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The Role of Silymarin in the Protection of Mice Liver Damage Against Microcystin-LR Toxicity

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Abstract

The presence of cyanobacterial toxins in drinking and recreational waters represents a potential risk to public health. Microcystin-LR (MC-LR) is a potent cyclic heptapeptide hepatotoxin produced by the blue-green algae *Microcystis aeruginosa* (*M. aeruginosa*). Chemoprotectant studies suggest that membrane-active antioxidants may offer a protection against microcystin toxicity. The aim of this study is to investigate the potential benefits of dietary supplementation of silymarin as antioxidant on microcystin toxicity in mouse livers. A group of Balb/c mice was pre-treated for ten days with silymarin (extracted from milk thistle seeds collected from local areas of north Jordan) (400 mg of silymarin /Kg mouse body weight given orally once a day for 10 days), before an intraperitoneal injection (i.p) with 200 µg toxin/kg mouse body weight of MC-LR (according to LD_{50} value). Pre-treatment of mice with a single dose of silymarin, aflavonolignane (Silybum marianum L. Geartin) completely abolished the lethal effects and significantly decreased the levels of serum enzymes, alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and gamma glutamyl transferase (GGT), inhibition of Protein phosphatase (PP1) , level of methylglyoxal (MG) and lipid peroxidation (LPO) as MDA amount induced by MC-LR. Therefore, silymarin supplied as dietary supplement may have protective effects against chronic exposure to MC-LR.

الملخص

يشكل وجود سموم الطحالب الزرقاء الخضراء (السيانوبكتريا) في مياه الشرب والري مشكلة صحية خطيرة للغاية . يمتاز هذا النوع من السموم بانه ذا سمية عالية الكفاءة وخاصة لنسيج الكبد خيث وجد انه يسبب تلف نسيج الكبد بسرعة فائقة اضافة الى ما يسببة من اورام سرطانية . وهذا النوع من السموم الطحلبية هو عبارة عن ببتيد سباعي حلقي يقوم بتكوينه نوع من الطحالب الزرقاء -الخضراء والمسماة بالمايكروسستس اريجينوزا . وقد اثبتت الدراسات الخاصة باستخدام الغذائية التي تساعد في حماية الانسجة الحية بان مضادات الاكسدة قد تساعد في الحماية من هذا النوع من السموم . ان هدف هذه الدراسة هو البحث عن امكانية استخدام مضادات الاكسدة الغذائية كالسليمارين (المستخلص من نبات حليب مريم الموجود في المناطق الشمالية من المملكة) في حماية كبد الفار من التلف الذي تسببه له هذه السموم . لقد تم اخذ مجموعة من الفئران من نوع Balb/c وتم اطعمها بمستخص السليمارين بمقدار 400 ملغم لكل كغم من جسم الفأر ولمدة عشرة ايام (مرة واحدة يوميا ولمرة واحدة) ومن ثم حقنها في اليوم الحادي عشر بسموم المايكروسستين تحت الجلد بجرعة مقدارها 200 مايكروغرام لكل كغم من وزن الفار (وذلك نسبة الى قيمة LD50) التي تم قياسها . ومن ثم تم قياس كفاءة السليمارين عن طريق قياس كمية كل من الانزيمات الالانين ترانسفيريز ، اللاكتيت ديهيدروجينيز والكلوتاميل ترانسفيريز في مصل الدم . وقياس كل من البروتين فوسفاتيز والمثيل كلايوكزاليز ، ومقدار اكسدة الليبيدات (قياس كمية المالون ثنائي الالدهيد في مستخلص نسيج الكبد .وقد وجد في هذه الدراسة ان السليمارين يتمتع بكفاءة عالية كمضاد للاكسدة التي يسببها هذا النوع من السموم وبذلك يوفر الحماية المطلوبة لكبد الفأر من التلف.

Keywords: Chemoprotection; Toxicity; Microcystin; Silymarin.

1. Introduction

The rising eutrophication by human activities of fresh waters, including drinking water reservoirs, has increased the occurrence and intensity of cyanobacterial blooms (Ordorika *et al*. 2004; Antoniou *et al*., 2008). Microcystins (MCs), specific hepatotoxins produced by numerous cyanobacterial species (primarily *M. aeruginosa*) in eutrophic surface waters have risen, increasing worldwide concern (Andrinolo *et al*., 2008; Billam *et al*., 2008). MCs are characterized as monocyclic heptapeptide with over 70 different MCs isoforms identified (Spoof and Meriluoto, 2002). The general structure of microcystin is cyclo (D-Ala-L-X-D-MeAsp-L-Z-Adda-D-Glu-Mdha) where X and Z represent. The two variable amino acids and Adda is 3-amino-9-methoxy- 2, 6, 8-trimethyl-10-phenyl-deca-4, 6-dienoic acid (Ordorika *et al*., 2004; Oriola and Lawton,2005).

Microcystin-LR (MC-LR) is the most widely distributed and studied MCs variant (Gupta and Guha, 2006). It has the amino acids leucine and arginine at positions 2 and 4, respectively (Spoof and Merilluoto, 2002; Antoniou *et al*., 2008). MC-LR has been found to be a potent inhibitor of protein phosphatase type 1 and type 2A (Andrinoloa *et al*.,2008), resulting in the disturbance of many important cellular processes (Oriola and Lawton, 2005). Exposure to MC-LR has been shown

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to cause oxidative stress in various organisms (Leao *et al*., 2008). Formation of reactive oxygen species (ROS) and oxidative stress is associated with the development of many pathological states (Gupta and Guha,2006; Billam *et al*., 2008). Oxidative stress may occur either due to the decrease of cellular antioxidant level, or due to the overproduction of ROS (Jayaraj *et al*.,2006). Exposure to MC-LR has been linked with increase of ROS production not only in domestic and wildlife animals, but also in human (Weng *et al*., 2007).

Recently, interest has increased considerably in finding naturally occurring antioxidants to replace synthetic antioxidants, which are being restricted due to their carcinogenicity (Looa *et al*., 2007). Silymarin was shown to be an antihepatotoxic agent against MC-LR toxicity in cultured rat hepatocytes(Mereish and Solowe, 1990). Mereish et al., (1991) extended their studies to *in vivo* and tested the use of silymarin against MC-LR toxicity in mice and rats, found that this compound acted potentially against it. Rao et al., (2004) concluded that silymarin could offer great protection against MC-LR toxicity.

The goal of this study is to investigate the role of naturally- extracted silymarin (one of the active polyphenol antioxidants in milk thistle seeds) pre-treatment in protection against MC-LR (extracted from King Talal Reservoir, Jordan)-induced liver injury in Balb/c mice.

2. Materials and Methods

2.1. Chemicals

All chemicals used in this study were of analytical grade and were purchased from Sigma Chemical Co. USA.

2.2. Samples

Samples of *M. aeruginosa* cells were collected according to (Al-Jassabi and Khalil, 2006) from selected sites of KTR in Jordan during blooming season (July, August and September, 2007). Microcystin was extracted from the lyophilized cells of *M. aeruginosa* according to Lawton *et al*., (1994), and the LD_{50} of the toxin extract was determined according to Fawell *et al*., (1999), in which LD_{50} is determined as the value between the lowest toxin dose at which mice die and the highest toxin dose at which mice live after administration of a toxin.

2.3. Silymarin Extraction

Milk thistle seeds were collected from specific areas in Jordan (Ajlun & Irbid) during July and August 2007. The silymarin was extracted according to the method reported by Duan *et al*., (2004).

2.4. Animal Treatment and Sample Collection

Male Balb/c mice 6-7 weeks old (average body weight 30 g) were used in this study. Mice were obtained from animal house/ Yarmouk University and were maintained on standard laboratory diet and tab water throughout the experiments. Five animals were maintained in each cage (stainless) under a 12-12h light-dark cycle and room temperatures of 23-26^{o}C.

Sixty mice were used in this study, and they were assigned into 4 groups (10 mice each) as follows: Group 1 was the control group (C), without supplementation of silymarin or treatment with toxin; Group 2 was the toxin control group (with 2 sub groups, 10 mice each) (T_6 and T_{12}), treated with toxin only, intraperitoneal injection (i.p) with 200 µg toxin / kg mouse body weight. 10 mice were killed after 6 h and 10 mice were killed after 12 h; Group 3 was the silymarin control group (SC), supplemented orally with 400 mg silymarin /kg mouse body weight daily for 10 days according to Lakshmana *et al*., (2004), then they were killed; Group 4 (silymarin and toxin) with two sub group (10 mice each) supplemented orally with 400 mg silymarin / kg mouse body weight daily for 10 days, then were injected i.p. with 200 µg toxin/kg mouse body weight. 10 mice were killed after 6 h (ST_6) and 10 mice were killed after 12 h (ST_{12}). Blood was collected immediately after sacrification of mice, and serum was isolated and stored at -20ºC for the biochemical tests. Livers were removed, immediately after death, perfused with normal saline containing heparin, weighed, 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 -20^{o}C.

2.5. Alanine Aminotransferase (ALT) Assay

Determination of ALT activity in the serum sample was measured according to the procedure recommended by Gehringer *et al*., (2003).

2.6. Cytotoxicity Assay

Serum lactate dehydrogenase (LDH) activity was measured according to Doyle and Griffiths, (1998).

2.7. Gamma Glutamyl Transferase Assay (GGT):

Determination of GGT activity in the serum sample was measured based on Szasz (1974) method .

2.8. Protein Phosphatase (PP1) Assay

Depending on the procedure described by Yuan *et al*., (2006), PP1 activity in liver homogenate was assayed by measuring the rate of formation of the yellow color of p-nitrophenol (*p*-NP) produced by hydrolysis of p-nitro-phenylphosphate (*p*-NPP) in an alkaline solution spectrophoto-metrically.

2.9. Lipid Peroxidation (LPO) Assay

The lipid peroxidation level of the hepatocyte was measured according to the method described by Hosseinzadeh *et al*., (2007).

2.10. Methylglyoxal Assay (MG)

The glyoxalase system breaks down the toxic methylglyoxal, which is formed as a by-product of the triosephosphate isomerase reaction in glycolysis. Methylglyoxal was determined from liver homogenate according to the method described by Ratliff *et al*., (1996).

2.11. 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.

3. 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, as shown in Table 1. The livers of mice which received silymarin were within the normal value, but this value increased slightly after the exposure to the toxin.

Treatment with MC-LR resulted in a significant increase in levels of ALT activity compared to the saline-treated group (Table 1). The elevated levels of serum ALT were significantly reduced in mice received toxin and silymarin supplementation. An increased ALT value was revealed in sera by folds of 1.5 in ST_6. A further increase was shown as 4.2, folds for ST_{12} compared to controls ($P<0.05$).

As shown in Table 1, MC-LR alone produced after 6 h of exposure nearly three-fold and five-fold after 12 h increases in serum LDH level compared to control mice, indicating severe liver injury. The mice receiving toxin and silymarin supplementation revealed an increased LDH value in sera by folds of 2 in ST_6. A further increase was shown as 2.5 folds for ST_{12} compared to controls ($P<0.05$).

Mice group, which received silymarin only, show levels of GGT which are within the normal value (39±4U/mg). Furthermore MC-LR administration (200mg toxin/kg mouse body weight) of 6 and 12 h increased serum values of GGT about 3.8 and 5.8 fold, respectively, compared with control mice ($P<0.05$), while the increase was dramatically diminished by silymarin pre-treatment, and it could significantly inhibit the increase of GGT induced by MC-LR as shown in Table 1.

Results of spectrophotometric measurements of protein phosphatase activity of liver homogenates for all groups are presented in Table 1. PP1 activity was significantly inhibited in group 2; 0.46% inhibition has occurred for those of 6 h and 72% for those of 12 h when compared with control. Supplementations with silymarin caused a partial protection of PP1 activity against the action of MC-LR, of almost three-fold compared with the levels in toxin control group.

Control mice exhibited normal levels of lipid peroxidation (LPO) measured as amount of MDA it was 0.067µM in hepatocytes homogenate. There was a dramatic increase in MDA level in liver homogenate from toxin-treated groups by 16 fold in case of T_6 and 34 fold in case of T_{12} when compared with those of control mice ($P<0.05$), as reflected in the elevation of TBA values, which appear time-dependent (Table 1). The mice receiving toxin and silymarin supplementation revealed an increased TBA value by 3 folds in ST_6 and further increase shown as 10, fold for ST_{12} in compared to controls.

The MG concentration in the liver homogenates was determined at 6 and 12h after i.p injection of MC-LR and results were compared with controls as shown in Table 1. It was clear that the effect of MC-LR on MG was time dependent; for mice exposed only to the toxin; results were found to be higher at 12 h compared to those at 6 h. However, pre-treated silymarin group showed a significant protection against accumulation of MG in the hepatocytes.

4. Discussion

M. aeruginosa dominates the cyanobacterial communities of KTR during the warmer season (specifically, from June to October, 2007) (AlJassabi, 2004). Our microscopic investigations ensured that the major cyanobacterial species found in the collected samples were *M. aeruginosa* in agreement with our previous study (Al-Jassabi and Khalil, 2006). The phosphatase inhibitory activity proved the bioactivity of the toxin while the spectrophotometric analysis proved that the extracted toxin was MC-LR (Spoof and Meriluoto, 2002 & Oriola and Lawton, 2005).

The present investigation examined the hepatotoxic effects of MC-LR isolated from *M. aeruginosa* of KTR, in Balb/c mice after intraperitoneal route of exposure to the toxin. Besides we investigated the potential hepatoprotective efficacy of silymarin as naturally isolated antioxidant against MC-LR effects. The LD_{50} concentration of MC-LR by i.p route was determined as 200 µg toxin/kg mouse body weight by a modified Fawell's up-and-down method (Fawell *et al*., 1999) which is at variation with some earlier reports (Ding *et al*., 2006 and Lombardo *et al*., 2006). A wide range of LD_{50} values have been reported for MC-LR by administration in mice.

In agreement with previous studies (Dawson, 1998; Weng *et al*., 2007; Xu *et al*., 2007) the results in toxin group indicate that severe liver damage accompanied by marked change in colour and weight can occur by i.p injection of LD_{50} dose. Hepatocellular damage was first noticed by the increase in total liver size, due to intrahepatic haemorrhage and accumulation of fluids caused by the action of MC-LR (Dufour and Clavien, 2005).

Exposure to MC-LR causes a disturbance of cellular iron homeostasis as a result of ferritin inhibition (Chen *et al*., 2005). Mackintosh *et al*., (1990) reported that Protein phosphatases are inhibited with high affinity by MC-LR. Thus, MC-LR completely blocks access to the active centre of the enzyme using Fe^{+3} as a catalyst (Lohse *et al*., 1995), thereby causing hyperphosphorylation of the cell and a massive disruption of a number of important cellular mechanisms (Yang *et al*., 1997). Silymarin in the mice pre-treated group could partially hinder the inhibition of PP1 binding to Fe^{+3} (Kanaze *et a*l., 2005).

One important consequence of excessive free radical production after exposure to MC-LR is the toxin ability to attack many organic molecules, including polyunsaturated fatty acids in the cell membrane (Denisov *et al*., 2003 and Pinho *et al*., 2005), leading to lipid peroxidation. Several studies reported that ROS can initiate lipid peroxidation through the action of hydroxyl radicals (Joshi *et al*., 2005). Our analysis of hepatocytes for lipid peroxidation showed that silymarin could bring a decrease in the formation of lipid peroxidation through their ability to scavenge the hydroxyl radicals. The activity of silymarin as scavenger of free radicals and ROS has been described by others (Mereish et al., 1991; Rao et al., 2004)

Methylglyoxal is considered to be toxic for mammals (Kalapos, 2008). The cytotoxicity associated with the accumulation of MG after exposure to MC-LR is due to the inhibition of glyoxalase I and the depletion of the action of the antioxidant defense system found in the liver

Table 1. Summary of results of the effect of silymarin supplementation on mice receiving single lethal dose of MC-LR

	C	T_6	T_{12}	SC	ST_6	ST_{12}
Livers weight (g)	1.47 ± 0.02	2 ± 0.057	2.74 ± 0.06	1.45 ± 0.02	1.55 ± 0.05	1.87 ± 0.09
ALT (U/L)	574.6±6.95	1649±43	2461±51	533±5	884±6	1202±39
LDH (U/mg)	987 ± 207	3352± 123	4868 ±46	885 ± 2	2057 ± 67	2471 ± 112
GGT (U/mg)	39 ± 4	132 ± 5	226 ± 7	32 ±4	95 ± 3	163 ±3
PP1 (U/mg)	0.583 ± 0.01	.316± 0.01	0.165± 0.002	0.562±0.02	0.414±0.021	0.31±0.007
LPO (μM)	0.067 ± 0.01	1.3 ± 0.04	2.292 ± 0.04	0.013± 0.001	0.212 ± 0.002	0.705 ±0.027
MG (μM)	1.61±0.075	29.03±0.39	41.94±0.797	1.58±0.0411	16.13±0.345	16.94±0.334

(Aljassabi, 2004). The increase in MG levels in the liver caused significant generation of free radicals which might further strengthen the damage and affect the hepatocyte function (Kalapos, 2008). This was noticed in the toxin group which received the toxin only as shown in the results. Silymarin pre-treated group showed a significant decrease in the accumulation of MG.

The antagonistic effect of silymarin could be similar to that of dithioerythritol by stabilizing protein-thiol, which may be important to the structure of liver cell (Mereish et al., 1991). Previous studies and the present investigation show that among the various chemoprotectants that have been screened till date, silymarin is the most effective in preventing the MC-LR-induced lethality in mice.

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