EFFECT OF GREEN TEA EXTRACT ON MITOCHONDRIAL ENZYMES AND IL-6

USHARANI, S.¹ AND CHITRA, M.²

¹Department of Biochemistry, S.T.E.T. Women’s College, Mannargudi- 641001, Tamilnadu,
²Bharathidasan University, Thiruchirappalli. E. mail: mschitra21@yahoo.com Cell: 09788545071.

Received: October 7, 2010; Revised: November 9, 2010; Accepted: November 19, 2010

Abstract: Hepato-protective activity of the green tea extract against mercuric chloride induced toxicity was investigated in this study. The levels of mitochondrial enzymes were significantly decreased during mercuric chloride treatment and these alterations were reversed by administration of the green tea extract. The present study also appraised alteration in the level of collagen and cytokine such as IL-6. The expressions of collagen and cytokine levels are maintained in green tea extract treated rats when compared to mercuric chloride treated rats.

Key words: Mercuric chloride, green tea extract, collagen, IL-6, mitochondrial enzymes.

INTRODUCTION

Heavy metals are placed under environmental pollutant category due to their toxic effects on plants, animals and human beings. Heavy metals are persistent in nature, therefore get accumulated in soil and plants. Dietary intake of many heavy metals through consumption of plants has long-term detrimental effects on human health [1]. Heavy metals can be toxic if consumed in large amount. Therefore, a continuous intake of low amounts of heavy metal over a long period could cause metal accumulation and damage of all organs including kidney and liver [2]. The main threats to human health from heavy metals are associated with exposure to lead, cadmium, mercury, chromium and arsenic etc. These metals have been extensively studied and their effects on human health regularly reviewed by international bodies such as the WHO [3].

Mercury, a highly toxic metal induces oxidative stress as well as a variety of adverse health effects including renal, neurological, respiratory, immune, dermatological, reproductive and developmental sequel [5]. It is well known that inorganic mercury causes severe kidney damage after acute and chronic exposure. It primarily affects the renal cortex causing dysuria, proteinuria, hematuria, oliguria and uremia leading to renal failure. Along with several other health problems it causes gastrointestinal troubles (colitis, gingivitis, stomatitis and excessive salivation), irritability and occasionally acrodynia [4].

Tea, a product made up from leaf and bud of the plant Camellia sinensis, is the second most consumed beverage in the world, well ahead of coffee, beer and wine and carbonated soft drinks [6]. Depending on the manufacturing process, tea is classified into three major types: ‘non-fermented’ green tea (produced by drying and steaming the fresh leaves to inactivate the polyphenol oxidase and thus, non-oxidation occurs); ‘semi-fermented’ oolong tea (produced when the fresh leaves are subjected to a partial fermentation stage before drying); and ‘fermented’ black and red teas which undergo a post-harvest fermentation stage before drying and steaming, although the fermentation of black tea is due to an oxidation catalyzed by polyphenol oxidase, and that of red tea is attained by using microorganisms [7]. Reported a per capita mean consumption of tea in the world of 120 ml/ day. Green tea (Camellia sinensis) extract is fast becoming ubiquitous in consumer products supplemented with green tea such as shampoos, creams, soaps, cosmetics, vitamins,
drinks, lollipops and ice creams [8]. Fresh tea leaves are rich in flavanol monomers (catechins such as epicatechins) [9].

The main flavonoids present in green tea include catechins (flavan-3-ols). The four major catechins are epigallocatechin-3-gallate (EGCG), that represents approximately 59% of the total of catechins; epigallocatechin (EGC) (19% approximately), (–)-epicatechin-3-gallate (ECG) (13.6% approximately); and (–)-epicatechin (EC) (6.4% approximately). Green tea also contains gallic acid (GA) and other phenolic acids such as chlorogenic acid and caffeic acid, and flavonols such as kaempferol, mycertin and quercetin [10].

The antioxidant capacity of green tea is more potent than that of black tea [11]. If consumed properly, green tea is a potent antioxidant that protects the humans against oxidative damage. Green tea extract can eliminate lipid peroxidation in plasma, and destroy formation of erythrocyte ROS in rats challenged with iron [12]. Ounjaijean et al. [13] studied the antioxidative property of leaf green tea, leaf and granulate of black tea and white tea at levels 1, 2, 4-g/150 ml of water. In the present investigation, effect of green tea extract on the mitochondrial enzymes and IL-6 was studied.

MATERIALS AND METHODS

Animals: Adult male Wister albino rats (weight 180 to 200 g) bred in the central animal house, Rajah Muthiah Medical College, Annamalai University were used in this study. The animals were housed in polypropylene cages and provided with food and water ad libitum.

During the experimental period, food and water consumption were measured every day and the body weight was measured at the initial (day 1) and final day (day 46) of the experiment. At the end of the experimental period, animals in different groups were sacrificed by cervical decapitation under pentobarbitone sodium (60 mg/kg body weight) anesthesia. Liver was taken for further analysis.

Chemicals: Green tea extract was purchased from Sigma Aldrich, U.S.A. Mercuric chloride, other chemicals used in this study were of analytical grade.

Experimental design: Animals were divided into 4 groups (n=6). Animals of first group were kept as control. Animals of group II received green tea (1.5g/kg b.w) for 45 days. The rats of group III were treated at a daily dose of mercuric chloride 1.25mg/kg for 45 days. Group VI was given mercuric chloride 1.25mg/kg as well as green tea (1.5 g/kg) throughout 45 days treatment [14].

Isolation of mitochondria: The liver was dried on blotting paper, weighed and homogenized at high speed. The liver mitochondria were isolated from cell debris, nuclei, microsomes, soluble components and contaminant RBC using differential centrifugation by the method of [15]. A 20% (w/v) homogenate was prepared in 0.25 M sucrose containing 0.05 M tris-HCl buffer and 5.0 mM EDTA. To remove cell debris, tissue fragments and cell nuclei (nuclei pellet), the homogenate was centrifuged at 600-x g for 10 min. The supernatant fraction was centrifuged (Himac SCP 70G, Hitachi, Japan) for 5 min at 4 °C to bring down the mitochondrial pellet. After removing the last bit of liquid, the remaining mitochondrial pellet was resuspended in KCl and used for the estimation of various parameters.

Mitochondrial Enzymes

Isocitrate dehydrogenase: The test tube contained 0.4 ml of tris-HCl, 0.2 ml of trisodium isocitrate, 0.3 ml of manganese chloride and 0.2 ml of mitochondrial suspension. A control tube was also prepared simultaneously. 0.2 ml of coenzyme solution (NADP) was added to the test and 0.2 ml saline was added in control tubes. Both the tubes were incubated 60 min 37 °C. 1.0 ml of colouring reagent (DNPH) was added following by 0.5 ml of EDTA. The contents were mixed well and kept at room temperature for 20 min. Then 10 ml of NaOH was added and the colour developed was read at 420 nm after 10 min. A calibration curve was established with a-ketoglutarate as standard [16].

a-ketoglutarate dehydrogenase: To 0.15 ml of phosphate buffer, 0.1 ml each of thiamine pyrophosphate, magnesium sulphate, a-ketoglutarate and potassium ferricyanide was added. The total volume was made up to 1.2 ml with water and 0.2 ml of mitochondrial ferricyanide was added. The reaction was terminated by the addition of 1 ml of TCA. 0.2 ml of mitochondrial suspension was added to the control after the addition of TCA. The tubes were centrifuged and to 1 ml of supernatant, 0.1 ml of potassium ferricyanide, 1 ml of dupanol and 0.5
ml of ferric ammonium sulphate-dupanol reagent were added and then incubated at room temperature for 30 min. The colour developed was measured at 540 nm. A standard containing potassium ferrocyanide was carried out simultaneously [17].

**Succinate dehydrogenase:** The reaction mixture containing 1.0 ml of phosphate buffer, 0.1 ml of EDTA, 0.1 ml of BSA, 0.3 ml of sodium succinate and 0.2 ml of potassium ferricyanide was made up to 2.8 ml with double distilled water. The reaction was started by the addition of 0.2 ml of mitochondrial suspension [18].

**Malate dehydrogenase** To 0.3 ml of phosphate buffer, 0.1 ml of NADH and 0.1 ml of oxaloacetate were added and the total volume was made up to 2.9 ml with water. The reaction was started by the addition of 0.2 ml mitochondrial suspension. The control tubes contained all reagents except NADH. The change in OD at 340 nm was measured for 2 min at an interval of 15 sec in UV spectrophotometer [19].

**NADH dehydrogenase** The reaction mixture contained 1.0 ml of phosphate buffer, 0.1 ml of potassium ferricyanide, 0.1 ml of NADH and 0.2 ml of mitochondrial suspension. The total volume was made up to 3.0 ml with water. NADH was added just before the addition of the enzyme source (mitochondrial suspension). A control was also treated similarly without NADH. The change in OD was measured at 420 nm for 3 min at 15 s intervals [20].

**Cytochrome C oxidase:** To 1.0 ml of phosphate buffer, 0.2 ml of 0.2% N-phenyl-p-phenylene diamine, and 0.1 ml of cytochrome c and 0.5 ml of distilled water was added. It was incubated at 25 °C for 5 min. Then 0.2 ml of enzyme preparation was added and the change in OD was recorded at 550 nm for 5 min at an interval of 15 s each [21].

**Reverse transcriptase polymerase chain reaction:** All glassware’s were rinsed with diethyl-pyrocarbonate (DEPC) treated water to inhibit RNases. Total RNA was isolated from liver tissue using guanidium thiocyanate-chloroform-phenol method [22]. It was quantified by UV-absorbance spectrophotometer. Total RNA (2 μg) was reverse transcribed and 4 μL cDNA obtained was used for polymerase chain reaction (PCR) amplification. To estimate the expression of interleukin (IL-6), glyceraldehyde 3-phosphate dehydrogenase was used as an internal standard.

**Statistical Analysis:** All data were expressed as mean ± SD (n=6). The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 9 (SPSS, Cary, NC, and USA) and Duncan’s Multiple Range Test (DMRT) obtained the individual comparisons. A value of p<0.05 was considered to indicate a significant difference between groups. Values sharing a common superscript letter do not differ significantly.

**Table 1:** Effect of green tea extract and mercuric chloride on the liver tissue of isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and malate dehydrogenase of control and experimental rats. Values are means ± S.D. for eight rats in each group. Values not sharing a common superscript letter differ significantly at p < 0.05 (Duncan’s multiple range test). Isocitrate dehydrogenase = nmoles of α-ketoglutarate liberated/hr/mg protein; α-ketoglutarate dehydrogenase = nmoles of ferrocyanide formed/hr/mg protein; Malate dehydrogenase = μmoles of NADH oxidized/min/mg protein.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Parameters</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Isocitrate DHase</td>
<td>828.38±79.73</td>
<td>827.22±79.62</td>
<td>721.14±69.41</td>
<td>816.00±78.54</td>
</tr>
<tr>
<td>2</td>
<td>α-ketoglutarate DHase</td>
<td>71.76±6.90</td>
<td>71.17±6.85</td>
<td>51.40±4.94</td>
<td>62.61±6.02</td>
</tr>
<tr>
<td>3</td>
<td>Malate DHase</td>
<td>362.67±34.90</td>
<td>363.5±34.9</td>
<td>255.52±24.59</td>
<td>311.4±29.98</td>
</tr>
</tbody>
</table>

**Table 2:** Effect of green tea extract and mercuric chloride on the liver tissue of NADH dehydrogenase, cytochrome c oxidase and succinate dehydrogenase of control and experimental rats. Values are means ± S.D. for eight rats in each group. Values not sharing a common superscript letter differ significantly at p < 0.05 (Duncan’s multiple range test). U* nmol of NADH oxidized/min U*/ change in OD X10^ {-2}/minU*/nmol of succinate oxidized/min

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Parameters</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NADH DHase(U*/mg protein)</td>
<td>32.58±3.13</td>
<td>30.70±2.95</td>
<td>18.61±1.79</td>
<td>29.88±2.87</td>
</tr>
<tr>
<td>2</td>
<td>cytochrome c oxidase (U*/mg protein)</td>
<td>5.91±0.56</td>
<td>5.11±0.49</td>
<td>2.86±0.27</td>
<td>4.89±0.47</td>
</tr>
<tr>
<td>3</td>
<td>succinate DHase (U*/mg protein)</td>
<td>31.48±3.03</td>
<td>30.62±2.94</td>
<td>11.11±1.07</td>
<td>22.35±2.15</td>
</tr>
</tbody>
</table>
Results

The activities of isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, malate dehydrogenase and succinate dehydrogenase in the mitochondria of liver of rat treated with mercuric chloride in group 3 animals were significantly lower as compared to control animals of group 1 (p<0.05). The administration of green tea extract to mercuric chloride treated rats (group 4) significantly recovered activities of all the enzymes as compared to mercuric chloride treatment alone (Tables 1 and 2). NADH dehydrogenase and cytochrome c oxidase activities on mercuric chloride treatment were also significantly lowered as compared to control rats. The treatment with green tea significantly elevated their activities as compared to group 3.

The transcript analysis of 4 different groups revealed notable increase in the mRNA expression of IL-6 in the liver of HgCl₂ treated rats as compared to control. While green tea supplementation showed significant down regulation of IL-6 levels when compared with HgCl₂ group rats (Tables 3 and 4).

Discussion

Under normal physiological conditions the mitochondria is the major source of ROS production in the hepatocyte [23]. Approximately 80 – 90% of the oxygen utilized by hepatocytes is metabolised by mitochondria [24] and it is estimated that 2% of the oxygen consumed is converted to superoxide anions [25], due to the “leakage” of unpaired electrons to molecular oxygen as they are being transported down the respiration. The life span of a species is to be determined by the rate of mitochondria damage inflicted by oxygen free radicals in the mitochondria during the course of normal cellular metabolism [26]. Impairment and oxidative stress induced by HgCl₂ in rats produces oxidative stress and the production of free radicals have been suggested to be involved in mercury induced injury [27] and the high affinity between mercury and endogenous thiol-containing molecules, such as GSH and α-aminolevulinate dehydratase seems to contribute to this process.

The decreased levels of mitochondrial enzymes such as isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase in the mercuric chloride treated rats were observed. Isocitrate dehydrogenase is involved in supply of NADPH needed for GSH production against cytosolic and mitochondrial oxidative damage [28]. Hence, the damage of isocitrate dehydrogenase may result in the perturbation of the balance between oxidants and antioxidants and subsequently lead to a pro-oxidant of green tea extract improved the activities of TCA cycle enzymes, probably by improving the mitochondrial antioxidant defence system, and prevailed over the complications associated with the decreased TCA cycle operation.

Cytochrome C-oxidase plays an important role in the mitochondrial respiratory chain that converts molecular oxygen into water [29]. NADH-dehydrogenase constitute complex I of the electron transport chain, which passes electron from NADH to coenzyme Q. Cytochrome C-oxidase donates electrons directly to molecular oxygen and constitutes complex IV. The decrease in the activities of these enzymes may be due to the increased oxidative stress after mercuric chloride administration. Thus, mercuric chloride limiting the ability of the mitochondria to meet the energy demands of the cell and affecting cellular energy homeostasis. This condition was normalised administering green tea to toxicated rats.

Table 3: Primer sequence and resultant PCR products: The PCR thermocycling conditions for IL-6

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X</td>
<td>1</td>
<td>95 ºC</td>
<td>5 min</td>
<td>Initial denaturation</td>
</tr>
<tr>
<td>30X</td>
<td>2</td>
<td>95 ºC</td>
<td>10 s</td>
<td>Template denaturation</td>
</tr>
<tr>
<td>3</td>
<td>55 ºC</td>
<td>10 s</td>
<td>Primer annealing</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>72 ºC</td>
<td>30 s</td>
<td>Primer extension</td>
<td></td>
</tr>
<tr>
<td>1X</td>
<td>5</td>
<td>72 ºC</td>
<td>7 min</td>
<td>Final extension</td>
</tr>
</tbody>
</table>

Table 4: Primer sequences and the resulting polymerase chain reaction products. IL-6-Interleukin-6 GAPDH-glyceraldehyde 3-phosphated dehydratase

<table>
<thead>
<tr>
<th>PCR products</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>Sense: 5’ TTG CCG AGT AGA CCT CAT AGT GAC C -3’&lt;br&gt;Antisense: 5’T GAG ACT TCC AGC CAG TG</td>
</tr>
</tbody>
</table>
**Fig. 1**: Effect of drug on IL-6 mRNA level in the liver of hepatotoxicity rats showing agarose gel lectrophorotogram of mRNA level.

**Fig. 2**: Band intensities were scanned by densitometer. The data were expressed as percentage of IL-6 /GAPDH ratio and given as means ± S.D. for six experiments. 

- \( P < 0.05 \) compared with control rats; 
- \( b \ P < 0.05 \) compared with mercuric chloride rats.

Lanes-1: Control; 2: Control + Drug; 3: Mercuric chloride; 4: Mercuric chloride + Drug; GAPDH - glyceraldehyde 3-phosphate dehydrogenase (internal standard).

**Fig. 3 to 6** are histological sections of liver Sirius Red Staining (40x)

**Fig. 3**: Liver of control rat showing normal struction.

**Fig. 4**: Liver of drug treated rat, which shows no histological alteration.

**Fig. 5**: Liver of mercuric chloride treated rats showing thickening and marked increase of collagen fiber bundles (arrows).

**Fig. 6**: Liver of mercuric chloride + green tea extract treated rat showing normal quantum of collagen fibers.
Chronic liver injury induced by mercuric chloride decreases in IL-6 expression. Although it may injure hepatocytes directly, recent evidence suggests that inflammatory cells and their products also contribute to hepatic necrosis [30]. The early response also includes interleukin-6 (IL-6), a cytokine that triggers the acute phase reaction and liver regeneration [31]. In this study, plasma levels of IL-6, were increased that showed that HgCl$_2$ stimulates VEGF and IL-6 release from human mast cells. Increased IL-6 expression in HgCl$_2$ treated rats may be due to inflammation, necrosis and the oxidative stress. Supplementation of green tea extract effectively decreased IL-6 expression in hepatic mercuric chloride rats.

In conclusion, green tea acts as potential therapeutic agent that reduces mitochondrial oxidative stress in mercuric chloride treated rats. However, more precise mechanism of action of green tea on molecular targets may be delineated in future studies.

ACKNOWLEDGMENTS

Authors acknowledge Dr. T. Manivasagam, Assistant Professor, Annamalai University and Dr. V. Dhivakaran, Secretary, S.T.E.T. Women's College, Mannargudi for support during this project work.

REFERENCES
