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Does Polydatin Have A Favorable Contribution To Liver Preservation In Ischemic Preconditioning Model?

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Abstract **Original Research Article**

Aim: The study aimed to investigate the antioxidant and anti-inflammatory effects of polydatin (PD) in liver preservation in an experimental early- and late-phase ischemic preconditioning (IP) model in rats. ***Materials and Methods:*** A total of 50 Wistar Albino rats were randomly divided into 5 equal groups. (I) The control group, received intraperitoneal saline injection only. (II) Early-phase IP (IE) group was formed at the end of second hour after the I/R procedure, (III) Early-phase IP + polydatin (IEP) group received intraperitoneal PD 40 mg/kg/day for 3 days after the I/R procedure, (IV) Late-phase IP (IL) group was formed at the end of day 3 after the I/R procedure, and (V) Late-phase IP + polydatin (ILP) group received intraperitoneal PD 40 mg/kg/day for 3 days and underwent hepatectomy at the end of day 3 after the I/R procedure. After blood and tissue sampling, all the rats were decapitated. Serum levels of Total antioxidant status (TAS), total oxidant status (TOS), alanine transaminase (ALT), aspartate transaminase (AST), hypoxia-inducible factor 1-alpha (HIF-1α), catalase (CAT), superoxide dismutase (SOD), and Glutathione Peroxidase (GSH-Px) in tissue samples were measured. Histopathological examination was performed using a light microscope. ***Results:*** CAT, SOD, and GSH-Px levels were increased in the PD groups (IEP and ILP) compared to the non-PD groups (IE and IL) and the increase in the CAT levels was statistically significant ($p<0.05$). HIF-1α levels were significantly lower in the PD groups compared to the non-PD groups ($p<0.05$). TOS levels were lower and TAS levels were higher in the PD groups compared to the non-PD groups although no significant difference was established in the two parameters ($p>0.05$). AST and ALT levels were lower in the PD groups compared to the non-PD groups and the ALT levels in the ILP group were significantly lower than in the IL group ($p<0.05$). Sinusoidal congestion, cytoplasmic vacuolization, polymorphonuclear leukocyte (PMN) infiltration, necrosis, and portal inflammation scores were significantly lower in the PD groups compared to the non-PD groups and the difference between ILP and IL groups was statistically significant ($p<0.001$). ***Conclusion:*** The results indicated that polydatin makes a favorable contribution to liver preservation in IP model by preventing hepatocyte injury.

Key words: Liver, ischemic preconditioning, polydatin, antioxidant.

INTRODUCTION

Safe and long-term preservation in liver transplantation is highly essential for the interventions facilitating the transport, quality, and vitality of the organ. The primary aim in organ preservation is to increase organ viability by preventing irreversible organelle damage caused by hypoxia/ischemia by downregulating the cellular metabolic rate via hypothermia [1, 2]. A 10 °C drop in temperature resulting from this downregulation leads to a 1.5- to 2.5-fold decrease in the metabolic rate [3]. Ischemia/reperfusion injury (IRI) refers to a series of events leading to injury in the affected cells and the organs, or in those reached by the resultant radicals washed through the circulatory system. Ischemic preconditioning (IP) is a protective mechanism created by a single or recurrent brief ischemia/reperfusion (I/R) periods against the tissue or organ injury resulting from subsequent I/R insult [4]. Remote ischemic preconditioning, on the other hand, is the protection provided by brief I/R periods applied in distant tissues or organs against the I/R injury [5].

Ischemic preconditioning (IP) occurs in two phases: early and late IP. Early-phase IP (IE) occurs within several minutes and disappears within several hours after the I/R periods. IE exerts its protective effect through the activation of a complex cascade involved in secondary transduction of the IP effect triggered by mediators such as adenosine and bradykinin [6]. In contrast, late-phase IP (IL) occurs within 24 h and disappears within 48-72 h after the I/R periods. IL exerts its protective effect through protein induction [6]. This phase is highly critical since it induces RICP and leads to a significant reduction in morbidity and mortality after liver transplantation or major liver resection by reducing IRI [7]. Compared to continuous clamping, IP is tolerated better and also reduces the requirement for perioperative transfusion and postoperative complications in liver surgery [8].

Polydatin (PD) is a natural precursor of resveratrol with antioxidant and anti-inflammatory activity [9]. Moreover, PD reduces the production of reactive oxygen species (ROS) in mitochondrial electron transport chain complex III, thereby suppressing oxidative stress-related inflammatory response and reducing IRI through its oxidative activity [10, 11]. PD exerts its hepatoprotective effect by inhibiting the release of glutamic-pyruvic transaminase, accumulation of glutathione, and the formation of malondialdehyde (MDA) and nitric oxide while activating superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and reversing the activation of TNF-α, interleukin 1-β, cyclooxygenase-2 (COX-2), and inducible NO synthase (iNOS) in the liver [12]. In liver tissue, early-phase IRI occurs within 0-2 hours after IRI as a result of increased Ca+ concentration and ROS production in the cell, primarily leading to hepatocyte injury. In contrast, late-phase IRI occurs within 6-48 h as a result of the migration of neutrophils, macrophages, lymphocytes, thrombocytes to the liver, leading to an inflammatory response and alterations in sinusoidal blood flow [13].

BACKGROUND

In this study, we aimed to investigate the antioxidant and anti-inflammatory effects of PD in liver preservation in the lower extremities in the rats induced with recurrent I/R followed by early- and late-phase IP.

MATERIALS AND METHODS

After obtaining an approval from Mustafa Kemal University Animal Experimentations Local Ethics Board, the study was conducted at Mustafa Kemal University Medical School Experimental Animals Laboratory with the financial support from Mustafa Kemal University Scientific Research Projects Directory. A total of 50 adult male Wistar Albino rats weighing 275-300 g were used for the experiment. The rats were randomly divided into 5 groups including 4 experimental groups and 1 control group. Before the experiment, the rats were allowed a 7-days adaptation period under a 12 h light/dark cycle.

Experimental Protocol and Groups (Table-1)

(I) Control group: Only intraperitoneal saline was injected. In all 4 experimental groups, I/R was induced by a 40-min occlusion of the proximal left lower extremity with continuous cycles of 10-min on and 10-min off using an elastic bandage (1 cm width x 30 cm length) under ketamine (4 mg/100 g) anesthesia [14]. (II) Early-phase IP (IE) group was formed at the end of second hour after the I/R procedure, (III) Early-phase IP + polydatin (IEP) group received intraperitoneal PD 40 mg/kg/day for 3 days after the I/R procedure, (IV) Late-phase IP (IL) group was formed at the end of day 3 after the I/R procedure, and (V) Late-phase IP + polydatin (ILP) group received intraperitoneal PD 40 mg/kg/day for 3 days and underwent hepatectomy at the end of day 3 after the I/R procedure. A median laparotomy followed by total hepatectomy was performed after blood collection from the tail vein under ketamine (4 mg/100 g) and xylazine (1.5 mg/100 g) anesthesia. Liver samples were immersed in UW solution and kept in an organ transport container at 10 °C for 6 h and then taken for biochemical and histopathological examinations [15].

Homogenization of Liver Tissue and Tissue Parameters

Resected liver tissues were washed with physiological saline and then divided into two parts, of which one part was wrapped in aluminum foil and stored at -80 °C until analysis. The tissues were divided into portions of 0.5-0.9 g and then homogenized in a Tris-HCl buffer (pH 7.4) at 16,000 rpm for 3 min in an ice bath. The resulting homogenate was centrifuged at 5,000 x g, +4 °C for 1 h and then the supernatants were separated. The CAT, GSH-Px, and SOD concentrations in tissue homogenate and the serum HIF-1α levels were measured at a wavelength of 450 nm using a commercially available ELISA kit (Multiskan™ GO, Thermo Scientific, USA) with the immunoassay method.

Measurement of ALT, AST, TAS/TOS and oxidative stress index (OSI)

Serum was separated by centrifuging at 1,500 x g and then portioned and stored at 80 °C until analysis. Serum alanine transaminase (ALT) and aspartate transaminase (AST) activities were measured spectrophotometrically using an autoanalyzer (Abbott Architect c8000). Total antioxidant status (TAS) and total oxidant status (TOS) levels were measured using he colorimetric method developed by Erel [16, 17]. Oxidative stress index (OSI) was calculated as the liver TOS-to-liver TAS ratio, with the TAS values changed to mmol/l. OSI was calculated depending on the following formula: OSI (AU-arbitrary units) = TOS (mmolH2O2/l)/TAS (mmol Trolox/l) [18].

Histopathological Examination

After the storage of liver tissues in the organ transport container for 6 h [15], the right lobe of each liver was removed for light microscopy analysis and the tissue sections were fixed in 10% formaldehyde solution. The tissues were cut into 4 μm thick sections, stained with hematoxylin-eosin, and then examined using a light microscope (Olympus BX 51, Tokyo, Japan) by a pathologist blinded to the study groups. Based on the study reported by Giovanardi *et al*., [19], hepatocyte injury was assessed semiquantitatively according to the sinusoidal congestion (0-3), cytoplasmic vacuolation (0-3), polymorphonuclear leukocyte (PMN) infiltration (0-4), liver necrosis (0-4), and portal inflammation (0-2) scores in liver tissue (Table-2) [15, 19].

Statistical Analysis

Data were analyzed using SPSS for Windows Version 21.0 (Armonk, NY: IBM Corp.). Quantitative variables were expressed as mean ± standard deviation (SD). Group means were compared using nonparametric tests. The groups were compared using Kruskal–Wallis test and binary comparisons were performed with Mann Whitney-U test.

RESULTS

Table-3 presents the concentrations of biochemical parameters measured in each group. The groups were initially compared with the control group and then with the PD (IEP and ILP) and non-PD (IE and IL) groups.

Assessment of antioxidant enzymes and oxidative stress parameters (Table-3)

The CAT, SOD, and GSH-Px levels in the IEP and ILP groups were higher than those in the IE and IL groups and the increase in the CAT levels was statistically significant (Figure 1) (p<0.05). HIF-1α levels were significantly lower in the IEP and ILP groups compared to the IE and IL groups (p<0.05). TAS levels were higher and TOS levels were insignificantly lower in the IEP and ILP groups compared to the IE and IL groups (p>0.05). OSI (AU) levels were significantly lower in the IEP and ILP groups compared to the IE and IL groups (p<0.05) (Figure-2).

Assessment of biochemical and histopathological parameters

The AST and ALT levels in the IEP and ILP groups were lower than those in the IE and IL groups and the ALT levels in the ILP group were significantly lower than in the IL group (p<0.05). Albumin levels were significantly increased in the IEP and ILP groups compared to the IE and IL groups, with a significant increase found in the IEP group (p<0.05) (Figure-3). The groups showed severe histopathological alterations including congestion, necrosis, and PMN infiltration. The highest mean congestion score (MCS) was in the IL group (Table-4). However, sinusoidal congestion, cytoplasmic vacuolization, PMN infiltration, necrosis, and portal inflammation scores were significantly lower in the IEP and ILP groups compared to the IE and IL groups (p<0.001).

Table-1: Scheme of study groups

	Control	IE	IEP	IL	ILP
n	10	10	10	10	10
IPt.	-	3h.	3h.	>48h	>48h
PDt.	-	-	3 day/b	-	3 day/a
BSt.	0.h	3.h	3.h	>48h	>48h
Portal inflammation	17.00	35.50	24.30	31.80	18.90

IP: ischemic preconditioning, IE: early ischemic preconditioning IEP: ischemic preconditioning+Polydatin, IL: late ischemic preconditioning, ILP: ischemic preconditioning+polydatin. IPt: ischemic preconditioning time, PDt: Polydatin implementation time, b: before, a: after, BS: Bloom samples time.

Table-2: Histopathological evaluation [18]

Sinusoidal congestion (score 0-3)	
0	none
1	mild (dilation of the centrilobular vein)
2	moderate (dilation of the centrilobular vein plus sinusoidal dilation of zone 3)
3	severe (dilation of the centrolobular vein sinusoidal dilation of zone 3 and zone 2)
Cytoplasmic vacuolation (score 0-3)	
0	none
1	mild (rare perivenular hepatocytes)
2	moderate (numerous perivenular hepatocytes)
3	severe (alterations of the hepatocytes beyond 2 one 3)
PMN infiltration (score 0-4)	
0	none
1	rare cells
2	focal
3	multi-focal
4	diffuse and uniformly intense
Liver necrosis (score 0 -4)	
0	absence of necrosis
1	spotty necrosis (scattered necrotic hepatocytes at zone 3)
2	focal necrosis (periportal or perivenular or mid acinar necrosis)
3	multifocal necrosis (necrosis in mere than one acinar zone)
4	lobular necrosis (necrosis bridging between vascular inflow and outflow structures - diffuse, zones 1,2,3)
Portal inflammation (score 0-2)	
0	none
1	mild /moderate (mild or moderate inflammation in some portal area)
2	severe (moderate or severe inflammation in most portal area)

Table-3: Concentrations of biochemical parameters in the groups

Enzyme/unit	**Control**	**IE**	**IEP**	**IL**	**ILP**
CAT (pg/ml)	84.16± 34.39	116.19±39.77	126.65±86.78	94.05 ± 31.83	122.83±91.07
SOD (pg/ml)	280 ± 190	720 ± 320	810 ± 510	570 ± 270	603 ± 190
GSH-Px (pg/ ml)	228.25 ± 42.82	206.59 ± 90.23	257.90 ± 22.41	222.78 ± 67.88	244.04±104.88
HIF-1α (pg/ ml)	57.76 ± 16.96	90.23 ± 21.60	48.93 ± 24.33	76.30 ± 17.76	45.05 ± 18.89
TAS (mmol Trolox equ./L)	1.32 ± 0.31	1.64 ± 0.43	1.86 ±0.41	2.78±0.76	2.95±0.6
TOS (mmol Trolox equ./L)	0.357 ± 0.07	0.43 ± 0.07	0.4 ± 0.07	0.56 ± 015	0.51 ± 0.16
OSI (AU)	0.27	0.26	0.213	0.21	0.17
AST-IU/L	142.90 ± 54.1	329.80 ± 156.6	235.2 ±62	153.50 ±82.3	105.50±23.1
ALT-IU/L	55.00 ± 14.05	65.10 ± 18.90	63.00 ± 11.28	45.30 ± 11.70	37.30 ± 7.40
*ALB- g/dl**	*2.50 ± 0.24*	*1.79 ± 0.11*	*2.30 ± 030*	*2.17 ± 0.29*	*2.39 ±0.20*

*cl was used in place for dl

Table-4: Histopathological scores in the groups

	Control	**IE**	**IEP**	**IL**	**ILP**
Sinusoidal congestion (score 0-3)	11.15	34.70	24.25	38.60	18.80
Cytoplasmic vacuolation (score 0-3)	5.50	25.50	18.50	27.50	20.50
PMN infiltration (score 0-4)	9.15	32.80	24.00	39.25	22.30
Liver necrosis (score 0-4)	15.60	31.10	28.95	35.20	16.65
Portal inflammation	17.00	35.50	24.30	31.80	18.90

IE: early ischemic preconditioning IEP: ischemic preconditioning+Polydatin, IL: late ischemic preconditioning, ILP: ischemic preconditioning+polydatin. IEP/IE, $p<0.001$; ILP/IL, $p<0.001$

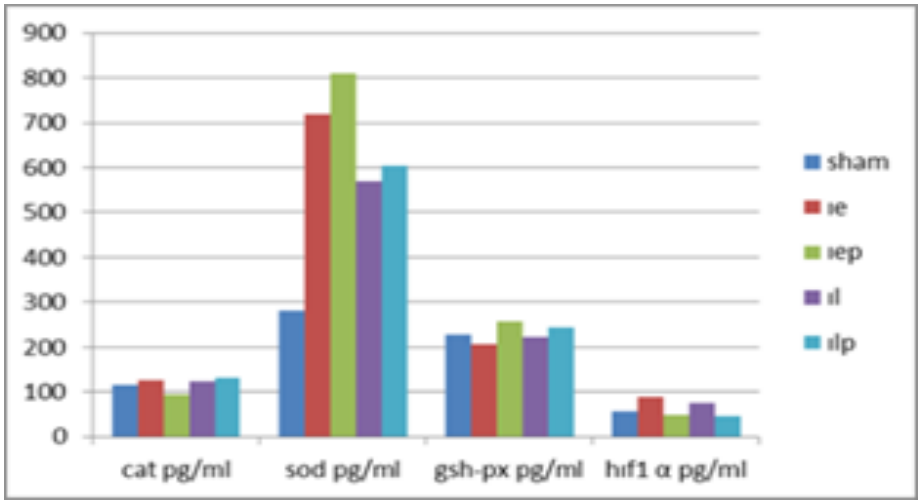

Fig-1: Assessment of antioxidative enzyms parameters. cat:catalase, sod:superoxidedismutase, gsh-px: glutation peroxidase, hıf 1α: hypoxic ischeamic factor alfa

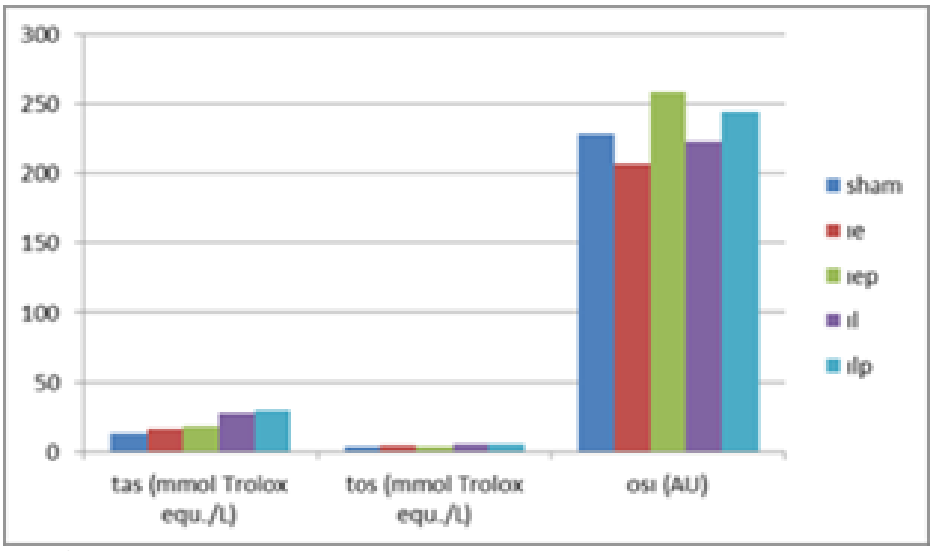

Fig-2: Assessment of oxidative stress parameters. tas: total antioxidant status, tos: total oxidant status, osı: oxidative stress index

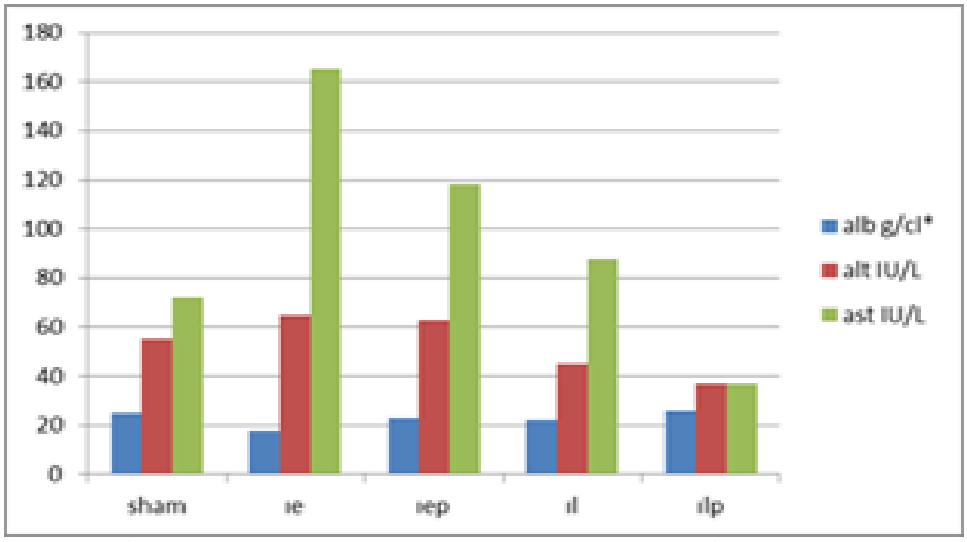

Fig-3: Assessment of biochemical parameters. alb: albumin, alt: alanine amino transferase, ast: aspartate transaminase

DISCUSSION

Reactive oxygen species (ROS), i.e. superoxide anion radical ($O2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), are produced by I/R injury and lead to the impairment of the cell membrane, resulting in cell lysis [20]. In the lower extremities induced with recurrent ischemia/reperfusion injury (IRI), early-phase IP (IE) occurs within 3 h and late-phase IP (IL) occurs within 24 h and disappears within 48-72 h after the I/R periods [6]. The signaling cascade of IP is initiated through the activation of G-protein-coupled receptors (GPCRs) by locally produced agonists including adenosine, bradykinin, catecholamines, acetylcholine, angiotensin II, and opioids. Following receptor activation, protein kinase C (PKC) initiates the antioxidative response after being phosphorylated. PKC plays a key role in IP, also activating the antioxidant mechanisms in response to cell-level oxidative stress [21-24].

Polydatin, with its antioxidant and anti-inflammatory activity, is a natural precursor of resveratrol. PD exerts its protective effect on the cells and tissues by triggering H_2O_2, preventing the oxidative stress injury leading to endothelial dysfunction, decreasing lactate dehydrogenase and ROS production, increasing SOD and GSH-Px activity, and regulating the PKC signal pathway [9, 25]. Moreover, PD also reduces ROS production in mitochondrial electron transport chain complex III [11]. In our study, CAT, SOD, and GSH-Px levels were increased in the IEP and ILP groups compared to the IE and IL groups. This findings, could be attributed to the induction of hepatic antioxidant enzymes [26]. Meaningfully, literature indicates that PD leads to a significant increase in the mRNA expression of hepatic antioxidant enzymes including CAT, SOD, and GSH-Px following oxidative events [26] and also leads to a significant decrease in the MDA levels in plasma and inflamed tissues [27]. On the other hand, in our study, HIF-1α levels were significantly lower in the PD groups (IEP and ILP) compared to the non-PD groups (IE and IL). This finding indicates that PD decreased the HIF-1α expression in inflamed and ischemic tissues. HIF-1α is a dimeric protein complex playing a pivotal role in the body's response to low oxygen concentrations, or hypoxia. Moreover, HIF-1α is significantly increased in hypoxic areas such as localized ischemia and tumors and it is also crucial for immunological responses and is an essential physiological regulator of vascularization, homeostasis, and anaerobic metabolism [28]. In our study, HIF-1α was increased in the experimental groups and was significantly decreased in the IEP and ILP groups, which suggests that PD prevented the oxidative stress and HIF-1α caused by hypoxia [10, 22, 27].

Total antioxidant status, TOS, and OSI are commonly used for the assessment of oxidative stress activity. TOS indicates the concentration of all free oxidant radicals caused by ischemia against oxidative damage. In contrast, TAS is a key indicator of the activities of antioxidant defense system against cell damage [29]. In our study, TOS levels were lower and TAS levels were higher in the PD groups compared to the non-PD groups although no significant difference was established in the two parameters. This outcome could be attributed to not only the anti-inflammatory and antioxidant activity of PD but also the elimination of ROS as a result of increased CAT, SOD, and GSH-Px levels induced by PD [9, 10, 12, 26, 27]. On the other hand, biochemical assessment of hepatocyte injury was performed based on the AST, ALT, and albumin levels. In the PD groups, the ALT levels were significantly lower ($p<0.05$). while the AST levels were insignificantly lower compared to the non-PD groups ($p>0.05$). We consider that the increased AST and ALT levels in the IE and IL groups occurred secondary to hepatocyte injury and decreased AST and ALT levels in the IEP and ILP groups, which was consistent with the literature, resulted from the prevention of hepatocyte injury by PD [12]. It is widely known that an increase in AST and ALT levels may indicate liver damage [30, 31]. In our study, albumin levels were lower in the IE and IL groups in which the extent of tissue damage was greater, whereas albumin levels were higher in the early- and late-phase PD groups in which tissue damage was prevented. This finding suggests that PD prevented tissue damage by preventing hepatocyte injury [32].

In liver tissue, early-phase IRI occurs within 0-2 h and late-phase IRI occurs within 6-48 h after the onset of IRI [13]. Histopathological evaluation of hepatocyte injury is performed based on the sinusoidal congestion, cytoplasmic vacuolation, PMNs, liver necrosis, and portal inflammation which result from IRI [19] (Table-2). In the early phase of hepatic IRI, the released damage-associated molecular patterns caused by ischemia injury bind to the Toll-like receptor on Kupffer cells, thereby leading to kupffer cell activation [33]. To further enhance the inflammatory reaction, the activated kupffer cells respond by releasing a large amount of inflammatory cytokines including IL-6, TNF-α, IL-12, IL-1β, chemokines, and endogenous ROS [34, 35]. In our study, the relatively lower sinusoidal congestion and hepatocyte injury observed in the IEP and ILP groups is likely to be a result of the prevention of kupffer cell activation, lipid peroxidation, and oxidative-stress-related gene expression by PD [10, 26, 27]. Moreover, PD is also likely to have contributed to the effectivity of remote ischemic preconditioning in the reduction of hepatocyte necrosis, cytoplasmic vacuolization, and sinusoidal congestion [9, 10, 12, 28].

CONCLUSION

Reactive oxygen species and cytokines resulting from recurrent IRI in lower extremities result in IP and tissue damage. PD reduces tissue/parenchymal damage in the liver, thereby leading to decreased AST and ALT levels. Antioxidant enzymes (i.e. CAT, SOD, and GSH-Px), which participate in the detoxification of

ROS, lead to a significant reduction in histopathological findings in liver tissue such as sinusoidal congestion, PMN infiltration, and cellular necrosis, thus making a significant contribution to liver preservation in IP model.

Conflicts of interest: There is no conflicts of interest.

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Openly available data

All data created during this research are openly available from the thesis of Erhan Kızılkaya, in national thesis center of Turkey. https://tez.yok.gov.tr/UlusalTezMerkezi/tezSorguSonucYeni.jsp

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