

## Does melatonin alleviate cold preservation injury of the liver?

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**Aim:** To investigate the effect of melatonin added to preservation solution on liver cold ischemia.

**Materials and methods:** Twenty male Wistar albino rats were divided equally into groups treated with University of Wisconsin (UW) solution with and without melatonin. The LDH, AST, and ACP activities of the preservation solutions were determined in samples collected at 2, 24, 36, and 48 h. Tissue malondialdehyde and heat shock protein (HSP 70) levels were also measured and a histological examination was performed at 48 h.

**Results:** Melatonin prevented enzyme elevation, induced heat shock protein (HSP 70), and decreased lipid peroxidation effectively in comparison to the control group ( $P < 0.05$ ). The histological examination revealed that UW solution containing melatonin prevented Kupffer cell activation and inflammation significantly ( $P < 0.05$ ).

**Conclusion:** Melatonin in preservation solutions such as UW solution may protect tissue effectively from damage during cold ischemia for up to 48 h.

**Key words:** Antioxidant, cold preservation, heat shock protein, liver, melatonin, University of Wisconsin solution

### Melatonin soğuk iskeminin karaciğer üzerindeki etkisini azaltıyormu?

**Amaç:** Bu çalışmada melatonin ilave edilen koruma solüsyonlarının karaciğer soğuk iskemisi üzerindeki etkisi araştırıldı.

**Yöntem ve gereç:** Yirmi erkek Wistar albino rat iki eşit gruba bölünerek melatonin içeren ve içermeyen University of Wisconsin solüsyonu ile muamele edildi. 2, 24, 36 ve 48. saatlerde koruma solüsyonlarından alınan numunelerde LDH, AST ve ACP aktiviteleri ölçüldü. 48. saatte doku malondialdehit, ısı şok protein (HSP 70) düzeyi ve histolojik inceleme yapıldı.

**Bulgular:** Melatonin, kontrol grubuna göre ısı şok proteinini anlamlı derecede indükleyerek enzim artışını ve lipid peroksidasyonunu azalttı ( $P < 0,05$ ). Histolojik incelemede melatonin içeren University of Wisconsin solüsyonunun Kupffer hücre aktivasyonunu ve inflamasyonu anlamlı düzeyde önlediği belirlendi ( $P < 0,05$ ).

**Sonuç:** University of Wisconsin gibi koruma solüsyonlarına melatoninin ilave edilmesi 48. saate kadar dokuyu soğuk iskemisi hasarından koruyabilir.

**Anahtar sözcükler:** Antioksidanlar, soğuk iskemisi, ısı şok proteini, karaciğer, melatonin, University of Wisconsin solüsyonu

### Introduction

Preservation injury has been described as early hepatic dysfunction occurring after transplantation, usually within the first 2 weeks (1). After organ harvesting, with the onset of ischemia, the organ is viable for only 30 to 60 min due to the deficiency of oxygen, substrates, and energy together with the accumulation of metabolic end products. To prolong the survival capacity

Received: 19.11.2009 – Accepted: 14.01.2010

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and to ensure the rapid resumption of function after transplantation, an important preservation strategy is to use hypothermia and pharmacological inhibition to slow the metabolic processes in the ischemic/anoxic organ (2).

Simple hypothermic organ preservation is an uncomplicated, cost-effective procedure that can be used for almost all solid organs. In 1987, Belzer and Southard developed University of Wisconsin (UW) solution for preserving the pancreas (2). It is the preferred perfusion solution and the 'gold standard' for multiple organ procurement (3). During hypothermic ischemia, UW solution minimizes cell swelling, prevents acidosis, averts interstitial edema, captures oxygen radicals, and supplies the organ with precursors for energy metabolism (2). However, prolongation of the organ preservation period is a challenge and success would open new frontiers in the development of organ transplantation. Altering the content of preservation solutions is the most commonly applied strategy in the development of new solutions.

Melatonin (N-acetyl-5-methoxytryptamine) is a highly lipophilic molecule secreted from the pineal gland, retina, and digestive tract (4,5), and the highest melatonin concentration is found in the hepatobiliary system (6). Melatonin given in combination with a variety of antioxidants acts synergistically to suppress the formation of free radicals (7). In addition to its direct free radical scavenging action, melatonin may prevent tissue damage by acting cooperatively with other antioxidants and also induce heat shock protein (HSP) synthesis (8-10). This study investigated the protective role of melatonin as an adjunct to UW solution in a liver cold preservation model.

## Materials and methods

Twenty male Wistar albino rats were divided into 2 groups: the first was given University of Wisconsin (UW) solution (n = 10) and the second was given UW solution containing melatonin (UW-M) (n = 10). The study was approved by the local ethics committee and the principles of laboratory animal care were followed.

### Anesthesia and Surgery

Rats were operated on under intraperitoneal (IP) ketamine (50 mg/kg) (Eczacıbaşı, İstanbul, Turkey)

anesthesia. All midline laparotomy was performed, and the abdominal aorta was cannulated with 23-gauge polyethylene tubing for the purpose of hydration and removing all the blood during liver perfusion prior to death. Two milliliters of physiological saline were infused through the abdominal aorta before blood sampling from the suprahepatic inferior vena cava. Another indwelling 23-gauge polyethylene cannula was fixed in the portal vein. The liver was perfused with UW or UW-M solution. The perfusion continued simultaneously with aortic exsanguination until the liver paled and the output from the aortic cannula was as clear as the perfusate. Following cardiac arrest, the porta hepatis was skeletonized and the liver was dissected free from all its attachments and removed from the abdomen with minimal manipulation. The portal vein cannula was left in place to flush the liver with additional preservation solution. The graft was weighed and placed in a container filled with 40 mL of preservation solution. Melatonin was added to the preservation solution at a concentration of 30 mg/L and prepared as 40-mL aliquots (Sigma, St Louis, MO, USA). All the graft tissues were immersed and preserved in preservation solution immediately after harvesting. The grafts were kept in sterile plastic containers measuring 4 cm in diameter and 6 cm in depth at 4 °C in a refrigerator. One milliliter of a mixed sample of preservation and percolate fluid was taken from the container for enzyme measurements at 2, 24, 36, and 48 h.

### Biochemical Measurements

The aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and acid phosphatase (ACP) activities in the preservation solutions were determined by standard clinical chemistry methods using an Olympus AU 640 autoanalyzer (Olympus, Tokyo, Japan). ACP activity was measured to evaluate Kupffer cell activity. Tissue malondialdehyde levels were measured at 48 h using the method described by Uchiyama et al. (11).

### Immunohistochemistry

Immunohistochemical staining for HSP 70 was performed on 5- $\mu$ m thick paraffin sections by the conventional avidin-biotin complex (ABC) method. A semiquantitative scoring system for the staining intensity was used. According to this scoring system:

- 0 = No staining
- 1 = Minimal staining
- 2 = Moderate staining
- 3 = Severe staining

**Histological Evaluation**

Liver injury was analyzed under light microscopy using hematoxylin–eosin (H&E) stained paraffin sections. Tissue samples were examined at 400’ magnification. Congestion, central and portal venous dilatation, sinusoidal dilatation, edema, Kupffer cell activation, inflammation, and autolysis were scored as 0 (no change), 1 (minimal change), 2 (moderate change), and 3 (severe change).

**Statistical Analysis**

The data were expressed as means ± SD. The significance of differences between the 2 groups was analyzed using a 2-tailed Student’s t-test. Immunohistochemical staining intensity and histopathological findings at 48 h are given as median values and were evaluated using the Mann–Whitney U-test. Values of P < 0.05 were considered statistically significant.

**Results**

**Enzyme Release**

As shown in Table 1, melatonin significantly decreased the AST and LDH release into the preservation solution in the UW-M group. In contrast to the UW group, ACP activity was not detected in the UW-M group (Table 1).

**Lipid Peroxidation**

Tissue malondialdehyde (MDA) levels in the UW-M group (2.0 ± 0.8 nmol/mg wet tissue) were significantly lower than those in the UW (3.6 ± 0.6 nmol/mg wet tissue) group (P < 0.001) (Figure 1).

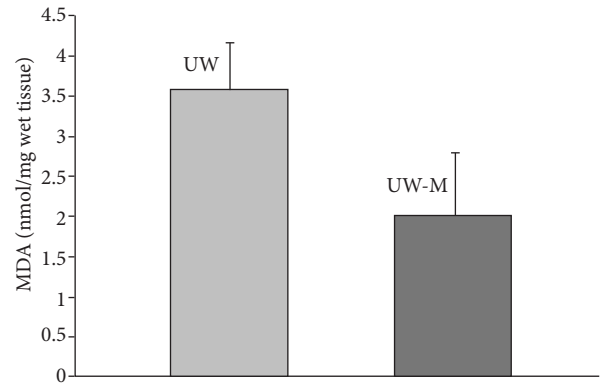


Figure 1. Malondialdehyde levels of liver tissue preserved in UW and UW-M solutions at 48 h.

**Expression of HSP 70 in Hepatocytes**

Addition of melatonin to UW solution induced HSP 70 expression significantly (P < 0.05). The median level of HSP-70 expression in the melatonin added group was 1 (Figure 2), whereas in the control group it was 2 (Figure 3).

**Histological Examination**

The median and overall histological scores of the preserved liver tissues are shown in Tables 2 and 3, respectively. The histological examination revealed that, in comparison to plain UW solution, the UW solution containing melatonin decreased inflammation and Kupffer cell activation significantly at 48 h.

**Discussion**

Since the beginning of the 1990s, UW solution has been used in heart, lung, liver, pancreas, kidney, and intestinal transplants with great success. Using UW solution for preservation, the ischemia tolerance limit has been extended to 18 h for liver and 36 h for kidney

Table 1. Enzyme activities (IU/L) of UW and UW-M preservation solutions at different times.

Enzyme	2h			24h			36h			48h		
	UW	UW-M	P	UW	UW-M	P	UW	UW-M	P	UW	UW-M	p
AST	40 ± 27	28 ± 5.6	0.186	200 ± 111	73 ± 43	0.003	245 ± 120	90 ± 45	0.001	303 ± 132	108 ± 46	0.000
LDH	200 ± 65	135 ± 36	0.013	972 ± 345	350 ± 121	0.000	1201 ± 363	429 ± 116	0.000	1441 ± 397	484 ± 142	0.000
ACP	0.0	0.0		0.9 ± 0.5	0.0	0.000	1.5 ± 0.6	0.0	0.000	2.4 ± 0.6	0.0	0.000

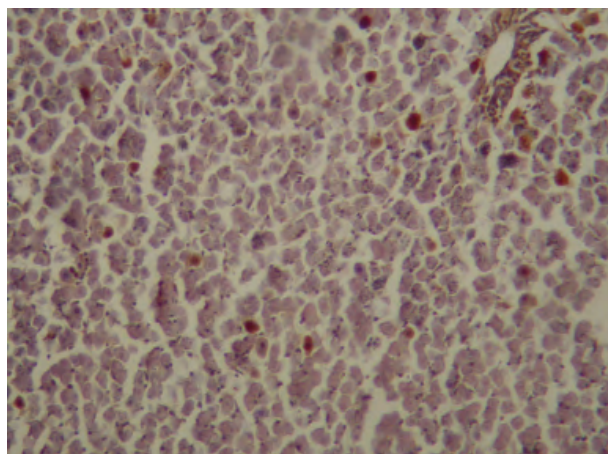


Figure 2. Immunohistochemical staining of liver tissue preserved in UW solution at 48 h. Hepatocytes were weak positive for HSP 70.

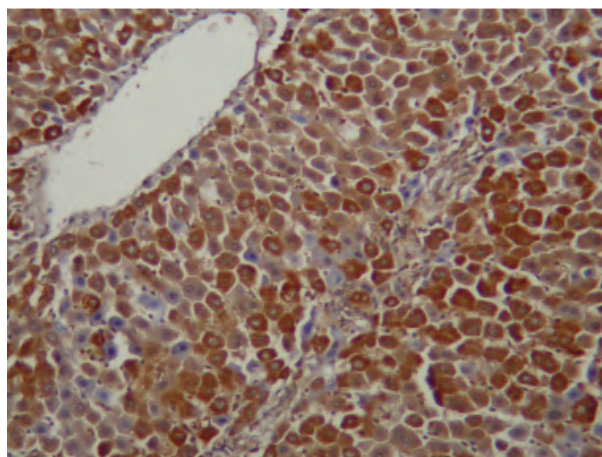


Figure 3. Immunohistochemical staining of liver tissue preserved in melatonin added UW solution at 48 h. Hepatocytes were strong positive for HSP 70.

Table 2. The median score of the injury parameters for liver tissue at 48 h.

Group	UW	UW-M
Congestion	0	0
Sinusoidal dilatation	3	3
Central vein dilatation	2	2
Portal vein dilatation	3	2
Inflammation	2	1 <sup>a</sup>
Kupffer cell activation	3	2 <sup>a</sup>
Edema	2	2
Autolysis	2	2

Values are given as medians. a: significantly different from the control (UW) group ( $P < 0.05$ ).

Table 3. Overall pathologic injury scores.

Score	UW	UW-M
0	12	9
1	16	16
2	33	55
3	29	10

(12). Despite this improvement in organ preservation, studies to extend the cold ischemia time are ongoing. One strategy to achieve this goal is to modify the gold standard solution, UW.

During preservation and re-anastomosis of the bile duct and blood vessels, liver allografts are exposed to variable periods of cold and warm ischemia. The mechanism of ischemic injury involves the loss of mitochondrial respiration and, consequently, ATP depletion, followed by the deterioration of energy-dependent metabolic pathways (13). ATP depletion is generally regarded as the primary factor causing irreversible cell injury, by altering membrane permeability. Therefore, the maintenance of adequate ATP levels is critical for liver cell viability during cold preservation. Hypothermia reduces the tissue metabolic rate, prolonging the time during which anoxic cells can retain essential metabolic functions. However, hypothermia itself can induce cell injury independent of hypoxia, which is attributed to an influx of sodium and chloride, leading to cell swelling. UW solution deals with all of these issues and is presently preferred as a cold storage solution for livers (3). Improving UW solution may prolong the graft preservation time and cell life. Melatonin seems to be a powerful candidate as an adjunct to preservation solutions.

In this study, melatonin decreased the AST and LDH release from liver tissue, reducing the AST and

LDH activity in the UW-M group more than 3-fold at 48 h. These data imply that melatonin exerts its effect via processes acting on cell membranes. Melatonin is found in extremely high concentrations in bile and this is thought to prevent oxidative damage to the intestinal epithelium by bile acids (14). Melatonin interacts with lipid bilayers (15) and stabilizes microsomal membranes (16). Furthermore, melatonin increases the activity of complexes I and IV in the mitochondrial respiratory chain, which increases ATP levels and accelerates electron flow, due to the capacity of indole to accept and donate electrons (17).

Melatonin reacts with hydroxyl radicals, singlet oxygen, peroxynitrite, and nitric oxide (18,19). It is a powerful scavenger of free radicals and its action mechanism differs from that of vitamins E and C, and glutathione (GSH) (8). Melatonin increases the activity of the antioxidative enzymes superoxide dismutase, GSH reductase, and GSH peroxidase (20). In our study, melatonin significantly decreased the lipid peroxidation product MDA in preserved liver tissue. Vairetti et al. suggested that melatonin significantly improves the restoration of liver function after cold storage (21). The restoration of biliary secretion is an important index of hepatic functional restoration after cold ischemic injury (22). Vairetti et al. showed that the administration of melatonin enhanced bile flow in the isolated liver after cold storage and reperfusion (21). They also demonstrated that melatonin increased the production of bile in the isolated perfused rat liver quantitatively and qualitatively. Therefore, melatonin seems to have both protective and function-improving effects on liver grafts, and may be used as an adjunct to preservation solutions or even administered as an improver drug after graft replantation.

In our study we have shown that addition of melatonin increased HSP 70 expression significantly in the liver tissue. We thought that in preserved liver during cold storage elevation of HSP 70 by melatonin is important because HSPs have strong cytoprotective effects and play a variety of roles in fundamental cellular response, especially in protein folding and

translocation. During acute or chronic stress, inappropriate activation of signaling pathways may occur as a result of protein misfolding, aggregation, or disruption of regulatory mechanisms. During this period, HSPs, through their properties in protein homeostasis, are thought to restore the existent imbalance (23). The HSP 70 family is the most highly conserved of the HSPs. It has also been shown that the protective effect of HSP 70 is at least partially mediated by the suppression of apoptosis. In particular, HSP 70 protects against multiple apoptotic stimuli, including DNA damage, UV irradiation, and a variety of chemotherapeutic agents (24,25).

The most important histological finding in the UW-M group was the decreased activation of Kupffer cells and inflammation (Table 2). We observed Kupffer cells microscopically and determined their activity by measuring acid phosphatase in the preservation solution (26). We did not detect ACP activity with the UW solution containing melatonin. Kupffer cells can be activated by subjecting them to hypoxia and subsequent reoxygenation (27). Activated Kupffer cells release reactive oxygen species into the vascular space and activate cytokines, which participate in the sinusoidal accumulation of granulocytes and cause microcirculatory failure (3). In pigs, Tsukamoto et al. showed that the elimination of Kupffer cells and the administration of a protease inhibitor improved graft viability and prevented reperfusion injury in non-heartbeating donors (28).

## Conclusion

Melatonin possesses tissue protective properties, which is promising for future organ preservation. This study revealed that melatonin added to UW solution contributes to its preservative function and may extend the cold ischemia time effectively.

## Acknowledgment

This study was supported by Abant İzzet Baysal University Department of Scientific and Research Projects (2005-07-01-236), Bolu, Turkey.

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