Araştırma Makalesi (Research Article)

Transplantasyonda Koruma Solüsyonlarına Eklenen P-Coumaric Asit ve Ellagic Asit Etkinliği

Efficiency of P-Coumaric Acid and Ellagic Acid Added to Preservation Solutions in Transplantation

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Özet

Amaç: Bu çalışmada University of Wisconsin (UW) and histidine–tryptophan–ketoglutarate(HTK) prezervasyon solüsyonları üzerine P-coumaric asit ve Elagic asidin koruyucu etkinliğinin değerlendirilmesi amaçlandı.

Gereç ve Yöntemler: Otuz altı Wistar albino erkek sıçan eşit 6 gruba ayrıldı: (Grup 1) UW; (Grup 2) UW + P-coumaric; (Grup 3) UW+Elagic; (Grup 4) HTK; (Grup 5) HTK + P-Coumaric; (Grup 6) HTK + Elagic. Karaciğer (KC)perfüzyonu yapıldıktan sonra 0, 6 ve 12. saatte karaciğer dokusundan ve perfüzyon sıvısından örnekler alındı. Altıncı saatte histopatolojik ve elektron mikroskopik inceleme için KC biyopsileri yapıldı.

Bulgular: P-coumaric asit ile zenginleştirilmiş gruplarda (grup 2 ve grup5) 0. saatte kontrol gruplarına göre yüksek olan antioksidan enzim aktivitelerinin azaldığı ve 6 ve 12. saatte farkın ortadan kalktığı görüldü. Ellagic asit ile zenginleştirilen gruplarda ise en yüksek en yüksek antioksidan enzim aktivitesinin grup 6'da olduğu, gruplar inflamatuar parametreler olan sitokin aktivitesi açısından değerlendirildiğinde antiinflamatuar sitokinler olan IL4 ve IL10 değerinin en yüksek olduğu grubun grup 6 olduğu, grup 3 de de sonuçların grup 6 ile benzer olduğu görüldü. İnflamatuar sitokinler olan TNF- α, IL1 ve IL6 açısından en düşük değerlerin grup 3'de olduğu grup 6 daki sonuçların ise grup 3'e göre yüksek olmakla birlikte özellikle IL6 değerleri açısından kontrol grubuna göre anlamlı oranda düşük olduğu görüldü ($P<0.05$). Elektron mikroskobik incelemede, ellagic asit eklenen grupların kontrol gruplarına göre hücresel düzeyde en düşük hasar düzeyine sahip olduğu görüldü ($p<0.001$).

Sonuç: Elagic asit ve P-Coumaric asitin prezervasyon solusyonlarına eklenmesi transplante edilecek KC dokusu üzerine koruyucu etkilere neden olmuştur.

Anahtar Kelimeler: Karaciğer transplantasyon, Prezervasyon solusyonları, Histidine–tryptophan–ketoglutarate, University of Wisconsin, Antioksidan, Elagic asit, P-Coumaric asit

Abstract

Objective: This study aimed to evaluate the preservative efficacy of University of Wisconsin (UW) and histidine-tryptophan-ketoglutarate (HTK) preservation solutions enriched with P-coumaric acid and ellagic acid.

Materials and Methods: Thirty-six Wistar albino male rats were divided into 6 equal groups; (Group 1) UW; (Group 2) UW + P-coumaric; (Group 3) UW+Ellagic; (Group 4) HTK; (Group 5) HTK + P-Coumaric; and (Group 6) HTK + Ellagic. After liver perfusion, at 0, 6 and 12 hours samples were taken from liver tissue and perfusion fluid. In the 6th hour, liver biopsy was performed for histopathologic and electron microscope investigation.

Results: In group 2 and group 5, high antioxidant enzyme activity compared to the control group was reduced at 0 hour and by 6th and 12th hours the difference was no longer observed. The highest antioxidant enzyme activity was in Group 6. The highest values for IL-4 and IL-10 anti-inflammatory cytokines were in Group 6, with similar results observed in Group 3. The lowest value for the inflammatory cytokines of TNF- α, IL-1 and IL-6 were in Group 3. Though the results in Group 6 were high compared to Group 3, they were observed to be significantly low compared to the control groups, especially IL-6 values ($p<0.05$). Electron microscope investigations observed that lowest damage at the cellular level compared to the control group was observed in the groups with added ellagic acid ($p<0.001$).

Conclusion: Adding ellagic acid and p-coumaric acid to preservation solutions causes protective effects on liver tissue for transplantation.

Key words: Liver transplantation, Preservation solutions, Histidine-tryptophan-ketoglutarate, University of Wisconsin, Antioxidant, Ellagic acid, P-coumaric acid.

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INTRODUCTION

Like all transplantation programs, in liver (L) transplantation one of the critical points in determining whether the graft will be functional when transplanted is preservation of the harvested liver tissue until transplantation occurs (1). It may be necessary to transport liver tissue long distances for transplantation, this means a long cold ischemia process. One of the primary stages of organ preservation is ensuring hypothermia to prevent cellular edema. In this way, release of free oxygen radicals is minimized. An attempt is made to delay structural changes linked to ischemia in organelles like mitochondria, lysosomes and the nucleus. If by chance these changes are not prevented, it is impossible to prevent development of primary non-function of the liver (2,3). With the aim of preventing this situation and preserving the tissue, the most commonly used solutions are University of Wisconsin (UW) and histidine-tryptophan-ketoglutarate (HTK). Due to the materials contained in the preservation solutions, cellular swelling is prevented and due to antioxidant and cytoprotective effects, the cell membrane is stabilized and cellular destruction delayed. However, in spite of the use of these solutions, sufficiently long preservation is not provided each time. Some researchers have enriched these solutions with a variety of antioxidant agents in attempts to lengthen the effect of preserving organ function (3-5).

P-coumaric acid (p-CA) is an organic compound that is a hydroxy derivative of cinnamic acid. It is found in plants like peanut, tomatoes and garlic. The phenyl hydroxyl group is responsible for antioxidant activity; however the antioxidant effect of p-CA is reported to be lower compared to its derivatives (6). Additionally p-CA shows antimutagenic and anticarcinogenic effects (7,8), with its anti-inflammatory and inhibiting effect on platelet aggregation emphasized (9). In recent times in the literature, there is one study on rats related to the effect of p-CA on the liver (10). In this study, though it was reported that buckwheat honey containing p-CA had a protective effect against liver tissue injury induced by carbon tetrachloride, there were more than twenty antioxidant agents found within this honey. Our study is the first to evaluate the effect of pure p-CA on the liver.

Ellagic acid (EA) is found in fruits like raspberry, strawberry and pomegranate and is a plant-sourced phytochemical containing four phenolic groups. Studies have reported that EA has strong antioxidant (11), antimutagenic (12), anticarcinogenic (13), anti-inflammatory (14) and anti-apoptotic effects (13), in addition to hepatoprotective properties (15).

After optimizing factors like surgical technique and hot ischemia, the aim of this study is to assess the protective effects on transplanted liver tissue of different preservation solutions enriched with agents with strong (EA) and weak (p-CA) antioxidant properties.

MATERIAL and METHODS

Study design and groups

The study was planned according to the principles of the Helsinki Declaration. The study was completed after receiving permission from Kahramanmaraş Sütçü Imam University (KSU) Animal Local Ethics Committee (KSU; Protocol No. 2014/03-02). All treatments and applications to experimental animals were completed in accordance with ethical rules (Institute of Laboratory Animal Resources. Guide for the Care and Use of Laboratory Animals, 8th edition, 2011, The National Academies Press, Washington D.C.). In the experiments, 36 adult male Wistar albino rats weighing 300±50 g were used. The rats were habituated to the laboratory conditions of 22 ± 2°C room temperature, 60±5% humidity, periodic (12 hours dark, 12 hours light) white fluorescent light and fed with standard pellet feed (Bil-Yem Lt., Ankara, Turkey) for 1 week. During the experiment all rats were given feed and drinking water ad libitum. Six groups were formed each containing 6 rats.

Biochemical agents used for experiments

The chemicals p-CA 5 g and EA 5 g were obtained from Sigma- Aldrich Chemical Co. (St Louis, MO, USA).

Experimental design

Rats were randomly separated into 6 groups and to prevent bias in the study, the groups were numbered by Aykut Urfalıoğlu (AU), who would not participate in experimental and analysis stages of the study, and placed in separate containers. The groups were; Group 1 (n=6): UW, Group 2 (n=6): UW + p-CA, Group 3 (n=6): UW+EA, Group 4 (n=6): HTK, Group 5 (n=6): HTK + p-CA, and Group 6 (n=6): HTK + EA. AU, again, prepared perfusion solutions with UW solution (Viaspan; DuPont Merck Pharmaceutical Company, Wilmington, DE, United States) for Group 1, UW solution containing 1 mg/l p-CA for Group 2, UW solution containing 1 mg/L EA for Group 3, HTK solution (Custodiol; Odyssey Pharmaceutical Inc., East Hanover, NJ, United States) for Group 4, HTK solution containing 1 mg/l p-CA for Group 5 and HTK solution containing 1 mg/l EA for Group 6 with the solutions given to the groups with the same numbers. In this way, the team completing the experimental protocol and the team completing biochemical and histopathologic assessment were blinded to the study.

General anesthesia was ensured by administering 50 mg/kg dose of ketamine hydrochloride (Ketalarflk, Eczacıbaşı, İstanbul, Turkey) by the ip route and laparotomy was performed with a medial line incision. The portal pedicle was identified and tied above the abdominal aorta truncusceliacus and below the SMA. The IVC was tied before the hepatic vein branches and after arterial and venous circulation was cut to the liver, the portal vein was cannulized for liver perfusion. For Group 1 UW (Viaspan; DuPont Merck Pharmaceu-

tical Company, Wilmington, DE, United States) solution was used, for Group 2 UW solution with 1 mg/l p-coumaric acid added, and for Group 3 UW solution with 1 mg/L ellagic acid added was used for perfusion. For Group 4 HTK (Custodiol; Odyssey Pharmaceutical Inc., East Hanover, NJ, United States) solution was used, for Group 5 HTK with 1 mg/l p-coumaric acid added was used, and for Group 6 HTK solution with 1 mg/L ellagic acid added was used for perfusion. Perfusion continued until clear fluid came from the hepatic vein and hepatectomy was performed. Liver tissue was placed in bags containing the preservation fluids they were washed in, with the bags placed in ice-filled storage containers.

For biochemical investigation liver biopsies were obtained from the perfused liver tissue at 0, 6 and 12 hours and fluid samples of the solution the liver tissue was stored in were taken in biochemical tubes. Liver biopsies were obtained in the 6th hour for histopathological investigation and electron microscope assessment.

For assessment of tumor necrosis factor α (TNF-α), interleukin 1 (IL-1), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 10 (IL-10), and alanin transaminase (ALT) levels, fluids were taken from the preservation solutions at 0, 6 and 12 hours. For assessment of catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA) and glutathion peroxidase (GSH-Px) levels, liver tissue samples were obtained at 0, 6 and 12 hours. All samples were stored at -70 °C until study.

Preparation of homogenate

Liver tissue samples were homogenized (Miccra D-8 homogenizer tool (ART, Germany)) in 1.15% KCI cold ice and centrifuged at 14,000 rpm to prepare for measurement of MDA, SOD, GSH-Px and CAT activity.

Biochemical analysis

Identification of MDA, SOD, CAT and GSH-Px levels

MDA levels in tissue were measured spectrophotometrically by absorbance at 532 nm of the pink-red color of the compound formed by MDA with thiobarbituric acid (TBA) in acidic pH and hot environment, based on the method used by Ohkawa et al. (16). SOD activity in tissue samples was determined by the transformation of the superoxide radical to hydrogen peroxide and the degree of inhibition of this reaction by SOD. There is an inverse relationship between SOD activity and amount of color with the reaction assessed from a standard curve of initial absorbance in the first 30 seconds of exposure to air at 37 °C in cuvettes with 1 cm at 505 nm wavelength. As described by Sun et al. (17), CAT catalyzes the destruction of H2O2. The destruction rate of H2O2 by CAT is measured spectrometrically by using the light absorbed by H2O2 at 230 nm. Sinha (18) used the method of Beutler (19) to measure GSH-Px activities. GPx catalyzes the oxidation of reduced gluthathione (GSH) via H2O2 to oxide glutathione (GSSG). In environments with H2O2 t-butyl hydroperoxide, the GSSG formed by GPx is reduced to GSH with the aid of glutathione reductase and NADPH. GPx activity is spectrophotometrically read as the absorption difference at 340 nm during the oxidation of NADPH to NADP (20).

Identification of TNF-α, IL-1, IL-4, IL 6, and IL-10 levels

Fluid samples taken from preservation solutions at 0. 6 and 12 hours for TNF-α, IL-1, IL-4, IL 6 and IL-10 measurements were placed in eppendorf tubes and stored at -70 °C until analysis. IL-1β, IL-4, IL 6 and IL-10 levels were identified as pg/ml using rat IL-1β, IL-4, IL 6 and IL-10 ELISA kits (IL-1β, IL-4, IL 6, IL-10 Rat Enzyme-Linked Immunosorbent Assay (ELISA) Kits, respectively, Catalog numbers: KRC0011-0041-0061-0102, NovexTM, ThermoFisher Scientific, USA). Serum TNF–α level was measured in pg/ml using a rat TNF–α ELISA kit (Rat TNF–α ELISA Kit, Catalog no: KRC3011, NovexTM, ThermoFisher Scientific, USA).

Identification of ALT levels

ALT enzymes in preservation solution were studied with a Siemens brand (Germany) Advia 1800 Chemistry system autoanalyzer in the biochemistry laboratory. Results were quantitated according to a calibration curve.

Histopathological parameters

Liver tissue samples were fixated in 10% formaldehyde then submerged in paraffin blocks and prepared in 5 µm sections with a Leica RM2145 microtome. Tissue sections were stained with hematoxylin eosin (H&E) and investigated with a Nikon DS-Fi2 microscope at 400x magnification. Under 400x magnification 4 areas accepted as 1 mm2 had broad eosinophilic cytoplasm apoptotic cells with small piknotic nuclei counted.

Caspase 3 Immunohistochemical staining

Apoptosis and protein expression with a role in regulation of apoptosis occurring in liver cells was assessed. Sections prepared after fixation in formaldehyde in paraffin blocks were stained with caspase-3 antibody (BosterBio, 1:100 dilution) and immunohistochemical stain (Ventana Bench Mark XT IHC/ISH). Increased (high) caspase-3 expression was accepted as positive with strong cytoplasmic and nuclear staining. Each slide had positive cells counted in 4 different fields (nearly 1 mm2 area) at 400x magnification.

Electron Microscope Investigation

Liver tissue samples taken for electron microscope investigation were fixated in 2.5% gluteraldehyde (0.2 M NaH2PO4 + NaHPO4 (pH: 7.2–7.3)) and 1% OsO4 then dehydrated with acetone and submerged in araldite blocks. Thin sections with 80 nm thickness were sliced from the araldite (Araldite Cy 212) blocks with an ultramicrotome and placed on copper grids. After contrast processes with uranyl acetate and lead citrate, sections were investigated with a Zeiss Libra 120 Transmission electron microscope (Carl Zeiss NTS GmBH; Oberkochen, Germany) and photographed.

Statistical Analyses

Data were analyzed using the SPSS 22 software (SPSS® version 22.0; SPSS, Chicago, IL, USA). Numeric variables

are presented as mean ± standard deviation. Groups were compared using the ANOVA test, while assessments within groups used the Tukey HSD method. The data were analyzed by a Repeated measure ANOVA followed by the Bonferroni test for between-group comparisons. We analyzed interaction between two factors (time by group interaction and the effects of time after drug administration). When Mauchly test was significant, normality and equality of variance were not violated in groups and the Greenhouse-Geisser adjustment was performed to determine the statistical significance of the factors (time after treatment and time group interaction). An overall %5 type-1 error level was used to infer statistical significance. Significance was accepted as $p<0.05$.

Sample Size

The pilot study results were analyzed by repeated measure ANOVA test. The sample size was determined based on the IL 1 since IL 1 values were the lowest significance value. At one time point (12 hour), the change in IL1 (Mean 0.42, SD: 0.12) results in F: 3.15 effect size ≥ 0.25; a $p<0.05$) the number of 6 animals per group provided sufficient statistical power ($1 - \beta \geq 0.90$). Base on the pilot study, 6 animals were calculated in each group according to the sample calculated with repeated measure ANOVA test. Study statistical power was confirmed via a sensitivity analysis performed with G*Power 3.1.9.2 software (University of Dusseldorf; http://www.gpower.hhu.de/en.html).

RESULTS

Results of biochemical analyses

MDA values

A one-way repeated measured analysis of variance (ANOVA) (followed by Bonferroni's correction) was conducted to evaluate the null hypothesis that there is no change in groups 'MDA hour 1, hour 6 and hour 12'. The results of the ANOVA indicated a significant time effect, by time [$F(2, 60) = 10{,}04$ $p<0.001$ $\eta 2 = 0{,}251$] and by time * group interactions [$F(10, 60) = 3{,}90$ $p<0.001$ $\eta 2 = 0{,}394$]. Thus, there is significant evidence to reject the null hypothesis.

With the aim of assessing the degree of oxidation, MDA measurement was performed. When the 1st and 4th groups are assessed, there was no difference observed between the groups at the 0 and 12th hours ($p=0.827$, $p=0.956$, respectively). In the 6th hour, the MDA values in the 4th group were lower ($p=0.039$). When the treatment groups are assessed in terms of MDA values, no group was different in the 12th hour, while in the 0 and 6th hours the values in Group 2 and Group 3 were significantly lower compared to the control groups ($p<0.001$ for all). Though the MDA values in Group 6 at 0 hour were low compared to the control group, there was no statistically significant difference observed in the 6th and 12th hours ($p=0.001$, $p=0.998$, $p=0.273$, respectively). The variation of MDA values over time is shown in **Figure 1.**

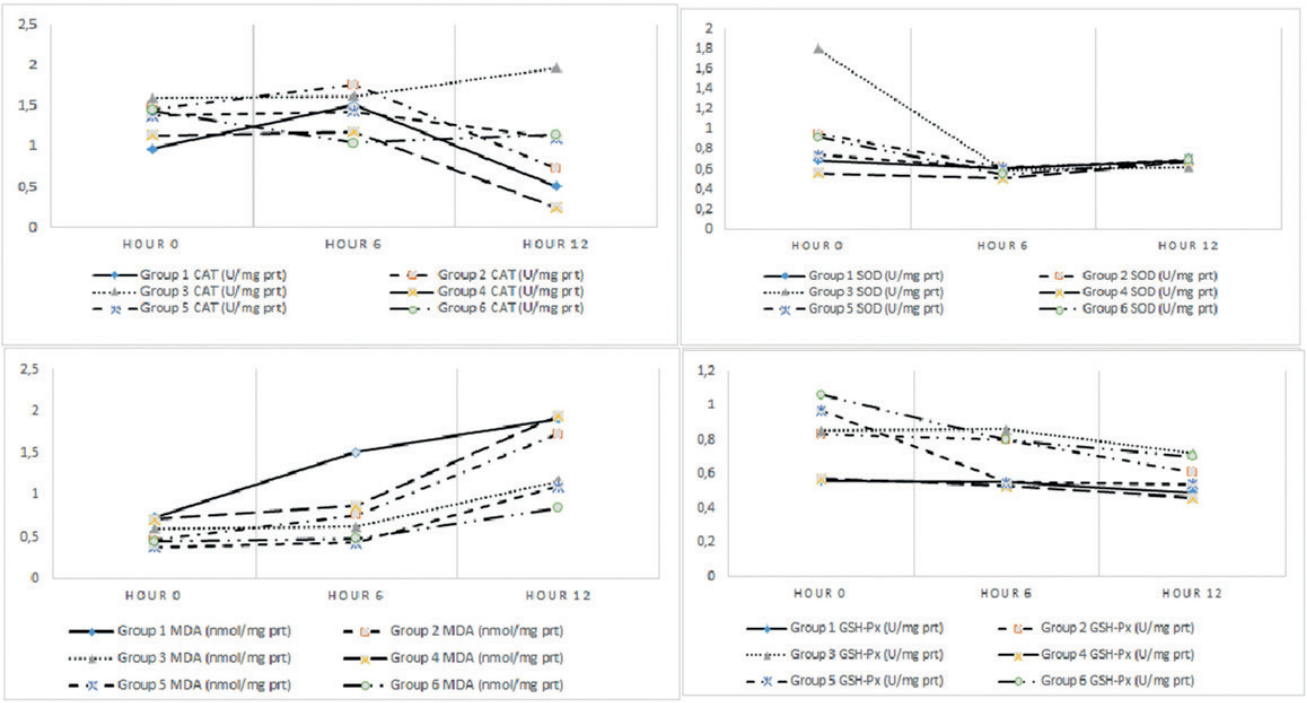

Figure 1. Variation in antioxidant enzyme levels and MDA levels over time in the groups

CAT values

The results of the ANOVA indicated a significant time effect, by time[F (2, 58) =77,68p<0.001 η2=0,728] and by time * group interactions [F (10, 58) =0,90 p=0.537 η2 =0,135]. There was a reduction in all groups with time, while generally the CAT values in the treatment groups were higher than the control groups. However, during the study period this increase was not identified to be statistically significant (p>0.05 for all). The variation in CAT values over time is shown in **Figure 1.**

SOD values

The results of the ANOVA indicated a significant time effect, by time [F (2, 60) =9,76 p<0.001 η2=0,259] and by time * group interactions [F (10, 60) =1,10 p=0.378 η2 =0,164]. The SOD values in Group 1 were higher than the SOD values in Group 4, though the difference did not appear to be statistically significant (p<0.05, for all). At all times the highest SOD values were in Group 5 and Group 6; however the difference was not statistically significant compared to other groups (p>0.05 for all). The variation of SOD values with time is shown in **Figure 1**.

GSH-Pxvalues

The results of the ANOVA indicated a significant time effect, by time [F (2, 60) =36,11 p<0.001 η2=0,555] and by time * group interactions [F (10, 60) =13,34 p<0.001 η2 =0,697]. The GSH-Px values in Group 1 and GSH-Px values in Group 4 were not different at all times (p>0.05 for all). The highest GSH-Px values were in Group 2 and Group 3; however including in these groups the difference was not observed to be statistically significant (p<0.05 for all). The difference in GSH-Px values with time is shown in **Figure 1.**

TNF-α values

A one-way repeated measured analysis of variance (ANOVA) (followed by Bonferroni's correction) was conducted to evaluate the null hypothesis that there is no change in groups ' TNF–α hour 1, hour 6 and hour 12' . The results of the ANOVA indicated a significant time effect, by time [F (2, 60) =10,07 p<0.001 η2=0,305] and by time * group interactions [F (10, 60) =3,17 p=0.004 η2 =0,409]. Thus, there is significant evidence to reject the null hypothesis. When the TNF–α values studied in preservation solutions are compared, there was no significant difference between Group 1 and Group 4 at 0 and 6 hours (p=0.972, p=0.983, respectively). However, in the 12th hour the TNF–α values in Group 1 were observed to be higher, but did not reach significance (p=0.146). When assessed in other groups, the TNF–α values in Group 2, Group 3 and Group 5 were significantly low in the 0 and 12th hours compared to the control group (p<0.05 for all). In the 6th hour, no group was observed to be different from the control groups (p<0.05 for all). The variation in TNF–α values with time is shown in **Figure 2.**

IL-1 values in Group 1 and Group 4 were not different between the groups (p>0.05 for all). The variation in IL-1 values with time is shown in **Figure 2.**

IL-4 values

We used a repeated measure ANOVA followed by Bonferroni's correction to analyze the significance of the data. There are statistically significant differences produced by time [F (2, 60) =67,13 p<0.001 η2=0,691] and by time * group interactions [F (10, 60) =12,14 p<0.001 η2 =0,669] on IL4. The IL-4 values in Group 3 and Group 6 were higher than the control group at all three time points and the diffe-

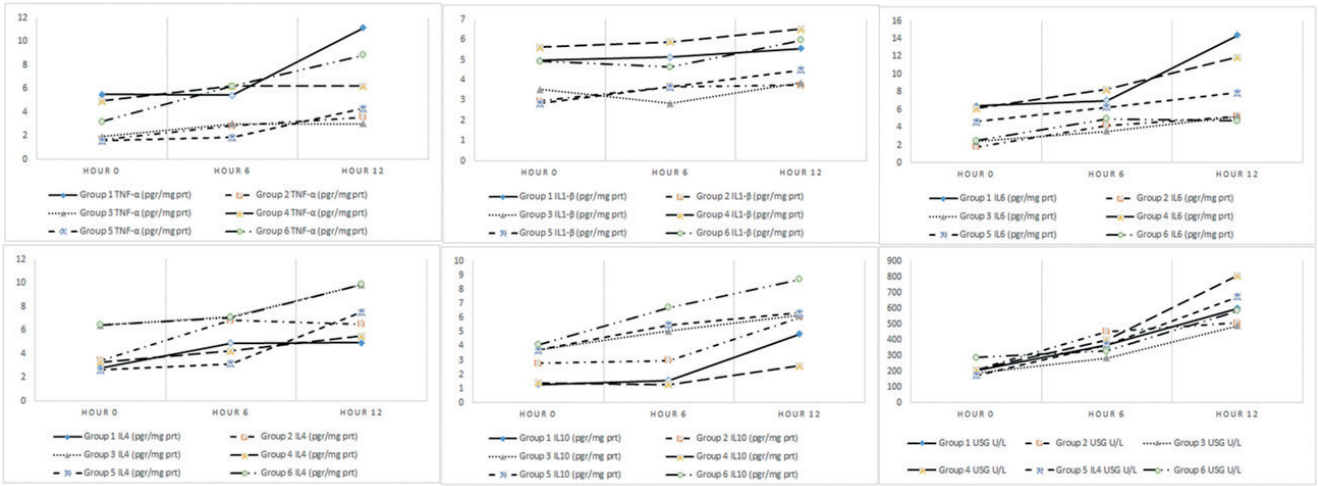

Figure 2. Comparison of inflammatory, anti-inflammatory and ALT levels in the groups

rence was significant (all p<0.001). The IL-4 values in Group 2 and Group 5 were not different from the control group at any time (p>0.05). The variation with time of IL-4 values is shown in Figure 2.

IL 6 values

There are statistically significant differences produced by time [F (2, 60) =37,79 p<0.001 η2=0,622] and by time * group interactions [F (10, 60) =8,02 p<0.001 η2 =0,636] on IL6. When the IL-6 values in Group 1 and Group 4 are compared, there was no different at all times within the study duration (p>0.05 for all). While the IL-6 values in no group were different in the 6th hour (p>0.05 for all), in the 12th hour the IL-6 values in Group 2, Group 3 and Group 6 were observed to be statistically significantly low compared to the control groups (p<0.001, p<0.001, p<0.001, respectively). The variation in IL-6 values with time is shown in **Figure 2.**

IL-10 values

There are statistically significant differences produced by time [F (2, 60) =18,55 p<0.001 η2=0,447] and by time * group interactions [F (10, 60) =3,08 P=0.004 η2 =0,401] on IL10. When Group 1 and Group 4 are compared, there was no significant difference between the groups at 0 and 6 hours (p=0.978, p=0.935, respectively). In the 12th hour the IL-10 values in Group 1 were higher, but this difference did not reach statistical significance (p=0.791). While no group was different in the 0 and 6 hours (p>0.05, for all), in the 6th hour the IL-10 values in Group 2 and Group 6 were higher compared to the control groups (p<0.05, P<0.001, respectively). The variation with time of IL-10 values is shown in Figure 2.

ALT values

There are statistically significant differences produced by time [F (2, 46) =54,92 p<0.001 η2=0,774] and by time * group interactions [F (10, 60) =5,29 P=0.001 η2 =0,623] on AST. While there was no difference identified between the groups at the initial time, at the end of the 12th hour the lowest ALT values were in Group 3. However, the difference was not significant compared to Group 1 (P>0.05). When compared to Group 4, the results in both Group 3 and Group 6 were significantly lower (P<0.05).

Results of Histopathological Investigation

H&E histopathologic assessment of apoptotic cells in liver tissue observed no difference between Group 1 and Group 4 (P=0.566). In Group 2, Group 3 and Group 6, the apoptotic cell counts were observed to be lower compared to the control groups (P=0.001, P=0.001, P=0.001, P=0.001, respectively) **Figure 3** and **4.** The apoptotic cell count in Group 5 was slightly lower than Group 4, though the difference was not significant (P=0.082). According to the results of caspase 3 immunohistopathological assessment, the apoptotic cell counts found in Group 2, Group 3, Group 5 and Group 6 were observed to be lower compared to the control groups (P=0.001, P=0.001, P=0.002, P=0.001, respectively) The results are shown in **Figure 3** and **5.**

Results of Electron Microscope investigation

Ultrastructural investigation of liver tissue was completed with statistical evaluation in terms of total injury score. The lowest cellular damage occurred in Group 3 and Group 6 and the difference was observed to be significant compared to the control group (P=0.001, P=0.01, respectively). In Group 2 and Group 5 with p-CA added, the injury was less compared to the control groups, but the results were not observed to be significant (P=0.851, P=0.151, respectively). When Group 1 and Group 4 are compared, there was no difference observed in total injury score (P=0.997)(**Figure 3**). When the groups are assessed in terms of ultrastructure, there was moderate degree of boundary irregularity in the nuclei of hepatocytes in Group 1. In some areas of hepatocyte and sinusoidal endothelium sporadic necrotic degeneration and minimal dilatation in the perinuclear area was observed. In Group 4, there was a moderate degree of perinuclear dilatation, with crista loss observed in some hepatocyte mitochondria. In both groups, there was a density increase observed in the interstitial area generally.

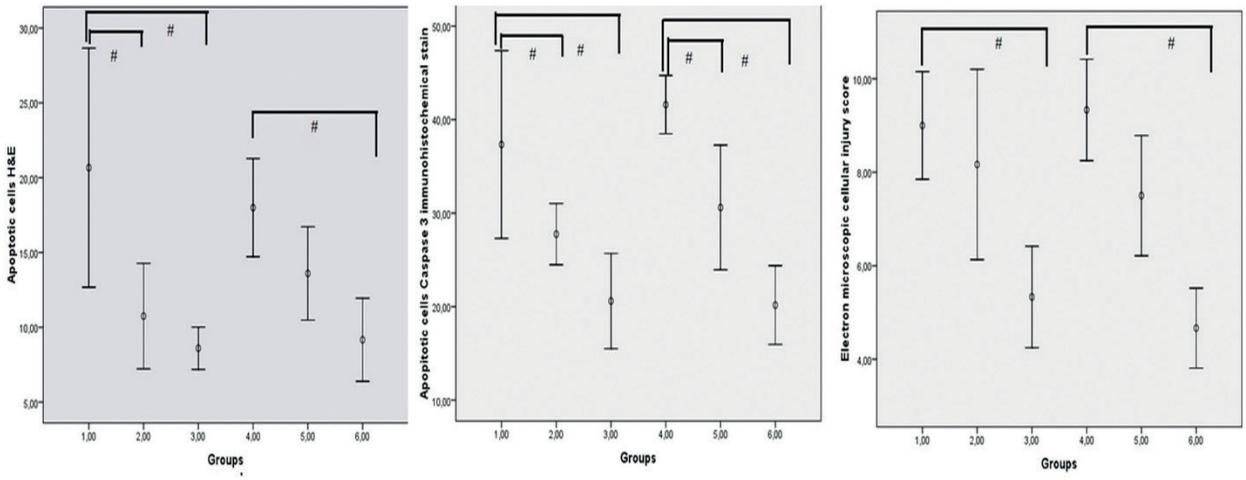

Figure 3. Statistical comparison of apoptotic cells H&E (A), caspase 3 immunohistochemical and electron microscope cellular injury values *: p<0.05 #: p<0.001

In Group 2 and Group 5 hepatocytes generally had normal ultrastructure, with sporadic small lysosomes and lipid vacuoles observed at intervals.

For liver tissue in preservation solutions with ellagic acid added (Group 3 and Group 6), electron microscopic investigation found hepatocytes generally had normal ultrastructure, with some hepatocytes identified to have minimal sporadic intrastoplasmic edema in the perinuclear area. However, there was no organelle injury in these hepatocytes. The interstitial area was open and sinusoidal endothelium appeard to have normal ultrastructure **(Figure 6)**.

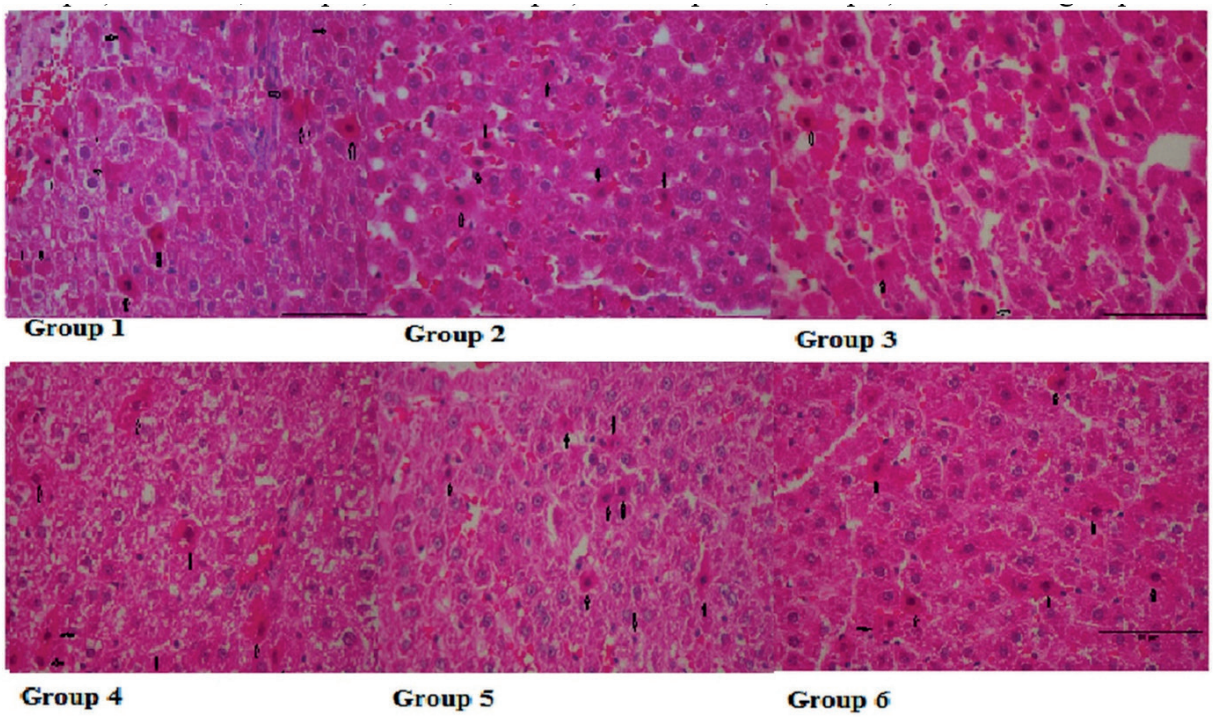

Figure 4. H&E staining in the groups (pink eosinophilic cytoplasm piknotic nuclei apoptotic hepatocytes shown with arrows) 400×magnification. Group 1) UW; Group 2) UW + p-CA; Group 3) UW+EA; Group 4) HTK; Group 5) HTK + p-CA; Group 6) HTK + EA group

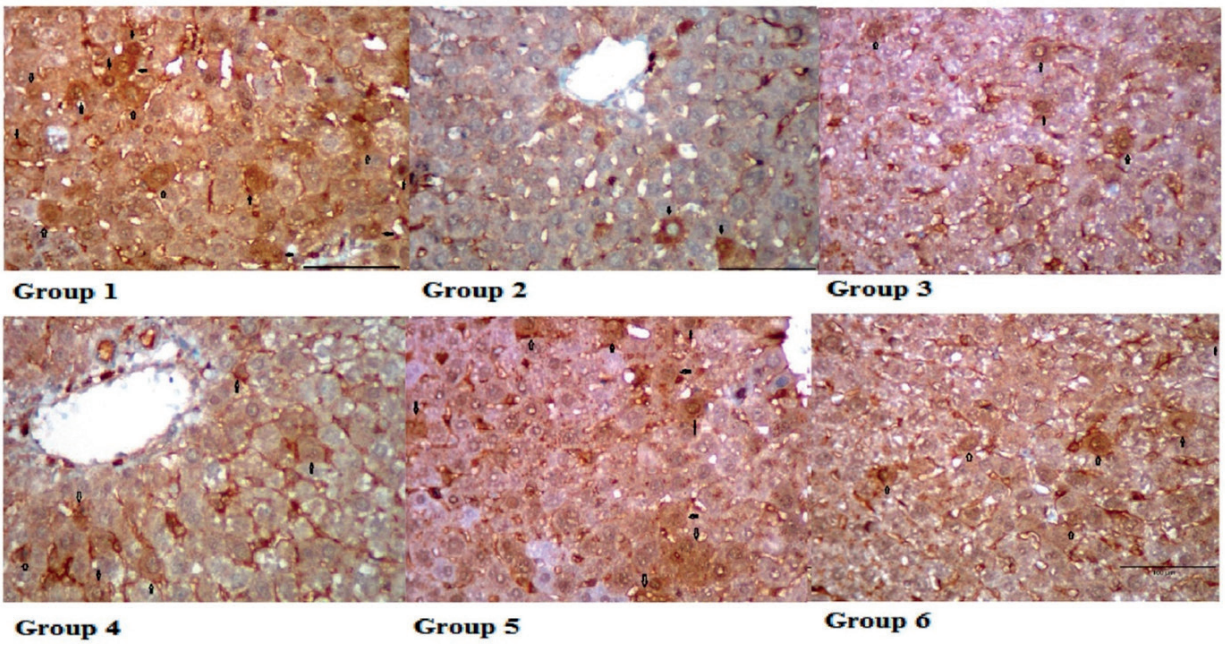

Figure 5. Caspase 3 immunohistochemcial staining in groups (activated caspase 3 and strong cytoplasmic or nuclear staining accepted as positive, apoptotic cells shown with arrows) 400×magnification. Group 1) UW; Group 2) UW + p-CA; Group 3) UW+EA; Group 4) HTK; Group 5) HTK + p-CA; Group 6) HTK + EA group

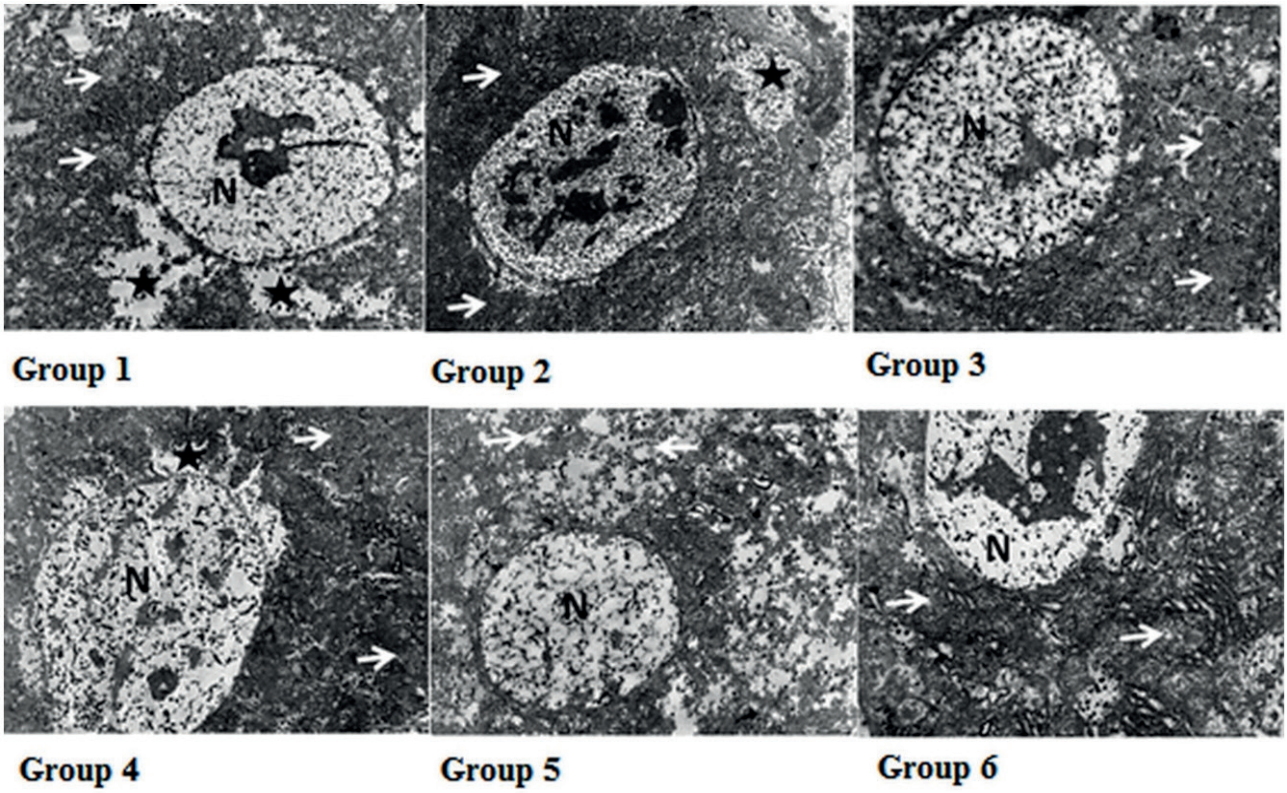

Figure 6. Electron microscope images of liver tissue in different preservation solutions
Group 1. Lipid vacuoles (short arrows), nucleus (N), mitochondria (arrow), intracellular edema (star). Scale bar = 2 µm
Group 2. Minimal intracellular edema (star), mitochondria (arrow). Scale bar = 2 µm
Group 3. Minimal intracellular edema with relative normal liver cells compared to other groups. Scale bar = 2 µm
Group 4. Nuclear chromatolysis (N) in nucleus of hepatocytes and intracellular edema (star). Scale bar = 2 µm
Group 5. Minimal intracellular edema (star), mitochondria (arrow). Scale bar = 2 µm
Group 6. Relatively normal ultrastructure liver cells compared to other groups, minimal intracellular edema (star), mitochondria (arrow). Scale bar = 2 µm

DISCUSSION

Completed for the first time in 1963, liver transplantation in the terminal period is used for a variety of liver diseases (21). In spite of increasing success linked to the use of immunosuppressive agents, intensive care facilities and experience, failure is still a serious problem (22). One of the most important causes of failure is primary non-function (PNF). Development of PNF in liver transplantation is blamed on a variety of factors like surgical technique, preservation damage, hot ischemia and histopathologic structure of the donor organ. Of these factors perhaps the only one that can be changed is preservation (23). A variety of studies have shown that free oxygen radicals released from Kupffer cells and endothelium during ischemia cause cellular damage (23, 24). After optimizing factors like surgical technique and hot ischemia, the aim of this study is to assess the effects on liver damage of enriching preservation solutions with agents with weak and strong antioxidant capacity.

When the study results are assessed in short, in groups enriched with p-CA (Group 2 and Group 5) antioxidant enzyme activities were high at 0 hour compared to the control group, especially in Group 5. However, over time this difference disappeared. In the groups enriched with EA, though the enzyme activities reduced over time, especially in Group 6, the antioxidant enzyme activity was observed to be high compared to the other groups. When groups are assessed in terms of cytokine activity for inflammatory parameters, the anti-inflammatory cytokines of IL-4 and IL-10 were highest in Group 6, while the results in Group 3 were similar to Group 6. The results in Group 2 and Group 5 were not found to be different to the control groups. In terms of the inflammatory cytokines of TNF-α, IL-1 and IL-6 the lowest values were in Group 3, with higher values in Group 6 compared to Group 3 and IL-6 values especially observed to be significantly low compared to the control group ($P<0.05$) . In our study the objective marker of EM investigation allowing evaluation of damage at the cellular level found the groups with EA added had lower damage levels at the cellular level compared to the control group. The cellular damage results in Group 2 and Group 5 were low compared to the control groups, but the difference was not statistically significant ($P>0.05$).

In the literature there are many studies evaluating the effects of UW and HTK preservation solutions on liver preservation. The basic emphasis in these studies is that as the UW solution is a solution with high viscosity due to the hydroxyethyl starch (HES) it contains and shows a hyperaggregating effect on erythrocytes, it is reported that it increases the liver perfusion duration, lengthens the total cooling time and increases the hepatic artery resistance. As a result, just as there are experimental studies reporting negative effects in the early period (25, 26), there is a study reported it more effectively reduces edema compared with HTK solutions. While there is no difference in terms of transplantation results in the HTK and UW groups, the biliary tract complications in the HTK group are stated to be higher compared to the UW group (27). In our study, the MDA values at 0 and 12 hours were similar in the UW and HTK groups but at the 6th hour the value in the HTK group was lower compared to the UW group (P=0.039). In terms of antioxidant enzyme activities, inflammatory and anti-inflammatory cytokine levels, there was no difference observed between the two groups. In our study the most objective marker of injury was histopathologic and electron microscopic investigations, with no difference between the two groups in terms of histopathologic apoptotic cells and electron microscopic damage.

The basic mechanism of the hepatoprotective effect of p-CA is its anti-oxidant effect. In studies of rats with induced cardiac pathology, p-CA is cardiac protective and this is stated to be due to the free radical scavenger effect lowering MDA levels and antilipidemic and direct antioxidant effects (28). Another study identified a fall in MDA levels and increase in SOD and GSH levels in rats treated with p-CA after a cardiotoxicity model was induced by antineoplastic DOX (doxorubicin) (29). In our study addition of p-CA to the preservation solutions reduced MDA levels; however only the reduction in Group 2 was observed to be statistically significant. When evaluated in terms of antioxidant enzyme activity, the CAT, SOD and GSH-Px levels in Group 2 and Group 5 were high compared to the control groups; however the difference was not significant. When the inflammatory cytokine values in Group 2 and Group 5 (TNF–α, IL-1, and IL-6) are generally assessed, the results in both groups were significantly low compared to the control group at 0 and 12 hours (P<0.05 for all). In the 6th hour, there was no difference in any group compared to the control groups (P>0.05 for all). When the results are assessed in terms of immunohistopathological apoptotic cells and electron microscope damage, the results in both groups were lower compared to the control groups; however a statistical difference was only observed in Group 2 in terms of immunohistochemical staining results. Another striking point of the study was that the use of p-coumaric acid, which has lower antioxidant capacity than ellagic acid, showed no difference from the control group in respect of both antioxidation markers and histological changes. This finding supports the aim of the study.

Çelik et al. reported that EA, especially used at high dose, has antioxidant efficacy in the liver (30). Salem et al. (31) administered aluminum to rats to induce oxidative stress in an experimental model and found that EA reduced the MDA levels and increased the antioxidant efficacy of GSH, GSH-Px and CAT. They stated it had a protective effect on liver cells against oxidative damage. In accordance with the literature, in our study the addition of EA to preservation solutions reduced the MDA levels, a marker of oxidation in the environment, and increased the GSH, GSH-Px and CAT levels, markers of antioxidant capacity. Similar results were observed to anti-inflammatory cytokines. When the results are assessed in terms of immunohistopathologic apoptotic cells and electron microscopic damage, in both groups the results were observed to be statistically significantly lower compared to the control groups.

When the treatment groups are compared, in the groups enriched with EA antioxidant enzymes and anti-inflammatory cytokines were higher, while immunohistopathologic apoptotic cell numbers were lower and there was more limited cellular damage observed. Additionally, moderate boundary irregularity was observed in the hepatocyte nuclei, sporadic necrotic degeneration in some areas of hepatocyte and sinusoidal endothelium and minimal dilatation was observed in the perinuclear areas in the UW and HTK groups. In the groups with p-CA added though sporadic small lysosomes and lipid vacuoles were observed at intervals, the hepatocytes were generally observed to have normal ultrastructure. In the groups with EA added, the ultrastructure was observed to be close to normal. This leads to the consideration that p-coumaric acid and ellagic acid neutralize free oxygen radicals leading to protection against the destructive effects of free oxygen radicals on the cellular membrane and cellular structure.

Due to limited organ reserves and the waiting time because harvested organs may not be transplanted immediately, the storage of organs is important. Therefore, studies related to this subject are important. The results of this study have shown that increasing the antioxidant capacity of preservation solutions provides longer protection for the cells. Furthermore, it was determined that agents with high antioxidant properties showed a greater protective effect. Nevertheless, as this was an experimental animal study, there is a need for further studies to support these results.

Conflicts of interest

Drs. Author 1, Author 2, Author 3, Author 4Author 5, Author 6, Author 7 and Author 8 have no conflicts of interest or financial ties to disclose.

Compliance with Ethical Requirements

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Research Contribution Rate Statement Summary

The authors declare that, they have contributed equally to the manuscript

REFERENCES

1. Guibert EE, Petrenko AY, Balaban CL, Somov AY, Rodriguez JV, Fuller BJ. Organ Preservation: Current Concepts and New Strategies for the Next Decade. Transfus Med Hemother. 2011;38:125-142.
2. Mangus RS, Wisecarver JL, Radio SJ, Stratta RJ, Langnas AN, Hirst K, et al. Frozen section evaluation of donor livers before transplantation. Transplantation 1993;56:1403–1409.
3. Mangus RS, Fridell JA, Vianna RM, Milgrom MA, Chestovich P, Chihara RK,et al. Comparison of histidine-tryptophan-ketoglutarate solution and University of Wisconsin solution in extended criteria liver donors. Liver Transpl 2008;14:365–373.
4. Feng L, Zhao N, Yao X, Sun X, Du L, Diao X, et al. Histidine-tryptophanketoglutarate solution vs. University of Wisconsin solution for liver transplantation: a systematic review. Liver Transpl. 2007;13:1125–1136.
5. Akbulut S, Sevmis Ş, Karakayali H, Bayraktar N, Unlukaplan M, Oksuz E, et al. Amifostine enhances the antioxidant and hepatoprotective effects of UW and HTK preservation solutions. World J Gastroenterol. 2014;Sep 14;20(34):12292-300.
6. Mathew S, Abraham TE, Zakaria ZA. Reactivity of phenolic compounds towards free radicals under in vitro conditions. J Food Sci Technol. 2015;52:5790–5798.
7. Stojković D, Petrovic´ J, Soković M, Glamočlija J, Kukić-Marković J, Petrović S. In situ antioxidant and antimicrobial activities of naturally occuring caffeic acid, p-coumaric acid and rutin, using food systems. J Sci Food Agric. 2013;93(13): 3205-3208.
8. Guven M, Yuksel Y, Sehitoglu MH, Tokmak M, Aras AB, Akman T, Golge UH, Goksel F, Karavelioglu E. The effect of coumaric acid on ischemia-reperfusion injury of sciatic nerve in rats. Inflammation. 2015;38(6): 2124-2132.
9. Pei K, Ou J, Huang J, Ou S. p-Coumaric acid and its conjugates: dietary sources, pharmacokinetic properties and biological activities. J Sci Food Agric. 2016;96(9):2952-2962.
10. Cheng N, Wu L, Zheng J, Cao W. 2015 Buckwheat Honey Attenuates Carbon Tetrachloride-Induced Liver and DNA Damage in Mice. Evid Based Complement Alternat Med. 2015:987385.
11. García-Niño WR, Zazueta C. Ellagic acid: Pharmacological activities and molecular mechanisms involved in liver protection. Pharmacol Res. 2015;97: 84-103.
12. Zahin M, Ahmad I, Gupta R.C, Aqil F. Punicalagin and ellagic acid demon-strate antimutagenic activity and inhibition of benzo(a)pyrene induced DNA adducts, Biomed Res Int . 2014;2014:467465.
13. Li TM, Chen GW, Su CC, Lin JG, Yeh CC, Cheng KC, et al. Ellagic acid induced p53/p21 expression, G1 arrest and apoptosis in human bladder cancer T24 cells. Anticancer Res. 2005;25(2A):971-979.
14. El-Shitany NA, El-Bastawissy EA, El-desoky K. Ellagic acid protects against carrageenan-induced acute inflammation through inhibition of nuclear factor kappa B, inducible cyclooxygenase and proinflammatory cytokines and enhancement of interleukin-10 via an antioxidant mechanism. Int Immunopharmacol. 2014;19(2):290-299.
15. Lee JH, Won JH, Choi JM, Cha HH, Jang YJ, Park S, et al. Protective effect of ellagic acid on concanavalin A-induced hepatitis via toll-like receptor and mitogen-activated protein kinase/nuclear factor κB signaling pathways. J Agric Food Chem. 2014;15;62(41):10110-10117.
16. Ohkawa H, Ohishi N, Tagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979;95:351–358.
17. Sun Y, Oberley LW, Li Y. A simple method for clinical assay of superoxide dismutase. Clin Chem. 1988;34(3):497-500
18. Sinha AK. Colorimetric assay of catalase. Anal Biochem.1972;47(2):389-394.
19. Beutler E, Matsumoto F. Ethnic variation in red cell glutathione peroxidase activity. Blood. 1975 Jul;46(1):103-110.
20. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. 1973 Selenium: biochemical role as a component of glutathione peroxidase. Science. 9;179(4073):588-590.
21. Busuttil RW, De Carlis LG, Mihaylov PV, Gridelli B, Fassati LR, Starzl TE. The first report of orthotopic liver transplantation in the Western world. Am J Transplant.2012;12:1385–1387.
22. Müller SA, Mehrabi A, Schmied BM, Welsch T, Fonouni H, Engelmann G, et al. Partial liver transplantation-living donor liver transplantation and split liver transplantation. Nephrol Dial Transplant. 2007;22 Supp l:viii13–viii22.
23. Li W, Meng Z, Liu Y, Patel RP, Lang JD. The hepatoprotective effect of sodium nitrite on cold ischemia-reperfusion injury. J Transplant. 2012;635179.
24. Stahl JE, Kreke JE, Malek FA, Schaefer AJ, Vacanti J. Consequences of coldischemia time on primary nonfunction and patient and graft survival in liver transplantation: a meta-analysis. PLoS One.2008;3:e2468.
25. Lynch RJ, Kubus J, Chenault RH, Pelletier SJ, Campbell DA, Englesbe MJ. Comparison of histidine-tryptophan-ketoglutarate and University of Wisconsin preservation in renal transplantation. Am J Transplant. 2008;8: 567-573
26. Jain A, Mohanka R, Orloff M, Abt P, Kashyap R, Cullen J, et al. University of Wisconsin versus histidine-tryptophan-ketoglutarate for tissue preservation in live donor liver transplantation. Exp Clin Transplant. 2006;4: 451-457
27. Avolio AW, Agnes S, Nure E, Maria G, Barbarino R, Pepe G, et al. Comparative evaluation of two perfusion solutions for liver preservation and transplantation. Transplant Proc.2006;8:1066–1067.
28. Prasanna N, Krishnan DN, Rasool M. 2013 Sodium arsenite-induced cardiotoxicity in rats: protective role of p-coumaric acid, a common dietary polyphenol. Toxicol Mech Methods. 2013;23(4): 255-262.
29. Shiromwar SS, Chidrawar VR. Combined effects of p-coumaric acid and naringenin against doxorubicin-induced cardiotoxicity in rats. Pharmacognosy Res. 2011;3(3):214-219.
30. Celik G, Semiz A, Karakurt S, Arslan S, Adali O, Sen A. A comparative study for the evaluation of two doses of ellagic acid on hepatic drug metabolizing and antioxidant enzymes in the rat. Biomed Res Int. 2013;2013:358945.
31. Salem AM, Mohammaden TF, Ali MA, Mohamed EA, Hassan HF. Ellagic and ferulic acids alleviate gamma radiation and aluminium chloride-induced oxidative damage. Life Sci. 2016;1;160:2-11.