Evaluation of silybum marinaum efficacy on University of Wisconsin and histidine-tryptophan-ketoglutarate solutions latter the damage of the perfused liver

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Abstract

Purpose: To investigate the hepatoprotective and antioxidant efficacies of Silybum marianum's (silymarin, S) on University of Wisconsin (UW) and histidine-tryptophan-ketoglutarate (HTK) preservation solutions.

Methods: Thirty two Wistar albino adult male rats were used. Group 1: UW group, Group 2: UW + Silymarin group (S), Group 3: HTK group, Group 4: HTK + silymarin group (S), respectively. Silymarin was enforced intraperitoneally before the surgery. Biopsies were enforced in 0, 6 and 12 hours to investigate.

Results: Biochemical parameters examined in alanine aminotransferase (ALT), furthermore superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) in rats were also evaluated. Detected histopathological changings were substantially declining in the groups that received silymarin, cellular damage was decreased significantly in HTK + Silymarin group, according to other groups. It has been identified as the most effective group was HTK + silymarin group in evaluation of ALT, electron microscopic results, also decreased MDA and elevated in SOD, and CAT activity. Caspase 3 analysis showed a substantial lower apoptosis ratio in the silymarin groups than in the non-performed groups (p<0.05).

Conclusion: Histidine-tryptophan-ketoglutarate+silymarin group provides better hepatoprotection than other groups, by decreasing the hepatic pathologic damage, delayed changes that arise under cold ischemic terms.

Key words: Liver. Histidine. Tryptophan. Antioxidants. Milk Thistle. Rats.
Introduction

Protecting the hepatic functions after the cross clamping and harvesting the liver is so significant and vital for donor tissues, otherwise it may cause rejection in the tissue. Organ preservation is needed for inhibiting the possible injuries of free radicals, provide hypothermia, and minimizing the functions and to provide vitality. Its injury absolutely connected with primary nonfunction (PNF) in liver transplantation. Although various molecules have been tried in recent decades. The University of Wisconsin (UW) and histidine-tryptophan-ketoglutarate (HTK) are common used solutions in clinical practice. They also include some substance for preventing cellular disorganization, stabilize cell membrane structure, provide intracellular electrolyte balance, and cytoprotective shield.

The UW solution contains adenosine (for ATP requirement), Hydroxy-ethyl-starch (Interstitial edema inhibitor), glutathione, phosphate for pH stabilization and allopurinol (duty for antioxidant and preserving cell structure) agents whereas the HTK solution uses mannitol, histidine (antioxidant and osmotic, effect), tryptophan (membrano, protective effect), ketoglutarate (membrano protective effect, duty in crebs cyclus), and mannitol.

A novel approach in the transplant society is to increase cytoprotective effects of solutions. Silybum Marinaum (Silymarin) is a plant which contains flavonolignan molecule, also protect liver parenchyma from the free radicals exerts membrane stabilizing and provide the detoxification of liver. Different from other studied molecules silybum marinaum has glutathion which supply cytoprotective effect reduces the inflammatory reaction and inhibit the fibrogenesis of liver. The aim of this research was to evaluate the applying of Silybum Marinaum (silymarin, S) in the aspect of improving the antioxidant and cellular preserving efficacies of HTK and UW solutions.

Methods

Surgical procedures

The tertiary center local ethics committee for experimental animal research confirmed this study. Research group obeyed the rules of rat procedures for experimental guidelines founded by the National Institutes of Health by the “Guide for the Care and Use of Laboratory Animals” (2013/3-28D). Thirty two wistar albino rats (average weight: 250-300g) were utilized for this experimental study. Of the 32 rats were separated randomly into four groups by equally. These groups are named as follows:

- Group-I: UW group
- Group-II: UW+Silymarin group (UW+S)
- Group-III: HTK group (HTK)
- Group-IV: HTK+Silymarin group (HTK+S)

Rats were anesthesiad by applying 30 mg/kg hydrochloridic ketamine (Ketalar, Eczacibasi, Turkey) and 10 mg/kg hydrochloridic xylazine (Rompon, Bayer, Turkey) before the conventional surgery. Wistar rats in groups II and IV were performed silymarin 100 mg/kg (S0292-10G, Sigma aldrich laboratories, Netherlands) via intraperitoneal performing half an hour ago before the surgery. After the verifying, rats were equally and balanced anesthetized. The surgical procedure was performed in a closed environment under controlled temperature, conventional laparotomy was applied by the incision midline.

The portal pedicle ligation performed after the cannula settled in portal vein lumen. Group-I rats were perfusated by solution of UW(Viaspan; DuPont Merck Pharmaceutical Company, Wilmington, DE, United States) which reserved at 4 °C Via the catheter inside the portal vein. Group-II rats were medicated silymarin prior to laparotomy later this application, perfusion started with UW, alike in first group (UW+S). Group-III were perfusated by HTK (Custodiol; Odyssey Pharmaceutical Inc, United States).
preserved at 4°C. Group-IV rats were firstly perfused silymarin prior to laparotomy, after this perfusion started by HTK (HTK+S)

Pending the applying of perfusion, the suprahepatic part of vena cava was dissected and isolated by cutting whereas discharging of liquid was clear in the liver. The perfusion finished when the liquid becomes clear. Secondly of the perfusion, a regular hepatectomy was applied. The parts of split hepatic tissue were put into bags including UW or HTK solutions and covered in ice slush-contained cases. In follow-up periods at 0, 6, and 12 hour after the hepatectomy (AH), tissue biopsy examples were taken from the dormant hepatic tissues for histopathological and immunohistochemical assessment, and liquid examples were taken for biochemical determination. Hepatic tissue biopsy was also applied 6 hour later the hepatectomy for examination by electron microscopy.

**Biochemical analysis**

Examples acquired from the stored liquid were frozen at -80°C in nitrogen liquid for consecutive assessments of alanine aminotransferase (ALT) levels. ALT was evaluated with a Ultraviolet (UV) absorbance procedure which is set like a standardized method. Both of the enzyme levels were stated in unit/liter (U/L).

**Pathologic evaluation**

**Liver histopathological examination**

After finishing the perfusion of hepatic tissues, weight measurement were enforced and covered at -80°C until use or fixed in 10% buffered formalin and buried in paraffin. Assessment of histopathologic damage was enforced in hematoxylin-eosin staining sections utilized to evaluate four diverse parameters by light microscope. The samples were assessed semi-quantitatively by ranking tissue lesion severity. Range from 0 to 3 depending on the degree and rank of the changings as follows:

Ballooning was evaluated as 0 : ¼ absent, 1 :¼ mild, 2: ¼ moderate, and 3: ¼ severe. Fat deposition was evaluated as a percentage: 0%-30%, 31%-60%, 61%-100%. Pericentral hydropic degeneration and sinusoidal dilation were evaluated as absent, focal, or diffuse FC; absent or present: 1%-30%, 31%-60%, 61%-100%), hepatocyte ballooning degeneration (BD; absent or present: mild, moderate and severe), centrilobular hydropic change (CLHC; absent or present: focal or diffuse), and sinusoidal dilatation (SD; absent or present: focal or diffuse)\(^5,6\).

**Determination of tissue levels of oxidative stress markers**

All biochemical evaluations were applied on the tissue obtained after centrifugation at 14000 rpm using spectrophotometric methods. In order to assess the lipid peroxidation in free radicals (malondialdehyde, MDA), and efficiency of any enzymatic antioxidants (superoxide dismutase, SOD and catalase, CAT)\(^7\).

All of the hepatic tissues were centrifuged in ice-cold 140 mM KCl at 16,000 rpm for 2 min using a centrifugator (IKA Ultra-Turrax T25 basic homogenizer, Germany). All of the procedures were performed at 4°C. The results were expressed appropriately to a regular graphic which was equipped from a regular solution by commercial kits( Diagnostics, USA).

**Determination of SOD activity**

Every piece of tissue centrifuged homogenate was diluted 1:40 with 10 mM phosphate buffer (pH 7.0). Twenty-five microliters of diluted example was mixed with 850 μL of substrate suspension including 0.05 mmol/L xanthine sodium and 0.025 mmol/L INT (2-[4—iodophenyl]-3-[4-nitrophenol]-5-phenyltetrazolium chloride) in a buffer solution including 50 mmol/L CAPS (N-cyclohexyl-3-aminopropane sulfonic acid) and 0.94 mmol/L EDTA disodium dihydrate (pH 10.2). Then, 125 μL of xanthine oxidase (80 U/L) was attached to the
mixture and absorbance elevation was pursued at 505 nm for 3 min against air. Standard solutions were prepared from stock solution of SOD from bovine erythrocytes in the solution range of 0.217 and 5.2 kU/L and phosphate buffer was utilized as the blank solution.

### Determination of CAT activity

For determination of the CAT activity, the obtained blend was formed of 50 mM phosphate buffer (pH 7.0), 10 mM H$_2$O$_2$, and tissue homogenate sample. The declining rate of H$_2$O$_2$ was pursued at 240 nm for 45s against air at cell temperature (Park and others 2010). CAT activity was expressed as kU/g protein. Phosphate buffer was used as the blank solution and the calibration curve was plotted with standard solutions (1.25 to 15 U/mL) prepared from CAT stock lyophilized powder.

### Measurement of MDA level

Level of MDA was determined as a thiobarbituric acid reactive substance (TBARS) in the liver and brain homogenates according to the method described by Jamall and Smith (1985). Tetramethoxypropane solution was used as the standard for plotting the calibration slope and the results in the sample were expressed as nmol/g protein. Briefly, 200 μL of sample or standard solution, 200 μL of sodium dodecyl sulfate (8.1%), 1500 μL of acetic acid solution (20%), and 1500 μL of thiobarbituric acid solution (0.8%) were mixed. The solution was completed to 4000 μL with distilled water. The tubes were kept at 95°C for 1h. Later, they were refrigerated under tap water and 2000 μL of the mixture was added to 2000 μL of trichloroacetic acid. They were centrifuged at 1017 × g for 10 min. The rate of supernatant absorbance was measured at 532 nm.

### Histologic and ultrastructural evaluation

Evaluations were done between groups. All examples were stabled at 2.5% glutaldehyde solution with phosphate buffer for 3 hours. Later the destabilization process with 1% osmium tetroxide, they were dried in scaled alcohol baths. Examples passed by propylene oxide were immersed in araldite CY 212, 2-dodecenyl succinic anhydride, benzylidimethylamine and dibutylphthalate.

Thin slices spotted with toluidine blue were evaluated below microscopy. Subsequently, thin slices cut and covered with uranyl acetate and lead citrate were assesses by Zeiss Libra 120 transmission electron microscopy. Also Changes in nucleus, endothelial cells, cell membrane, mitochondrium, and endoplasmic reticulum composition were evaluated. The outcomes were assessed by an specialised histologist.

### Assessment of apoptosis in hepatocytes

Three micron transvers-parts from paraffin-coated samples were detected by caspase - 3 method evaluated samples were examined in at least 10 Various sites of view under × 400 magnification, with at least 1000 hepatocytes evaluated. The number of cells with caspase 3 -positive were saved.

### Statistical analysis

The outcomes of statistical analysis was enforced by the SPSS 17.0 (IBM, Chicago, IL, United States) software program. Chi-square and Fisher exact tests were used to analyze histopathologic disperancies between the groups. A result of P < 0.05 was accepted to be statistically significant. Intergroup benchmarks of biochemical outcomes were applied utilizing mann whitney u test. Suitable, results submitted in tables are displayed as the mean ± SD.

### Results

**Evaluation of liver samples histopathology**

Hepatic tissue biopsies fixed with
hematoxylin and eosin were applied to detect the injury parameters as FC, BD, CLHC, or SD. A substantial difference was detected among groups UW and UW+S in evaluation of SD and CLHC (both of parameters at 6, 12 hours, P < 0.05). Also, this difference detected between HTK and HTK+S groups in SD and CLHC outcomes (p< 0.05 at both time points and parameters) (Figure 1, Table 1).

Furthermore, the examination of Silymarin applied groups, HTK+S group had the best outcomes and also significant difference detected than UW+S group. More pathological changings occur for SD, CLHC in UW+S group. FC and BD histology was not altered between the groups.

**Table 1** - Pathological changings after the procedure.

| Groups  | Time Hours | SD  | CLHC | BD  | FC  |
|---------|------------|-----|------|-----|-----|
| UW      | 0          | 2   | 6    | 1   | 7   |
|         | 6          | 4   | 4    | 4   | 0   | 8   |
|         | 12         | 3   | 5    | 3   | 5   | 1   | 7   |
| HTK     | 0          | 1   | 7    | 0   | 8   |
|         | 6          | 4   | 4    | 4   | 1   | 7   |
|         | 12         | 3   | 5    | 3   | 5   | 2   | 6   | 1   | 7   |
| UW+S    | 0          | 0   | 8    | 0   | 8   |
|         | 6          | 1   | 7    | 2   | 6   |
|         | 12         | 1   | 7    | 2   | 6   |
| HTK+S   | 0          | 0   | 8    | 0   | 8   |
|         | 6          | 2   | 6    | 1   | 7   |
|         | 12         | 1   | 7    | 2   | 6   | 1   | 7   |

**Evaluation of apoptosis**

According to Caspase -3 results used to assess apoptosis, in the postoperatively baseline to 6 hours no significant differences detected in apoptosis for any groups. On the other hand significant decline was detected in groups which applied Silymarin than non applied groups. UW group had a significantly higher rate for apoptosis (p<0.05), also secondly higher rate was detected in HTK and respectively lower rates in UW+S and HTK+S Groups. There was no significant difference detected in HTK+S group than UW+S group for apoptosis (p>0.05) (Table 2, Figure 2).
Table 2 - Outcomes of apoptotic cells detected with Caspase-3 method.

| Groups  | 0 h     | 6 h     | 12 h    |
|---------|---------|---------|---------|
| UW      | 125 ±2.1| 142±18  | 149±10.5|
| UW+S    | 102±5.1 | 108±16.4| 110±8.5 |
| P value | >0.05   | <0.05   | <0.05   |
| HTK     | 122±9.1 | 142±9.9 | 188±6.7 |
| HTK+S   | 90±14   | 124±7.3 | 130±20  |
| P value | >0.05   | <0.05   | <0.05   |

HTK: Histidine-tryptophan-ketoglutarate; UW: University of Wisconsin; S: Silybum marinaum.

Evaluation of SOD, CAT and MDA levels

SOD, CAT and MDA levels from perfused liquids were evaluated at 0, 6, and 12 hours after the procedure. The correlations of groups showed that HTK+S group had the highest in SOD and CAT, lowest in MDA scores when has compared to other groups (Figures 3 to 5).

Figure 3 - MDA level of liver tissues of rats perfused by Solutions and/or silymarine, MDA level is expressed as nmol/g protein. Significant differences and decreasing detected between 0-6, 0-12 and 6-12 hours in all groups, especially in HTK+S group (p<0.05).

Figure 4 - MDA level of liver tissues of rats perfused by Solutions and/or silymarine, MDA level is expressed as nmol/g protein. Significant differences and decreasing detected between 0-6, 0-12 and 6-12 hours in all groups, especially in HTK+S group (p<0.05).

Figure 5 - SOD enzyme activity in liver tissues of rats perfused by solutions, SOD activity is expressed as U/mg protein. Significant differences and increasing detected between 0-6, 0-12 and 6-12 hours in all groups, especially in HTK+S group (p<0.05).

Outcomes of ALT levels

Comparisons in ALT hepatic function tests were enforced in groups at specific hours as shown in Table 1. The elevated ALT levels were significantly higher in HTK and UW group versus to other groups (P<0.05). However ALT levels were significantly lower...
in groups of HTK+S and UW+S (P < 0.05) (UW vs UW + S: P < 0.05; HTK vs HTK + S: P < 0.05). The record low ALT levels were detected in the HTK + S group whereas the most elevated levels were observed in the HTK group (Table 3).

| Hours | UW(IU/L)  | HTK  | UW+S   | HTK+S  | P       |
|-------|-----------|------|--------|--------|---------|
| 0     | 99.5±64.5 | 285.5±140.3 | 126.1±11.9 | 84.2±34.2 | NS      |
| 6     | 688.2±28.0 | 1169.7±172.7 | 520.7±270.0 | 359±127.4 | <0.05   |
| 12    | 1033.1±282.7 | 1424.8±221.5 | 661.3±350.3 | 333.1±172.0 | <0.05   |

ALT, alanine aminotransferase; HTK, histidine-tryptophan-ketoglutarate; UW, university of Wisconsin; S, Silybum marinaum; NS, not significant.

a Data were given as mean standard deviation.
b Significantly different when compared with UW group (P < 0.05)
c Significantly different when compared with HTK group (P < 0.05)
d Significantly different when compared with UW+S group (P <0.05)
x Significantly different when compared with 0 hour (P < 0.05)
y Unsignificant when compared with 6th hour result (P < 0.05)

Table 3 - Comparison of obtained Alt (IU/L) levels detected in study groups according to period.

Evaluation of outcomes with transmission electron microscopy

Assessment of the ultrastructure of all groups obtained that the HTK+S group were best preserved, like as standard non pathologic liver example. The UW+S, HTK, and UW groups had a lower preservation result, with some ultrastructural injuries. Furthermore HTK and UW groups were lower preserved. Obtained lipid droplets in liver cells of some groups, make us think about any external nutritent types may effects this sample (Figure 6).

Figure 6 - The electron microscopic figures in the liver in groups. E1: Dilation in the perinuclear space (arrows), dilatation and loss of crystals in mitochondria (arrows). E2: Minimal levels of intracellular edema in hepatocytes (stars). E3: Hepatocyte in normal ultrastructural structure. E4: Lipid vacuoles in the hepatocyte cytoplasm (arrows).

Discussion

Transplantation is the life saving and also one of the rare procedure that give a curative result especially in hepatic end stage diseases. Inspite of the novel researchs in surgical procedures, increase in intensive care quality and immunosuppressive drugs, rejection of organs nevertheless arise in early or late time interval of transplantation. In this case situations transplant performed patients have losing time, and lose their chance for a lively life temporarily. The term “Primary non-function” (PNF) was utilized to explain this event diagnosed with postoperative blood hepatic function enzyme increasing, decline in gall secretion, also serious coagulopathy. Furthermore some causes like as equipmentless team, extended warm ischemia period, ischemia-reperfusion damage, and poor preservation solutions related with PNF after the transplant process. After this, some negative cyclic occurs such as thromocyte adhesion, which lead to endothelial damage, reducing in cold presevation period and drop the materials cause to trombosis.
Protecting the tissue and organ, starts when the donor patient is diagnosed as death of brain and also has living healthy, useful organs, and period ends after the organ is starts to work totally functional. At this moment, preservation solutions come to the scene, aim to provide the organ after cross clamping and harvesting for functioning by keeping hypothermic and diminishing the free radical induced damage\textsuperscript{11,12}.

The utilizing of preservation liquors has help for conveyance of organs in a easy and feasible way. Nowadays, UW solution is the common used for conveyed abdominal organs and especially, liver. Furthermore, HTK solution, firstly used for heart donors with its low potassium ingredient, has demonstrated effective in organ transplantations. However UW solution includes, electrolytes especially potassium in elevated concentration, also UW solution is increase the vessel efficacies\textsuperscript{13,14}.

Many solutions used for transplantation processess however the best are UW and HTK solutions with minimal side effects and ensure osmotic balance by erasing the free radical effects\textsuperscript{15}. UW solution was found and improved by Belzer et al.\textsuperscript{16}, this solution includes glutathion for protected than free radical effects, hydroxyethyl starch for decreasing interstitial edema and phospate for ph equalization. However it has some bad sides such as increased viscosity, more gall complication proportion, elevated artery capacitance, lead to aggregation.

On the other hand HTK is an another solution for transplantation, firstly founded for cardioplegia later noticed that may use in transplants. It includes mannitol histidin combination for antioxidant effects, ketoglutarat and tryptophan for cell protection also have some plus force like decreased viscosity, quick cooling time, lower capillary permeability, elevated tissue oxygenation. Moray et al.\textsuperscript{17} found that, statistically un

significant differences detected among the UW and HTK groups related after the transplantation hepatic biochemistry, complication ratios, acute rejection possibility, and mortality outcomes\textsuperscript{18}.

In present study, HTK+S group have better results than UW+S and non applied drugs groups. Histopathologic outcomes were obtained preferable in HTK+S than others. Silymarin (the extract of Silybum marianum) is an antioxidant, anti-inflammatory, antifibrotic and hepatoprotective agent which has power of diminishing free oxygen radicals. Due to silymarin’s detoxifying effect, it is used as an anti-hepatotoxic molecule in some poisonings such as acetaminophen, arsenic, carbon tetrachloride, and phenothiazines. In hypercholesterolemia, silymarin diminishes the HMG-CoA reductase (3-hydroxy-3-methylglutaryl coenzyme A), and degrades formation of cholesterol\textsuperscript{19,20}.

The mitochondrial dysfunction effects the variation of lipid metabolism, also cause some cycles that allows the progression of chronic liver damage, such secondary biliary cirrhosis. Furthermore mitochondrial dysfunction leads to increase in ROS production, leading to a advanced stage of cell damage. In this respect, silymarin trigger mitochondrial functions by encouraging an recovery in mitochondrial citrate transport and, triggering ROS (reactive oxygen species) eradication\textsuperscript{21,22}.

Oxidative stress induced by ROS plays a substantial role in hepatotoxicity. Furthermore Reactive oxygen species induces the oxidative stress and this cycle leads to liver injury by lipid peroxidation, and destroying the enzyme activity. MDA is the result of lipid peroxida–tion, and the elevation of MDA in plasma is an triggering factor for hepatic damage. On the other hand SOD and CAT were the significant pieces for antioxidant sys–tem, and the declined levels shows the hepatic damage\textsuperscript{23,24}.

In present study, Silymarin drug
caused significant decline in MDA and major increase of SOD, and CAT after the treatment of silymarin (P<0.05). These outcomes showed that silymarin may be feasible hepatoprotective molecule for possible injury. The action of defence may cover the elevating in antioxidant enzyme level and the diminishing in lipid peroxidation procedure 

Silymarin’s hepatopreservative effect has been showed by different researchers in experimental partial hepatectomy models. Ligeret et al. studied with silibinin which is the component of Silymarin. They researched the efficacy of silibinin on rat liver at cold preservation-warm reperfusion damage. At the end they obtained that silibinin diminish the findings of oxidative stress in the hepatic cells and elevate the mitochondrial ATP amount than non applied livers. In an experimental renal damage model on rats, found the protective effect on ischemia/reperfusion damages. Mourelle et al. also found that silymarin cotreatment absolutely protect the liver from the CCL4 effects in cirrhotic rats.

In present research, two silymarin performed group were compared with non performed groups. Biochemical outcomes find out that drug performed groups have lower ALT levels, less vacuolization and epithelial degeneration in the hepatocytes. Also according to immunohistochemical results lower hepatocellular damage detected after the silymarin applying, Ghobadi Pour et al. studied the effects of lactulose and silymarin on hepatic enzymes in cirrhotic rats and found that alone silymarin or combined drugs have decreased the levels of hepatic enzymes as a companion to our work.

Caspase proteins shows a serious act in apoptosis and are liable for many of biochemical and also morphological situations. For this reason, an increased level of activated caspase proteins is one of the most common apoptosis markers that has been used to indicate apoptosis phenotype. Sang et al. already used caspase-3 for evaluating apoptosis by applying Intraportal mesenchymal stem cell. Consistent with this study, our data showed that the treatment of hepatic cells with silymarin for 6 and 12 hours can elevate the proportion of caspase-3 with an declined number of apoptotic cells. Present study, applying of silymarin obtained significantly declined apoptosis ratios compared to non applied silymarin groups. However, the molecular mechanisms underlying apoptosis in the partial hepatectomy experimental model need more research.

### Conclusions

The demand for donor livers is in high proportion furthermore the need for modified preservation solutions is increasing for donor organs. The main aim for research in this field is to diminish cold ischemic injury before the transplantation. We seek a remedy for curing the influences of preservation solutions recently utilized in the surgical process through applying of hepatoprotective agent silymarin.

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