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Prevention of Formation of Toxic Carbonyl and Aldehyde Compounds in BALB/C Micehepatocytes Caused by the Toxicity of Microcystin-LR Using Hydroxytyrosol Extracted From *Zizyphusspina-christi*

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Abstract: Microcystin-LR (MC-LR) is a potent cyclic heptapeptidehepatotoxin produced by the blue-green algae *Microcystisaeruginosa* (*M.aeruginosa*). It has been reported that oxidative stress plays a vital role in MC-LR-induced biochemical and molecular alterations. Chemoprotectant studies suggest that membrane-active antioxidants may offer a protection against microcystin toxicity. The aim of this study was to investigate the potential benefits of dietary supplementation of hydroxytyrosol extracted from *Zizyphus spina-christi* as antioxidant on microcystin toxicity inmouse liver. A group of Balb/c mice were pre-treated for 14 days with hydroxytyrosol (extracted from *Zizyphus spina-christi*) (220mg/kg mouse body weight given orally once a day for 14 days) before an intraperitoneal injection (i.p.) with 95 µg MC-LR/ kg mouse body weight (according to LD_{50} value). The potential benefits of hydroxytyrosol were evaluated based on alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and gamma glutamyl transferase (GGT) in the serum. Protein phosphatase (PP 1), methylglyoxal (MG), ROS, carbonyl contents (CC) and lipid peroxidation (LPO) as malondialdehyde (MDA) amount in the liver homogenate. Co-administration of hydroxytyrosol to MC-LR treated mice showed substantial reduction in the production of carbonyl and aldehyde groups.Therefore, hydroxytyrosol supplied as dietary supplement may have protective effects against chronic exposure to MC-LR and may prevent the formation of carbonyl and aldehyde compounds.

Key words: Chemoprotection · Microcystin-LR · Hydroxytyrosol · Carbonyl and Aldehyde Compounds

INTRODUCTION

The increasing eutrophication by human activities of fresh water including drinking water reservoirs has increased the spread and intensity of cyanobacterial blooms [1, 2]. Microcystins (MCs), specific hepatotoxins produced by numerous cyanobacterial species (*Microcystisaeruginosa*, *M.aeruginosa*) in eutrophic surface water, have raised, increasing worldwide concern [3]. Previous results [4,5], have shown that MC-LR induced the production of reactive oxygen species (ROS) which causes the oxidative stress because the balance of oxidants within the cell exceeds the levels of antioxidants present [6]. This imbalance can potentially lead to damage in a variety of disease conditions. An increased level of ROS can cause damage of macromolecules within the cell and it is this damage to lipids, proteins and DNA that can give rise to pathological consequences [7]. In many cases, reactive carbonyls are produced as a consequence of oxidative stress and considerable evidence is now emerging that it is the presence of these carbonyls rather than the initial oxidative insult that leads to the cellular damage observed [8].

In recent years, attention has been paid to the role of diet in human health [9]. Several epidemiological studies have indicated that a high intake of plant products is associated with a reduced risk of a number of chronic diseases, such as atherosclerosis and cancer [10]. Polyphenols are a wide group of secondary metabolites that can range from simple molecules, such as phenolic acids to highly polymerized constituents such as tannins [11]. All the phenolic compounds, especially flavonoids, have been reported to have multiple biologicaleffects such as antioxidant activity [12, 13], which can terminate

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or retard the oxidation process by scavenging free radicals [14]. These antioxidants are considered as possible protective agents against oxidative damage in human body from ROS [15]. It has been reported that Hydroxytyrosol decreases the intracellular ROS level [16]. Hydroxytyrosol, HT, is a phenylethanoid (4-(2-Hydroxyethyl)-1,2-benzenediol) and it was found that it is one of the essential antioxidants and it was listed in WHO list of main nutrients. Therefore, a large number of studies have been carried out to characterize its role in various diseases and exploit it for the prevention and treatment of carcinogenesis, hypertention, diabetes, atherosclerosis and other diseases [17, 18].

The goal of this study was to investigate the role of hydroxytyrosol extracted from *Zizyphusspina-christi* as antioxidant to preventthe formation of toxic carbonyl and aldehydic compounds in Balb/c mice hepatocytes.

MATERIALS AND METHODS

Chemicals: All chemicals used in this study were of analytical grade and purchased from Sigma-Aldrich Co. USA. Microcystin-LR was purchased from Cyano Biotech GmbH, Germany.

Extraction of Hydroxytyrosol: Healthy ripe fruits of *Zizyphus spina-christi* were collected in Irbid area in Jordan. Fruits were soaked in phosphate buffer (pH 7.4) for 4h, then dried in reflex air for 30h. 300 g ofdry fruits were homogenized in 500 ml of Me OH/H_2O (4:1) mixture using Ultra homogenizer for 20min, then centrifuged. The supernatant was freeze-dried for 24h, kept at -80° for 24h and then used.

In order to obtain a hydroxytyrosol-rich extract, 100g of freeze-dried extract was dissolved in 400 ml of a Me OH/H_2O (4:1) mixture. The solution was hydrolyzed at 100°C for 1h using 25 ml of HCL (2M), then cooled and diluted with water (100 ml). The hydrophobic fraction was extracted by a separatory funnel three times with 200 ml of ethyl acetate, which was subsequently removed by evaporation. A reversed phase HPLC technique was developed to identify and quantify the major phenolic compounds contained in the hydrolyzed extract using the method reported before [19].

The free radical scavenging activity (FRSA) of extracted HT was measured by the decrease in the absorbance of methanol solution of 1,1 diphenyl-2 picryl-hydrazyl (DPPH) [20]. The antioxidant activity was calculated using the following equation:

FRSA % = A sample 517nm/ A control 517 nm X 100

Biochemical Studies: Healthy sixty male Balb/c mice (6-7 weeks old, with an average weight was 28-30g) were housed in stainless metal cages under laboratory conditions (23-25°C and 12-12 light/ dark). Animals were divided into six groups; control group (C), mice of this group kept on standard diet without any supplementation, HT or MC-LR. The second group was hydroxytyrosol control (CHT), mice were supplemented orally with 220mg/kg body weight for 14 days prior to toxin treatment. The third group was toxin group (CT_6), mice of this group were supplemented i.p 95 µg toxin/kg body weight (according to LD_{50} [21]) and decapitated after 6h. Fourth group was also toxin group (CT_{12}), mice were treated in the same way as in case of group 3 and then killed after 12h. Fifth group (HTT_6), mice were supplemented with 220mg HT/kg body weight daily for 14 days then injected with 95 µg toxin/kg body weight and scarified after 6h. The 6th group (HTT_{12}) was treated like group 5 and scarified after 12h. Blood was collected immediately after decapitation, serum was isolated and stored at -20°C for the biochemical tests. Livers were removed, perfused with normal saline containing heparin and homogenized with phosphate buffer saline (pH 7.2) using Ultra Turax homogenizer, centrifuged at 3000g for 30min. The supernatant was removed and stored at -80°C.

ALT activity in the serum sample was measured following the procedure reported in literatures [22]. LDH activity was determined according to the method reported before [23]. Gamma glutamyl transferase assay in the serum was performed according to the method recommended before [24].

Protein phosphatase (PP1) was assayed depending on the procedure described before [25], its activity was assayed by measuring the rate of formation of the yellow color of *P*-nitro- phenyl phosphate (*P*-NPP) in an alkaline solution spectrophotometrically.

The lipid peroxidation (LPO) level of the hepatocytes was measured as malondialdehyde (MDA) according to the method described before [26]. LPO level was considered as aldehyde marker.

Carbonyl contents (CC) were measured using standard method reported before [27].

ROS determined in hepatocyte homogenate, were quantified by the dichlorofluorescein diacetate (DCFH-DA) assay using microplate reader [28].

Methylglyoxal (MG) was assayed in the liver homogenate by the method reported in literature [29].

Statistical Analysis: All results were expressed as the mean ± S.E.M. from ten mice per group. One way analysis of variance (ANOVA) followed by a Tukey test was used to determine the significance of the differences between the groups. Statistical significance was declared when P value was equal to or less than 0.05. The statistical analysis was performed using the Sigma stat Statistical Software version 3.5.

RESULTS

In this study, MC-LR treated mice showed increased liver body mass index ratio due to massive intrahepatic hemorrhage and pooling of blood in the liver (Table 1). Livers of mice receivinghydroxytyrosol (220mg HT/kg body weight) were within the normal weight value.

Toxin-treated groups (T_6 and T_{12}) supplemented with 95 µgMC-LR/kg body weight i.p as LD_{50} according to Fawell [21],showed a significant increased activity of ALT in the serum compared with C group($P \leq 0.05$) (about 2.3 fold in T_6 and 4.2 fold in T_{12}). This value was significantly reduced in experimental groups which were supplemented with HT (HTT6 and HTT12) (Table 2).

As shown in Table 2, LDH level in the sera of mice treated with MC-LR(T_6 and T_{12}) increased significantly ($P \leq 0.05$)(3 folds and 5 fold respectively as compared with C group). In the experimental mice (HTT_6 and HTT_{12}) the value of LDH was reduced by about 70% (Table 2).

GGT level in the serum of HT control group was very close to its value in sera of control mice. GGT level was increased significantly ($P \leq 0.05$) in the sera of toxin groups (T_6 and T_{12}) (3.8 and 5.6 folds respectively). Our results showed that this elevation of GGT was dramatically diminished in the sera of HTT_6 and HTT_{12} after administration of HT (Table 2).

Results of spectrophotometric measurements of protein phosphatase (PP1) activity of the liver homogenates of all groups are presented in Table 3. PP1 activity was significantly ($P \leq 0.05$) inhibited in toxin groups (T_6 and T_{12}) (by 55% and 76% respectively). Supplementation with HT (as in experimental groups, HTT6 and HTT12) reduced the inhibition and PP1 activity was retained against MC-LR (Table 3).

Lipid peroxidation was measured spectrophotometrically as MDA in hepatocytes homogenate. Our results showed a dramatic increase in MDA level (elevation of TBA values) in hepatocytes of toxin groups (T_6 and T_{12}) (16 and 34 folds respectively) when compared with C group. HT supplemented groups (HTT_6 and HTT_{12}) revealed a decrement of TBA significantly ($P \leq 0.05$) as in Table 3.

The MG concentration in the liver homogenates showed that MG generation was increased by 5 and 7 folds in T_6 and T_{12} groups respectively. Pre-treatment mice of experimental groups (HTT_6 and HTT_{12}) with hydroxytyrosol showed significant decrement of MG in hepatocytes.

Carbonyl contents (CCs) in the hepatocytes homogenate increased by 21 fold in $T_6$and 91 fold in T_{12}groups and these values reduced near to normal after HT supplementation.

ROS value was increased in the serum by 10 fold in group T_6 and 19 fold in $T_{12}$and these values were reduced near to normal after supplementation with HT.

Table 1: Results of the effect of MC-LR and HR on Balb/c liver weight

	C	T_6	T_{12}	HT	HTT_6	HTT_{12}
Liver weight (g)	1.66±0.07	2.24±0.09	2.91±0.11	1.64±0.07	1.88±0.08	1.92±0.09

Table 2: Summary of results of the effect of HT supplementation on serum enzymes and ROS value of Balb/c mice receiving single dose of MC-LR

	C	T_6	T_{12}	HT	HTT_6	HTT_{12}
ALT (U/L)	9.77±0.07	31.81±2.05	58.62±2.88	6.12±0.04	15.11±1.02	18.04±1.11
LDH (U/L)	228.33±2.09	528.13±4.16	923.44±8.03	201.21±2.12	368.64±2.48	478.12±5.16
GGT (U/mg)	101±7.81	703±11.31	1306±22.88	102±7.66	348±3.99	481±4.98
ROS in serum µM/ml blood	4.2	41.11	76.08	2.2	11.7	17.8

Table 3: Summary of results of the effect of HT supplementation on hepatocytes of Balb/c mice receiving 95 µg MC-LR/kg body weight

	C	T_6	T_{12}	HT	HTT_6	HTT_{12}
PP1 (U/mg)	9.04±0.04	4.11±0.02	2.31±o.02	9.62±0.04	5.66±0.02	4.08±0.02
LPO (nmol/mg)	0.84±0.01	7.31±0.08	13.66±0.11	0.79±0.01	2.88±0.03	5.18±0.08
MG (µM)	1.62±0.01	11.61±0.07	29.04±1.01	0.88±0.01	3.92±0.01	6.99±0.05
CC (nmol)	0.37±0.01	9.66±1.09	42.11±4.33	0.31±0.01	3.12±0.03	5.88±0.04
ROS in liver (FIU)	133	664	871	78	176	211

Also, in liver homogenate, ROS were increased by 6 folds in T_6 and 8 fold in T_{12} and reduced to the normal after HT supplementation.

DISCUSSION

Our previous results elucidated that MC-LR is highly liver-specific causing irreversible hepatotoxicity indicated by increment in the weight of livers of toxin supplemented groups because of the intrahepatic haemorrhage and induced characteristic liver injuries [30].

Microcystin -LR is a potent toxin produced worldwide by cyanobacteria*M.aeruginosa*during boom events [31]. Phosphatases inhibition and oxidative stress are well-recognized effects of this kind of toxin [32]. However, it is not fully understood why and how MC-LR exposure can lead toformation of an excessive of reactive oxygen species (ROS) that culminate in oxidative damage [33]. Some evidence suggests a close connection between

cellular hyperphosphorylation state and oxidative stress generation induced by MC-LR exposure [34]. It is shown, based on literature data [35], that MC-LR incorporation per se can be the first event that triggers glutathione depletion and the consequent increase the ROS concentration [28]. Also, literature data [33,36] suggest that hyperphosphorylated cellular environment induced by MC-LR exposure can modulate antioxidant enzymes, contributing to the generation of oxidative damage.

Oxidative stress is classically defined as a disturbance in the prooxidant/ antioxidant balance in favour of the former, leading to potential molecular damage. Generated ROS may damages all types of biological molecules, proteins, lipids, or DNA and may be deleterious and could be concomitant [37].

A large body of evidence has implicated reactive carbonyl compounds generated in high amount through oxidative stress. Our results showed high level of carbonyl compounds (aldehyde and ketones) in toxin groups which agreed with other results [38] and confirmed this statement. Carbonyl groups are produced on protein side chains (especially of Pro, Arg, Lys and Thr) when they are oxidized. CC derivatives can also be generated through oxidative cleavage of proteins by either the α-amidation pathway or by oxidation of glutamyl side chains, leading to formation of a peptide in which the N-terminal amino acid is blocked by anα-ketoacyl derivative [39]. In addition, CO groups may be introduced into proteinby secondary reaction of the nucleophile side chains of Cys, His and Lys residues, with aldehydes (MDA) produced during lipid peroxidation or with reactive carbonyl derivatives generated as a consequence of the reaction of reducing sugar [37]. Our results showed that all these alteration may lead to high production of carbonyl contents and aldehyde which were ameliorated significantly by using the strong antioxidant, hydroxytyrosol extracted from *Zizyphus spina-christi.*

Methylglyoxal (MG) is a distinctive representative of α-oxoaldehyde compounds, generated intracellularly in all mammalian cells, through b oth enzymatic and non-enzymatic pathways [40]. The majority of MG production occurs from the intermediates in the glycolytic pathway, in the ketone body metabolism and catabolism of threonine. It is very reactive compound, but its toxicity is usually controlled by glyoxalase system (glyoxalase I and II) and a catalytic amount of reduced glutathione (GSH) [41]. Our previous results showed that MG increased to significant level in oxidative stress mainly because of significant inhibition of glyoxalase enzyme system [42].Intracellularly produced MG crosses cell membrane, probably by passive diffusion and humeral MG is mainly cell – derived. Additionally, some of MG might arise from ketone body metabolism, degradation of threonine and lipid peroxidation [43]. Enzymatic oxidation of ketone bodies can also generate MG. Its production from acetoacetate is mediated by myeloperoxidase, whereas P450 2E1 cytochrome catalyzes acetone conversion into MG [44]. Our results showed that use of hydroxytyrosol can attenuate the production of MG via the reaction of hydroxyl groups with MC-LR and prevent the inhibition of glyoxalase system and activate GSH formation.

Recently, much attention has been focused on antioxidants in food that are potential compounds for preventing diseases caused by oxidative stress and its products, carbonyl and aldehydes compounds [43]. Others studies have shown that hydroxytyrosol extracted from olive oil were endowed with important antioxidant activities [45]. In this work we have extracted hydroxytyrosol from *Zizyphus spina-christi* for the first time using modified method and measured its antioxidant potency using standard methods based on our previous calculations [46]. This study has proved that it is a very potent antioxidant, able to prevent the production of oxidative stress and formation of carbonyl and aldehyde compounds in hepatocyte of Balb/c mice exposed to MC-LR.

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