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Original Article

Does sirolimus attenuate the damage induced by partial warm ischemia/reperfusion injury in rat livers?

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A B S T R A C T

Ischemia/reperfusion (I/R) injury of liver occurs in a number of clinical settings as hepatic resection surgery. In this study we have addressed the possible protective effect of sirolimus, a potent novel immunosuppressant which acts on signal transduction pathways in CD4+ T-cells, and could potentially modulate immune/inflammatory cellular reactions involved in hepatic injury induced by I/R in rats.

Rat livers were subjected to partial warm hepatic ischemia for 30 min followed by 0, 1, 3 and 7 days reperfusion. I/R-induced liver damage was characterized both histologically and biochemically. Histologically I/R induced necrosis and inflammatory cell infiltration during different reperfusion time intervals. Moreover, biochemical investigation showed significant elevation in serum aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transferase and lactate dehydrogenase levels as compared with sham-operated group.

Pretreatment with sirolimus (1mg/kg/day for 4 days before I/R) did not improve histological manifestations but caused gradual improvement of liver function tests. To evaluate the possible mechanisms involved in I/R injury myeloperoxidase (MPO) malondialdehyde (MDA), tumor necrosis factor alpha (TNF-α) and reduced glutathione (GSH) were measured in liver homogenates. I/R disturbed the redox state, increased neutrophil infiltration and raised TNF-α. Sirolimus improved MDA, MPO and TNF-α, but did not induce any change in GSH level. It might be concluded from the current study that sirolimus exerts some degree of hepatic protection and improved hepatocyte membrane integrity. This effect may be attributed to its immunosuppressive action through modulating the release of the inflammatory cytokine TNF-α rather than its antioxidant property

Key Words: Sirolimus, ischemia/reperfusion, myeloperoxidase, malondialdehyde, tumor necrosis factor-alpha, oxidative stress, liver injury

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1. INTRODUCTION

I/R can occur during resection of large hepatic tumors, management of hepatic trauma of diverse origins, vascular reconstructions, and liver procurement for transplantation *[Delva et al., 1989; Huguet et al., 1992; Powner, 2004]*. Cellular injury is linked to activation of several inflammatory pathways, which include free radicals *[Gonzalez-Flecha et al., 1993]*, cytokines *[Colleti et al., 1996]* and neutrophil infiltration *[Jaeschke and Farhood, 1991]*.

Events occurring during early phase of reperfusion injury include activation of Kupffer cells and infiltration of $CD4^+$T-lymphocytes. These cells initiate a complex inflammatory pathway by generating a number of cytokines that culminate in the hepatic accumulation of neutrophils *[Jaeschke and Farhood 1991; Colletti et al., 1990; Lentsch et al., 2003]*. Recruited neutrophils directly damage hepatocytes by releasing reactive oxidants and proteases (such as myeloperoxidase, elastase and collagenase) *[Granger, 1988]*. These in turn are responsible for the later phase of liver injury induced by I/R *[Jaeschke et al., 1990 and 1992; Lentsch et al., 2000; Jaeschke and Hasegawa 2006]*.

Protection against I/R injury by sirolimus is reported from some animal models like pancreatic I/R injury *[Serr et al., 2007]* and small bowel ischemic injury *[Puglisi et al., 1996]*; little is known on its effect on liver I/R injury. This study was undertaken to investigate the possible hepatoprotective action of sirolimus on a model of partial warm hepatic I/R injury.

2. MATERIALS AND METHODS

2.1 Animals

Adult male albino Wistar rats, purchased from Schistosoma Biological Supply Centre at Theodor Bilharz Research Institute (TBRI, Giza, Egypt), weighing 150-250 g were used. They were housed in Theodor Bilharz Research Institute under suitable laboratory conditions to keep their body temperature during the whole experiment. The animals were fed a standard commercial pellet diet (El Kahira Company for Oils and Soap, Cairo, Egypt) and allowed free access to water.

2.2 Drug used

Sirolimus (Wyeth-company, USA) was given orally in a dose of 1 mg/kg 4 days before ischemia/reperfusion *[DiJoseph et al., 1992*; *Serr et al., 2007*]. The drug was suspended in 2% cremophore-El (Sigma Chemical Company St Louis, Missouri).

2.3 *Hepatic ischemia/reperfusion*

Rats were anesthetized with intraperitoneal thiopental (25 mg/kg) *[Urakami et al., 1997]*. An upper abdominal midline laparotomy was performed. The ligament attachments connecting the liver, diaphragm, abdominal wall, and neighboring organs were dissected *[Mota-Filipe et al., 2002]*. Liver hilus was exposed to find the common hepatic artery and the portal vein. Ischemia was induced by clamping the portal vein and hepatic artery with a vascular microclip to the left lateral and median lobes of the liver. This procedure yields approximately 70% partial ischemia *[Nishimura et al., 1986; Colletti et al., 1996]*. The right lateral, caudate lobes and papillary processes (30% of liver mass) retain intact portal and arterial inflow and venous outflow, preventing intestinal congestion *[Colletti et al., 1996]*. Clamping induced immediate discoloration of hepatic lobes. After 30 min ischemia, reperfusion of the ischemic lobes was performed by removing the clamp *[Papadopoulos et al., 2005]*, the colour of the ischemic lobes (ILs) was restored gradually during 1 to 1.5 minutes. Laparotomy was closed with silk 3/0 immediately after the termination of 30 minutes of ischemia and removing the clip. During ischemia, the abdominal incision was closed temporarily to prevent loss of body temperature and insensible perspiration.

2.4 Experimental design

Animals were randomly allocated into three groups (35 rats each). These were a sham group where animals underwent laparotomy with the same anesthesia duration but without liver I/R, an I/R group where animals were exposed to I/R but were not treated and a sirolimus-treated group where animals were given sirolimus orally in a dose 1 mg/kg/day for 4 days before I/R *[DiJoseph et al., 1992; Serr et al., 2007]*. The drug was suspended in 2% Cremophore-El. Each group was further divided into five subgroups. Rats of four subgroups were sacrificed either immediately after the 30 minutes ischemia (zero group) or after 1, 3 and 7 days of reperfusion respectively. The fifth subgroup (five to ten rats) was kept to record the number of dead animals every week for a period of 5 months to find out the survival rate of animals in all experimental groups.

2.5 Blood and Tissue preparation

Immediately after sacrificing the rats, blood was collected and serum was separated by centrifugation at 3000 r.p.m for 10 min. Serum was used for the estimation of liver function tests: alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT) and lactate dehydrogenase (LDH). After collecting the blood the entire liver was removed, both the ischemic lobes (ILs) and the non ischemic lobes (NILs) were weighed separately. Each was homogenized in ice-cold saline to obtain a 25% w/v homogenate using a glass homogenizer (B. Braun Melsungen AG). The homogenate was then frozen rapidly at -70°C after being divided into several fractions. These fractions were used for the determination of thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), myeloperoxidase (MPO) and tumor necrosis factor-alpha (TNF-α).

2.6 Histological studies

Parts of ILs and NILs were fixed in 10% formalin and embedded in paraffin. Sections of 4 microns were stained with hematoxylin and eosin *[Banchroft et al., 1996]* for microscopic examination.

2.7 Measurement of serum enzyme activities

Serum **ALT and AST** activities were determined using a test reagent kit (Spectrum Diagnostics kit)

according to the method described by Reitman and Frankel [1957]. **LDH** was determined kinetically using a test reagent kit (Greiner) according to the method of Buhl and Jackson [1978]. **GGT** assay was performed kinetically using a test reagent kit (Greiner) according to the method of Szasz [1969].

2.8 Measurement of hepatic reduced glutathione (GSH)

The level of glutathione was determined in liver homogenate according to the method described by Ahmed et al., [1991]. Protein was precipitated in liver tissue homogenate by adding 0.5 ml of precipitating reagent (10 g of Trichloroacetic acid and 0.186 g of disodium edetate dissolved in 100 bidistilled water) to 0.5 ml homogenate. The mixture was centrifugated at 4000 r.p.m for 10 minutes. The supernatant was used for the assay of glutathione. To 0.1ml of the resulting clear supernatant, or standard glutathione solution, 0.85 ml of phosphate buffer was added followed by 0.05 ml Ellman's reagent. The absorbance of yellow color was measured within 5 minutes at 412 nm using double beam spectrophotometer against blank. The blank was treated exactly as the sample but using bidistilled water. GSH level in liver homogenate was first obtained from the standard curve as mg/ml.

This was then converted to mg/g tissue according to the following equation:

GSH (mg/g wet tissue) = GSH (mg/ml) (tissue weight) x dilution factor

2.9 Measurement of hepatic lipid peroxidation

Lipid peroxidation was assayed as the generation of thiobarbituric acid-reactive substances according to Uchiyama and Mihara [1978]. Briefly, after preparation of liver homogenate, in a centrifuge tube, 3 ml of 1 % ortho-phosphoric acid and 1 ml of 0.6 % thiobarbituric acid were added to 0.5 ml sample either liver homogenate or standard. The mixture was heated for 45 min in a boiling water bath. After cooling, 4 ml n-butanol were added and mixed vigorously. The n-butanol layer was separated by centrifugation at 4000 r.p.m for 15 min. The absorbance of the pink colored product was measured at 535 and 520 nm against a blank containing 0.5 ml distilled water instead of the sample. The difference in absorbance between the two readings ($\Delta A_{535-520}$) was taken as the level of TBARS in the sample. Results were expressed as nmol/ml from the standard curve then converted to nmol/g wet tissue according to the following equation:

TBARS (nmol/g wet tissue) =

$$\text{TBARS} \times \text{dilution factor}/(\text{tissue weight})$$

3. Measurement of hepatic myeloperoxidase

Tissue MPO was extracted according to the method described by Mancuso *et al*., [1997]. A volume of 0.25 ml of 10% aqueous homogenate was treated with 1 ml of phosphate buffer (pH 7.4), and centrifuged at 14000 r.p.m for 15 minutes at 4 ˚C (Megafuge 1.OR, Heraeus, Germany). The supernatant was discarded and the pellets were resuspended in 1.25 ml of phosphate HTAB buffer (pH 6), sonicated for 1 minute in an ice bath. The specimens were freeze-thawed 3 times [Bradley *et al.*, 1982] after which the sample tubes were centrifuged at 4000 r.p.m for 20 minutes to remove any particulate matter (Megafuge 1.OR, Heraeus, Germany). The resulting supernatant was assayed for MPO activity. In a glass microcuvette, 30 µl of the supernatant were added to 870 µl of the reaction mixture. The change in absorbance at 460 nm was measured at 1 minute intervals for 3 minutes using a double beam computerized spectrophotometer. One unit of MPO activity was defined as that degrading one micromole of peroxide per minute at 25˚C. Then MPO activity in U/g tissue was obtained by the following equation=

$$\frac{\Delta A_{\text{Sample}} \times \text{U in ml standard used}}{\Delta A_{\text{Standard}}} \times \text{dilution factor}$$

3.1 Measurement of hepatic Tumor necrosis factor-alpha

TNF-α was measured by a quantitative sandwich enzyme immunoassay technique with microplates pre-coated with a rat TNFα-specific monoclonal antibody (AssayMax Rat TNF-alpha ELISA kit). This was according to the method described by Caso *et al.*, [2001].

3.2 Reagents

All chemicals were obtained from Sigma-Aldrich Chemical Company, USA, Riedel-de-Haën, Germany and Fluka, USA. Sirolimus was obtained from Wyeth-company, USA.

3.3 Statistical Analyses

Values were expressed as mean ± SEM. Statistical differences between groups were computed by one-way analysis of variance **(ANOVA).** Tukey-Kramer multiple comparison tests were used to compare between treated and control groups. The level of significance was accepted at $p<0.05$. Statistical analysis was performed by the aid of Instate version 2 computer programs (Graphpad Software, Inc., San Diego, USA).

4. RESULTS

4.1. Effect of sirolimus on serum levels of ALT, AST, LDH and GGT

I/R-induced hepatic injury was indicated by an immediate increase in serum levels of ALT, AST, GGT and LDH at zero time. This, elevation dramatically peaked at 1 day of reperfusion. Thereafter, the levels began to decrease gradually to levels slightly greater than those in the sham groups at the respective time intervals. Pretreatment with sirolimus reduced ALT activity to half the value of I/R at zero time (21.40 ± 2.39) and then declined to about third the value on the 1st day (23.72 ± 3.21), finally, at 3rd and 7th day of reperfusion the activity of ALT reached corresponding sham activity (Figure 1A).

Moreover, sirolimus reduced AST activity to less than half the value in I/R groups at zero time (34.22 ± 1.65) and showed further reduction such that at the 3rd and 7th day of reperfusion it reached the corresponding sham activity (Figure 1B). In addition, sirolimus lowered LDH and GGT levels to various degrees, and their levels approached the normal level at the 3rd and 7th days of reperfusion (Figure 1Cand 1D respectively).

4.2. Effect of sirolimus on hepatic GSH

In the group subjected to I/R, the GSH contents of ischemic lobes were markedly decreased during reperfusion at zero time (4.55 ± 0.47). They were then elevated to about the level in the sham group at 1st day (7.89 ± 0.51) and then declined on the 3rd (4.62 ± 0.38) and 7th days (4.33 ± 0.46) of reperfusion. Pretreatment with sirolimus did not produce any alteration in GSH level during various reperfusion times (Table 1).

4.3. Effect of sirolimus on hepatic lipid peroxidation

In I/R group, TBARS content of ischemic lobes were markedly increased during reperfusion and reached more than twice the sham content after 1st day (273.57 ± 28.94), then declined to about half the sham value at 7th day (187.13 ±16.11). Pretreatment with sirolimus lowered to various degrees, the peak reached at 1 day after I/R but all values approached the normal level at the 3rd and 7th days of reperfusion (Table 2).

4.4. Effect of sirolimus on the activity of hepatic myeloperoxidase (MPO)

I/R caused infiltration of neutrophils as determined by MPO activity. MPO reached more than twice the sham value after the 1st day (0.220 ± 0.01), then declined to about half the sham activity at the 7th day of reperfusion (0.149 ±0.01). Pretreatment with sirolimus reduced the maximal elevation in MPO that was observed on the 1st day of I/R, then all values approached the normal level at the 3rd and 7th days of reperfusion (Figure 2).

4.5. Effect of sirolimus on hepatic TNF-α

In the group subjected to I/R, marked elevation in TNF-α was recorded at zero time (9.62 ± 0.50), then it started to decline on the 1st day (9.45 ± 0.61) till 7 days (5.84 ± 0.38) of reperfusion. Pretreatment with sirolimus produce a slight significant reduction in the value of TNF-α at zero time (7.64 ± 0.27) and declined to about half the level of I/R at 1day (6.89 ± 0.29), and reached corresponding sham value at 7th day (Table 3).

4.6. *Non-ischemic lobes*

There was no significant difference in all the parameters measured between the non-ischemic lobes of the sham group, the I/R group and the sirolimus pretreated group.

4.7. *Histopathological examination*

The sham groups and the non-ischemic lobes of all groups in the study showed a normal histological structure normal histological structure of the central vein and surrounding hepatocytes (Fig 3A) at zero, 1, 3 and 7 days of reperfusion. In the ischemic lobes of I/R group there was fatty changes in between hepatocytes at zero time, mild necrosis at 1 day (Fig 3B), mild necrosis with fatty changes at 3 days (Fig 3C) and mild necrosis, inflammatory cell infiltration in the hepatic capsule at 7 days of reperfusion (Fig 3D). Sirolimus pretreated groups showed mild apoptosis in hepatocytes at zero time, mild necrosis underneath the hepatic capsule at 1 day (Fig 3E), moderate necrosis in the deep hepatic parenchyma at 3 days (Fig 3F). Moreover, there was mononuclear leucocytes inflammatory cell infiltration with fibrosis in the portal area at 7 days of reperfusion (Fig 3G).

Table 1: Effect of sirolimus pretreatment on GSH content in ischemic lobes of rat livers subjected to partial warm hepatic ischemia for 30 min followed by 0, 1, 3 and 7 days reperfusion

Groups	GSH (mg/g liver) at corresponding times after reperfusion			
	Zero	1 day	3 days	7 days
Sham	7.06 ± 0.33	6.99 ± 0.50	7.25 ± 0.34	6.94 ± 0.42
I/R	4.55 ± 0.47*	7.89 ± 0.51	4.62 ± 0.38*	4.33 ± 0.46*
Sirolimus (1 mg/kg/day)	4.45 ± 0.55	7.49 ± 0.49	5.70 ± 0.55	5.86 ± 0.49

Sirolimus was given orally for 4 days before I/R.
Each value represents the mean of 5-7 observations ± SEM
Statistical differences between groups were computed by one-way analysis of variance (ANOVA). Tukey-Kramer multiple comparison tests were used to compare between treated and control groups. The level of significance was accepted at $p<0.05$.
*Significant difference from sham group at $P< 0.05$
** Significant difference from I/R group at $P< 0.05$

Table 2: Effect of sirolimus pretreatment on TBARS content in ischemic lobes of rat livers subjected to partial warm hepatic ischemia for 30 min followed by 0, 1, 3 and 7 days reperfusion

Groups	TBARS (nmol/mg) at corresponding times after reperfusion			
	Zero	1 day	3 days	7 days
Sham	91.46 ± 5.60	96.99 ± 3.47	97.06 ± 13.06	99.50 ± 14.76
I/R	177.88 ± 13.79*	273.57 ± 28.94*	192.31 ± 12.23*	187.13 ±16.11*
Sirolimus (1 mg/kg/day)	100.52 ± 13.29**	124.48 ± 16.05**	109.34 ± 10.84**	124.80 ± 10.65**

Sirolimus was given orally for 4 days before I/R
Each value represents the mean of 5-7 observations ± SEM
Statistical differences between groups were computed by one-way analysis of variance (ANOVA). Tukey-Kramer multiple comparison tests were used to compare between treated and control groups. The level of significance was accepted at $p<0.05$.
*Significant difference from sham group at $P< 0.05$
** Significant difference from I/R group at $P< 0.05$

Table 3: Effect of pretreatment with sirolimus on tumor necrosis factor alpha (TNF-α) content in ischemic lobes of rats subjected to 30 min partial hepatic ischemia followed by 0, 1, 3 and 7 days reperfusion.

Groups	TNF-α (pg/ml) at corresponding times after reperfusion			
	Zero	1 day	3 days	7 days
Sham	5.33 ± 0.83	5.70 ± 0.05	5.46 ± 0.46	5.25 ± 0.75
I/R	9.62 ± 0.50*	9.45 ± 0.61*	8.23 ± 0.0*	5.84 ± 0.38
Sirolimus (1 mg/kg/day)	6.98 ± 0.51**	6.48 ± 0.31**	6.20 ± 0.33**	6.71± 0.77

Sirolimus was given orally for 4 days before I/R.
Each value represents the mean of 5-7 observations ± SEM.
Statistical differences between groups were computed by one-way analysis of variance (ANOVA). Tukey-Kramer multiple.
comparison tests were used to compare between treated and control groups. The level of significance was accepted at $p<0.05$.
*Significant difference from sham group at $P< 0.05$.
** Significant difference from I/R group at $P< 0.05$.

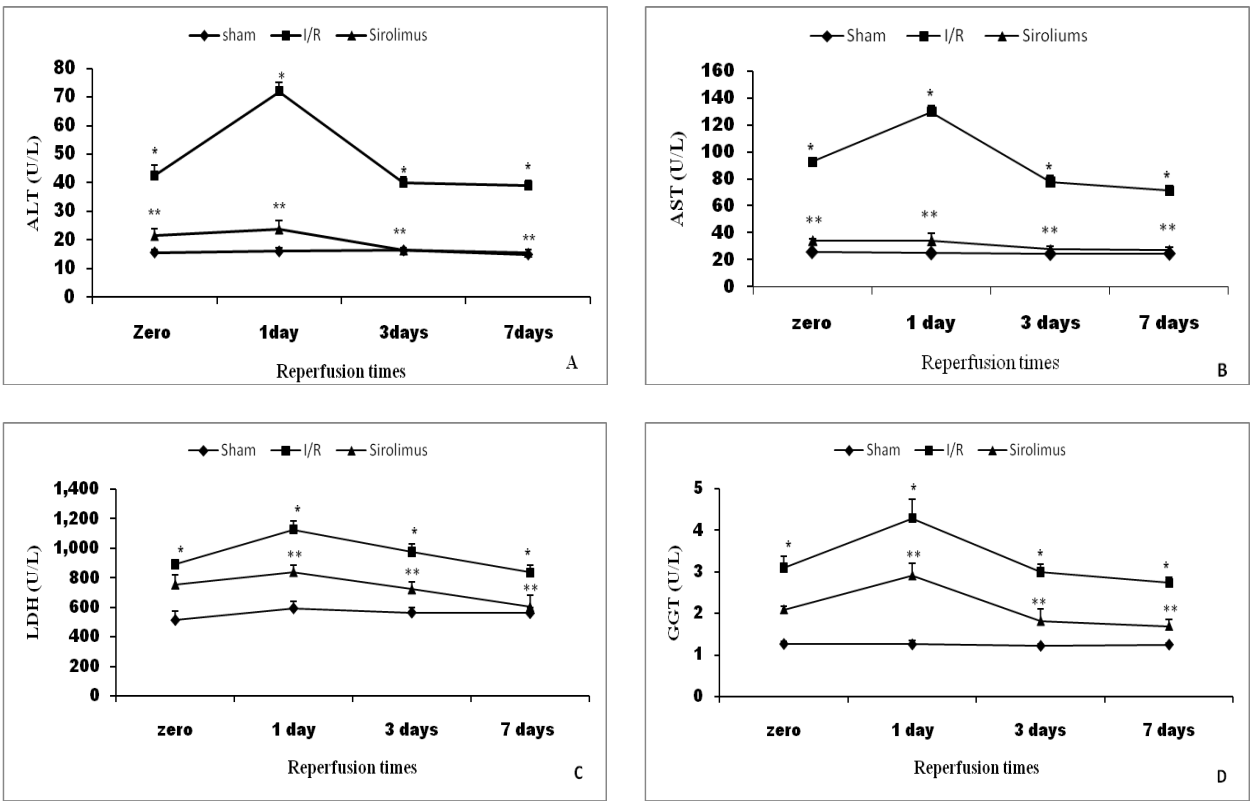

Figure 1: The effect of pretreatment with sirolimus on ALT(A), AST(B), LDH(C) and GGT (D) activities in serum of rats subjected to 30 min partial hepatic ischemia followed by zero, 1 day, 3 and 7 days reperfusion as compared to I/R group
Sirolimus was given orally (1 mg/kg/day) for 4 days before I/R
Statistical differences between groups were computed by one-way analysis of variance (ANOVA). Tukey-Kramer multiple comparison tests were used to compare between treated and control groups.
*Significant difference from sham group at P< 0.05
**Significant difference from I/R group at P< 0.05

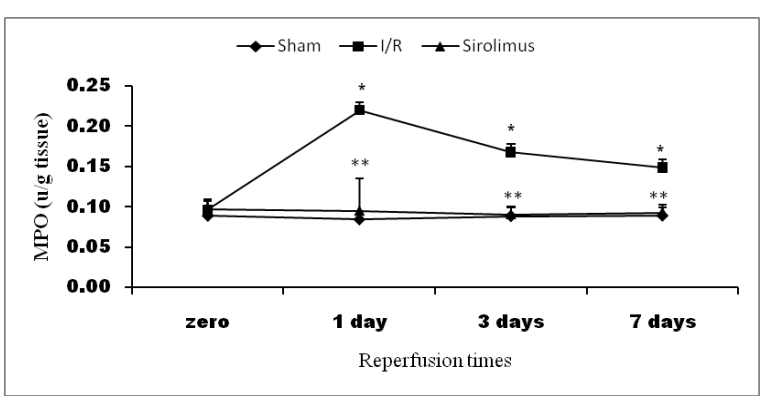

Figure 2: The effect of pretreatment with sirolimus on MPO activity in ischemic lobes of rats subjected to 30 min partial hepatic ischemia followed by zero, 1 day, 3 and 7 days reperfusion as compared to I/R group
Sirolimus was given orally (1 mg/kg/day) for 4 days before I/R
Each value represents the mean of 5-7 observations ± SEM
Statistical differences between groups were computed by one-way analysis of variance (ANOVA). Tukey-Kramer multiple comparison tests were used to compare between treated and control groups. The level of significance was accepted at p<0.05.
*Significant difference from sham group at P < 0.05
** Significant difference from I/R group at P < 0.05

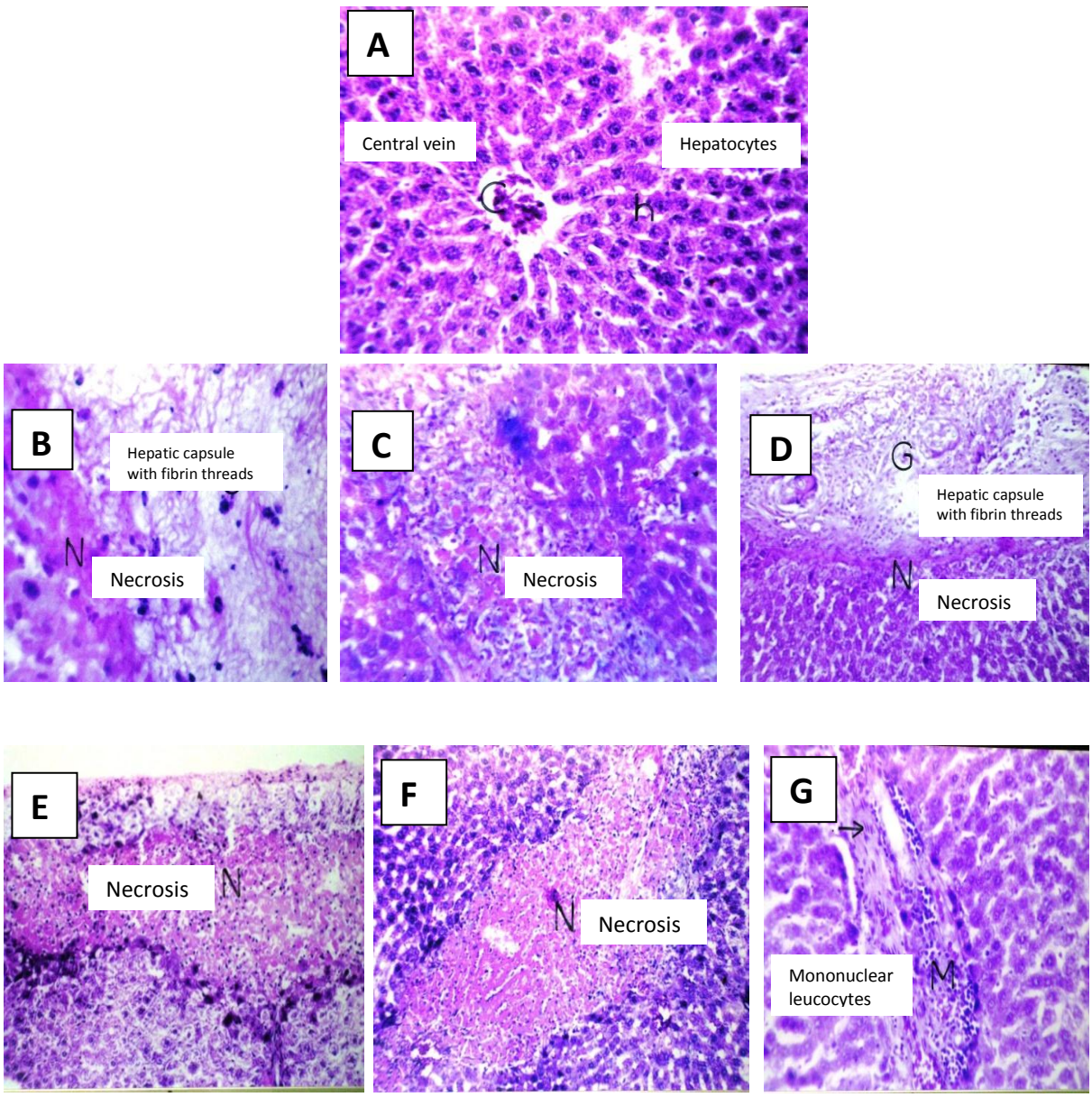

Figure 3: Representative micrographs of liver tissue from I/R and sirolimus treated rats at various reperfusion times (H & E X100)

A) Normal histological structure of the central vein (C) and surrounding hepatocytes (h) observed in the sham groups and the non-ischemic lobes of all groups at zero, 1, 3 and 7 days of reperfusion.

B) Fatty changes in between hepatocytes in the ischemic lobes of I/R group at zero time, and mild necrosis in the hepatic capsule with fibrin threads (G) 1 day after I/R.

C) Mild necrosis (N) with fatty changes in the ischemic lobes of I/R group 3 days after I/R.

D) Mild necrosis (N), inflammatory cell infiltration and fibrin threads exudation in the hepatic capsule (G) in the ischemic lobes of I/R group at 7 days after I/R.

E) Mild apoptosis in hepatocytes at zero time in the sirolimus pretreated groups and mild necrosis underneath the hepatic capsule at 1 day after I/R.

F) Moderate necrosis in the deep hepatic parenchyma at 3 days in the sirolimus pretreated groups.

G) Mononuclear leucocytes inflammatory cell infiltration (M) with fibrosis in the portal area at 7 days in the sirolimus pretreated groups.

5. DISCUSSION

Liver I/R injury represents a complex interplay between mediators, cellular, vascular endothelial and tissue specific factors with inflammation being a common feature *[Serracino-Inglott et al., 2001; Banga et al., 2005; Huang et al., 2007]*. All these interrelated processes caused microvascular and hepatocellular dysfunction which was shown in this as an immediate increase in serum activity of ALT, AST, GGT and LDH at zero time in the I/R group. This upsurge dramatically peaked at 1 day of reperfusion. Thereafter, the activity began to decline gradually to levels slightly greater than those in the sham groups at the respective time intervals. This may be due to ROS generated by Kupffer cells at the early phase of reperfusion, which destroy hepatocyte cell membranes through lipid peroxidation *[Jaeschke et al*., 1990]. Pretreatment with sirolimus displayed a significant reduction in ALT, AST, GGT and LDH as compared to untreated I/R group at respective time intervals. This effect might be an evidence for to the ability of sirolimus to restore hepatocyte membrane integrity. This finding is in harmony with those of Matsuda *et al* [1998] and Lu *et al* [2009].

There are two distinct phases of liver reperfusion injury *[Lentsch et al., 2000]*. The intermediate phase (1-6 h after reperfusion) is characterized by oxidant stress, where the production and release of reactive oxygen species (ROS) appears to directly bring about hepatocellular injury. Sometimes this phase is known as early phase of liver reperfusion injury. In this investigation, hepatic GSH and MDA were measured to reveal the remarkable damage induced by I/R. Concerning hepatic GSH content, it was reduced immediately at zero time then elevated at 1 day then declined gradually at the 3rd and 7th day of reperfusion. The immediate drop in GSH level at zero time may reflect the oxidative stress that develops in the liver during ischemia. This result is in accordance with those of Grattagliano *et al* [1999], Settaf *et al* [2000] and Fukai *et al* [2005]. In the liver, oxidative stress results from Kupffer cell activation during ischemia which overwhelms the antioxidant ability, resulting in enhanced lipid peroxidation and diminution of total and reduced glutathione. As for the gradual lowering in GSH content during the 3th and 7th day of reperfusion, this may be brought about by oxidative stress induced by neutrophil infiltration at that time, leading to ROS production. In this study, MDA started to escalate from zero time, reached its peak at 1 day then decreased gradually during the 3rd and 7th day of reperfusion. The rapid ascent of MDA reflects the existence of oxidative stress during the early phase of reperfusion by Kupffer cells. This induces hepatocellular injury and lipid peroxidation to hepatocytes membrane through ROS and proteolytic enzymes *[Dieter et al., 1988; Jaeschke et al., 1990]*. This finding is in harmony with Fukai *et al* [2005].

As for the effect of sirolimus on hepatic GSH, no significant difference was observed between sirolimus groups and I/R groups during the various reperfusion times. In spite of this, sirolimus pretreatment relegated MDA at all reperfusion times .This may give an indication that sirolimus has low antioxidant scavenger ability if compared with other antioxidant drugs.

The late phase of liver injury, from 6 to 48 h after hepatic reperfusion, is an inflammatory disorder mediated by recruited neutrophils that damage hepatocytes through release of ROS *[Teoh and Farrell, 2003]*. Neutrophil infiltration was determined as MPO activity. In the I/R group, there was no change in MPO activity at zero time, indicating that no neutrophil infiltration takes place at that time. Accumulation of PMNs started from 1 to 7 days of reperfusion, where MPO activity reached the maximal level during the 1st day and then was driven down gradually. Sirolimus pretreatment downgraded neutrophil accumulation in the rat liver after I/R; this was revealed by a decrease in MPO level during various reperfusion times. This result is consistent with the work of Matsuda *et al* [1998]. These researchers reported that the immunosuppressants azathioprine, cyclosporine A, tacrolimus, and rapamycin reduced the expression of cytokine-induced neutrophil chemoattractant (CINC) 24 hours after ischemia/reperfusion of the liver. Therefore, one possible explanation for lowered MPO activity in the sirolimus-treated group is that, by reducing CINC production by Kupffer cells, sirolimus attenuates neutrophil accumulation and reperfusion injury *[Matsuda et al., 1998; Serr et al., 2007]*. Another indirect pathway is through blocking the effector function of CD4+ T helper cells and CD8+ cytotoxic T cells, activation of monocytes and proliferation and differentiation of B cells *[Schmidbauer et al., 1994; Abraham and Wiederrrecht, 1996; Schuler et al., 1997]*. It has been reported that CD4+ T lymphocytes are a key regulators in initiating I/R- induced inflammatory response in the liver *[Lu et al., 2009]* through liver recruitment of neutrophils following I/R *[Caldwell et al., 2005]*. Accordingly, neutrophil infiltration will be reduced as was shown by the lowering MPO level during various reperfusion times. Histopathological findings confirm these biochemical findings.

TNF-α also contributes to I/R injury. The present study showed that TNF-α, in the I/R group, spilled over significantly and nearly reached its maximum at zero time, remained high at 1st day and then it dropped off gradually during the following time intervals. On

the other hand, sirolimus treatment brought about a reduction in the raised TNF-α when compared to I/R group. Since I/R injury directly activates the resident CD4+T cells, which once activated, secrete a number of cytokines including IFN-*γ*, TNF-*β*, and GM-CSF. These, in turn, either directly or indirectly (through Kupffer cell–secreted cytokines as TNF-α) activate neutrophils to infiltrate the liver. Accordingly, sirolimus by blocking CD4+T cell function will decrease TNF-α release from Kupffer cells.

Concerning the histopathological findings, rat livers in the I/R group showed mild necrosis after 1 and 3 days of reperfusion; this was amended on the 7th day. Many studies demonstrated that ROS and proteases produced by Kupffer cells and neutrophils cause hepatocellular necrosis *[Mavier et al., 1988; Li et al., 1993; Nieminen et al., 1995; 1997; Jaeschke et al., 1999]*. Activated neutrophils play a central role in I/R injury. Jaeschke and Smith [1997] reviewed the mechanisms of liver parenchymal cell injury by neutrophils starting from sequestration in the hepatic vasculature, transendothelial migration, adherence to hepatic parenchymal cells and ending by cytotoxicity. Yabe *et al* [2001] examined the effect of catalase and SOD derivatives on the late phase of I/R injury and proved that they are effective in preventing neutrophil-mediated hepatic I/R injury.

Histopathological findings of the sirolimus-pretreated group revealed apoptosis and necrosis during various reperfusion times. Similarly, Puglisi *et al* (1996) using a model of small bowel ischemic injury failed to record any decrease in ischemia TNF-α after giving either cyclosporine or rapamycin. They came to the conclusion that each drug plays a significant role in attenuating I/R injury in the gut and that there are cytoprotective and anti-inflammatory mechanisms of these drugs independent of T-cell signal transduction that provide some protective effect in small bowel ischemia. Furthermore, T-cell-mediated immune mechanisms may not be associated with the adverse effects of small bowel ischemia/reperfusion injury.

Moreover, the data of Serr et al (2007) indicate that administration of sirolimus improves microcirculation at a very early stage after pancreatic I/R injury, but results in an impairment of the recovery phase. They found that 90 minutes after ischemia, intravital microscopy revealed an improved functional capillary density and reduction of adherent leucocytes and platelets in the sirolimus-treated group compared to the vehicle-treated controls. In contrast, on day 5 after ischemia, the pancreatic tissue of sirolimus-treated animals showed a higher grade of histological injury and higher rate of apoptotic cells than the vehicle controls.

It should be noted that TNF-α triggers programmed cell death (PCD) either apoptosis or necrosis, only when new protein or RNA synthesis is inhibited. Besides, it induces ROS accumulation in many cell types, and these ROS are important mediators of PCD *[Fiers et al., 1999]*. For that reason, pretreatment with sirolimus would lead to reduction or inhibition of protein synthesis giving a chance for TNF-α to induce PCD during I/R. This obviously indicates that TNF-α production was not shut off by sirolimus. *In vitro* studies demonstrated that sirolimus possesses apoptosis-promoting effects on immune cells *[Woltman et al., 2001]* and on non- immune cells *[Migita et al., 1997; Lieberthal et al., 2001]*. In fact, it has been reported that sirolimus seems to interfere with cell survival signaling mediated via the Akt-mTOR-p70S6k pathway which compromises the inhibition of proapototic protein BAD *[Lieberthal et al., 2001]*. Furthermore, sirolimus treatment resulted in higher rate of apoptosis in murine vein grafts 4 weeks after transplantation *[Schachner et al., 2005]*.

6. CONCLUSION

Sirolimus pretreatment resulted in amelioration of elevated ALT, AST, LDH and GGT during various reperfusion times. Besides, it reduced the raised MDA, MPO and TNF-α production. However, histopathological examination revealed some degree of necrosis and apoptosis. It should be noted that sirolimus did not improve GSH level at any time during reperfusion. Sirolimus pretreatment had the ability to improve hepatocyte membrane integrity and had minor antioxidant activity. This may be considered one of the causes of cell death induced by sirolimus. Moreover, Sirolimus hepatoprotection may result from its ability to interfere with T cell response to cytokines and accordingly blocks their proliferation and differentiation. Therefore, one can conclude that sirolimus exerts only minor hepatoprotective action during hepatic I/R.

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