HTK-Alcar, a Modified Organ Transplantation Solution, Decreases Ischemic Injury in the Rat Kidney Tissue

Modifiye Organ TtransplantasyonÇ olan HTK-Alcar, Sıçan Böbrek Dokusunda İskemik Hasarı Azaltır

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ABSTRACT

Objectives: Histidine-tryptophan-ketoglutarate (HTK) solution is the storage solution used in organ transplantation. However, such solutions cannot completely eliminate tissue damage. Acetyl L-carnitine (Alcar) is a strong antioxidant. In this study we aimed to determine the protective effects of HTK solution prepared with Alcar in kidney tissue.

Methods: Twenty-four rats used in this study were divided into 4 groups. Kidneys of rats in groups 1 and 2 were stored for 4 hours in HTK and HTK+Alcar solutions, respectively. Kidneys of rats in groups 3 and 4 were stored for 24 hours in HTK and HTK+Alcar solutions, respectively. Histological and immunohistochemical examinations of the kidneys were performed. In addition, TUNEL analysis was performed for the evaluation of apoptosis.

Results: The findings of histomorphological damage in short-term HTK and HTK+Alcar groups were mild, but it was found widely in long-term HTK and ong-term HTK+Alcar groups on histologic evaluation. When histological scoring was made from kidney sections stained with H&E, the scores in HTK+Alcar groups decreased significantly compared to HTK groups. It was also seen that the score level increased significantly in long-term groups. According to immunohistochemical evaluation, in short- and long-term HTK+Alcar groups, the acetyl-L-carnitine prevented the antiapoptotic mechanisms to be activated and the intense expression of Bcl-2 has not occurred. In short- and long-term HTK groups, osteopontin showed more immunopositive result.

Conclusion: It was determined that the modified HTK solution prevented the increase of the activation of the expected oxidant mechanisms resulting in ischemia. This contribution of acetyl-L-carnitine was also found in long-term group findings.

Key Words: Kidney, ischemia, histidine-tryptophan-ketoglutarate, acetyl L-carnitine, apoptosis

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ÖZET

Amaç: Histidin-triptofan-ketoglutarat (HTK) çözeltisi, organ naklinde kullanılan depolama solüsyonudur. Bununla birlikte, bu tür çözeltiler doku hasarını tamamen ortadan kaldıramaz. Asetil L-karnitin (Alkar) güçlü bir antioksidandır. Bu çalışmada, Alkar ile hazırlanan HTK çözeltisinin böbrek dokusunda koruyucu etkilerini belirlemeyi amaçladık.

Yöntemler: Bu çalışmada kullanılan 24 sıçan 4 gruba ayrıldı. Grup 1 ve 2'deki sıçanların böbrekleri, sırasıyla HTK ve HTK+Alkar çözeltilerinde 4 saat bekletildi. Grup 3 ve 4'teki sıçanların böbrekleri sırasıyla HTK ve HTK+Alkar çözeltilerinde 24 saat bekletildi. Böbreklerin histolojik ve immünohistokimyasal incelemeleri yapıldı. Ayrıca apoptozis değerlendirilmesi için TUNEL analizi yapıldı.

Bulgular: Kısa süreli HTK ve HTK+Alkar gruplarında histomorfolojik hasar bulguları hafifti, ancak histolojik değerlendirmede uzun süreli HTK ve uzun süreli HTK+Alkar gruplarında bu bulgular yaygın olarak saptandı. H&E ile boyanmış böbrek kesitlerinden histolojik skorlama yapıldığında HTK+Alkar gruplarındaki skorlar HTK gruplarına göre anlamlı olarak azaldı. Uzun dönemli gruplarda da skor düzeyinin önemli ölçüde arttığı görülmüştür. İmmünohistokimyasal değerlendirmeye göre, kısa ve uzun süreli HTK+Alkar gruplarında asetil-L-karnitin, antiapoptotik mekanizmaların aktive olmasını önledi ve Bcl-2'nin yoğun ekspresyonu gözlenmedi. Kısa ve uzun süreli HTK gruplarında osteopontin daha fazla immünopozitif sonuç gösterdi.

Sonuç: Modifiye edilmiş HTK çözeltisinin, iskemi ile sonuçlanan beklenen oksidan mekanizmaların aktivasyonunun artmasını engellediği belirlenmiştir. Asetil L-karnitinin bu katkısı uzun dönem grup bulgularında da bulunmuştur.

Anahtar Sözcükler: Böbrek, iskemi, histidin-triptofan-ketoglutarat, asetil L-karnitin, apoptoz

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INTRODUCTION

The rapid increase in the number of patients waiting for organ transplantation every year has made it important to protect the few organs obtained. For this reason, many techniques and different content solutions are being developed to protect these organs (1, 2). The purpose of preservation solutions is to protect the morphological and biochemical properties of organs by reducing ischemia and hypothermia damage during and after transplantation of organs. In addition, these preservation solutions optimize organ function and graft survival time. Organ transplantion is main solution for organ failure. Transplant organ must be protected from hypoxia to avoid ischemia. Ischemia is a restriction in blood flows to tissues, trigger to produce of reactive oxygen species (ROS), proinflammatory cytokine production and caused activation of apoptotic pathway (3-5). In order to prevent these conditions, organ preservation solutions are used. Although the contents of organ preservation solutions are different, the common purpose of all is to prevent cell swelling, weakening of energy metabolism, and accumulation of acidosis and reactive oxygen precursors (6, 7). Histidine-tryptophan-ketoglutarate (HTK) solution, which is a protective agent, is a solution that plays an important role in mitochondrial functions and provides metabolism and vitality of the organ during organ storage. However, these solutions cannot completely eliminate the tissue damage (5, 8). Therefore, in our study; we investigated the effects of acetyl L-carnitine (Alcar) which is a strong antioxidant to HTK solution on the kidneys kept in different cold storage periods and investigated the protection of Alcar added to HTK solution against ischemic damage.

MATERIALS and METHODS

Animals

The experimental part of this work was carried out at Gazi University Laboratory Animals and Experimental Research Center (GUDAM). The animals were sacrificed under high anesthesia and their kidneys were removed. These healthy kidneys were divided into four groups.

Group 1 (HTK-4h) (n=6): The kidneys were stored in cold storage (+4°C) for 4 hours in HTK solution.

Group 2 (HTK+Alcar-4h) (n=6): The kidneys were stored in cold storage for 4 hours in HTK solution added with Alcar.

Group 3 (HTK-24h) (n=6): The kidneys were strored in cold storage for 24-hours in HTK solution,

Group 4 (HTK+Alcar-24h) (n=6): The kidneys were stored in cold storage for 24-hours in HTK solution added with Alcar.

Histological analysis

The kidney of each animal fixed in 10% buffered formalin were dehydrated with gradual concentrations of alcohol and then embedded in paraffin. Serial paraffin sections were stained with hematoxylin and eosin (H&E) for examination under light microscopy brand Leica DM 4000.

TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method was used to assess DNA fragmentation in the cells. The ApopTag Plus Peroxidase In Situ Apoptosis Kit (Millipore, cat no: 7102) was used for detects apoptotic cells, following the manufacturer's protocol. Briefly, four µm-thick cross-sections obtained from the kidney tissue blocks were incubated at 61 ˚C. After deparaffinization, tissues were incubated with 20 µg/ml proteinase K at room temprature for 15 minutes. Then, tissue sections were incubated with 3% hydrogen peroxide for the inhibition of endogenous peroxidase activity, in a humid environment for 5 min at room temperature. Equilibration buffer was applied for 5 min at room temperature. After the excess liquid was aspirated, the slides were incubated in TdT enzyme solution for 1 hour at 37°C in a humidity chamber. The slides were incubated at room temperature in the stop/wash buffer for 10 min, then slides were incubated in anti-digoxigenin peroxidase solution at room temperature for 30 min in a humidity chamber. Then, staining with DAB was performed to identify TUNEL-positive cells. Mayer's haematoxylin was used for background. Photomicrographs were taken using a light microscope.

Immunohistochemical analysis

Following deparaffinization, the cross-sections were incubated in citrate buffer (pH: 6.0) and 3% hydrogen peroxide. Ultra V block (Genemed Biotechnology, Cat: 54-0003) was applied for blocking. After the blocking stage, sections were incubated with Bcl-2 (rabbit polyclonal; SantaCruz, cat no: sc-783) and osteopontin (OPN) (mouse polyclonal; SantaCruz, cat no: sc-21742) primer antibody in different dilutions (1:100; 1:200, respectively) for overnight at 4 °C. Subsequently, tissue sections were incubated with secondary antibody (Genemed Biotechnology, Cat: 54-0003), and then immunoreaction was made visible with streptavidin peroxidase & diaminobenzidin (DAB) (Lab Vision, cat no: TA-125-HD) complex. Mayer's haematoxylin was used for background staining. Photomicrographs were taken using a light microscope. At each preparation of Bcl-2 and OPN immunohistochemical stainings, 6 areas were determined randomly at x400 magnification and the immunological involvement was determined as (%) in the ImageJ program.

Ethical approval

This study was conducted in the GUDAM Laboratory of Gazi University with the consent of the Experimental Animals Ethics Committee of Gazi University (G. Ü. ET-13.040).

Statistical analysis

Data distribution was assessed by Shapiro-Wilk test. To compare continuous variables, the post-hoc paired comparisons after Kruskal–Wallis test were used for non-normally distributed data. Statistical analyses were performed using IBM SPSS Statistics 21. $P < 0.05$ was accepted as statistically significant.

RESULTS

Histomorphological results

Diffuse glomerular sclerosis in a small number of glomeruli, dilatation in a small number of tubules, and dilatation in a small number of veins were observed in the HTK-4h group. Other kidney structures were observed to have normal histological structure. In the HTK Alcar-4h group, all structures in the renal tissue were observed as in the normal histological structure. In the HTK-24h group, dilatation in the vessels, dilatation in the tubules, diffuse glomerular sclerosis, and atypical dilatation in the tubule epithelial cells, cellular debris in the tubule lumen, peripheral localization in the glomeruli and mononuclear cell infiltration in the intersitium were observed. In the HTK+Alcar-24h showed diffuse glomerular sclerosis in some glomeruli and dilatation in vessels, however dilatation and mononuclear cell infiltration was not observed in the tubules. When tubular and glomerular damage were evaluated histomorphological, the first and second groups were similar, but the third group had prominent tubular damage. Tubular damage in group 4 was less than group 3 (Figure 1).

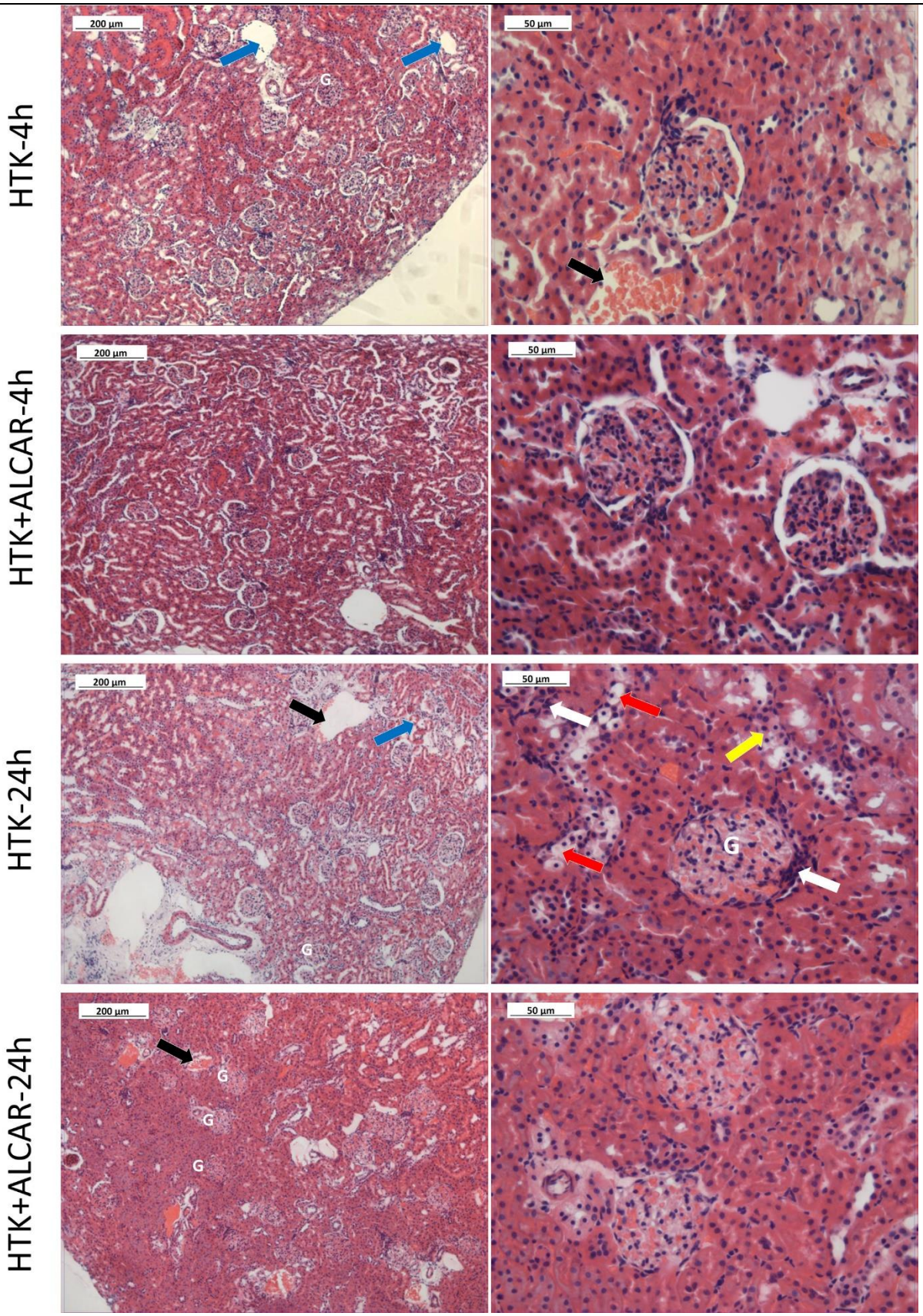

Figure 1. Histological findings of all the groups (H&E). Diffuse glomerular sclerosis (G); dilatation in tubules (blue arrow); dilatation in the vessel (black arrow); atypical dilatation in the tubule epithelial cells (red arrow); cellular debris in the tubular lumen (yellow arrows); mononuclear cell infiltration (white arrows)

When the histological parameters defined with H&E staining were scored, a statistically significant difference was determined between the groups (Table 1). When HTK-4h group and HTK+Alcar-4h group were compared, the score level of the Alcar added group decreased statistically significantly (p<0.001). This comparison was the same in 24-hour groups. When the HTK-4h group and the HTK-24h group were compared, the score in the 24-hour group increased statistically significantly (p<0.001). This comparison was the same for the groups that was added to the Alcar.

Table 1. The percentage of histological score in each group. P<0.001, HTK-4h vs. HTK+Alcar-4h group; HTK-24h vs. HTK+Alcar-24h group; HTK-4h vs. HTK-24h group and HTK+Alcar-4h vs. HTK+Alcar-24h group; post-hoc paired comparisons after Kruskal–Wallis test.

	GRADE 0	GRADE 1	GRADE 2
HTK-4h	18,3 %	68,3 %	13,3 %
HTK+ALCAR-4h	70 %	25 %	5 %
HTK-24h	0 %	23,3 %	76,7 %
HTK+ALCAR-24h	23,3 %	36,7 %	40 %

TUNEL results

When TUNEL positive cell numbers were evaluated statistically, there was no significant difference between the HTK-4h and the HTK+Alcar-4h groups (p=0.166), but there was a statistically significant difference between the other groups. In the HTK+Alcar-24h group, TUNEL positive cell number decreased significantly compared to the in HTK-24h group (p=0.013). In addition, the HTK-24h group showed a statistically significant increase compared to the HTK-4h group (p<0.001) and also the HTK+Alcar-24h group showed a statistically significant increase compared to the HTK+Alcar-4h group (p<0.001) (Table 2; Figure 2,3).

Table 2. Descriptive data of TUNEL and immunohistochemical results for all the groups

		HTK-4h	HTK+Alcar-4h	HTK-24h	HTK+Alcar-24h
TUNEL (number of cells)	Mean±SD	9,92±4,05	7,17±2,63	39,72±14,65	24,28±8,51
	Median	10,50	7,00	42,00	24,00
	Min-Max	1-18	2-13	10-75	9-45
OPN (%)	Mean±SD	11,5±3,99	5,79±3,52	22,64±6,45	12,64±4,62
	Median	10,75	5,07	23,07	12,24
	Min-Max	5,18-25,47	1,23-15,36	9,34-42,58	4,51-24,17
BCL-2 (%)	Mean±SD	9,65±3,78	4,86±2,52	13,04±4,33	8,61±2,95
	Median	9,31	4,35	12,51	8,04
	Min-Max	2,74-19,24	1,69-12,17	5,36-24,35	4,54-15,66

SD: Standart Deviation; Min: Minimum; Max: Maximum

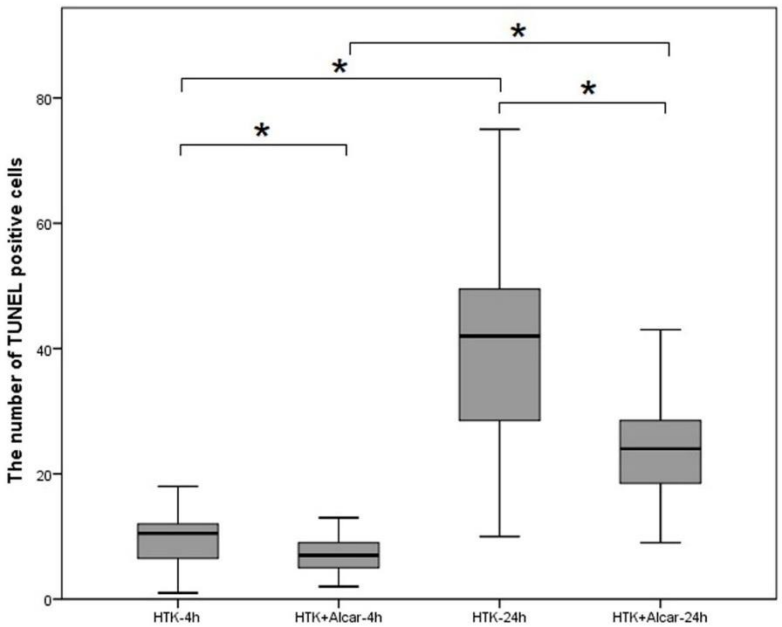

Figure 2. The number of TUNEL positive cells for all the groups; *p<0,05

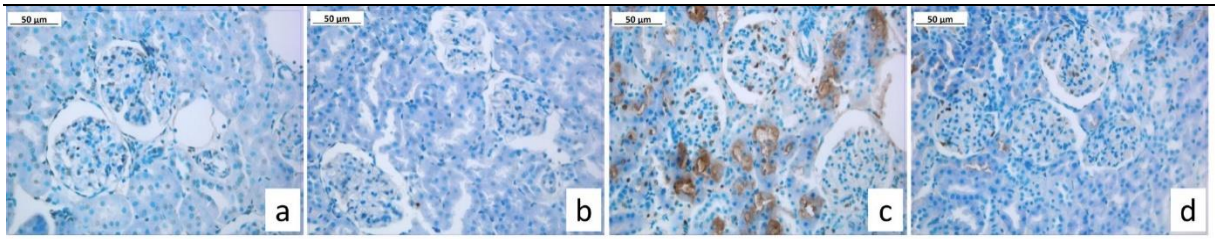

Figure 3. Apoptotic findings of all the groups, (a) HTK-4h; (b) HTK+Alcar-4h; (c) HTK-24h; (d) HTK+Alcar-24h (DAB, Haematoxylin)

Immunohistochemical results

Bcl-2 results

In the HTK-4h group, moderate Bcl-2 immunoreactivity was observed in the proximal tubules, whereas in the HTK+Alcar-4h group, moderate immunreactivite was observed in the few number of proximal tubules and mild immunreactivity was observed in the few number of distal tubule. In the HTK-24h group, moderate Bcl-2 immunreactivity was observed in tubular cells, however in the HTK+Alcar-24h group, mild immunreactivity were observed in the proximal tubule and mild immunreactivity in the distal tubule cells (Figure 5).

When Bcl2 immunpositivity rates were evaluated statistically, there was a statistically significant difference between all groups. In the HTK+Alcar-4h group, Bcl2 immunpositivity rates decreased significantly compared to the in HTK-4h group ($p<0.001$). In the HTK+Alcar-24h group, Bcl2 immunpositivity rates decreased significantly compared to the in HTK-24h group ($p<0.001$). In addition, the HTK-24h group showed a statistically significant increase compared to the HTK-4h group ($p=0.002$) and also the HTK+Alcar-24h group showed a statistically significant increase compared to the HTK+Alcar-4h group ($p<0.001$) (Table 2; Figure 4).

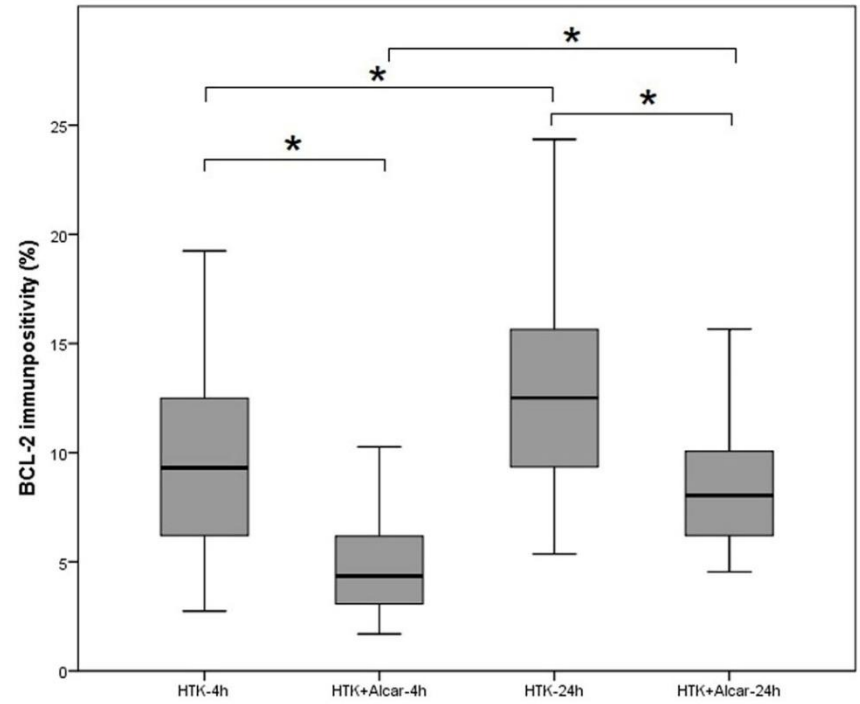

Figure 4. The percentage of Bcl-2 immunpositivity in each group; *p<0,05.

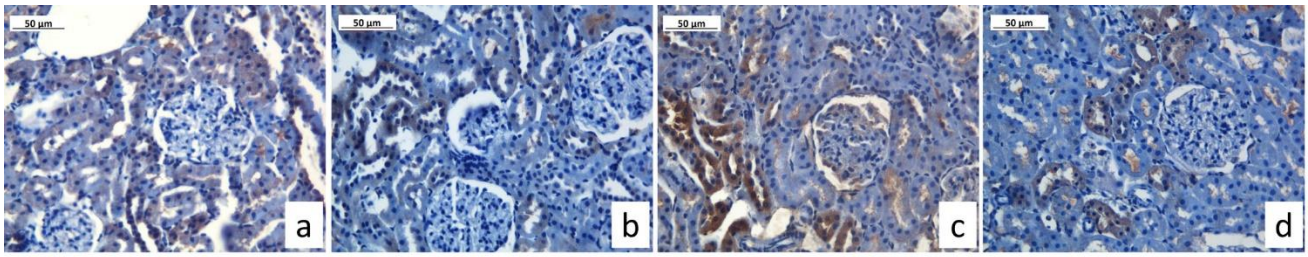

Figure 5. Bcl-2 immunohistochemical findings of all the groups (a) HTK-4h; (b) HTK+Alcar-4h; (c) HTK-24h; (d) HTK+Alcar-24h (DAB, Haematoxylin)

OPN results

In the HTK-4h group, immunoreactivity was observed in the cytoplasmic granules in the proximal and mostly distal tubules, whereas in the HTK+Alcar-4h group, less intensity immunoreaction was observed in the proximal and distal tubules. In the HTK-24h group, all of the proximal distal tubules in the cortex showed strong immunoreactivity. In the HTK+Alcar-24h group, less density granular involvement was observed in the apical cytoplasm in the proximal and mostly distal tubules (Figure 7).

When OPN immunpositivity rates were evaluated statistically, there was a statistically significant difference between all groups. In the HTK+Alcar-4h group, OPN immunpositivity rates decreased significantly compared to the in HTK-4h group ($p<0.001$). In the HTK+Alcar-24h group, Bcl2 immunpositivity rates decreased significantly compared to the in HTK-24h group ($p<0.001$). In addition, the HTK-24h group showed a statistically significant increase compared to the HTK-4h group ($p<0.001$) and also the HTK+Alcar-24h group showed a statistically significant increase compared to the HTK+Alcar-4h group ($p<0.001$) (Table 2; Figure 6).

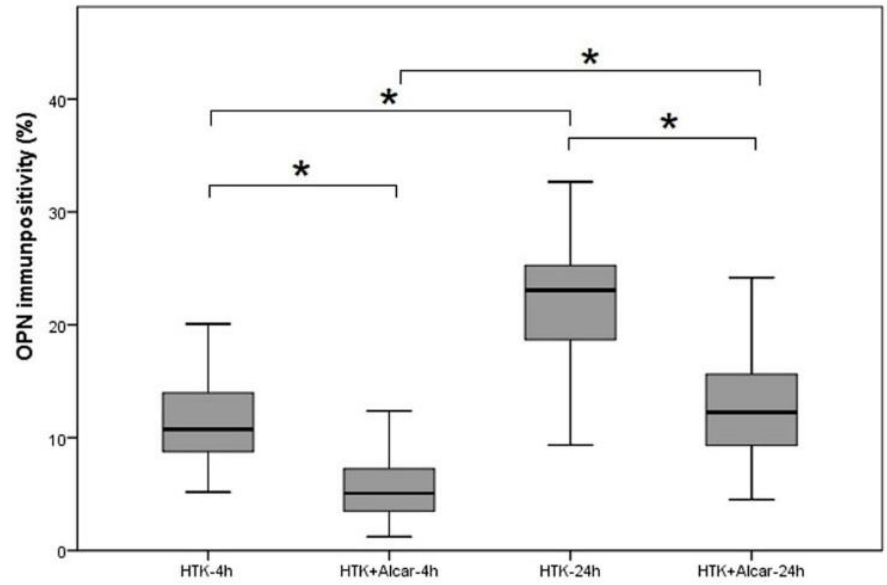

Figure 6. The percentage of OPN immunpositivity in each group; *p<0,05.

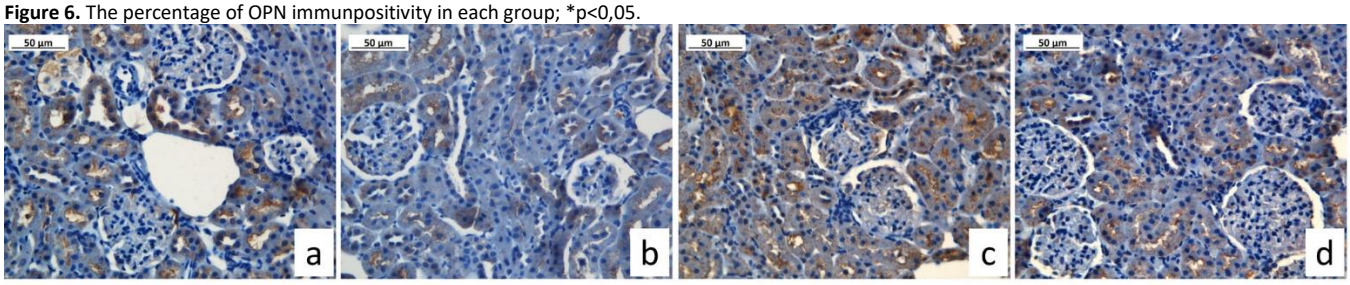

Figure 7. OPN immunohistochemical findings of all the groups (a) HTK-4h; (b) HTK+Alcar-4h; (c) HTK-24h; (d) HTK+Alcar-24h (DAB, Haematoxylin)

DISCUSSION

There are many different transport solutions available for use on the market during organ transplantation (9, 10). These solutions has different structure and content, however the purpose of all is to prevent cell swelling, weakening of energy metabolism, acidosis and accumulation of reactive oxygen precursors (6, 7). One of these solutions HTK is a buffer solution with a high histidine concentration. The first experimental studies on the renal preservation of HTK started in 1978. It was started to be used in heart transplantation in 1985 and it has been shown that it has a good protective effect in lung and liver transplantations in the following years (5, 11-14). Damage due to hypoxia and hypothermia can be prevented by means of organ transport solutions and simple cold storage-continuous hypothermic perfusion techniques (5). However, most of the damage to the transplanted organ is observed during re-perfusion of the organ's blood supply. With the reperfusion, free radicals, cytokines and nitric oxide are released, and the oxygen content in the tissue falls below the level that will allow the xanthine oxidase to be metabolized to xanthine and hypoxanthine, in addition to the decrease in the formation of antioxidant enzymes (15). A method of completely eliminating reperfusion damage was not found. This requires more work. One of the methods that reduce the harmful effects of free radicals are antioxidants. For this reason, the addition of antioxidant agent supplements to transport solutions is gaining importance. Acetyl-L-carnitine which is used in this study has been shown that it prevents free radical production, protects against degradation of ß-oxidant fatty acid in mitochondria, prevents platelet activation, maintains cell membrane integrity and causes vasodilatation (16).

The beneficial effects of carnitine on ischemia-reperfusion injury in many organs such as heart, testes, brain, and intestine have been documented in animal models (17-21). In our study; We investigated the potential positive effects of Alcar which is a strong antioxidant added to HTK solution on the kidneys kept in different cold storage periods and we investigated the possible protective effects of Alcar. Similarly, Dolińska et al. (22) investigated the effect of selected antioxidants in 2006 on kinetic changes in HTK solution. As a result, vitamin C is a powerful antioxidant, added to the HTK solution at 5 ° C, observed that the solution can keep the solution stable for a long time. Vitamin C by acting like a hydrophilic antioxidant, it protects the aqueous environment around the cell, can prevent oxidative damage in the tissue DNA, and similar to the study we have done, the strong antioxidants added to the protective solutions can prevent the reperfusion injury that may occur in the tissue. Caban et al. (23) in their study in 2010, biochemically demonstrated the prevention of modified HTK solution against ischemic injury on swine kidneys by adding prolactin. They observed that during 24 hours of cold ischemia, 0.02 mg/dl of prolactin-containing solution had a cell-protective effect on perfused kidney tissue. In a study performed by Ugurlu et al. (24), uterine tissues were stored in Alcar-added HTK solution for short and long term. The researchers said that this storage solution minimizes ischemic damage.

In this study, when tubular and glomerular damage were evaluated histomorphological, the first and second groups were similar, but the third group had significant tubular damage.

In the histological findings of our study, diffuse glomerular sclerosis in a small number of glomeruli, dilatation in a small number of tubules, and dilatation in a small number of veins were observed in the HTK-4h group. In the HTK+Alcar-4h, all elements of renal tissue were observed in normal histological structure. Dilatation in the vessels, dilatation in the tubules, diffuse glomerular sclerosis, and atypical dilatation in the tubule epithelial cells, cellular debris in the tubule lumen, peripheral localization in the glomeruli and mononuclear cell infiltration in the intersitium were observed in the HTK-24h group. In the HTK+Alcar-24h group, showed diffuse glomerular sclerosis in some glomeruli and dilatation in vessels, however dilatation and mononuclear cell infiltration was not observed in the tubules.

Apoptosis is a form of genetically controlled cell death in both normal physiological processes and in toxic damage and/or disease states. Renal tubular epithelial cells are the primary targets for apoptotic cell death after ischemic or toxic damage (25). When the Bcl-2 results were evaluated, moderate immunoreactivity was observed in the HTK-4h group whereas mild immunreactivite was observed in the HTK + Alcar-4h group. In the HTK-24h group, moderate Bcl-2 immunreactivity was observed, however mild immunreactivity were observed in the HTK+Alcar-24h group. When the Bcl-2 results were evaluated, in acetyl-L-carnitine groups no intensive expression of Bcl 2 so it can prevents the activation of anti-apoptotic mechanisms. When we confirm Bcl-2 results with TUNEL methods, tubular damage in group 4 was less than group 3. Also, in group 4, TUNEL positive cell number decreased significantly compared to the in group 3. In addition, the group 3 showed a statistically significant increase compared to the 1st group. There was also no statistically significant difference between the apoptotic cell numbers of group 1 and group 2. As a result of Bcl-2 and TUNEL findings, we think that acetyl-l-carnitine prevents the activation of anti-apoptotic mechanisms for a long-term survival and protect toxic damage. Ischemia/reperfusion injury remains unresolved problem in clinical organ transplantation. Likewise, Czigany et al. (26) in their study in 2018, flushed livers and stored in HTK-solution in cold storage for 8 hours before transplantation. In the study, RIC was applied to the recipient before hepatectomy or after reperfusion. RIC groups showed that reduced tissue injury according to results of histopathological evaluation and reduced TUNEL-staining. These results suggest that RIC might confer potent protection against the detrimental effects of ischemia/reperfusion injury including tissue damage and apoptosis.

OPN is an extracellular matrix cell adhesion phosphoglycoprotein and generally produced by osteoblasts and also produced by brain and kidney. It is a secreted phosphoprotein that is constitutıvely expressed in the normal kidney and is induced by various experimental and pathologic conditions during inflammation, cancer, and various other conditions. Increased production due to some renoprotective actions in kidney damage, such as increasing tolerance to acute ischemia, reducing cell apoptosis, and participating in cell regeneration (27-30). In our study, when the OPN results were evaluated, moderate immunoreactivity was observed in the HTK-4h group whereas mild immunreactivite was observed in the HTK + Alcar-4h group. In the HTK-24h group, strong OPN immunreactivity was observed, however moderate immunreactivity were observed in the HTK+Alcar-24h group. These findings have shown that acetyl-1carnitine is protective against ischemic damage.

In conclusion, in short and long term HTK+Alcar groups, acetyl-L-carnitine prevented the antiapoptotic mechanisms to be activated and the intense expression of Bcl-2 has not occurred. In short- and long-term HTK groups, osteopontin showed more immunopositive result, as a marker of tissue damage. It was determined that the modified HTK solution prevented the increase of the activation of the expected oxidant mechanisms resulting in ischemia. This contribution of acetyl-L-carnitine was also found in long-term group findings. It has been determined that acetyl-L-carnitine added to the HTK solution used for organ transplantation has a protective effect against ischemia-reperfusion damage in the kidney during the 24-hour at long cold period.

Conflict of interest

No conflict of interest was declared by the authors.

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