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Prevention of Hepatorenal Syndrome by Green Tea, Branched Chain Amino Acids and Mannitol in Rat Model

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ABSTRACT

In the present research the protective effect of green tea ethanol extract (TE) with or without mannitol and branched chain amino acids (TAM) were evaluated in a rat model of hepatorenal syndrome (HRS). HRS was induced in rats by intraperitoneal injection of D-(+)-galactosamine hydrochloride. Control HRS rats showed significant increase in plasma bilirubin and transaminases activities with reduction in albumin compared with normal rats. Plasma creatinine, urea, endotheline-1, cholesterol, malondialdehyde, phenylalanine and tumor necrosis factor-α increased significantly with significant decrease in high density lipoprotein cholesterol, total antioxidant capacity and calcium in HRS model. Creatinine clearance was reduced while urinary N-acetyl-β-D-glucosaminidase was elevated in HRS rats. Pretreatment of rats by TE and TAM separately produced significant prevention of the induced biochemical changes by galactosamine hydrochloride. Interlukin-6 gene expression was significantly enhanced in the liver and kidney of HRS model and significantly down regulated on treatment with either TE or TAM. Liver histopathology was severely affected without any change in kidney histology in HRS rats; TAM treatment prevented the changes in liver histopathology more than TE. It can be concluded that pretreatment with TE or TAM afforded protection from HRS; TAM was more efficient.

Keywords: Biochemical changes, interlukin 6 gene expression, histopathology, hepatorenal syndrome, phenolic contents, green tea extract.

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INTRODUCTION

Green tea (*Camellia sinensis* L.) is one of the most popular beverages around the world. Its consumption in Egypt is becoming increased due to awareness of consumers about its health benefits. It is a source of different active constituents that posses variable health effects and could be used as functional food or nutraceutical. The most bioactive constituent present in green tea is the phenolic compounds [1]. Green tea was shown to possess high antioxidant activity [2] pointed to its importance in prevention of chronic diseases. Among the reported health benefits are anti-inflammatory, anti-proliferative and anti-atherosclerotic activities [3]. Green tea was reported to improve liver function [4] and to possess reno-protective effect [5]. So, it is expected to have good health effect towards HRS. Branched chain amino acids (leucine, isoleucine and valine) supplementation has been shown to improve the nutritional status and prevent encephalopathy in liver cirrhotic patients (6, 7). Branched chain amino acids (BCAA) are metabolized extrahepatic so they do not represent any load on the diseased liver [8]. Cha *et al*. [9] reported that BCAA ameliorate fibrosis and Liver cirrhosis, as well as decreasing the ammonia level. BCAA significantly decreases the expression of mRNA for fibrosis markers (TGF-b1, Col1a2, Col3a1, TIMP-1, TIMP-2). Also, oral and parentral mannitol was reported to impart health effect in hepatic cirrhotic patients and in patients with renal dysfunction [10-12].

Hepatorenal syndrome (HRS) is a functional renal failure that frequently develops in patients with advanced cirrhosis and severe impairment in systemic circulatory function. It has been considered to be the consequence of a progression of the splanchnic arterial vasodilation occurring in these patients. Arroyo *et al*. [13] reported that a reduction in cardiac output also plays a significant role in the etiology of HRS. We hypothesized that supplementation of phenolic compound rich extract like green tea ethanol extract that possess anti-inflammatory and antioxidant activity with or without BCAA and mannitol could improve liver function and prevent functional renal failure. The objective of the present research is to study the protective effect of ethanol extract of tea alone or in combination with mannitol and branched chain amino acids towards the induced HRS in rats.

MATERIALS AND METHODS

Plant materials

Green tea, *Camellia sinensis,* Family, Theaceae was purchased from local market, Cairo, Egypt.

Animals

Male Sprague Dawley rats weighing 140 g ± 18.1 as mean ± SD were used in the HRS experiment. Mice of 23-25 g were used in the acute toxicity test. Animals were obtained from Animal house of National Research Centre, Cairo, Egypt. Rats were kept individually in stainless steel metabolic cages; water and food were given *ad-libtium*. Each group of mice (8 mice) were kept in a stainless steel cage, water and food were given *ad-libtium*. Animal experiments were carried out according to the Medical Research Ethics Committee, National Research Centre; Cairo, Egypt.

Preparation of green tea extract

Green tea was dried in hot air oven at 40 °C then placed in a continuous extraction apparatus and subjected to extraction by ethanol. The solvent was completely removed by evaporation under reduced pressure at a temperature not exceeding 40°C.

Diets

Two experimental diets were prepared. The first diet was balanced containing 12% casein, 10% corn oil, 47% starch, 23.5% sucrose, 3.5% salt mixture, 1% vitamin mixture and 3% cellulose. The second diet consisted of 10.2% casein, 10% corn oil, 1.5% branched chain amino acids, 0.6% mannitol, 46.8% starch, 23.4% sucrose, 3.5% salt mixture, 1% vitamin mixture and 3% cellulose. The added branched chain amino acids (BCAA) consist of leucine, isoleucine and valine as 2:1: 1.2).

Determination of total phenolic content in the extract

Total phenolics were determined colorimetric in the crude ethanol extract of green tea using Folin-Ciocalteu reagent [14]. Absorbance was measured at 765 nm using UVPC spectrophotometer. The total phenolic content was expressed as mg gallic acid equivalent (GAE)/g dry extract.

HPLC analysis of phenolic compounds in tea extract

Tea alcohol extract was prepared according to Kim *et al*. [15] for HPLC analysis using Agilent Technologies 1100 series liquid chromatograph equipped with an auto sampler and a diode-array detector. The analytical column was Eclipse XDB-C18 (150 X 4.6 µm; 5 µm) with a C18 guard column (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was kept at 0.8 ml/min for a total run time of 70 min and the gradient programme was as follows: 100% B to 85% B in 30 min, 85% B to 50% B in 20 min, 50% B to 0% B in 5 min and 0% B to 100% B in 5 min. All samples were filtered through a 0.45 µm Acrodisc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by retention times and UV spectra and compared with those of standards.

Experimental procedures of the animal study

Twenty four rats were fed on balanced diet for two weeks, and then divided into 4 groups (each of six rats). The first was healthy normal control group where rats received a balanced diet without any treatments. Rats of group two were fed on balanced diet and given crude ethanol extract of green tea (TE) as daily oral dose of 250 mg/kg rat body weight for three weeks. Rats of group three were fed on balanced diet containing branched chain amino acids and mannitol, and given crude ethanol extract of green tea as daily oral dose of 250 mg/kg rat body weight for three weeks (TAM). Rats of the fourth group were fed on balanced diet for three weeks. At the 34th day all rats except the normal healthy control (group: 1) were injected intraperitoneally by 1.1 g/kg rat body weight of D-(+)-galactosamine hydrochloride (product of Sigma, USA) as 200 mg/ml solution in saline to induce HRS according to Saracyn *et al*. [16]. So, groups 2 and 3 were test groups with HRS and treated with different functional food ingredients while group 4 served as control with HRS. The experiment period was 5 weeks. During the experiment, body weight and food intake were recorded weekly. At the end of the study, total food intake, body weight gain and food efficiency ratio (Body weight gain/total food intake) were calculated. Twenty-four-hours urine samples were collected from the galactosamine hydrochloride injection for determination of N-acetyl-β-D-Glucosaminidase (NAG) according to Price and Whiting [17]. Creatinine was determined in the collected 24 h urine [18]. Blood samples were collected from all rats after an overnight fast. Blood hemoglobin was determined according to Vankampen and Zijlstra, [19]. Plasma was separated for the determination of plasma total cholesterol (T-Ch) [20], and high density lipoprotein cholesterol (HDL-Ch) [21]. Malondialdehyde (MDA) and total antioxidant capacity (TAC) were assessed as indicator of lipid peroxidation and antioxidant state, respectively [22, 23]. Plasma tumor necrosis factor-α (TNF-α) (an inflammatory biomarker) was determined by the method of Stepaniak *et al*. [24]. The activity of plasma aspartate transaminase (AST) and alanine transaminase (ALT) [25], plasma albumin [26] and total and direct bilirubin [27] were estimated as indicator of liver function. Plasma creatinine [18] and urea [28] were determined to evaluate kidney function. Plasma endothelin 1 (ET-1) was assessed using ELISA [29]. Plasma calcium and phosphorus were estimated according to Gindler *et al*. [30] and Taussky and Shorr [31], respectively. Plasma total protein was determined according to Rheinhold [32]. Amino acids were assessed according to Spackman *et al.* [33] and Moor *et al.* [34]. Amino acids were determined in three pooled samples from each group. Creatinine clearance was calculated. Kidneys and livers were separated and weighed. About 40 mg was taken immediately from each tissue for assessing Interlukin 6 gene expression. For histopathological study, parts of livers and kidneys were placed in 10% formaldehyde, dehydrated in graded alcohol and embedded in paraffin. Fine sections were prepared, mounted on glass slides and counter-stained with hematoxylin and eosin for light microscopic analysis. Percentage Kidneys and livers/body weight were calculated.

Amino acid analysis

Amino acid analysis was performed in the National Research Centre Central Laboratory using LC3000 amino acid analyzer (Eppendorf-Biotronik, Germany). The technique was based on the separation of

the amino acids using strong cation exchange chromatography followed by the ninhydrine color reaction and photometric detection at 570 nm. Samples were hydrolyzed with 6 N HCL at 110 °C in teflon capped vials for 24 hours. After vaccum removal of HCL, the residues were dissolved in lithium citrate buffer, pH, 2.2. Twenty µl of the solution were loaded onto the cation exchange column (pre-equilibrated with the same buffer), then four lithium citrate buffers with pH values of 2.2, 2.8, 3.3 and 3.7, respectively were applied to the column at flow rate of 0.2 ml/min. The ninhydrin flow rate was 0.2ml/min and the pressure was 0-150 bar. The pressure of buffer was from 0 to 50 bar and reaction temperature was 130 °C.

Determination of gene expression of liver and kidney Interlukin 6 (IL-6)

Total RNA was isolated from <50 mg of liver and kidney tissue with *PureLink® RNA Mini Kit* (ambion® Life technologies™) according to the manufacturer's instructions. RNA concentrations were measured with a Nano-Drop spectrophotometer and Purity of the extracted RNA was assessed by the A_{260nm}/A_{280nm} ratio. The cDNA was synthesized from 1.5 µg of total RNA in 20 µl reaction with the High Capacity cDNA Reverse Transcription kit (ambion® Life technologies™) according to the manufacturer's instructions. RNA template was incubated for 10 min at 25°C followed by120 min at 37°C. The reaction was stopped by heating at 85°C for 5 min, finally 4°C.

Real-time PCR was performed as described previously [35] with Applied Biosystems 7500 Instrument. The RT-PCR reaction mixture (25 µl) contained 1 µl template cDNA, 1× the SYBR ®Green PCR master mix (ambion® Life technologiestm™) and 0.2 µM of the IL-6 primer pairs. Primers pairs sequence used for RT-PCR gene expression analysis were adapted from the literature [36], sequences were as follow: IL-6-FW (TGA TGG ATG CTT CCA AAC TG), IL-6-RW (GAG CAT TGG AAG TTG GGG TA); GAPDH-FW (GTA TTG GGC GCC TGG TCA CC), GAPDH-RW (CGC TCC TGG AAG ATG GTG ATG G).

PCR reactions were performed using the following protocol: 50°C for 2 min, 95°C for 10 min, 45 cycles of 15 seconds at 95°C, 60 seconds at 60°C, 15 seconds at 72°C, melting curve program (60-95°C) and finally a cooling step to 4°C. PCR water was used instead of cDNA templates as a negative. $2^{-\Delta\Delta CT}$ was used to calculate the relative expression ratio of the target gene [37]; the target gene expression was normalized to the expression of the house-keeping gene GAPDH.

Acute lethal toxicity test

Acute lethal toxicity test of crude ethanol extract of green tea was carried out according to Goodman *et al*. [38]. The 24 h mortality counts (if any) among equal sized groups of mice (8 animals/group) receiving progressively increasing oral dose levels of tea extract were recorded.

Statistical analysis

The results of animal experiments were expressed as the mean±SE and they were analyzed statistically using the one-way analysis of variance ANOVA followed by Duncan's test; $p<0.05$ was used as the criterion of statistical significance. Data from gene expression were analyzed by one-way ANOVA followed by the Dun-nett´s multiple comparison test using the SPSS statistical program. Differences were considered significant at $p < 0.05$. Total phenolic content was expressed as mean ±SD.

RESULTS

Results showed total phenolic content to be 234.53 ± 0.509 mg gallic acid equivalent/g dry green tea alcohol extract. HPLC analysis of different phenolic compounds showed the presence of gallic acid (26 mg/g), protocatechuic (1.532), catechin (1.207), syrngic (1.247), vanillic acid (0.109), sinapic acid (0.075), cummaric acid (0.049), cinnamic acid (0.269) and chrysin (0.685).

Table 1 showed the biochemical parameters of different experimental groups. Plasma levels of total and direct bilirubin and plasma activities of AST and ALT were increased significantly in HRS control, indicating liver dysfunction. Also plasma albumin and total protein reduced significantly in HRS rats compared with normal healthy rats. Plasma levels of creatinine, urea and endotheline-1 (ET-1) increased

significantly in hepatorenal rats compared with normal rats, indicating kidney dysfunction. Oral administration of crude ethanol extract of green tea with or without feeding rats on balanced diet containing branched chain amino acid and mannitol showed significant improvement in liver and kidney functions compared to HRS control. Plasma albumin and total protein showed significant elevation in the test groups compared with HRS control. HRS control rats exhibited a significant increase in plasma total cholesterol and significant reduction in HDL-Ch compared to normal healthy control. HRS rats with different treatments showed significant improvement in plasma cholesterol and HDL-Ch.

Table 1. Biochemical parameters of different experimental groups (Mean+SE)

Parameters	Normal control	Hepatorenal control	Rats given TAM	Rats given TE
Plasma parameters:				
Creatinine (mg/dl)	0.717^{a}±0.045	1.004^{b}±0.020	0.872^{c}±0.036	0.882^{c}±0.046
Urea (mg/dl)	27.1^{a}±1.940	43.4^{b}±2.200	28.1^{a}±2.509	31.0^{a}±2.390
Total protein (g/dl)	6.8^{a} ± 0.126	5.7^{b} ± 0.384	6.98^{c} ± 0.284	6.73^{a} ± 0.119
Albumin (g/dl)	4.2^{a}±0.210	2.74^{b}±0.171	4.0^{a}±0.199	3.6^{a}±0.231
ALT (U/l)	56.7^{a}±0.882	88.8^{b}±1.108	67.5^{c}±0.922	68.3^{c}±1.174
AST (U/l)	41.5^{a}±0.722	82.5^{b}±0.764	60.3^{c}±1.801	62.0^{c}±2.408
T. Bilirubin (mg/dl)	0.376^{a}±0.013	0.511^{b}±0.009	0.382^{c}±0.017	0.392^{c}±0.014
D. Bilirubin (mg/dl)	0.162^{a}±0.004	0.254^{b}±0.007	0.16^{c}±0.007	0.17^{c}±0.006
ET-1 (ng/ml)	23.5^{a}±1.607	40.8^{b}±1.851	28.8^{c}±1.014	29.5^{c}±1.784
T-Ch (mg/dl)	50.2^{a}±2.358	117.5^{b}±8.575	63.6^{c}±3.591	69.4^{c}±4.985
HDL-Ch (mg/dl)	29.9^{a}±1.731	4.9^{d}±0.217	18.9^{c}±2.775	19.5^{c}±1.985
TAC (mmol/l)	1.6^{a}±0.04	0.885^{b}±0.042	1.4^{c}±0.033	1.3^{c}±0.025
MDA (nmol/ml)	6.0^{a}±0.386	22.4^{b}±1.620	6.7^{a}±0.973	7.7^{a}±1.079
TNF-α (pg/ml)	19.4^{a}±0.566	32.1^{b}±0.596	26.2^{c}±1.661	27.3^{c}±1.453
Ca (mg/dl)	9.34^{a}±0.214	7.4^{b}±0.216	9.4^{a}±0.339	9.9^{a}±0.577
P (mg/dl)	4.2^{a}±0.204	3.4^{a}±0.419	4.3^{a}±0.253	5.2^{a}±0.558
Blood parameter:				
Hb (g/dl)	13.7^{a}±0.316	10.1^{b}±0.458	13.7^{a}±0.461	13.6^{a}±0.293
Urine parameter:				
NAG (IU/l)	37.4^{a}±0.526	47.4^{b}±1.351	40.7^{c} ±0.930	40.8^{c} ±0.730
Creatinine clearance (ml/min)	0.928^{a}±0.047	0.546^{b}±0.052	0.654^{b} ±0.045	0.621^{b} ±0.032

TAM: Green tea alcohol extract+ BCAA+ mannitol, TE: Green tea alcohol extract. In each row same letters means non-significant difference; different letters means significant difference at 0.05 probability.

Blood hemoglobin, plasma TAC and plasma Ca reduced significantly in HRS control rats compared with normal control and all the studied test groups. Plasma phosphorus showed non-significant changes in all groups. Plasma levels of MDA and TNF-α as indicator of lipid peroxidation and inflammation, respectively increased significantly in HRS control compared with normal control. All treatments showed significant reduction in MDA and TNF-α levels compared with HRS control group. Creatinine clearance was reduced significantly in HRS group compared to normal group. The test groups showed non-significant increase in creatinine clearance compared with HRS group. Urine level of NAG was elevated significantly in HRS control compared to normal group. Different treatments showed significant reduction in NAG compared with HRS control. Urea, total protein, albumin, MDA, Ca and hemoglobin were normalized with different treatments where they match the level in normal control rats.

Table 2 showed plasma amino acids of different groups. It could be noticed that plasma phenylalanine was the only affected amino acid in HRS control where it showed significant elevation compared to normal control, treatment with TAM normalized its level. No significant changes were noticed in other individual plasma amino acids, total essential amino acids, non essential amino acids and total amino acids among different groups. Fischer ratio (BCAA/aromatic amino acids) showed reduced level in HRS control compared to normal control but none significantly. Treatment with TAM and TE produced increase in

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the Fischer ratio compared to HRS control and normal control, the increase was only significant on TAM treatment.

Table 2. Plasma amino acids of different experimental groups

Amino acids (Umol/l)	Normal control	Hepatorenal Control	Rats given TAM	Rats given TE
Essential amino acids				
Valine	960.8[a]±67.04	1110[a]±285.1	987.1[a]±330.3	1294.6[a]±588.7
Methionine	504.1[a] ±111.6	476.7[a]±209.4	968.8[a]±356.3	441.2[a]±97.4
Isoleucine	362.1[a]±55.5	407.8[a]±143.9	1152.7[a]±599.2	331.1[a]±166.4
Leucine	1914.9[a]±199.3	2051.9[a]±447.6	1514.2[a]±218.6	2320.6[a]±167.7
Phenylalanine	2743.1[b]±354.6	2822.3[a]±519.2	1718.4[b]±114.5	2788.4[a]±303.8
Histidine	1393.3[a]±134.4	1499.1[a]±255.8	1226.7[a]±79.33	1111.9[a]±165.8
Lysine	1493.6[a]±131.7	1760.7[a]±272.4	1668.2[a]±180.1	1275.2[a]±397.8
Arginine	1384.7[a]±127.7	1555.6[a]±387.5	1410.8[a]±304.3	1116.7[a]±695.5
Threonine	682.6[a]±60.5	950.8[a]±187.39	633.3[a]±319.6	765[a]±385.1
Non- Essential amino acids				
Aspartic	1419.5[a]±155.7	1837.3[a]±277.4	1569[a]±158.9	1716.3[a]±250.8
Serine	1717.2[a]±127.9	2006.6[a]±310.3	2835.9[a]±923	1692.3[a]±560.6
Glutamic	3490[a]±2367.1	1273[a]±97.95	1394.2[a]±182.4	1182.9[a]±592.6
Glycine	932[a]±49.34	1165.1[a]±322.4	1750.2[a]±863.5	1973.3[a]±871.6
Tyrosine	1064[a]±311.71	1661.9[a]±453.9	719.4[a]±154.7	1187.5[a]±32.8
Proline	662.2[a]±100.5	541[a]±88.84	1164.7[a]±444.3	1456.1[a]±594.9
Alanine	3120.9[a]±254.7	2915.9[a]±470.3	790.9[b]±791.9	2368.1[a]±1195.9
Total essential amino acids	11439.3[a]±1203.9	12634.9[a]±2648.2	11280.2[a]±1778.2	11444.9[a]±1244.1
Total non- essential amino acids	12405.9[a]±1849.1	11405.8[a]±1433.2	10224.4[a]±2323.2	11576.6[a]±1747.8
Total amino acids	23845.2[a]±2431.3	24040.7[a]±4081.4	21504.6[a]±943.7	23021[a]±593.3
Fisher ratio	0.86[a]±0.056	0.78[a]±0.047	1.48[b]±0.286	1.01[a]±0.247

TAM: Green tea alcohol extract+ BCAA+ mannitol, TE: Green tea alcohol extract. In each row same letters means non-significant difference; different letters means significant difference at 0.05 probability.

Table 3. The relative expression of IL-6 gene in liver and kidney of different experimental groups (means± SE)

Groups	Expression of IL-6 gene in kidney	Expression of IL-6 gene in liver
HRS control rats	1.000±0.051	1.000±0.030
Normal control	0.007±0.004***	0.012±0.004***
Rats given green tea extract, BCAA, and mannitol (TAM)	0.037±0.012***	0.144±0.046***
Rats given green tea extract (TE)	0.355±0.065**	0.304±0.048**

† Values of HRS control showed 83 (in liver) -143 (in Kidney) fold changes compared to normal control. The mRNA expression of IL-6 in the kidney of TAM group was normalized with housekeeping gene (GAPDH). TE produced down regulation of IL 6 gene.**P<0·001; ***P<0·0001 compared to HRS control rats.

Table 3 showed that mRNA expression of IL-6 was significantly stimulated in the liver of rats on induction of HRS compared to control normal rats. Gene expression of IL-6 was significantly down-regulated in liver of rats consumed green tea extract by 69.6% compared to HRS control rats. The addition of branched chain amino acids and mannitol to the diet of rats given green tea extract significantly lowered the mRNA expression of IL-6 in the liver of rats by 85.6% compared to HRS control rats. Similarly, IL-6 gene expression in kidney was stimulated in HRS control rats. IL-6 mRNA level was suppressed in the kidney of rats consumed diet containing branched chain amino acids and mannitol concomitantly with green tea extract where gene expression of IL-6 was almost the same like normal control rats. Green tea extract lowered the mRNA expression of IL-6 in the kidney of rats by 64.5% compared to HRS control rats.

Nutritional parameters of all studied groups are shown in table 4. The results revealed non-significant changes in all nutritional parameters, liver weight/body weight % and kidney weight/body weight % among all the studied groups.

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Table 4. Nutritional parameters of different experimental groups (mean± SE)

Parameters	Normal control	Hepatorenal control	TAM	TE
Initial BW(g)	140.3[a] ±5.659	139.8[a]±7.267	139.8[a]±9.702	140[a]±8.044
Final BW (g)	220.3[a] ±5.991	219.5[a]±9.264	210.3[a]±11.595	215[a]±5.875
Body weight gain (g)	80[a]±2.129	79.7[a]±4.827	70.5[a]±2.837	75[a]±5.427
Total Food intake (g)	517[a]±5.214	516.3[a]±2.716	510.8[a]±6.638	511.7[a]±5.999
Food efficiency ratio	0.155[a]±0.004	0.154[a]±0.009	0.138[a] ±0.006	0.146[a] ±0.009
Liver weight/body weight %	2.7[a]±0.148	2.4[a]±0.263	2.6[a]±0.164	2.8[a]±0.126
Kidney weight/body weight %	0.687[a]±0.036	0.63[a]±0.048	0.596[a]±0.044	0.685[a]±0.028

TAM: Green tea alcohol extract+ BCAA+ mannitol, TE: Green tea alcohol extract. In each row same letters means non-significant difference; different letter means significant difference at 0.05 probabilities.

The acute lethal toxicity test revealed that ethanol extract of green tea was very safe up to 12g/kg mice body weight (the highest tested dose).

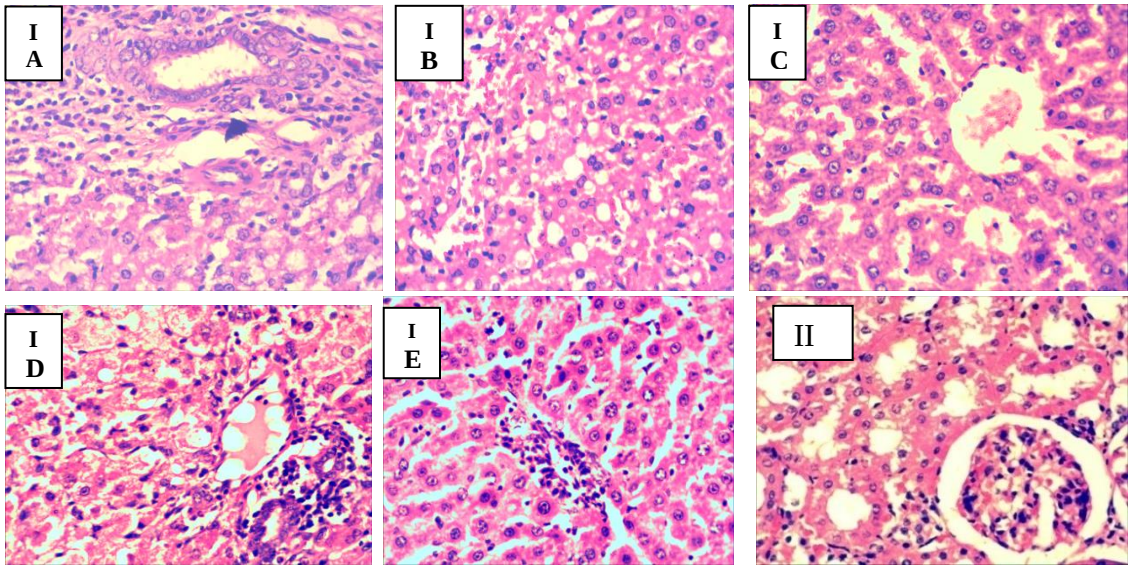

Figure 1: Sections of rats´ livers and kidneys (X400). I A-E: Sections of rat liver; A&B: Hepatorenal control, C: Control normal group, D: Rats given green tea extract, E: Rats given green tea extract with BCAA and mannitol. II: Section of rat kidney (x400): The kidney of all groups showed normal appearance.

The histopathological examination of livers and kidneys of rats (Figure 1/I and 1/II) revealed that the liver is the most affected organs in all treated groups while the kidney showed normal appearance. The liver of HRS control rats showed congestion of blood vessels, newly formed bile ductules and inflammatory cell infiltration in portal areas with beginning of liver failure (Figure 1/I A). Also it could also noticed that HRS control hepatocytes suffered from fatty changes (Figure 1/I, B).The kidneys of HRS control appeared normal (Figure, 1/II). The kidneys and liver of normal control rats showed normal histology (Figure 1/I C& Figure 1/II). Rats with hepatorenal syndrome treated with green tea extract showed normal kidneys and improved liver histopathology compared to control HRS except for few numbers of newly formed bile ductules and few numbers of inflammatory cells in portal area (Figure 1/I, D). Also, in this group, some hepatocytes suffered from degenerative changes either hydropic degeneration or fatty and some of them showed necrosis. Rats treated with green tea extract and fed on diet containing branched chain amino acids & mannitol showed normal kidneys and improved liver histopathology compared to HRS control and rats treated with green tea alone. The liver showed very few numbers of inflammatory cells around the central veins while there was hyperactivity to Kupfer cells (Figure 1/I, E).

DISCUSSION

The patho-physiology and plasma biomarkers in HRS are still not exactly known. It would be very important to study the biomarkers that could reflect the presence of HRS. Oxidative stress and inflammation

are involved in different liver diseases including liver cirrhosis and hepatic failure. Oxidative stress and inflammation during hepatic failure may be one of the factors that induce renal dysfunction, collectively known as HRS. Clinical and experimental studies linked renal disease with oxidative stress, which derived from various pathological conditions that lead to free radical-generating toxic substances, and inflammation. The free radicals are highly reactive and harmful to lipids, proteins, and nucleic acids, resulting in structural and functional impairment. Increased levels of end products mediated by the reactions between biomolecules and free radicals, such as malondialdehyde were observed with renal damage [39-43]. Glomerular damage is induced by oxidative stress [44] and the subsequent inflammatory lesions [45]. Oxidative stress may also mediate acute renal failure [46], obstructive nephropathy [47] and chronic renal failure associated with inflammation [41]. In the present study, the induced HRS produced high oxidative stress reflected in elevated MDA, reduced total antioxidant capacity along with increased the inflammatory biomarker TNF-α and enhanced gene expression of IL 6 in both liver and kidney. These effects were accompanied by liver and kidney dysfunction together with elevated ED-1 that was assumed previously as biomarker of HRS [48] since it is an important determinant of renal function in HRS model. Antioxidants and inhibitors of free radicals have been shown to protect against renal damage [49]. So, antioxidant and anti-inflammatory functional food ingredients could play an important role as hepato- and reno-protective in the model of HRS in rats. Based on this hypothesis, green tea might be a good source of such functional food ingredient according to their previously reported antioxidant and anti-inflammatory activity. Green tea was demonstrated to reduce oxidative stress [50, 51], increase activity of antioxidant enzymes [50], and decrease expression of proinflammatory mediators [50, 51]. These reports coincided with the reduction in oxidative stress (represented by the increased total antioxidant and the reduced MDA) that noticed in HRS rats together with down regulation of IL6gene expression and reduction of TNF-α on administration of TE in the present study. The extract of green tea showed in the present study to contain different phenolic compounds to which the therapeutic efficiency might be attributed. Green tea was reported previously to contain several polyphenolic components (catechins or tea polyphenols) including epigallocatechin gallate, epicatechin gallate, epicatechin, and epigallocatechin [52]. Green tea was shown to possess nephroprotective effects as they reduced the elevation in nonenzymatic kidney markers [53], which may support the improvement in renal dysfunction observed in TE treated HRS rats in the present study. In addition, the present results also showed improvement of enzymatic kidney biomarker represented by NAG. Reno-protective effects of epigallocatechingallate a bioactive component from green tea was proved in pigs which might be ascribed to its antioxidant, antiapoptotic and NO-scavenging properties [5]. A significant decrease in MDA concentrations and urea nitrogen values was reported in hypertensive rats given tannic acid which is one of the main components of green tea [54]. It was reported [4] that polyphenols enriched extract from green tea has hepatoprotective effect since it reduced the CCl_4-induced elevation of serum ALT, AST and ALP activities, and significantly prevented an increase in hepatic MDA levels. The extract displayed a better profile of hepatosomatic index and improved antioxidant enzymes in the liver, relative to CCl_4-intoxicated mice. Liver histopathological observation also confirmed the protection on CCl_4-caused histological alteration [4]. These results confirmed the improvement of liver function and histopathology in HRS group given TE in the present study. Tokunaga *et al*. [55] showed that consumption of green tea produced reduction in serum concentration of total cholesterol in Japanese subjects without change in serum HDL-cholesterol. Present research, confirmed the reduction in plasma cholesterol as that in Tokunaga *et al* study, but also showed elevation in HDL-Ch, this controversy could be due to difference in mammalian type, the studied disease and the type of the prepared extract of green tea. The induced HRS in the present study produced significant reduction in plasma Ca while P showed no change compared to normal healthy rats. The reduction of calcium supports the induced dysfunction of kidney in the present study. Previously, susceptibility to osteoporosis was reported in renal failure [56]. Hyperphosphatemia, is a nearly universal complication of kidney failure [57]. The non change in plasma P in the present study in HRS control might reflect that renal dysfunction did not reach a very late stage. Recent research has suggested that bone mineral density (BMD) is positively associated with green tea consumption, which may optimize bone health [58-61]; this may clarify the improved Ca level in HRS rats treated with TE.

Further improvement on feeding mannitol and BCAA containing diet together with administration of tea extracts reflects the therapeutic efficiency of these functional food ingredients.

Parentral and oral administration of mannitol were shown to have beneficial effect in both hepatic and renal failure in man and animal, so mannitol was used in combination with TE and branched chain amino acids in the present study for protection from HRS. In a previous study rats with D-galactosamine-induced

hepatic failure, mortality was decreased by mannitol infusion. Experimental data indicate the efficiency and safety of hypertonic mannitol therapy for cerebral edema in acute hepatic failure [11]. In a clinical study, mannitol increases brain capillary osmolality, drawing water from the brain tissue into the capillaries, and has been shown to significantly reduce the extent of cerebral oedema and improve survival [62, 63]. There has been considerable evidence of hydrogen's protective effect to diseases related to oxidative injury such as liver disease. Microflora in the large intestine can produce endogenous hydrogen, and it has been reported that oral administration of mannitol in humans and animals can significantly increase the level of endogenous hydrogen [12, 64-66]. Therefore, it could be speculated that oral administration of mannitol may be effective against hepatorenal syndrome. In addition, rats fed Mannitol as 20% of diet had significantly lower serum total cholesterol than control rats [67]. This might mean that mannitol in the present study could be an aid to TE in reducing plasma cholesterol. Mannitol was used previously for conditions like partial nephrectomy and hepatorenal syndrome [68].

Branched-Chain Amino Acids (BCAAs) are a good substrate for protein synthesis, both conserving and restoring muscle mass in advanced liver disease. In cirrhosis, poor dietary intake leads to a deficiency of BCAA and accumulation of aromatic amino acids, both worsening protein-energy deficits and glutaminergic neurotransmission. In "high protein diet" intolerant and severely malnourished patients, BCAA supplements may be useful to provide the necessary nitrogen intake without a decline in mental state. However, a number of meta analyses have failed to find consensus on the use of BCAAs in cirrhosis from a wealth of conflicting data [69, 70]. Hyperammonemia develops due to failure of ammonia detoxification during the progression of chronic liver disease or portal systemic shunting (71, 72). BCAA administration decreases blood levels of ammonia through a catabolic effect that influences glutamate dehydrogenase activity and reduces the rate of glutamate breakdown [72, 73]. TAM treatment in the present study succeeded to elevate Fischer ratio and significantly reduced the aromatic amino acid phenylalanine in HRS rats pointing to protection from encephalopathy due to high ammonia.

TE and TAM treatment showed improvement of the histopathology of liver compared to hepatorenal control rats. Better results of histopathology were shown by rats of TAM group followed by green tea extract. However no change was noticed in the histology of kidney of HRS control group compared to normal control which agreed with previous studies of that showed that renal failure in HRS is due to functional rather than histological change [74, 75].

All biochemical parameters were significantly improved on both treatments (TE and TAM) except for creatinine clearance compared to HRS control group that only showed non significant elevation. TAM was more efficient than TE in prevention of HRS specially in reducing plasma aromatic amino acid, phenylalanine, elevation of plasma Fischer ratio and further improvement of plasma total protein, in addition of liver histopathology.

Tea extract was very safe as shown from the result of the present study. The highest tested dose level in the acute toxicity test in the present study was 12 g/kg mice which correspond to 93g/70 kg man body weight for human when the dose of mice was extrapolated to corresponding estimates in human adopting interspecies dosage conversion scheme [76]. This reflects the highest safety of the green tea extract.

The results of the present research suggests a future study dealing with application of nutraceuticals containing green tea alcohol extract combined with both mannitol and BCAA in patients with liver dysfunction in the goal of preventing progression to HRS.

CONCLUSION

HRS induced in Sprague Dawley rats by intraperitoneal injection of galactosamine hydrochloride produced liver and kidney dysfunction, reduced hemoglobin, elevated oxidative stress, and increased inflammatory biomarker. Galactosamine also induced elevated ET-1, elevated plasma cholesterol, reduced plasma HDL-Ch, reduced plasma albumin, reduced total protein, reduced plasma Ca and elevated urinary NAG with no change in plasma P and nutritional parameters. Enhanced gene expression of IL6 was noticed in liver and kidney of HRS control. Plasma phenylalanine was significantly elevated in HRS control. All these biochemical changes could be considered collectively as biochemical profile of HRS. Histopathology of liver

of HRS control rats showed severe dysfunction and beginning of liver failure with normal appearance of kidney. Pretreatment with TE or TAM afforded protection from HRS; TAM was more efficient. The therapeutic efficiency of green tea alcohol extract might be attributed to its phenolic content. The present work could be applied in clinical study in patients suffering from severe liver dysfunction aiming at prevention of progression to HRS.

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