum GSH and GSSG were expressed in µmol/L of serum, while those of liver tissue were expressed as nmol/mg of protein at 37°C.

4.7.2. Lipid peroxidation

The level of lipid peroxidation in liver tissue was measured by the method of Ohkawa et al., (1979). The assay mixture containing 0.2 ml homogenate, 0.2 ml of 8.0% NaC12H25SO4, 1.5 ml of 20% CH3COOH and 1.5 ml of 0.8% thiobarbituric acid (TBA) was boiled for 60 min at 95ºC and then cooled on ice. 1 ml of double distilled water and 5 ml of n-butanol-pyridine (15:1 v/v) mixture were added to the tubes and centrifuged at 4000 rpm for 10 min. The absorbance of pinkish color developed at the organic layer was read at 532 nm. TBA reactive substances (TBARS) level was determined from the standard curve of TBA adduct formation. Series of dilutions of malondialdehyde were subjected to the above procedure and the values were plotted for standard graph. The level of TBARS was expressed as nmol of malondialdehyde formed /min/mg of protein at 37°C.

4.8. Western blot analysis

The expression levels of active caspase-3, N-terminal cleavage fragment of PARP-1, TNF-α, formation of protein carbonyls (PC) and 4-hydroxy nonenal (HNE) were analyzed by western blotting using standard protocol. The 10% homogenate of the fresh tissue sample was homogenized in ice-cold radio immuno precipitation assay (RIPA) buffer [50 mM Tris pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 0.5% sodium deoxycholate and 1% TritonX100]. Protease inhibitor cocktail from Calbiochem (1in 1000 dilution) was used to prevent proteolysis during
homogenization. The homogenates were stored immediately in -80°C as 10µl aliquots until further use. Protein concentrations of the samples were estimated by Bradford method (1976) (Section 4.5) employing bovine serum albumin (BSA) standards (Section 4.5). The samples (30µg) were subjected to sodium dodecyl sulphate- poly acrylamide gel electrophoresis (SDS-PAGE) on a 12% acrylamide gel and then transferred on to a polyvinylidene fluoride (PVDF) membrane using transfer buffer in a Hoefer –semi-dry blot apparatus (Hoefer Inc, Holliston, MA, USA).

The membranes were blocked with 1% BSA in TBST (Tris buffered saline, 0.01% Tween 20) for 2 h at RT and then incubated with appropriate primary antibodies for 2 h at RT, washed (3x15min each) with TBST and incubated with appropriate secondary antibody. The membranes were subjected to enhanced chemiluminescence reaction and densitometric analyses of the blots were done by ImageJ–analysis software (NIH, Bethesda, USA). β–actin expression was used as an internal control to confirm equal protein loading. The list of antibodies used for this study, their make and source of origin are provided in table 1.1.

4.9. Assessment of serum and liver TNF-α level

About 100 mg of frozen liver tissue was taken in 900 µl of tissue lysis buffer containing 50 mM HEPES, 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 10 mM Na₂HPO₄, 5 mM EDTA, 1mM Phenylmethane sulfonyl fluoride (PMSF) and 0.5% sodium azide. The tissue sample was homogenized and the cell debris were removed by centrifugation at 15,000×g at 4˚C for 15min. The protein concentration of the samples was estimated by Bradford method (1976) (Section 4.5). The TNF-α enzyme linked immunosorbent assay (ELISA) was performed by using Invitrogen™ ELISA kit for Rat TNF-α (KRC3011 -96 tests). The assay was performed in triplicates and the standards provided in the kit (11.7pg/ml to 750pg/ml) were used. The assay was performed according to the manual instructions and absorption was read at 450nm using Molecular devices™ single microplate reader (Molecular devices, LLC, USA). The TNF-α level of the samples were calibrated from standard values and expressed as pg/ml in serum and pg/g of liver tissue.
Table 1.1. Details of primary and secondary antibodies used in western blotting analysis

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<tr>
<td>Rabbit/anti-goat</td>
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</tbody>
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4.10. Terminal deoxynucleotide transferase dUTP Nick End Labeling (TUNEL) assay

The extent of apoptotic nuclear DNA damage in hepatocytes was analyzed by performing TUNEL staining in formalin fixed tissue sections. The formalin fixed paraffin sections were deparaffinised in 2 changes of xylene 5min each. The sections were rehydrated by immersion in descending series of alcohol (100%, 90%, 70% and 50%) and then in water. The sections were then subjected to TUNEL staining using
TACS® 2 TdT-Fluor *in situ* apoptosis detection kit, Trevigen Inc, MD, USA. Briefly, the sections were digested with 20µg/ml proteinase K solution for 15 to 20min followed by labeling with TdT labeling mixture (prepared as per manual instructions using contents provided in TACS® 2 TdT-Fluor *in situ* apoptosis detection kit) at 37°C for 1h. The slides were immersed in stop solution and then covered with Strep-fluor for 20min at room temperature in dark. The slides were then washed in PBS and mounted with aqueous mounting medium. The slides were observed under fluorescent field and 10 fields were observed per each slide. The apoptosis pattern was graded as average number of apoptotic nuclei observed per high power (400×magnification) field.

4.11. **Histopathological assessment**

Liver tissues fixed in neutral buffered formalin were dehydrated in ascending series of alcohol, cleared in xylene and embedded in paraffin. The section (4 µm thickness) were cut using Leica RM2125 rotatory microtome (Leica, Germany) and fixed on to gelatin-formaldehyde coated slides. The slides were deparaffinized in xylene and stained using Hematoxylin-eosin (H&E) staining protocol and were pictured under 10x objective field using an Olympus CX40 microscope with Progres™ image capture setup.

4.12. **Statistical analysis**

The data were analyzed by using Statistical Package for Social Sciences (SPSS 11.6 version). Statistical analyses was performed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. Differences were considered to be significant at $P \leq 0.05$ against control. Data were presented as mean ± SD (standard deviation).