

The preventive effect of sulforaphane on liver injury in a non-heart-beating donor model

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Abstract

Aim: In this preliminary study, we aimed to investigate the effect of sulforaphane (SFN) warm ischemia in a non-heart beat rat liver model (NHBD).

Materials and Methods: Livers taken from NHBD 15,30,60 min after warm ischemia were stored up at University of Wisconsin solution for 5 h at 4°C [cold storage (CS)] and were, then, exposed to 2 h of machine reperfusion (MRP) at 37°C. In the study group, SFN 5 mg/kg was intraperitoneal given 30 min before the introduction of NHB. Firstly, we divided the rats into the control and SFN groups. Then, each group was divided into three groups (n=10 in each) including control I, II, III and SFN I, II, III groups (15-, 30-, and 60-min warm ischemia period, respectively). Control and study groups at the same time of ischemia were compared with each other in terms of lactate, AST, ALT, IL-6, TNF-α and morphologically.

Results: In the group treated with SFN, AST, ALT, TNF-α, IL-6, and lactate levels were lower, compared to the control group. Histopathologically, Bcl-2 staining intensity and percentages of staining were statistically significant, compared to the control and study groups during 15-min warm ischemia. Although not statistically significant, the intensity and percentage of staining were higher at 30 min and identical at 60 min.

Conclusion: Our study results suggest that, despite prolonged warm ischemia time, SFN can biochemically and morphologically protect the liver from the hazardous effects of ischemia reperfusion in an experimentally-controlled NHB in rats, indicating an increased utilization rate of NHBDs.

Keywords: Hepatic injury; NHBDs; organ preservation; sulforaphane; warm ischemia

INTRODUCTION

The paucity of donor organs comprises one of the barriers in hepatic transplantation. In prior decades, organ donation from patients with brain death is the primary source for donor organs; however, today, the patients in waiting list for organ transplantation reaches up to substantial extent due to success in liver transplantation as well as recipient criteria expanded to use marginal organs such donation soon after cardiac death. In the transplantations from NHB donors, graft dysfunction rate and recipient mortality are higher than those with organs donated after brain death (1). One can attribute this to warm ischemia preceding cold storage in liver from NHB donor. In NHB organ donation, several experimental studies found that graft dysfunction is associated to depletion in energy during warm ischemia period, micro-circulation abnormalities, oxidative injury, activated Kuppfer cells with pro-inflammatory cytokine release including TNF-α, IL-1 and IL-6(1).

Sulforaphane (SFN) is a compound with high antioxidant properties present in broccoli. Sulforaphane is considered

as a potent fighter against oxidative stress with its antioxidant, anti-inflammatory and anti-tumor effects (2). SFN has been found to decrease hepatic damage, reducing oxidative stress through decreased accumulation of iron in the liver through the Nrf2 pathway (3). In addition, in rats, the protective effect of SFN and sulfur-radish extract against carbon tetrachloride (CCl₄)-induced hepatotoxicity in a hepatic injury model induced by CCl₄ has been examined, and SFN has been shown to be effective against hepatotoxicity (4).

This study aim of was to investigate the effect of warm ischemia and to improve liver use by protecting the liver from ischemia-reperfusion injury with SFN and an NHB liver perfusion model was used.

MATERIALS and METHODS

Experimental Animals

A total of 28 to 32-week-old 60 male Wistar Albino rats weighing 225 to 290 g were used. Animal use was confirmed the animal ethics committee. The animals were taken care of according to the National Institutes of

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Health guide for the care and use of Laboratory animals. This study was carried out in the Erciyes University's Local Ethical committee decision of date 14.11.2012 and the project number of 2012/113.

Sulforaphane was bought from Sigma; dimethyl sulfoxide was bought from Sigma; Hartmann's solution of Baxter Health Care Pty. Ltd., Krebs-Henseleit buffer and reduced glutathione of Sigma and Aldrich, Ross perfusion fluid of Orion Laboratories and Solution (UWS) of Bristol-Myers Squibb Co. were bought and used in this study.

NHB Rat Model

Anesthesia during liver harvest was performed by using 50 mg/kg ketamine hydrochloride and 10 mg/kg xylazine via intramuscular injection. The experimental procedure was initiated. The NHB donor model was created with modifications from Xianwa Niu et al.'s study(1). The experiment was performed by the same surgeon. In the study group, SFN 5 mg/kg was intraperitoneal given 30 min before the introduction of NHB. Solution of 10% povidone-iodine was used to clean the skin and then 3 cm long laparotomy was performed after shaving the anterior walls of the abdomen. During the procedure, splenic vein, the right renal artery, the gastroduodenal vein, left and right adrenal veins were ligated. Bile duct was inserted a catheter, and 200 U of heparin was administered via the tibial vein. An incision into the diaphragm was made to induce warm ischemia. The thoracic aorta was clamped, and the liver was covered with damp gauze in order to moisture with saline in a continuous manner. After 15, 30, 60 min of ischemia, we cut the intrathoracic vena cava, and flushed the liver with heparin (10 U/mL) and 50 mL of ice-cold Ross perfusion fluid through the aorta (40 mL/min). The dose of SFN administration was determined according to the previous studies with modification from preliminary experiments.

CS and Warm Machine Perfusion

The liver was kept in a container in cold UWS. The portal vein (inlet) and suprahepatic vena cava (outlet) were cannulated and the liver was flushed with 12 mL of cold UWS before storing at 2 to 4°C for 5 h. After CS, we warmed the liver till room temperature and flushed with heparin (5 U/mL) and 20 mL of Hartmann's solution. Later on we collected the effluent. The liver was linked to a rat liver perfusion system and perfused with a water-jacketed perfusion system (Radnoti Glass Technology, Inc., Monrovia, CA) with oxygenated (5% carbon dioxide and 95% oxygen) Krebs-Henseleit buffer at 20 mL/min and 37°C. Following 7.5 min of perfusion, the perfusate was filtered through a prefilter and filter (0.8 μ m/0.2 μ m, 32-mm outside diameter; Pall Life Sciences, Cheltenham, Australia), and was recirculated. Then, the liver was further perfused for 2 h. The mean volume of the perfusate after 2 h of machine reperfusion (MRP) was 353611 mL (n516). During MRP, perfusate samples (1.5 mL) and bile were collected every 30 min and kept on ice. The samples were centrifuged at 10,000g for 10 min at 4°C, and the supernatant was analyzed.

After a 2 h perfusion period, livers were taken into formaldehyde for pathological examination. Immunohistochemically the livers were stored under appropriate conditions to be evaluated for Bcl-2 positivity.

Experimental Groups and Use of SFN

Firstly, we divided the rats into control and SFN groups. Then, each group was divided into three groups (n=10 in each) including control I, II, and III (15-, 30-, and 60-min warm ischemia period, respectively) and SFN I, II, and III groups (15-, 30-, and 60-min warm ischemia period, respectively).

In the SFN groups, 5 mg/kg SFN was given via the intraperitoneal route after solving in 10% dimethyl sulfoxide (DMSO) to the rats 30 min before the introduction NHB. Perfusates from control and study groups were stored at -80°C, until tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), aspartate aminotransferase (AST), alanine aminotransferase (ALT) were measured. However, lactate levels were measured on the same day. Liver samples were stored for immunohistochemical staining with Bcl-2.

Biochemical parameters

Perfusate samples were centrifuged for 10 min at 3,000 rpm. Sera were, later, stored at -80°C, until assay. On the day of assays, the sera samples were thawed at room temperature. In addition, AST and ALT levels (IU/L) were measured using the Architect C-800 automated analyzer (Abbott Inc., USA). TNF- α level (pg/mL) and Interleukin-6 were quantified by using the enzyme-linked immunosorbent assay (ELISA) kits (Bioscience Inc., USA) (catalogue #: BMS625 and catalogue #: BMS622, respectively). Lactate levels (mmol/L) were measured on the day of sampling using blood samples drawn into the heparin tubes by Lactate Colorimetric (ab65331) assay kit (Abcam Inc., USA).

Histopathological Parameters

The mouse monoclonal anti-Bcl-2 α antibody kit (Thermo-Scientific/Labvision, USA) was used as the primary antibody. After waiting 30 min following primary antibody exposure, the avidin-biotin-peroxidase method was used to stain the samples immunohistochemically using the streptavidin-biotin kit. The cells stained with Bcl-2 in 30 magnification field were assessed in terms of staining intensity and percent staining in a semi-quantitative manner. All procedures were performed in a humid medium at room temperature to prevent drying of the sections. Sections were, then, assessed under a light microscope. All sections were randomly assessed by an experienced pathologist blinded to all groups.

Statistical Analysis

The data was analyzed using Statistical Package for Social Sciences (SPSS) for Windows version 15.0 (SPSS Inc., Chicago, IL, USA). Descriptive data were expressed in mean and standard deviation. Independent sample t-test was used to compare the groups at each time point. The chi-square test was used to compare categorical variables. Any p value of <0.05 was considered statistically significant.

RESULTS

Of all groups, TNF- α , IL-6, ALT, AST, and lactate levels were measured in perfusates obtained from the rats. There were statistically significant differences in all parameters between the groups at 15, 30, and 60 min with significantly lower levels in the SFN group ($p < 0.05$) (Table 1)(Figure 1-2-3-4-5).

Table 1. AST, ALT, IL-6, TNF- α and lactate levels in the perfusates of SFN and control groups				
	Time point	Control group	SFN group	P value
AST	Minute 15	757.8 \pm 311.5	362.6 \pm 160	0.003
	Minute 30	772.1 \pm 194	298.3 \pm 183	<0.001
	Minute 60	797 \pm 262	505.8 \pm 249.2	0.002
ALT	Minute 15	267.8 \pm 104	239 \pm 85	0.003
	Minute 30	739.6 \pm 268	221.1 \pm 113	0.001
	Minute 60	375.5 \pm 186	349 \pm 123	0.004
IL-6	Minute 15	976 \pm 406	138.8 \pm 125.6	<0.001
	Minute 30	800 \pm 328	212.2 \pm 196	<0.001
	Minute 60	110.0 \pm 41.0	82 \pm 41	0.001
TNF α	Minute 15	49.9 \pm 6.5	38.5 \pm 2.7	<0.001
	Minute 30	50.4 \pm 6.2	41.6 \pm 4.8	0.002
	Minute 60	93.5 \pm 39.9	33.9 \pm 7.5	0.001
Lactate	Minute 15	6 \pm 0.5	4.7 \pm 1.0	0.002
	Minute 30	5.9 \pm 1.1	2.7 \pm 1.0	<0.001
	Minute 60	9 \pm 0.8	6.8 \pm 1.4	0.001

ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; IL-6, Interleukin-6; SFN, Sulforaphane; TNF- α , Tumor Necrosis Factor-alpha

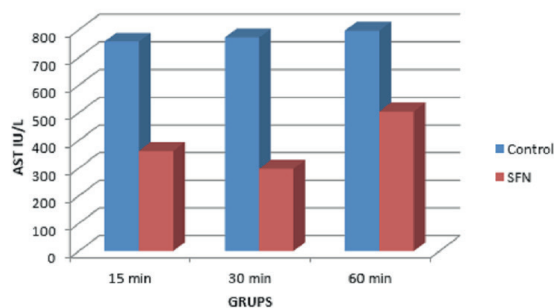


Figure 1. Comparison of AST values in Control and Study (SFN) Groups with the same warm ischemia times

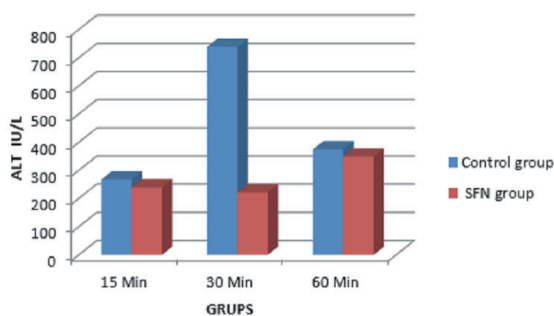


Figure 2. Comparison of ALT values in Control and Study (SFN) Groups with the same warm ischemia times

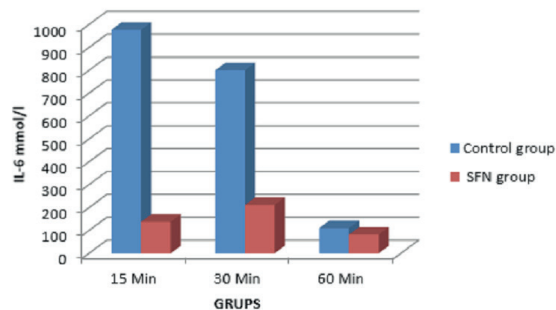


Figure 3. Comparison of IL-6 values in Control and Study (SFN) Groups with the same warm ischemia times

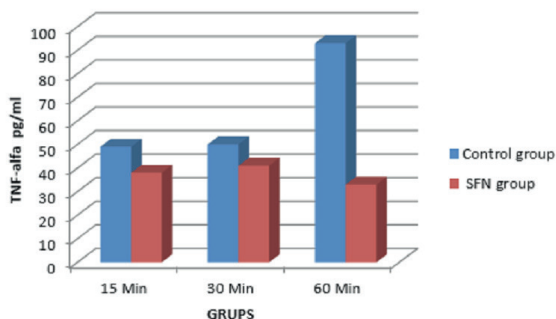


Figure 4. Comparison of TNF- alfa values in Control and Study (SFN) Groups with the same warm ischemia times

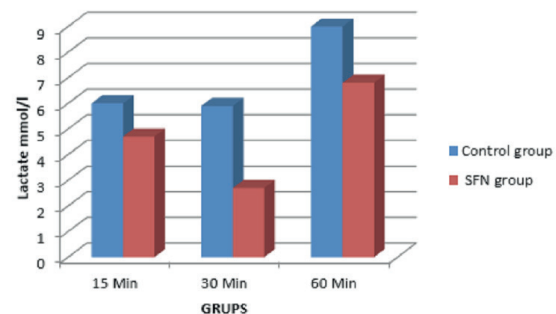


Figure 5. Comparison of Lactate values in Control and Study (SFN) Groups with the same warm ischemia times

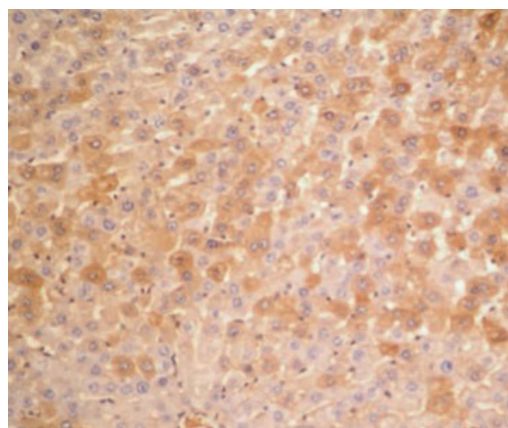


Figure 6. Photomicrograph showing strong cytoplasmic Bcl-2 staining in hepatocytes in 15 min SFN group (Bcl-2 immunoperoxidase; x200)

Table 2. Intensity and percent staining of Bcl-2 staining at 15, 30, and 60 min of SFN and control groups

		Time point					
		Minute 15		Minute 30		Minute 60	
		Control group	SFN group	Control group	SFN group	Control group	SFN group
Staining intensity	No	7 (70%)	0 (0%)	3 (30%)	1 (10%)	0 (0%)	0 (0%)
	Weak	2 (20%)	8 (80%)	4 (40%)	5 (50%)	4 (40%)	3 (30%)
	Strong	1 (10%)	2 (20%)	3 (30%)	4 (40%)	6 (60%)	7 (70%)
	p value	0.004		0.714		1.000	
Percent staining	0%	7(70%)	0(0%)	3(30%)	1(10%)	0(0%)	0(0%)
	33%	2(20%)	4(40%)	4(40%)	4(40%)	2(20%)	4(40%)
	66%	1(10%)	5(50%)	3(30%)	3(30%)	7(70%)	4(40%)
	99%	0(0%)	1(10%)	0(0%)	2(20%)	1(10%)	2(20%)
	p value	0.004		0.471		0.475	

SFN, Sulforaphane

Following staining with Bcl-2, the preparations were evaluated for staining intensity (score of 0, weakness: 1, severity: 2) and percentage of staining (1%: 0, 33%: 1, 66%: 2, 99%: 3) (Table 2). There was a significant difference in the staining intensity and percent staining between the control and SFN groups at 15 min ($p=0.004$ and $p=0.004$, respectively); however, no significant difference was observed at 30 min ($p=0.714$ and $p=0.471$, respectively). In addition, there was no significant difference between the control and SFN groups at 60 min ($p=1.00$ and $p=0.475$, respectively). However, a strong cytoplasmic staining with Bcl-2 at 15 min in the SFN group was observed (Figure 6), while weak cytoplasmic staining with Bcl-2 at 30 min in the SFN group was achieved (Figure 7).

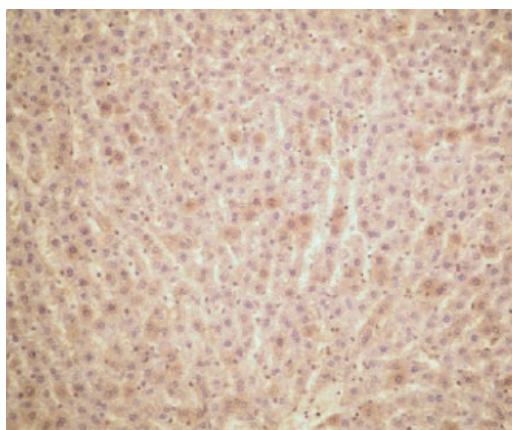


Figure 7. Photomicrograph showing weak cytoplasmic Bcl-2 staining in hepatocytes in 30 min SFN group (Bcl-2 immunoperoxidase; x200)

DISCUSSION

An insufficient number of grafts lead overcrowding of waiting list and loss of patients during donor waiting period. Therefore, several studies have currently addressed to the shortage of donor organ. An estimated average of 15% of liver transplantation pool will be provided to the liver pool (5).

Organs removed for transplant, another place or country it needs to be protected safely until it is delivered to the person to be transplanted. Acceptable organ preservation times vary according to the organs. The liver needs to be transplanted within 6-12 hours (6). It was observed that simple hypothermic protection was possible up to 48 hours after Ringer Lactate perfusion, after which ultrastructural changes occurred (7). Hypothermic protection and freezing-thawing are available as organ preservation methods. The freezing-thawing method has attracted the attention of scientists for many years, but it is not used today (8). Hypothermic protection has two methods, simple hypothermic protection and continuous hypothermic perfusion. In simple hypothermic preservation, the removed organ is washed intravenously with cold preservative solution ($+4^{\circ}\text{C}$) and placed in a sterile bag. This sterile bag is placed inside a second sterile bag with crushed ice particles. The most important advantage of this method is that it is simple, easy to carry and cheap. Simple hypothermic protection, the most widely used and is the preferred method. (9). The second form of hypothermic protection, It is a "preservation machine" developed by Belzer in 1967. Perfusion solution with a certain pressure is passed through the vessel placed in this machine (10). Approximately 25-30% of kidneys transplanted with simple hypothermic protection function late graft function. However, in less than 10% of the kidneys protected in the perfusion machine, late graft function occurs (10). Before the UW (University Wisconsin) solution, the liver had to be used within 6 hours with simple hypothermic protection. With the UW solution, the duration of simple hypothermic protection was extended up to 16 hours and the organs could be transported to remote places (6). In order to increase the protection time and quality of the organs, the hypothermia and perfusion technique found by Belzer, combined with the hypothermic perfusion machine, has not been found to be very common because it is complicated and expensive (11). In 2001,

Friend et al. Developed normothermic machine perfusion instead of hypothermia (12).

Sulforaphane cytoprotective effect is mediated by transcription factor NF-E2-related factor-2 (Nrf2), which binds to the antioxidant response element (ARE) in the promoter region of a number of genes, encoding for antioxidative and phase 2 enzymes, including heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase 1, glutathione reductase, and glutathione peroxidase (GSH-Px)(13).

The main difficulty is prolonged warm ischemia time in organs retrieved from NHBDS. It is well-understand that organ preservation and organ transplantation are directly related to ischemia-reperfusion injury (14, 15).

AST and ALT, which are key parameters of hepatocellular damage, are important routine hepatic tests assessing the presence and characteristics of the liver disease and the response to treatment (16). In their study by Zhao et al. (13), SFN (3 mg/kg) was given to the rats one hour before ischemia, and AST, ALT, and superoxide dismutase (SOD) measurements and immunohistochemical studies were performed one hour after ischemia. The authors found that SFN was associated with a significant decrease in ALT, AST, and SOD.

In our study, there was a significant difference in AST and ALT levels between both groups with identical warm ischemia time, which was measured at 15, 30, and 60 min. The levels of SFN groups were significantly lower than those in the control groups. Lower AST and ALT values in the SFN groups can be considered as an evidence for lesser hepatocellular injury due to the blockade of several inflammatory steps by effected SFN. Based on this finding, it can be estimated that cell viability is improved, and improved survival outcomes can be achieved in the SFN groups, in which AST and ALT levels were found to be lower.

In hepatic ischemia-reperfusion injury, hepatic lipid peroxidation increases, leading to hepatic injury due to oxidative stress during reperfusion. This is manifested by the leakage of liver enzymes into the plasma. Inflammatory mediators are produced by the Kupffer cells in oxidative stress-induced hepatic ischemia-reperfusion injury. Several studies showed that activated Kupffer cells caused increased oxygen-dependent free radicals and cytokine production (1,17). TNF- α is one of the most main cytokines which induces apoptosis of hepatocytes in hepatic ischemia-reperfusion injury (18). In an *in vitro* study by Kong et al. (19), SFN abolished the production of IL-6 and TNF- α in rheumatoid T cells.

In our study, we found that TNF- α level were significantly lower in the SFN groups, compared to that of the control groups with identical warm ischemia times. We also found that IL-6 levels were significantly lower in the SFN groups, compared to the control groups with identical warm ischemia times. This finding supports that SFN protects the liver against deleterious effects of reperfusion in

ischemia-reperfusion injury, preventing hepatocellular injury, Kupffer-cell activation, and excessive production of TNF- α and IL-6, and that SFN can improve the outcomes for the prevention of primary graft non-function. These findings are also consistent with the literature data (1).

Organ preparation in cold media (4°C) results in a slower metabolism, limiting the impact of ischemia. Reduced ATP and O₂ depletion activate anaerobic metabolism, resulting in the accumulation of lactate and hypoxanthine. This process leads to development of intracellular acidosis. Reperfusion causes transformation of hypoxanthine into xanthine and urate with reactive oxygen species (ROS), thereby, leading to lipid peroxidation. These non-physiological deviations comprise one of the main reasons of graft dysfunction (5,14).

In our study, lactate levels were significantly lower in the SFN groups, compared to the control groups with identical warm ischemia times. This finding suggests that SFN can minimize ischemic injury in graft and prevent lipid peroxidation which is considered as one of the main reasons of graft dysfunction.

The name of the antiapoptotic gene expressed in B cell lymphomas is bcl-2. Recent studies suggested that it had important roles in the regulation of mitochondrial function and metabolism assuming that its protective role may not be limited to an apoptotic effect. Ischemia and reperfusion (I/R) injury is a phenomenon in which cellular damage is caused by hypoxic situation and further damage is caused by the restoration of oxygen reperfusion in the liver. Decrement of nutrients, oxygen, or growth factors are important to cause ischemia and reperfusion injury that causes necrosis and apoptosis during hepatic I/R injury (20). In another study by Gapany et al.(21), apoptosis in the biliary duct epithelium were found to be associated with Bcl-2 levels in allograft rejection of the liver. The authors reported that apoptosis of hepatocytes and biliary duct epithelium played a key role in the graft rejection, and the number of apoptotic cells indicated the severity of rejection.

In our study, we found a significant difference at 15 min, but not at 30 and 60 min between the control and SFN groups following Bcl-2 staining. According to the SFN, Bcl-2 responded to early warm ischemia to induce anti-apoptotic mechanism and reduced tissue death. This effect was very strong at 15 min (100%-30%), it keeps protecting at 30 min(90%-70%). This protective mechanism was synchronized with the control group at 60 min (100% -100%). In our study, although there was no statistically significant difference in the intensity and percentage of staining at 30 and 60 min but the results were very similar to each other. However, according to the control group in the SFN group, higher values in terms of intensity of staining and percentage of staining were achieved. More staining of Bcl-2 in the SFN group than in the control group induced anti-apoptotic mechanism of SFN, suggesting that hepatocytes protect ischemia from adverse effects and that SFN can improve the outcomes of graft dysfunction and survival.

In a similar study conducted by Cetin et al.(22), experimental NHBDs Edaravon's liver injury inhibitory effects were investigated and showed that hepatocytes increased survival of ischemia in NHBDs by detecting that TNF- α , IL-6, AST, ALT levels were significantly reduced in the edaravone given study group

Nonetheless, our study is limited with one histopathological parameter. Despite the strengths of large sample size and perfusate analysis, only Bcl-2 was examined in the histopathological examination. Therefore, further studies with an increased number of histopathological parameters and molecular working would yield more definite results.

CONCLUSION

In conclusion, NHBD can be an alternative to expand the donor pool due to the increasing number of patients waiting for liver transplantation and insufficient number of organ donors. These donors may play a key role in the expanding donor pool, if disadvantageous aspects of complex process which adversely affect the graft survival and function can be improved by novel strategies. Based on our study results, we suggest that SFN can be a promising agent as a potent antioxidant in NHBD exposed to warm ischemia. We believe that the positive results of this study would shed light on cross-transplant experiments at the molecular level, which we would, then, do particularly in a pig model.

Conflict of interest : The authors declare that they have no competing interest.

Financial Disclosure: There are no financial supports.

Ethical approval: This study was carried out in the Erciyes University's Local Ethical committee decision of date 14.11.2012 and the project number of 2012/113.

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