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RESEARCH PAPER

# Inhibition of γ-glutamyltransferase ameliorates ischaemia-reoxygenation tissue damage in rats with hepatic steatosis

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**Background and Purpose:** Hepatic steatosis may be associated with an increased γ-glutamyltransferase (γ-GT) levels. Ischaemia-reoxygenation (IR) injury causes several deleterious effects. We evaluated the protective effects of a selective inhibitor of γ-GT in experimentally induced IR injury in rats with obesity and steatosis.

**Experimental Approach:** Otsuka Long-Evans Tokushima Fatty (OLETF) rats with hepatic steatosis were used in the current study. The portal vein and hepatic artery of left lateral and median lobes were clamped to induce ischaemia. Before clamping, 1 ml of saline (IR group) or 1-ml saline containing 1 mg·kg$^{-1}$ body weight of GGsTop (γ-GT inhibitor; IR-GGsTop group) was injected into the liver via the inferior vena cava. Blood flow was restored after at 30 min of the start of ischaemia. Blood was collected before, at 30 min after ischaemia and at 2 h and 6 h after reoxygenation. All the animals were killed at 6 h and the livers were collected.

**Key Results:** Treatment with GGsTop resulted in significant reduction of serum ALT, AST and γ-GT levels and hepatic γ-GT, malondialdehyde, 4-hydroxy-2-nonenal and HMGB1 at 6 h after reoxygenation. Inhibition of γ-GT retained normal hepatic glutathione levels. There was prominent hepatic necrosis in IR group, which is significantly reduced in IR-GGsTop group.

**Conclusion and Implications:** Treatment with GGsTop significantly increased hepatic glutathione content, reduced hepatic MDA, 4-HNE and HMGB1 levels and, remarkably, ameliorated hepatic necrosis after ischaemia-reoxygenation. The results indicated that GGsTop could be an appropriate therapeutic agent to reduce IR-induced liver injury in obesity and steatosis.

KEYWORDS

GGsTop, ischaemia, ischaemia-reoxygenation injury, steatosis, γ-glutamyl transpeptidase

**Abbreviations:** 4-HNE, , 4-hydroxy-2-nonenal; AEC, 3-amino-9-ethylcarbazole; ALT, alanine transaminase; AST, aspartate transaminase; CCK-1, cholecystokinin-1; GGsTop, 2-amino-4{[3-(carboxymethyl)phenyl](methyl)phosphono}butanoic acid; H&E, haematoxylin and eosin; HMGB1, high mobility group box 1; IR, ischaemia-reoxygenation; MDA, malondialdehyde; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OLETF, Otsuka Long-Evans Tokushima Fatty; γ-GT, γ-glutamyltransferase.

  

## 1 | INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease throughout the world with an estimate prevalence of 20%–25% people (Huang, Behary, & Zekry, 2020; Thapa et al., 2020). In individuals with non-alcoholic fatty liver disease, excess hepatic fat is associated with an increased risk of developing diabetes, hypertension, cardiovascular events, abnormal resting electrocardiography and endothelial dysfunction (Sanyal, 2011). When considered the hepatic manifestation of metabolic syndrome, the prevalence of non-alcoholic fatty liver disease is expected to rise in parallel with the increasing epidemic of obesity and metabolic syndrome (Kothari, Dhami-Shah, & Shah, 2019; Yki-Järvinen, 2014). This is especially true in economically developed countries where the imbalance between caloric intake and caloric output steadily increasing (Dite, Blaho, Bojkova, Jabandziev, & Kunovsky, 2020). The subtype of non-alcoholic fatty liver disease, characterized as non-alcoholic steatohepatitis (NASH), is a potentially progressive liver disease that can lead to cirrhosis, hepatocellular carcinoma, liver transplantation and eventually death (Younossi, 2019). This indicates that the number of patients with non-alcoholic fatty liver disease requiring liver surgery is likely to increase dramatically in the near future. The use of steatotic grafts for liver transplantation has long been associated with poor short-term and long-term outcomes (Behrns et al., 1998; Tevar et al., 2010). Complications following hepatic resection are twofold to threefold higher in patients with moderate to severe hepatic steatosis (Behrns et al., 1998; Belghiti et al., 2000). Steatotic liver responds with increased hepatocellular injury when exposed to ischaemic-reoxygenation (IR) insult, leading to worse clinical outcome (Jiménez-Castro, Cornide-Petronio, Gracia-Sancho, Casillas-Ramírez, & Peralta, 2019; Kolachala et al., 2017; Selzner & Clavien, 2001). Therefore, it is considerably important to find appropriate modalities to prevent hepatic ischaemia-reoxygenation (IR) injury in patients with non-alcoholic fatty liver disease.

**γ-Glutamyltransferase (γ-GT)** or γ-glutamyl transpeptidase catalyses the transfer of γ-glutamyl functional groups from molecules such as **glutathione (GSH)** to an acceptor like amino acid or peptide (Hiratake, Suzuki, & Kumagai, 2004). γ-GT catalyses the first step in GSH degradation and transfers the γ-glutamyl moiety of GSH to water and amino acids or peptides (transpeptidation) into glutamate and γ-glutamyl-amino acids or peptides, respectively, with a by-product cysteinyl-glycine (Perry, Wannamethee, & Shaper, 1998). The cysteinyl-glycine is one of the most reactive thiol compounds with potent physiological activity. It has been reported that this particular thiol can reduce oxygen under normal physiological conditions by reducing ferric iron $Fe^{3+}$ into $Fe^{2+}$ (Stark, Zeiger, & Pagano, 1993). This process is known as iron redox-cycling and produces reeactive oxygen species (ROS) leading to lipid peroxidation (Valko, Jomova, Rhodes, Kuča, & Musílek, 2016). GSH-driven oxidative damage generated by γ-GT could produce preneoplastic foci in liver and may lead to hepatocarcinogenesis (Stark et al., 1993). This is very significant in obesity and the increased blood γ-GT level is considered as a marker for steatosis.

### What is already known

- GGsTop is a potent inhibitor of γ-glutamyltransferase and could reduce hepatic ischaemia-reperfusion injury.

### What this study adds

- GGsTop can ameliorate oxidative stress and ischaemia-reoxygenation injury in rats with obesity and steatosis.

### What is the clinical significance

- GGsTop may serve as a potential therapeutic agent for ischaemia-reperfusion injury in obesity and steatosis.

**GGsTop** (2-amino-4{[3-(carboxymethyl)phenyl](methyl)phosphono} butanoic acid) is a novel phosphonate and a potent irreversible inhibitor of γ-GT (Han, Hiratake, Kamiyama, & Sakata, 2007). It specifically inhibits human γ-GT more than 100-fold compared to acivicin and does not affect glutamine amidotransferase (Smith, Ikeda, Fujii, Taniguchi, & Meister, 1995). GGsTop covalently binds between the side chain oxygen of Thr-381 of human γ-GT1 (hGGT1) and the phosphate of GGsTop resulting in an enzyme-inhibitor complex (Terzyan et al., 2015). No abnormalities were observed in behaviour, body weight and amount of food intake for 2 weeks after intravenous administration of GGsTop at a single dose of 30 or 100 $mg \cdot kg^{-1}$ in rats (Yamamoto et al., 2011). Recently, we have observed that treatment with GGsTop significantly reduces oxidative stress and formation of free radicals in the hepatic tissue that led to decreased IR-induced liver injury in healthy rats (Tamura et al., 2016). It is well known that serum γ-GT level increases in patients with non-alcoholic fatty liver disease as well as alcoholic liver disease (Sueyoshi et al., 2016). Otsuka Long-Evans Tokushima Fatty (OLETF) rat that has a deletion in the gene encoding (*Cckar*) **cholecystokinin ($CCK_1$) receptor** is a well-known animal model for type 2 diabetes mellitus and obesity (Sato, Asahi, Toide, & Nakayama, 1995). In the current study, we employed OLETF rats to investigate the effects of GGsTop to protect against ischaemia-reoxygenation injury in obesity-induced hepatic steatosis.

## 2 | METHODS

### 2.1 | Animals

Otsuka Long-Evans Tokushima Fatty (OLETF) rats were procured from Sankyo Labo Service Corporation (Tokyo, Japan). OLETF rats spontaneously develop obesity, steatosis, hyperglycaemia, hyperinsulinemia

and insulin resistance during their lifespan (Sato et al., 1995). They depict insulin resistance at about the age of 10–15 weeks and type 2 diabetes mellitus at about 25–30 weeks (Sato et al., 1995). The deletion of the gene encoding $CCK_1$ receptor makes the OLETF rats a $CCK_1$ receptor knockout model (Moran & Bi, 2006). Cholecystokinin, the brain-gut peptide, inhibits food intake by reducing the size and duration of a meal and its inhibitory actions are mediated through $CCK_1$ receptors. The biochemical and histopathological alterations observed in adult OLETF rats during obesity and steatosis are similar to the hepatic fatty degeneration present in obese individuals (Ota et al., 2007).

In the current study, 6-week-old male OLETF rats were housed in stainless steel wire mesh cages in air-conditioned rooms with a relative humidity of 50 ± 10% and 10–15 air changes per hour. All the animals had automatic 12-h light/dark cycles and access to general rat chow and drinking water ad libitum throughout the experiment. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

## 2.2 | Experimental design

The overall experimental protocol of present study is depicted in Figure 1. Experiments were designed to generate groups of equal size, using randomization and blinded analysis. About 41-week-old male OLETF rats were randomly divided into four groups of six rats each as sham group (body weight 652.9 ± 27.9 g), sham treated with GGsTop (sham-GGsTop group, body weight 645.8 ± 32.6 g), IR group (body weight 655.9 ± 25.6 g) and IR treated with GGsTop (IR-GGsTop group, body weight 641.5 ± 37.3 g). All animal experiments were carried out as per the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 86-23, revised 1996). The protocol was approved by the Animal Care and Research Committee of Kanazawa Medical University on the Ethics of Animal Experiments (#2015-90).

The animals were anaesthetized with intraperitoneal injections of pentobarbital (4.8 mg/100 g body weight), shaved the abdomen and disinfected with 70% ethanol. An upper abdominal ventral incision was made, without harming the internal organs, and 1 ml of blood was collected from the inferior vena cava of all rats in all the four groups. Then either 1 ml of normal saline (IR group) or 1 ml of normal saline containing 1 $mg{\cdot}kg^{-1}$ body weight of GGsTop (Wako Pure Chemical Industries, Osaka, Japan) (IR-GGsTop group) was injected into the inferior vena cava. Ischaemia was induced by clamping the portal vein and hepatic artery with a microvascular clip (30–60 $g{\cdot}mm^{-2}$, Cat# AM-1-30, Bear Medic Corporation, Ibaraki, Japan) to the left lateral and median lobes of the liver. This procedure yields approximately 70% partial ischaemia (Colletti et al., 1996; Hirakawa et al., 2019). The right and caudate lobes (30% of liver mass) retain intact portal and arterial inflow and venous outflow, preventing intestinal congestion. Laparotomy was closed by suturing immediately after the start of ischaemia and the animals were observed for 25 min maintaining at 37°C on a warm pad. At 30 min after ischaemia, abdominal incision was made again, 1 ml of blood was obtained from inferior vena cava and blood flow was restored by unclamping the vessels. The colour of ischaemic lobes was restored gradually within 1 to 1.5 min. The laparotomy was closed again using suture. The two groups of treated rats that are not subjected to IR were considered as sham group and sham-GGsTop group. The animals were spontaneously breathing during the whole procedure.

At 2 h after reoxygenation, 1 ml of blood was obtained from orbital venous plexus of each rat using capillary tube. At 6 h after reoxygenation, all the animals were killed and the blood was collected immediately. The livers were quickly removed and the median lobe was cut into 3 mm pieces and fixed in 10% phosphate-buffered formalin for histopathology and the remaining liver tissue was flash frozen in liquid nitrogen and stored at −80°C until assayed.

## 2.3 | Measurement of ALT, AST and γ-GT levels in serum

Blood was allowed to clot for 3–5 h at 37°C and serum was separated by the conventional method. Serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) were estimated using an auto-analyser that process only animal blood. Serum γ-GT activity was determined by using L-γ-glutamyl-3-carboxy-4-nitroanilide (Cat# FG11354, Funakoshi, Bunkyo-ku, Tokyo, Japan) according to the method of Theodorsen and Stromme (1976).

## 2.4 | Measurement of γ-GT, GSH and malondialdehyde (MDA) in the liver tissue

Hepatic levels of γ-GT, GSH and malondialdehyde (MDA) were measured in the liver tissues as described below. About 100 mg of frozen liver tissue was homogenized in 1 ml of ice-cold 50-mM

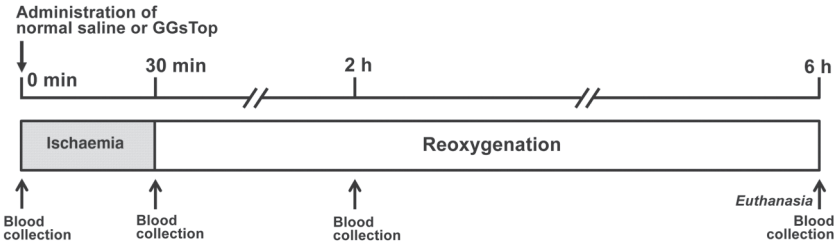


**FIGURE 1** Schematic presentation of the experimental design of ischaemia-reoxygenation (IR) and GGsTop treatment in rats with hepatic steatosis

Tris-HCl buffer (pH 8) containing 150 mmol·L$^{-1}$ NaCl, 1 mmol·L$^{-1}$ EDTA and 1% Triton X-100. Then 50 μl of the homogenate was treated with 150 μl of 5% 5-sulfosalicylic acid solution and vortexed well. It was allowed to stand on ice for 10 min and centrifuged at 12,000 *g* for 10 min at 4°C. Then, 50 μl of the supernatant was used to measure hepatic γ-GT activity as per the protocol used for serum (Theodorsen & Stromme, 1976). The activity of γ-GT in the liver homogenate was presented as units·g$^{-1}$ fresh liver tissue. One unit of γ-GT catalyses the transfer of 1 μmol of the glutamyl moiety from γ-glutamyl-3-carboxy-4-nitroanilide to glycylglycine per minute at 37°C.

Hepatic GSH content was measured using 50 μl of the above supernatant diluted with 50 μl of distilled water using a GSH assay kit (Cat# CS0260, Sigma-Aldrich, St. Louis, USA). Hepatic MDA content was determined with 30 μl of the supernatant diluted 1:1 with distilled water employing MDA assay kit (Cat# NWK-MDA01, Northwest Life Science, Vancouver, WA, USA). Hepatic GSH content is presented as nmol·mg$^{-1}$ protein and hepatic MDA levels as nmol·g$^{-1}$ fresh liver tissue.

## 2.5 | Immunohistochemical staining for 4-hydroxy-2-nonenal (4-HNE) and high mobility group box 1 (HMGB1)

Immunohistochemical staining for 4-hydroxy-2-nonenal (4-HNE) and high mobility group box 1 (HMGB1) was carried out on paraffin liver sections to evaluate the increased production of 4-HNE and HMGB1. The liver sections were deparaffinized by using xylene and alcohol, and hydrated to water. The antigen retrieval for HMGB1 was performed using 1-mM EDTA buffer (dissolved 0.37-g EDTA in 1-L distilled water, adjusted pH to 8.0 with 1-N NaOH and added 0.5-ml Tween 20) at 95°C water bath for 20 min. Immunohistochemistry was performed using a broad-spectrum histostain-plus kit (Invitrogen, Carlsbad, CA, USA). After blocking, the liver sections were treated with either 4-HNE mouse monoclonal antibody (Cat# HNE-J2, Nikken Seil, Shizuoka, Japan) or HMGB1 antibody (GeneTex Cat# GTX127344, RRID:AB_11164700) with appropriate dilutions and incubated in a moisturized chamber (Evergreen Scientific, Los Angeles, CA, USA) at 4°C overnight. The sections were then washed five times in cold PBS and incubated with broad-spectrum biotinylated secondary antibody for 2 h at room temperature. The slides were washed again and treated with streptavidin-peroxidase conjugate and incubated for another 1 h. The final stain was developed by using 3% 3-amino-9-ethylcarbazole (AEC) in *N*,*N*-dimethylformamide. The stained sections were washed and counterstained with Mayer's haematoxylin for 2 min and mounted by using aqueous-based mounting medium. The slides were examined under a microscope (Olympus BX51, Tokyo, Japan) attached with a digital camera (Olympus DP71) and photographed. In the case of treated animals, all the samples were from IR lobes. The stained area for HMGB1 and the staining intensity of 4-HNE were quantified in all the sample images using WinRoof image analysing software (Mitani, Fukui, Japan). The data are presented as square microns for HMGB1 (stained area) and percentage square microns for 4-HNE, where the total sample area was considered as 100%. The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

## 2.6 | Histopathological evaluation of the liver tissue

The formalin-fixed liver samples were processed in an automatic tissue processor optimized for liver tissue, embedded in paraffin blocks and cut into sections of 5-μm thickness. The sections were stained with haematoxylin and eosin (H&E) as per the standard protocol The stained sections were examined using an Olympus BX51 microscope attached with an Olympus DP71 digital camera and photographed. The degree of IR injury was quantified as per the method of Suzuki, Toledo-Pereyra, Rodriguez, and Cejalvo (1993) based on congestion, vacuolization and necrosis. The data are classified into five grades as 0–4, where 0 is no necrosis, 1 is minimal (focal, single cell necrosis), 2 is mild (focal necrosis, many cells), 3 is moderate (continuous, <50% of lobules) and 4 is severe (continuous, >50% of lobules). The total grade was calculated after examining 10 lobules in each liver section.

## 2.7 | Data analysis and statistics

The manuscript complies with the recommendations and requirements of the *British Journal of Pharmacology* on experimental design and data analysis (Curtis et al., 2018). Statistical analysis was undertaken only for the data set where each group size was at least $n = 5$. All declared group size is the number of independent values and all statistical analyses were done on the independent values. Outliers were not excluded from the data set. Arithmetic mean and standard error of the mean (SEM) were calculated for all the data and presented as Mean ± SE. Kruskal–Wallis test was used to compare intragroup differences between various time points. Bonferroni post hoc test was used to determine the level of significance between each time point. The difference between the IR and IR-GGsTop group at a given time point was assessed using Mann–Whitney *U* test. A value of $P < 0.05$ was considered statistically significant.

## 2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos, et al., 2019).

## 3 | RESULTS

### 3.1 | Treatment with GGsTop decreased serum ALT and AST levels and markedly inhibited serum γ-GT activity

The levels of ALT, AST and γ-GT in the serum before and after ischaemia-reoxygenation are presented in Figure 2. The mean serum ALT levels between IR and IR-GGsTop groups at 30 min after ischaemia and at 2 h after reoxygenation were not significantly different. At 6 h after reoxygenation, the mean serum ALT level was significantly reduced in GGsTop treated group (Figure 2a). Serum ALT level was markedly elevated in IR group at both 2 and 6 h, and the increased values were not significantly different. Treatment with GGsTop did not reduce increased serum ALT level at 2 h after reoxygenation (Figure 2a). There was no difference in the serum ALT levels between sham and sham-GGsTop groups. Serum AST levels depicted similar pattern as in the case of ALT before ischaemia and after ischaemia-reoxygenation in sham, sham-GGsTop, IR and IR-GGsTop groups (Figure 2b).

Serum γ-GT levels in ischaemia and after reoxygenation in sham, sham-GGsTop, IR and IR-GGsTop groups are depicted Figure 2c. Serum γ-GT levels were not altered between sham and sham-GGsTop groups at any time point. Serum γ-GT activities between IR and IR-GGsTop groups were not different before and at 30 min after ischaemia. However, in the IR group, serum γ-GT activity was significantly increased at 2 and 6 h after reoxygenation. Ischaemia-reoxygenation injury leads to hepatic necrosis and spillage of γ-GT into the blood stream. Treatment with GGsTop inhibited the activity of γ-GT in IR-GGsTop group and the level was significantly lower compared to IR group. There was no significant difference in the increased activities of γ-GT between 2 and 6 h after reoxygenation in the IR group (Figure 2c).

### 3.2 | Effect of GGsTop treatment on hepatic γ-GT, GSH and MDA after ischaemia-reoxygenation

The effect of the treatment of GGsTop on hepatic γ-GT, GSH and MDA during ischaemia-reoxygenation is demonstrated in Figure 3. Hepatic γ-GT activity was increased significantly in IR group compared to sham group, which was remarkably and significantly reduced in IR-GGsTop group (Figure 3a). There was no difference in hepatic γ-GT activity between sham and sham-GGsTop groups.

Hepatic GSH content in sham, sham-GGsTop, IR and IR-GGsTop groups is depicted in Figure 3b. GSH content in IR group was significantly lower compared to IR-GGsTop group. Treatment with GGsTop restored normal levels of GSH in IR-GGsTop group (Figure 3b). The difference between mean hepatic GSH content in sham and sham-GGsTop groups was not significant.

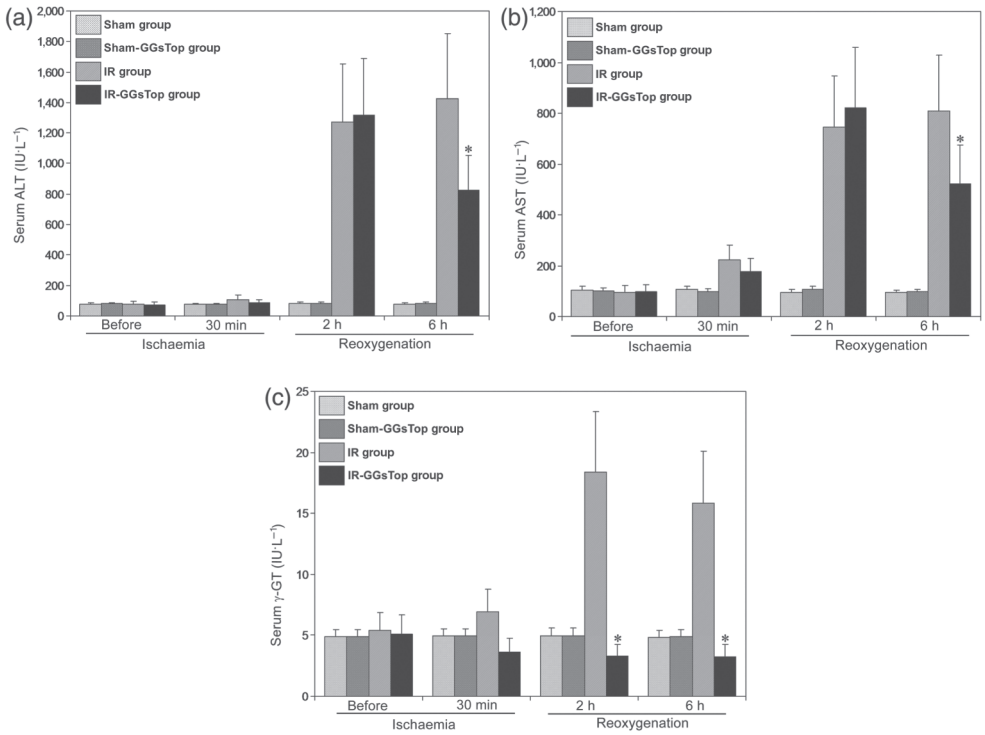


**FIGURE 2** Serum levels of alanine transaminase (ALT), aspartate transaminase (AST) and γ-glutamyltransferase (γ-GT) before and after ischaemia-reoxygenation and effects of GGsTop treatment in rats with hepatic steatosis. (a) Serum levels of ALT. The mean serum ALT level at 6 h after ischaemia-reoxygenation was significantly lower in IR-GGsTop group compared to ischaemia-reoxygenation (IR) group. (b) Serum levels of AST. The mean serum AST level at 6 h after ischaemia-reoxygenation was significantly less in IR-GGsTop group compared to IR group. (c) Serum levels of γ-GT. Serum γ-GT levels at 2 and 6 h after ischaemia-reoxygenation maintained same level as sham in GGsTop treated group compared to IR group. The values are mean ± SE ($n$ = 6). $^{**}P < 0.01$ and $^{***}P < 0.001$, when compared to the respective mean values in IR group

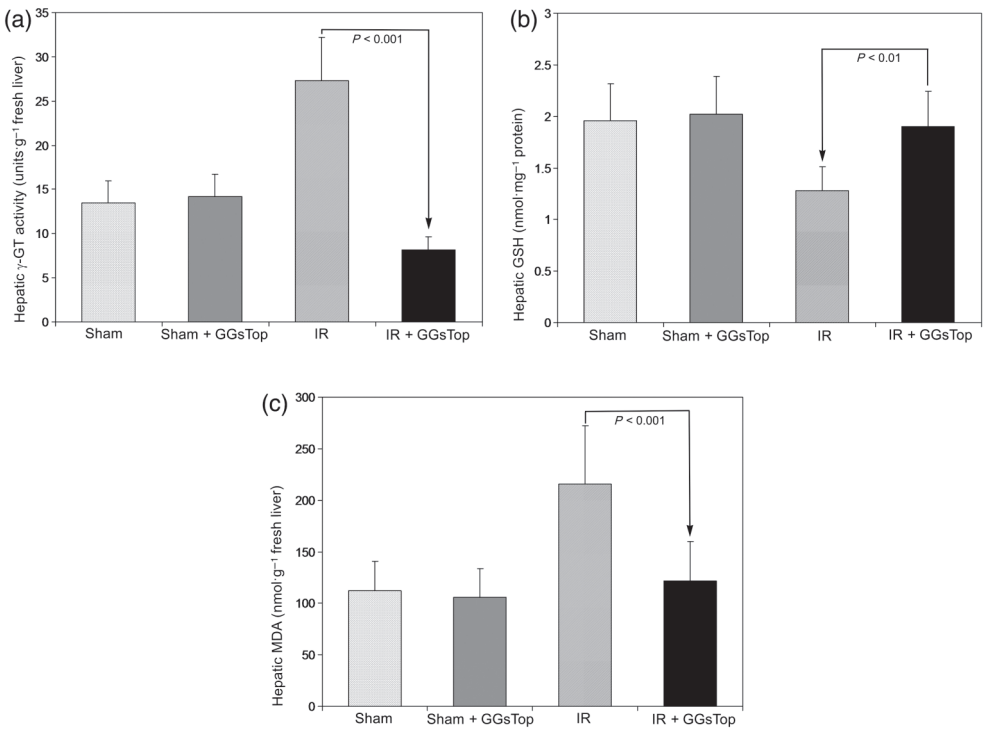


**FIGURE 3** Levels of γ-glutamyltransferase(γ-GT), glutathione (GSH) and malondialdehyde (MDA) in the liver tissue of sham, sham-GGsTop, ischaemia-reoxygenation (IR) and IR-GGsTop groups in rats with hepatic steatosis. (a) Hepatic γ-GT activities. Treatment with GGsTop inhibited the increase of hepatic γ-GT activity. (b) Hepatic GSH contents. Treatment with GGsTop restored significantly reduced hepatic GSH content in IR group. (c) Hepatic MDA levels. Treatment with GGsTop significantly reduced the elevated MDA levels in IR group. The values are mean ± SE ($n$ = 6)

Malondialdehyde (MDA) is an indicator of excessive generation of ROS that results in oxidative stress leading to cellular damage. The levels of hepatic MDA in ischaemia and after reoxygenation are presented in Figure 3c. The mean MDA level in the liver tissue was increased markedly in IR group compared to sham group. Treatment with GGsTop during ischaemia resulted in significant reduction of MDA levels in the hepatic tissue (Figure 3c). There was no significant difference between the mean hepatic MDA level in sham-GGsTop and IR-GGsTop groups.

## 3.3 | GGsTop decreased production of 4-HNE

Excessive production of ROS results in tissue oxidative stress that leads to lipid peroxidation and formation of a variety of aldehydes. 4-Hydroxy-2-nonenal is an α,β-unsaturated aldehyde formed by peroxidation of omega-6 unsaturated fatty acids such as linoleic acid and arachidonic acid (Hirakawa et al., 2019). The results of the immunohistochemical staining for 4-HNE in ischaemia and after reoxygenation are presented in Figure 4. Since the present experimental animals are obese rats with hepatic steatosis, there was mild to moderate staining for 4-HNE in the livers obtained from sham and sham-GGsTop groups, especially in pericentral zone and areas with fatty degeneration (Figure 4a). Remarkable and strong staining of 4-HNE was present in the necrotic zone with steatosis in the IR group (Figure 4a). On the other hand, only moderate staining was observed, especially in pericentral areas with steatosis in the liver sections from IR-GGsTop group. Quantification of the staining intensity of 4-HNE is presented in Figure 4b. The staining intensity of 4-HNE was significantly higher in IR group compared to IR-GGsTop group.

## 3.4 | Treatment with GGsTop reduced generation of HMGB1

HMGB1 promotes inflammatory response to sterile and infectious signals and is involved in the coordination and integration of innate and adaptive immune responses (Bianchi & Manfredi, 2007). During necrosis, the reduced HMGB1 moves to the extracellular compartment and acts as a chemokine. The results of the immunohistochemical staining for HMGB1 on paraffin liver sections after antigen retrieval using 1-mM EDTA buffer are presented in Figure 5. Staining for HMGB1 was completely absent in sham and sham-GGsTop group liver sections. Marked staining for HMGB1 was present as conspicuous spots on liver sections from IR group (Figure 5a). The staining was prominent in necrotic zone. HMGB1 translocates from nucleus to cytoplasm during ischaemia-reoxygenation injury (inset). Treatment with GGsTop resulted in significant reduction in HMGB1 staining and only few spots were present on liver sections from IR-GGsTop group animals. Quantification of HMGB1 stained area on liver sections using computer assisted image analysis software is depicted in Figure 5b. The area of HMGB1 staining in IR-GGsTop group was significantly lower compared to the IR group.

## 3.5 | Treatment with GGsTop prevented hepatic necrosis during ischaemia-reoxygenation

The histopathological alterations of liver tissue during IR injury and effects of GGsTop treatment are demonstrated in Figure 6. There was macrovesicular steatosis, hepatocyte ballooning and moderate

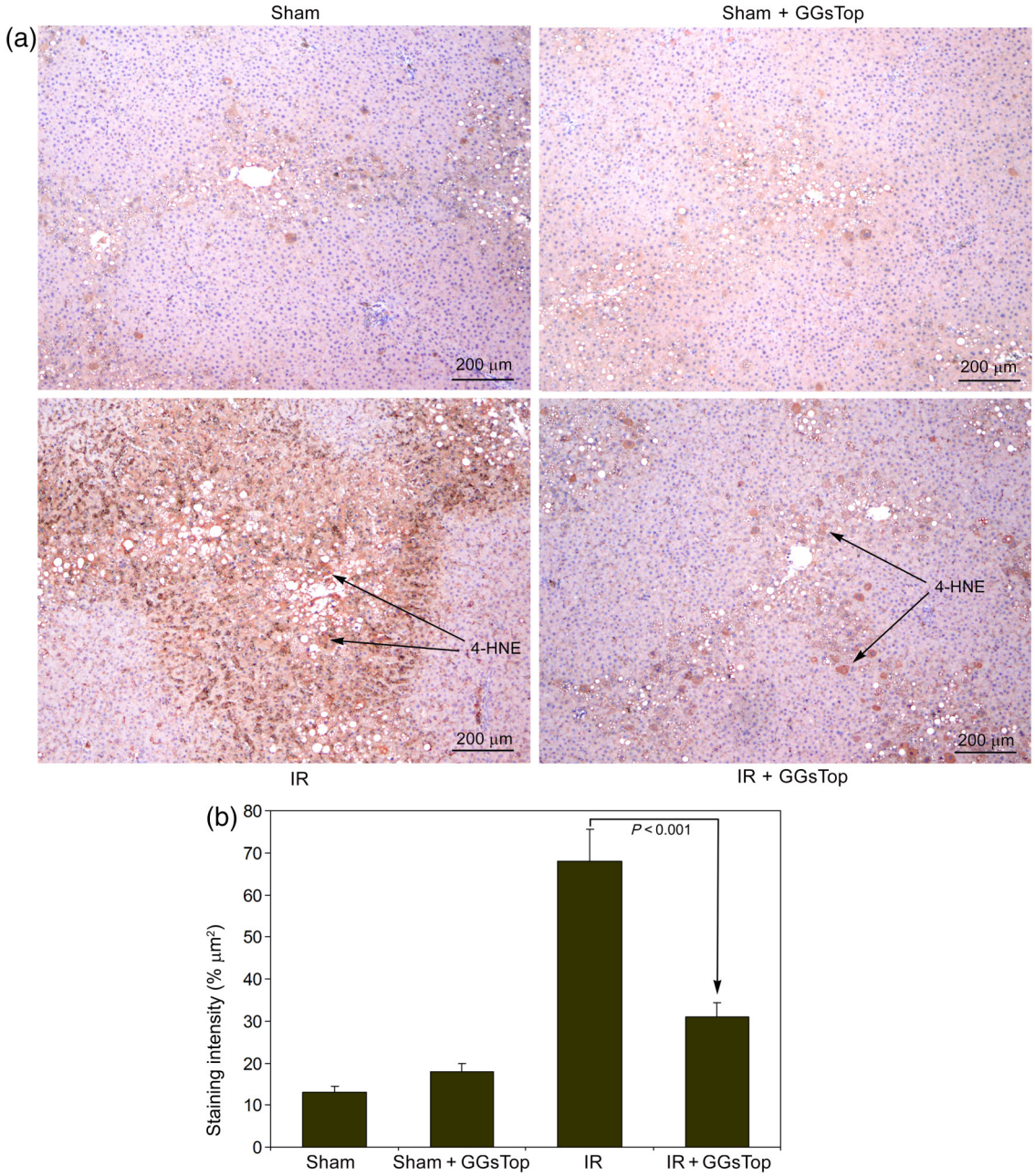


**FIGURE 4** Immunohistochemical staining for 4-hydroxy-2-nonenal (4-HNE) after ischaemia-reoxygenation and effects of GGsTop treatment in rats with hepatic steatosis. (a) Staining for 4-HNE in the liver sections from sham, sham-GGsTop, ischaemia-reoxygenation (IR) and IR-GGsTop groups. Moderate staining for 4-HNE was present in the liver sections from sham and sham-GGsTop groups in areas with fat globules and surrounding central veins. Marked and strong staining of 4-HNE was present in pericentral areas and necrotic zone in IR group. However, staining for 4-HNE was markedly and significantly reduced in IR-GGsTop group (×40). (b) Quantification of the staining intensity of 4-HNE. The data are mean ± SE ($n$ = 6)

necrosis, especially in pericentral areas in the liver sections from sham and sham-GGsTop group animals (Figure 6a). Massive hepatic necrosis with infiltration of mononuclear cells, sinusoidal congestion and vacuolization was present in many lobules in IR group (Figure 6a). Severe and haemorrhagic necrosis was prominent in pericentral areas. However, only mild necrosis and moderate congestion were present in livers obtained from IR-GGsTop group rats (Figure 6a). Treatment with GGsTop prevented intense hepatic necrosis and retained normal architecture of the liver after reoxygenation. Furthermore, there was decrease of fat globules after reoxygenation in GGsTop treated group. Figure 6b represents the degree of hepatic IR injury quantified by Suzuki's criteria based on congestion, vacuolization and necrosis. The degree of hepatic injury in IR group was significantly higher compared to IR-GGsTop group.

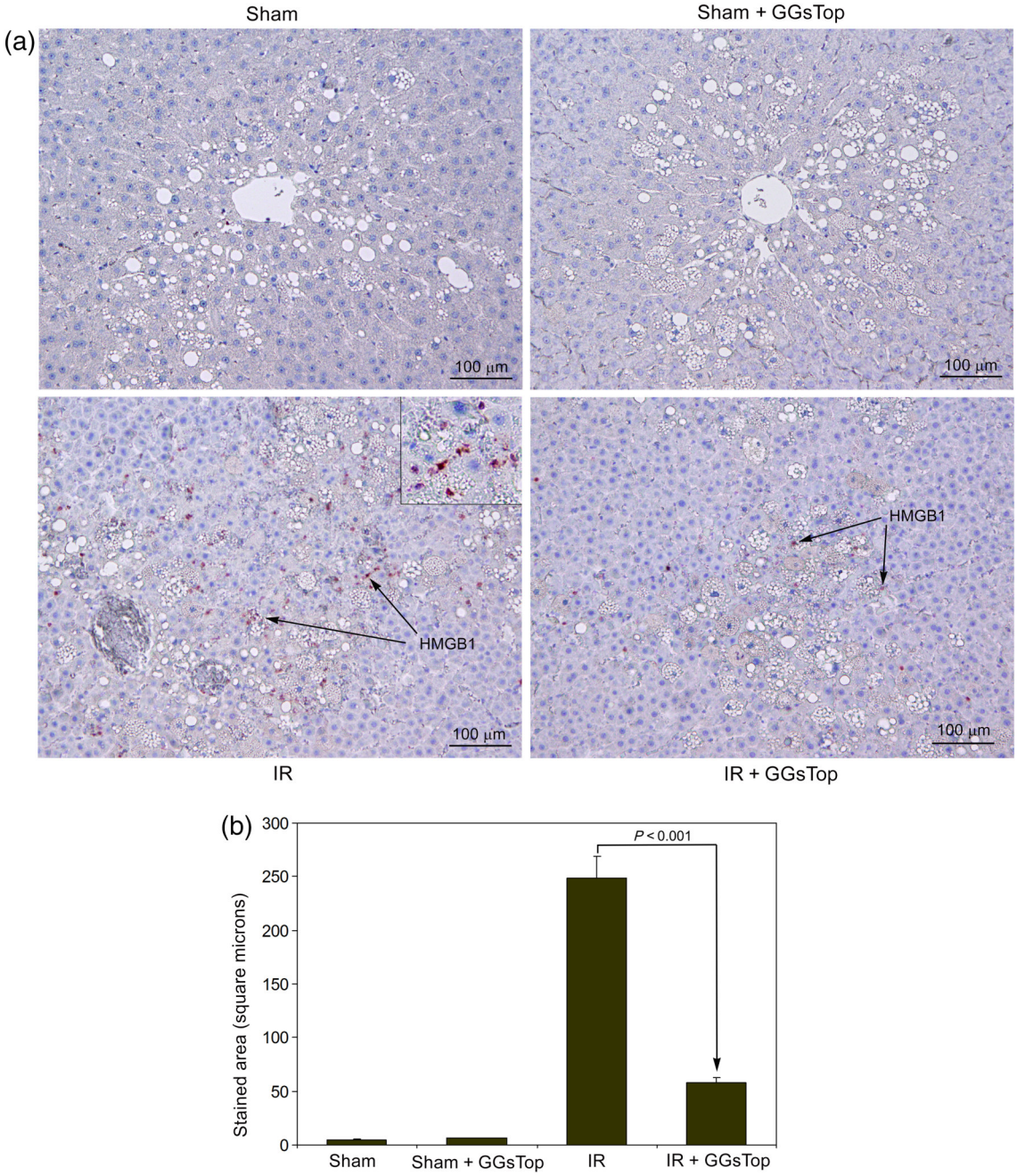


**FIGURE 5** Immunohistochemical staining of high mobility group box 1 (HMGB1) after ischaemia-reoxygenation and effects of GGsTop treatment in rats with hepatic steatosis. (a) Staining for HMGB1 in the liver sections from sham, sham-GGsTop, ischaemia-reoxygenation (IR) and IR-GGsTop groups. Staining for HMGB1 was completely absent in sham and sham-GGsTop groups. Conspicuous staining of HMGB1 was present in the necrotic zone with fat globules in IR group, while only few cells stained for HMGB1 in IR-GGsTop group (×100). Inset: higher magnification demonstrating translocation of HMGB1 from nucleus to cytoplasm during ischaemia-reoxygenation injury (×200). (b) Measurement of the staining area of HMGB1. The data are mean ± SE of six rats per group

## 4 | DISCUSSION

Hepatic steatosis, the accumulation of fat globules within the liver, is parallel with the increasing epidemic of obesity and metabolic syndrome in the Western world. Due to the economic development in many Asian countries during the two last decades, obesity, steatosis and the associated non-alcoholic fatty liver disease are dramatically increasing in Asian countries also in analogous with the West. It has been asserted that steatotic livers are less tolerant of ischaemia-reoxygenation injury leading to worst clinical outcome during liver transplantation (Chu, Hickey, Phillips, & Bartlett, 2013). Furthermore, the production of ROS would be high in obese individuals compared to healthy people, which could exacerbate the situation. In the current study, we used a rat model with steatosis in order to study the

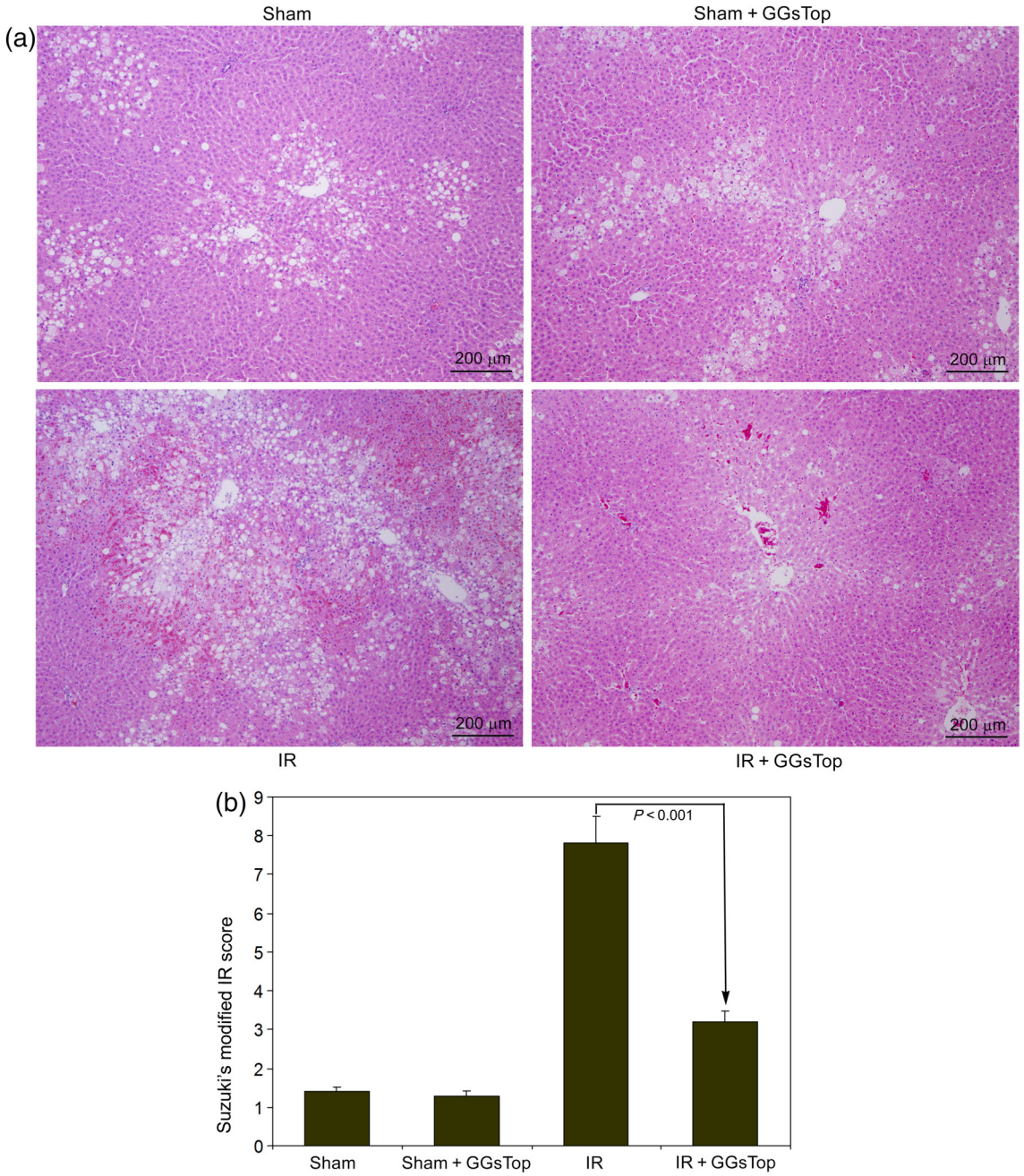


**FIGURE 6** Histopathological alterations in the liver tissue after ischaemia-reoxygenation and effects of GGsTop treatment in rats with hepatic steatosis. (a) Haematoxylin and eosin (H&E) staining of liver sections from sham, sham-GGsTop, ischaemia-reoxygenation (IR) and IR-GGsTop groups. Fatty degeneration, macrovesicular steatosis, hepatocyte ballooning and moderate necrosis were present especially in pericentral areas in sham and sham-GGsTop groups. Massive necrosis, sinusoidal congestion and vacuolization of hepatocytes were observed in IR group, which were prominent in pericentral areas. However, only mild congestion and slight necrosis were present in IR-GGsTop group. Treatment with GGsTop prevented intense hepatic necrosis and retained normal architecture of the liver after reoxygenation (×40). (b) Suzuki's modified IR score. The degree of hepatic IR injury was quantified following Suzuki's criteria based on congestion, vacuolization and necrosis. The data are mean ± SE ($n$ = 6)

beneficial effects of GGsTop to ameliorate the numerous adverse events associated with ischaemia-reoxygenation injury in obese individuals.

γ-GT is an important enzyme that involved in the transfer of γ-glutamyl functional groups from molecules such as GSH to an acceptor compound forming glutamate (Hiratake et al., 2004). Serum level of γ-GT increases in several pathological conditions and considered as a potent marker for alcoholic liver disease (Sueyoshi et al., 2016). In the liver, γ-GT is mainly present in hepatocyte microsomes and biliary epithelial cells. Ischaemia-reoxygenation injury leads

to extreme hepatic necrosis that results in spillage of γ-GT into the blood stream and subsequent elevation of serum γ-GT levels. In the current study, we also noticed a significant increase of hepatic γ-GT activity after IR-injury. Hepatic Kupffer cells are activated during IR-injury resulting in increased production of several pro-inflammatory cytokines, especially TNF-α (Hirakawa et al., 2019; Tamura et al., 2016). Hepatocytes and hepatic stellate cells could also produce TNF-α following IR-injury. Elevated levels of TNF-α along with other inflammatory cytokines cause marked increase of ROS levels, which in turn up-regulate nuclear transcription factors like NF-κB. During IR-injury, the up-regulated NF-κB translocates to nucleus and drive up the synthesis of several molecules including γ-GT that leads to an increased hepatic γ-GT activity during IR-injury (Konishi & Lentsch, 2017).

GGsTop is a potent, non-toxic, highly selective and irreversible inhibitor of γ-GT with inhibitor constant, $K_i$ of 170 μM for human γ-GT (Kamiyama et al., 2016). GGsTop depicted no cytotoxicity towards human fibroblasts and hepatic stellate cells up to 1-mM concentration (Kamiyama et al., 2016). X-ray crystallography with *Escherichia coli* γ-GT, sequence alignment and site-directed mutagenesis of human γ-GT revealed a critical electrostatic interaction between the terminal carboxylate of GGsTop and the active-site residue Lys562 of human γ-GT for potent inhibition (Kamiyama et al., 2016). GGsTop promoted collagen production in oral mucosa and has therapeutic effects on oral mucositis (Shimamura, Takeuchi, Terada, & Makino, 2019). It was reported that inhibition of γ-GT activity in lung lining fluid increased GSH levels and protected lung airway epithelial cells against oxidative stress injury and associated inflammation in a mouse of model of asthma and recommended GGsTop as a novel pharmacological agent for the treatment of asthma (Tuzova et al., 2014). It was demonstrated that enhanced γ-GT activity contributes to cardiac impairment after myocardial ischaemia/reperfusion through oxidative stress and subsequent norepinephrine overflow and treatment with GGsTop has potential therapeutic implications to prevent myocardial ischaemia/reperfusion injury (Koyama et al., 2019). In the present study, we have observed that treatment with GGsTop significantly reduced serum levels of ALT and AST at 6 h after ischaemia-reoxygenation. Furthermore, treatment with GGsTop completely prevented increase of serum γ-GT activity after ischaemia-reoxygenation at all time points. These data indicate that GGsTop could be used as a therapeutic agent to arrest IR-induced liver injury.

Generation of tissue oxidative stress from overproduction of ROS leads to lipid peroxidation that forms a variety of reactive aldehydes. Malondialdehyde (MDA) is a final product of lipid peroxidation of polyunsaturated fatty acids and one of the best markers of ROS and oxidative stress (Del Rio, Stewart, & Pellegrini, 2005). It is a highly reactive compound and mutagen that forms nucleic acid and protein adducts. The protein adducts formed from MDA are referred as advanced lipoxidation end-products (ALEs) analogous to advanced glycation end-products (AGEs) (Moldogazieva, Mokhosoev, Mel'nikova, Porozov, & Terentiev, 2019). Since increased levels of γ-GT degrade GSH and forms the highly reactive thiol compound cysteinyl-glycine, which in turn produce ROS, inhibition of increased activity of γ-GT is an important step to prevent cellular oxidative stress and subsequent membrane damage. In the current study, arrest of γ-GT activity employing GGsTop resulted in retaining normal GSH levels and prevented increase of hepatic MDA levels. This observation is in par with our previous report of maintaining normal hepatic GSH levels and blocking MDA formation during GGsTop treatment (Tamura et al., 2016).

The aldehyde, 4-hydroxy-2-nonenal (4-HNE) is a major end-product of peroxidation of omega-6 unsaturated fatty acids such as linoleic acid and arachidonic acid (Ayala, Muñoz, & Argüelles, 2014). It has three reactive groups consisting an aldehyde, a double-bond at carbon 2 and a hydroxyl group at carbon 4. The formation of excessive 4-HNE is considered as one of the most reliable biomarker of lipid peroxidation during liver injury (Poli, Biasi, & Leonarduzzi, 2008). In the present study, immunohistochemical staining for 4-HNE demonstrated that treatment with GGsTop significantly reduced ROS production and subsequent cellular membrane lipid peroxidation. Staining for 4-HNE will be completely absent in healthy normal rat liver. The current experiment was carried out in adult OLETF rats that have diabetes, obesity and steatosis, which are conditions accompanied with excessive ROS production and oxidative stress. Therefore, moderate staining for 4-HNE was present in liver sections from sham and sham-GGsTop groups, especially in pericentral areas where steatosis is prominent. This is in par with our previous study on oxidative stress and pathogenesis of NASH from obesity-induced simple steatosis in OLETF rats (Minato et al., 2014). Overall, the current data demonstrated that GGsTop could markedly reduce the formation of 4-HNE, which plays potent role in the pathogenesis and progression of liver diseases.

High mobility group box 1 is an abundant and conserved nuclear protein that is released by necrotic cells and acts in the extracellular environment as a primary proinflammatory signal (Dumitriu et al., 2005). HMGB1 actively secretes from a variety of immune and non-immune cells such as macrophages, monocytes, neutrophils, dendritic cells and natural killer (NK) cells in response to various stimuli such as LPSs and cytokines (Gardella et al., 2002). Tissue oxidative stress is one of the key factors that induce the secretion of HMGB1 from the nucleus and its relocation to extracellular matrix to play pivotal roles in regulation of the cellular response to stress (Yu, Tang, & Kang, 2015). It was reported that HMGB1 is important for oxidative stress-mediated autophagy and serves as a new target for the treatment of stress-associated disorders (Tang, Kang, Livesey, Zeh, & Lotze, 2011). In the present study, immunohistochemical staining for HMGB1 demonstrated that GGsTop treatment significantly reduced the secretion of HMGB1 to the extracellular compartment in ischaemic rat liver with steatosis. It was reported that ischaemia-reperfusion injury induces HMGB1 translocation and expression in ischaemic areas and also to the adjacent non-ischaemic lobes (Liu et al., 2011). In another study, hydrogen-enriched saline treatment protected hepatic IR injury by reducing oxidative stress and HMGB1 release (Liu et al., 2014). In the current study, inhibition of γ-GT employing GGsTop resulted in significant decrease of oxidative stress, which is evidenced through marked reduction in hepatic MDA and

4-HNE productions. This could explain the significant diminish in secretion of HMGB1 and apparent decrease of hepatic necrosis in the animals treated with GGsTop.

One of the reasons of hepatic necrosis and tissue injury is increased oxidative stress resulting from elevated levels of ROS that leads to damage to cellular components, especially to membrane lipids. Treatment with GSH, the most potent natural antioxidant, could reduce renal ischaemia-reperfusion injury in rats (Ahmadvand, Babaeenezhad, Nasri, Jafaripour, & Khorramabadi, 2019). It was observed that increased γ-GT activity contributes to cardiac damage after myocardial ischaemia/reperfusion due to increased oxidative stress (Koyama et al., 2019). In the current study, treatment with GGsTop protected hepatic parenchyma from necrotic damage after ischaemia-reoxygenation. Furthermore, inhibition of γ-GT activity reduced fat globules in IR-GGsTop group, which could be attributed to the decreased ROS and oxidative stress. Since the major cause of tissue damage during ischaemia-reoxygenation is due to oxidative stress, GGsTop may serve as an efficient modality to prevent ischaemia-reoxygenation associated hepatic injury in obesity and steatosis.

In conclusion, the results of the present study demonstrated that GGsTop treatment inhibits the elevation of γ-GT activity during ischaemia-reoxygenation that resulted in significant reduction of serum AST and ALT activity and increase of hepatic GSH levels. Furthermore, it depicted marked decrease in hepatic MDA and 4-HNE productions, drastic diminish in the secretion of HMGB1 and the ultimate protection of hepatic parenchyma in ischaemic rat liver. Thus, the data indicate that GGsTop could serve as a potential therapeutic agent to prevent IR-induced injury in livers with steatosis.

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## AUTHOR CONTRIBUTIONS

Mi.T. and Mu.T. involved in conception and design of the study, obtained funding and provided technical and material support. R.K., N.H., K.K., T.S. and K.O. carried out all the major experiments and collected the data. Y.U. carried out histopathological evaluation. J.G. analysed and interpreted the data and wrote the manuscript.

## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, Immunoblotting and Immunochemistry and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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