doi: 10.4149/gpb_2014038

Protective effect of allicin against glycidamide-induced toxicity in male and female mice

En-Ting Wang[#], Dong-yan Chen[#], Huang-you Liu, Hai-Yang Yan and Yuan Yuan

College of Quartermaster Technology, Jilin University, 130062 Changchun, China

Abstract. Acrylamide is known to be a neurotoxic, genotoxic, and carcinogenic compound. Glycidamide has a close relationship to the toxic mechanism of acrylamide. In order to explore the toxic mechanism of acrylamide, we further discussed the effects of oral administration of allicin on glycidamide-induced toxicity by determining the hematological parameters like AST, ALT, LDH, BUN, creatinine, ROS, and 8-OHdG, and biochemical parameters such as MDA, MPO, SOD, GST and GSH in the kidney, liver, brain and lung of male and female mice for the first time. We found that the same dose of glycidamide had more toxic effects and damage effects to the mice compared to the previous study of acrylamide. It could markedly increase the level of AST, ALT, LDH, BUN, ROS, 8-OHdG, MDA, MPO while decrease the SOD, GST and GSH. However, our data showed the oral administered allicin with a concentration of 5, 10, and 20 mg/kg b.w./day could significantly decrease the damage indexes of AST, ALT, LDH, BUN, ROS, 8-OHdG, MDA, and MPO, while increase the antioxidant indicators of SOD, GST and GSH. Thus allicin could be used as an effective dietary supplement for the chemoprevention of glycidamide genotoxicity internally, and to prevent the tissue damage and toxicity induced by glycidamide.

Key words: Allicin — Glycidamide — Acrylamide — Toxicity — Protective effect

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; GA, glycidamide; LDH, lactic dehydrogenase; MDA, maleic dialdehyde; MPO, myeloperoxidase; 8-OHdG, 8-hydroxy-desoxyguanosine; ROS, reactive oxygen species.

Introduction

From studies on laboratory animals it has long been known that the administration of acrylamide results in the formation of tumours in various tissues (Rice 2005). Acrylamide is classified as a probable human carcinogen by the International Agency for Research on Cancer (IARC, 1994), which can undergo oxidative biotransformation by cytochrome P450 2E1 (CYP2E1) to produce an epoxide derivative, glycidamide. Glycidamide is more reactive with cellular DNA compared with acrylamide, which could lead to multiple forms of toxicity including mutation and tumour induction (Koyama et al. 2006). Besaratinia and Pfeifer (2005) reported that the mutagenicity of acrylamide in human and mouse cells was based on the capacity of its epoxide metabolite glycidamide to form DNA adducts. Glycidamide induced DNA damage in human blood lymphocytes in a dose dependent manner at ≥300 μmol/μl (within 4 h), as measured by the comet assay under standard conditions. Similar results for glycidamide were obtained with the V79, Caco-2, and primary hepatic cell lines from rats (Puppel et al. 2005). Therefore, glycidamide is consequently involved in carcinogenic and mutagenic effects of acrylamide. The reduction or control of the toxicity of glycidamide to humans is very important because acrylamide can be easily transformed into glycidamide *in vivo.*

Allicin, as a diallyl thiosulfinate, is the main biologically active compound derived from garlic. It is reported that about 70% of the total thiosulfinates that are formed when the cloves are crushed (Han et al. 1995). Allicin has been suggested for use as an antibiotic, or for treating diseases such as cancer

Correspondence to: Yuan Yuan, College of Quartermaster Technology, Jilin University, 130062 Changchun, China
E-mail: yuanyuan1024@gmail.com
[#] Both authors contributed equally to this manuscript.

and diabetes. Yet, several lines of evidence indicate that allicin affects cells of the immune system and also has good anti-inflammatory properties (Haase et al. 2012; Shin et al. 2013). In addition, allicin is a natural antioxidant that scavenges the hydroxyl and oxygen free radicals as well as prevents the hydroxyl radical-induced lipid peroxidation of tissue homogenates (Kumar et al. 2009; Sirius Chung et al. 2013). In our previous study, allicin was reported for the first time to effectively prevent the acrylamide-induced hepatocyte damage and toxicity *in vitro* and *in vivo* (Zhang et al. 2012). Taubert et al. (2006) have shown that allicin could inhibit the activity of cytochrome P450 enzyme CYP2E1, which could reduce the conversion of acrylamide to glycidamide, and reduce the oxidative tissues damage and toxicity induced by acrylamide. Beyond that, our research is further to study the toxicity and tissues damage of glycidamide and whether the allicin can also protect against the glycidamide-induced tissues damage and toxicity *in vivo* of male and female mice. This is crucial to investigate the toxicity mechanism of acrylamide and its relationship with glycidamide, and the protection mechanism of allicin as well. In our present research, the activity of superoxide dismutase (SOD) as well as the myeloperoxidase (MPO) levels, glutathione (GSH), maleic dialdehyde (MDA) in the kidney, liver, brain and lung, aspartate aminotransferase (AST), and alanine aminotransferase (ALT), lactic dehydrogenase (LDH), blood urea nitrogen (BUN), creatinine, reactive oxygen species (ROS), and 8-hydroxy-desoxyguanosine (8-OHdG) in the serum of glycidamide-treated mice, where the absence or presence of allicin were determined to investigate the possible protective properties of allicin against hematological toxicities and various oxidative stresses in the kidney, liver, brain and lung of glycidamide intoxicated mice, and to evaluate the protective effects of allicin against glycidamide-induced toxicity at the same time.

Materials and Methods

Materials

Glycidamide (purity, >96.0%), dimethyl sulfoxide (DMSO), allicin and 3-(4,5-dimethylthiazol-2-y1)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Collagenase IV was obtained from Gibco-Invitrogen (Carlsbad, CA). DNase I was purchased from Applichem (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin for cell culture were purchased from Gibco BRL Co. Ltd. (Grand Island, NY, USA). The commercial assay kits for MDA, MPO, GSH, GST, total protein content, total SOD, ALT, AST, LDH, BUN, creatinine as well as a mouse 8-OHdG, ROS ELISA kits were obtained from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). All other chemicals and reagents were of the highest quality available. For the *in vitro* study, a stock solution of 15 mM for allicin was prepared in DMSO and stored at –20°C. For each experiment, the stock solution of allicin was diluted with DMEM to obtain a series of concentrations (15, 30 and 60 μM). The final DMSO concentration was 0.1% (v/v). For the *in vivo* study, allicin was dissolved in normal saline to final concentrations of 0.5, 1, and 2 mg/ml. The dosing volume of allicin was determined based on the body weight of each animal, for example, 0.2 ml for a 20 g mouse. Glycidamide was diluted in normal saline to a final concentration of 5 mg/ml.

Primary culture of hepatocytes and treatment of cells and cell viability assay in vitro study

The primary culture of hepatocytes and treatment of cells and cell viability assay were based on the methods that were previously described by Zhang et al. (2012) with some modifications. The primary liver cell suspensions were centrifuged two times at 50 g for 1 min. The pellet was discarded after each centrifugation, and the supernatant was again centrifuged two times at 50 g for 2 min. The cell pellet was suspended in DMEM with 10% FBS, penicillin (100 IU/ml), and streptomycin (100 mg/ml) (RPMI-FBS) and maintained at 37°C in a humidified atmosphere in a 5% CO_2 incubator. Hepatocytes were plated onto 6-well plates (10^5 cells/well) and incubated for 1 h. The cells were divided into eight groups, including the control group, the glycidamide group, three doses of allicin alone and three allicin with glycidamide groups. For the control group, hepatocytes were cultured in the base culture medium (DMEM) for 24 h. For the allicin groups, the cells were incubated with allicin at 37°C for 24 h with different concentrations at 3.75, 7.5, 15 μM (final concentration after dilution). For the allicin with glycidamide groups the cells were incubated with allicin at 37°C for 2 h with different concentrations at 3.75, 7.5, 15 μM. Then these allicin-treated cells were incubated with 3.5 mM glycidamide (final concentration after dilution) for other 24 h. For the glycidamide group, cells were cultured in the base culture medium supplemented with 3.5 mM of glycidamide for 24 h. The cells in all groups were finally collected and washed twice with cold PBS, suspended in 1 ml of PBS and used for future biochemical analyses.

Cell viability was evaluated by the MTT assay according to the method of Zhang et al. (2012). The blank wells without cell was used to zero adjustment and the wells containing medium only. The optical density was measured at 570 nm on a microplate reader (Tecan, Austria) finally, and the calculation of cell viability is based on the following formula:

Cell viability = (test well OD – blank well OD)/(control well OD – blank well OD).

Animals and experimental design in vivo study

Male and female BALB/c mice weighing 20 ± 2 g were provided by the Laboratory Animals Center of Jilin University (Changchun, China). The experiments were performed in accordance with the Guidelines for Animal Experimentation of Jilin University (Changchun, China). Animals were housed in an air-conditioned room at 22 ± 2°C and 30 ± 10% relative humidity. The general conditions of the animals were observed during a quarantine and acclimation period of 7 days to confirm that there were no abnormalities.

After the quarantine period, 25 male mice and 25 female mice were randomly divided into ten groups of five animals. The first five groups were male mice and the rest were female mice. Groups I, II, III, IV, V were control group, glycidamide group, low dose allicin group, middle dose group, and high dose group. Meanwhile, Groups VI, VIII, VIII, IX, X were the same grouping method with the former five ones. The control groups were given saline by oral gavage for 14 consecutive days. Glycidamide groups were intragastrically given saline by oral gavage for 7 consecutive days. On day 8, the mice were intraperitoneally injected with the glycidamide solution (50 mg/kg b.w./day) for another 7 days. The groups of low, middle, high dose allicin were intragastrically treated with allicin (5, 10, and 20 mg/kg b.w./day, respectively) once daily for the first 7 days. On day 8, they were intragastrically treated with allicin (5, 10, and 20 mg/kg b.w./day, respectively) and intraperitoneally injected with a single dose of glycidamide (50 mg/kg b.w./day) for another 7 days. The body weights of the animals were measured daily. The doses of glycidamide and allicin were administrated according to the body weights of the animals.

On day 15, all the animals were sacrificed within 24 h of the last treatment and the kidneys, livers, brains and lungs were collected. The weight of each tissue was measured immediately after its removal from the animal's body. The tissue samples were then washed thoroughly with ice-cold normal saline. The tissues were homogenised with 10% pre-chilled normal saline in a tissue homogeniser and centrifuged at 2500 × *g* for 10 min at 4°C. The resulting supernatant was used for the subsequent biochemical analyses.

Detection methods

The content of ROS, 8-OHdG, BUN and the activity of ALT, AST, LDH and creatinine in serum, the MPO content in tissue homogenates, protein in tissue supernatant, SOD and GST activity in tissue supernatant, GSH content in tissue supernatant, MDA level in tissue supernatant were determined using commercial kits according to the manufacturer's instructions. The detailed steps were based on the methods described in the study of Zhang et al. (2012, 2013).

Briefly, the content of ROS and 8-OHdG in serum was determined by the use of ELISA kits according to the manufacturer's instructions. These particular assay kits were selected because of their high degree of sensitivity, specificity, inter- and intra-assay precision and small amount of plasma sample required conducting the assay. The samples which were treated with streptavidin-horseradish peroxidase (HRP) were added to plate wells precoated with mouse monoclonal anti-cytokine antibody. Then a substrate containing 3,3′,5,5′-tetramethylbenzidine (TMB) was added, the color of the substrate turned into blue by the catalyzation of the peroxidase. The reaction was terminated by the addition of sulphuric acid. The blue color of the substrate was changed to yellow by the addition of sulphuric acid and the intensity of the color was measured at 450 nm using a spectrophotometer.

The measurement of BUN content in serum is based on the principle that urea nitrogen and diacetyl oxime could generate into red couplet azine by condensation reaction under the condition of strong acid, which also called Fearon reaction. The content of BUN was determined according to the absorbance at a wavelength 520 nm and was expressed as milligram *per* litre.

The measurement of LDH activity in serum is based on the principle that LDH catalyzes the oxidation of NAD to NADH. In brief, about 25 μl of matrix liquid, 20 μl of serum and 5 μl coenzyme I were added to the testing sample well. The mixture was incubated at 37°C for 15 min. Then, 25 μl of 2,4-dinitrophenylhydrazine solution was added to the testing sample wells and incubated at 37°C for 15 min. Finally, 250 μl of NaOH solution with a concentration of 0.4 mol/l was added to the wells. After thoroughly mixing and incubating for 15 min, the absorbance was determined at a wavelength 450 nm. Activity of LDH was expressed as unit *per* litre.

Similarly, the determination of ALT was in the condition of 37°C and pH 7.4, the ALT could react with the mixture of alanine and α-ketoglutaric acid, producing the acetylformic acid and glutamic acid. 2,4-DNPH hydrochloric acid was added to solution and reacted with acetylformic acid. This would generate reddish brown pyruvate phenylhydrazone and then the reaction was ended. The activity of ALT could be calculated by the absorbance in 505 nm.

The serum content of creatinine was determined by commercial kits according to the manufacturer's instructions. Briefly, about 200 μl serum and 2 ml tungstic acid protein precipitation agent were mixed and centrifuged at 3000 × *g* for 10 min. Then about 500 μl of picric acid solution and 500 μl of NaOH solution were added to 1.6 ml mixed supernatant solution which we got at the first step, and incubated at 37°C for 10 min. Finally, the content of creatinine was determined at a wavelength 510 nm. The content of creatinine was expressed as micromoles *per* liter.

Protein contents were determined based on the instructions of a Coomassie (Bradford) protein assay kit using 0.563 g/l bovine serum albumin as the standard. About 50 μl of the protein sample or cell suspension was mixed with 3 ml of

Coomassie brilliant blue solution. After 10 min of incubation, the absorbance was determined at a wavelength of 595 nm. The protein concentration was expressed as gram *per* liter.

The content of MPO was determined by commercial kits according to the manufacturer's instructions. The principle of the assay is based on using 4-aminoantipyrine/phenol solution as the substrate for MPO-mediated oxidation by H_2O_2. Briefly, 20 μl tissue homogenate was added to 3.2 ml of reaction mixture and incubated at 37°C for 30 min. Finally, 50 μl of working solution containing was added. The mixture was incubated at 60°C for 10 min and determined at a wavelength 460 nm. The content of MPO was expressed as unit *per* gram of protein.

Liver GSH levels were measured by colorimetric end point assay using dithionitrobenzoic acid (DTNB) method according to the manufacturer's instructions. DTNB reacts with hydrosulfuryl compound to form yellow compound, which can be determined by colorimetry. The content of GSH is reflected the intensity of the yellow color. Briefly, the standard and testing sample wells were set, and 100 μl of standard solution was added to the standard wells. The concentrations of the standard solution were 0, 5, 10, 20, 50, and 100 ng/ml. Then, 100 ul of liver homogenate or cell suspension was added to the testing sample wells. Finally, 100 μl of buffer liquid and 25 μl of color reagents were added to these wells. After thoroughly mixing and incubating for 5 min, the absorbance was determined at a wavelength 405 nm. The GSH concentration was expressed as nanomole *per* milligram of protein.

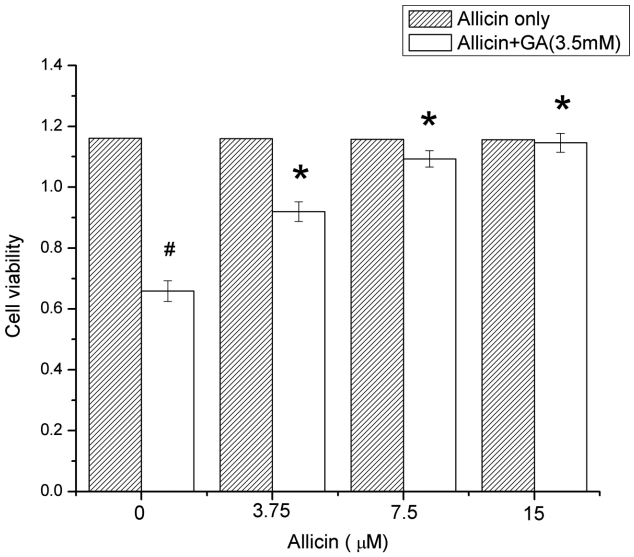

Figure 1. Effect of allicin on glycidamide (GA)-induced cytotoxicity in cultured mouse primary hepatocytes. Data are represented as the mean values ± S.D. of five independent experiments ($n = 5$). * statistically significant differences between the group treated with glycidamide ($p < 0.05$); # statistically significant differences with control ($p < 0.05$).

The determination method of GST is based on fact that GST can catalyze the conjugation of reduced glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB). The activity of GST is reflected the concentration of GSH. Briefly, 30 μl matrix liquid and 25 μl serum were mixed and incubated at 37°C for 30 min. Then, 2 ml of work solution was added and centrifuged at 4000 r/min for 10 min at 4°C to separate supernatant, Finally, 2 ml of supernatant was added to 4.5 ml of reaction mixture and determined at a wavelength 412 nm. The content of GSH was expressed as unit *per* milligram of protein.

Total SOD activity was assayed by detecting the superoxide radicals generated by xanthine oxidase and hypoxanthine according to the manufacturer's instructions. The reduction-oxidation reaction of hypoxanthine and xanthine oxidase produces superoxide, which oxidizes hydroxylamine by reacting with the reagent with a purple-red color. Given that superoxide can be consumed by SOD, different degrees of SOD activity can exhibit different shades of color. About 30 μl of tissue homogenate or cell suspension was mixed with 3.3 ml of reaction solution containing nitroblue tetrazolium chloride and incubated at 37°C for 40 min. The absorbance was measured at 560 nm. SOD activity was expressed as unit *per* milligram of protein.

MDA is a biomarker of lipid peroxidation that has been widely associated with food rancidity as well as many human diseases. The MDA level was examined by detecting the reaction of MDA with thiobarbituric acid (TBA), followed by UV-visible detection according to the manufacturer's instructions. Briefly, about 100 μl of tissue homogenate or cell suspension was mixed with 4.1 ml of work solution containing thiobarbituric acid. The mixture was incubated at 95°C for 40 min and cooled down using tap water. The samples were centrifuged at 2000 × *g* for 10 min, and the absorbance of the supernatant was measured at 532 nm. The concentration of MDA was expressed as nanomole *per* milligram of protein.

Statistical analysis

Statistical analysis was performed using the SPSS 11.5 software (SPSS Inc., Chicago, USA). The significant differences between treatment groups were determined using one-way ANOVA. Results with $p < 0.05$ were considered statistically significant. The graphs for data presentation were drawn using the OriginPro 8.0 software (OriginLab Corporation, Northampton, MA, USA).

Results

Effects of allicin on glycidamide cytotoxicity in hepatocytes

The protective effect of allicin on the cytotoxicity of glycidamide was shown in Fig. 1. According to the calculation of

cell viability, the cell viability of control well is 1. So the cell viability >1 indicates an increase in cell proliferation. Conversely, the cell viability <1 indicates a reduction in the rate of cell proliferation. Cells treated with 3.5 mM glycidamide showed evidently reduced cell viability. The cytotoxicity presented by allicin alone with a concentration of 0, 3.75, 7.5, 15 μM (final concentration after dilution) showed the cell viability had no significant difference in different allicin concentration. This suggested that all doses of allicin alone have no significant role in cell proliferation. However, the cytotoxic effect was significantly attenuated by pretreatment with allicin compared with the glycidamide group ($p < 0.05$). There was no significant difference between the hepatic cytotoxicity of the control and allicin (7.5, 15 μM) with glycidamide groups.

Effects of allicin on hematological parameters of glycidamide-treated mice

The changes in the ROS, 8-OHdG, AST, ALT, BUN, LDH and creatinine levels of the serum samples from all the control and experimental groups are shown in Table 1. Effect of glycidamide alone and supplementation of allicin individually during glycidamide exposes in some hematological profiles (Table 1). ROS and 8-OHdG levels, as an indicator of oxidative DNA damage showed a significant increase in the glycidamide groups ($p < 0.05$). After treatment with glycidamide, the ROS were up to 2.01 times larger than male control group, and 1.99 times larger than female control group respectively. As shown in Table 1, the BUN and creatinine levels also significantly increased in the glycidamide-treated groups compared with the control groups ($p < 0.05$), which are the important reliable markers for kidney damage induced by glycidamide. Similarly, serum LDH activity, as an indicator of generalized tissue damage showed a significant increase in the glycidamide groups ($p < 0.05$). However, the effect was reversed significantly by allicin treatment. In the groups treated with 20 mg/kg b.w./day of allicin, the ROS were reduced to 63.8% in male glycidamide group and 66.3% in female glycidamide group, and the BUN were reduced by 44.4%, 44.5% compared with that in male and female glycidamide group, respectively. The same trend was found with the 8-OHdG, LDH and creatinine levels. Both AST and ALT levels significantly increased in the glycidamide-treated groups compared with the control groups ($p < 0.05$, Table 1). After the addition of allicin, the AST and ALT levels was decreased with the concentration of allicin. The AST levels in the groups treated with 20 mg/kg b.w./day of allicin for 14 days reduced by 34.7% in male, and the 38.6% in female, respectively, whereas the AST levels were reduced by 32.8%, 13.5% after the treatment with 10 mg/kg b.w./day of allicin and 36.3%, 18.1% with 5 mg/kg b.w./day of allicin, respectively, compared with glycidamide groups. Meanwhile, the ALT levels in the groups treated with 20, 10, and 5 mg/kg.bw/day of allicin for 14 days reduced by

Table 1. Effects of allicin on level of 8-OHdG, ROS, LDH, BUN, creatinine AST, and ALT in the serum of glycidamide-treated mice

		Group				
		Control	GA	A5	A10	A20
8-OHdG (ng/ml)	M	11.25 ± 0.07*	29.64 ± 0.33#	22.77 ± 0.24*,#	17.82 ± 0.10*,#	13.72 ± 0.22*,#
	F	11.45 ± 0.23*	27.60 ± 0.20#	21.04 ± 0.13*,#	18.14 ± 0.27*,#	12.27 ± 0.16*,#
ROS (U/ml)	M	148.50 ± 3.80*	298.17 ± 2.89#	252.33 ± 1.61*,#	204.00 ± 2.18*,#	190.17 ± 1.76*,#
	F	140.17 ± 3.33*	280.00 ± 1.80#	249.50 ± 2.27*,#	192.20 ± 1.73*,#	185.50 ± 1.80*,#
LDH (U/l)	M	2041.84 ± 56.67*	3015.60 ± 93.75#	2785.82 ± 48.39*,#	2499.29 ± 35.4*,#	2255.32 ± 25.5*,#
	F	2104.97 ± 49.87*	3049.65 ± 42.84#	2785.82 ± 26.00*,#	2479.43 ± 17.7*,#	2266.67 ± 91.0*,#
BUN (mg/l)	M	4.48 ± 0.63*	9.47 ± 0.47#	7.88 ± 0.37*,#	5.95 ± 0.45*,#	5.27 ± 0.34*
	F	4.30 ± 0.88*	8.96 ± 0.26#	8.10 ± 0.29*,#	6.00 ± 0.22*,#	4.97 ± 0.10*
Creatinine (μmol/l)	M	57.86 ± 5.09*	165.00 ± 2.17#	120.95 ± 0.74*,#	97.86 ± 1.97*,#	72.86 ± 1.04*,#
	F	58.81 ± 1.31*	162.86 ± 1.13#	118.33 ± 2.10*,#	102.14 ± 1.31*,#	73.57 ± 0.67*,#
ALT (U/l)	M	4.93 ± 0.64*	13.46 ± 0.94#	11.38 ± 0.45*,#	8.81 ± 0.51*,#	6.94 ± 0.96*
	F	5.86 ± 0.64*	13.98 ± 0.14#	11.55 ± 0.40*,#	8.64 ± 0.53*,#	7.96 ± 0.30*
AST (U/l)	M	5.45 ± 0.20*	12.85 ± 0.40#	11.12 ± 0.68*,#	8.64 ± 0.39*,#	8.39 ± 1.54*,#
	F	6.18 ± 0.64*	14.24 ± 0.26#	11.65 ± 0.87*,#	9.07 ± 0.26*,#	8.73 ± 1.15*,#

All values are means ± SD ($n = 5$). * $p < 0.05$, statistical significant differences between the groups treated with glycidamide; # $p < 0.05$, statistically significant differences with control. Groups: GA, group with a dose 50 mg/kg b.w./day of glycidamide; A5, the low dose allicin group; A10, middle dose allicin group; A20, high dose allicin group; 8-OHdG, 8-hydroxy-desoxyguanosine; ROS, reactive oxygen species; LDH, lactic dehydrogenase; BUN, blood urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; M, male mice; F, female mice.

48.4%, 34.6%, 15.5% in male, and 43.1%, 38.3%, 17.4% in female, respectively, compared with glycidamide groups; the levels are closed to those in control groups.

Effects of allicin on biochemical parameters of glycidamide-treated mice

The GSH content, GST and SOD activity were measured in kidney, liver, brain and lung samples as the parameters of the antioxidant status of tissues, and the results were presented in Fig. 2. There were some differences in the content of GSH (Fig. 2A), the activity of GST (Fig. 2B) and SOD (Fig. 2C) in each group and variances were found in four tissues. All parameters have the same variation tendency in the four tissues. The highest activity of SOD and GST, the highest levels of GSH were shown in liver, the lowest in lung compared to the four tissues. Moreover, these three parameters were also changed most in liver after glycidamide and allicin treatment. The treatment of glycidamide induced significant decline of the GSH, GST and SOD compared with control groups. However, at the tested dose range (5 mg/kg b.w./day to 20 mg/kg b.w./day), allicin significantly enhanced the SOD activity, GST activity and GSH content compared with the glycidamide groups. With the highest allicin dose, the SOD activity was enhanced to 208.5%, 94.5%, 86.6%, 108.6% in the kidney, liver, brain, lung in male and to 142.5%, 61.3%, 123.3%, 92.3% in female, respectively. As GSH, GST, SOD were all the parameters related to the antioxidant ability, it indicated the effect of glycidamide on oxidative stress of displaying system *in vivo* and the antioxidant role of allicin *in vivo* indirectly. Thus, allicin can inhibit the decrease of antioxidant enzyme activity in the mouse kidney, liver, brain and lung which was induced by glycidamide.

The MDA and MPO levels in the glycidamide groups were higher than that of the control groups ($p < 0.05$) obviously. Fig. 2 shows that the MDA (Fig. 2D) and MPO (Fig. 2E) levels in the allicin-treated groups were significantly lower than that of the glycidamide-treated groups ($p < 0.05$). The addition of allicin could clearly decrease the MDA and MPO levels which were related to the lipid peroxidation in the mouse tissues. The results could infer that free radicals released in the kidney, liver, brain and lung tissues were effectively scavenged when the mice were treated with allicin.

Discussion

In our preliminary test, glycidamide administration at a concentration of 50 mg/kg b.w./day could cause the collapse and apoptosis in tissue cells based on the appearance of the cell. Moreover, further study showed that treatment with 50 mg/kg b.w./day of glycidamide could be used to generate the mouse model for tissue damage in kidney, liver, brain and lung. A large number of studies and reviews have reported the genotoxicity of acrylamide and its metabolite glycidamide (Dearfield et al. 1995, 1998; Besaratinia and Pfeifer 2007; Lee et al. 2007). Compared with acrylamide, glycidamide was mutagenic even at a 2 mmol/ml dose, which suggested that glycidamide is much more mutagenic than acrylamide.

The antioxidant effect of allicin

Large numbers of studies have reported the important influence of free radicals towards body damage (Fearon and Faux 2009; Muriel 2009; etc.). 8-OHdG induces DNA lesions that are formed by the oxidation of the C-8 position of 2′-deoxyguanosine, thereby leading to its use as a biomarker of oxidative DNA damage (Toyokuni et al. 1995; Rüdiger et al. 2006; Singh et al. 2007). 8-OHdG is produced by the oxidation of deoxyguanosine (dG), which is a component of DNA. The hydroxyl radicals directly react with dG to form 8-OHdG. Different physical and chemical factors produce the hydroxyl radicals that can directly and indirectly damage DNA through 8-OHdG formation (Chen et al. 2010). The study of Zhang et al. (2012, 2013) about acrylamide previously shown that after the treatment with acrylamide, the 8-OHdG levels in serum of male mice increased by 58.2%, which was lower than 62% after treatment with isodose glycidamide. Therefore, glycidamide may be more toxic than the same dose of acrylamide. In the present study, the 8-OHdG levels of the allicin-treated groups were significantly decreased as compared with the glycidamide groups. In addition, the highest dose of allicin (20 mg/kg b.w./day) showed a perfect protective effect ($p < 0.05$). Thus, allicin was highly active in the scavenging of free radicals. Similar findings were reported by the chemical studies of Rabinkov et al. (1998, 2000), which showed that allicin had a pronounced antioxidant effect on the hydroxyl radicals that were formed from hydrogen peroxide in the presence of Fe^{2+} during a Fenton's reaction using of electron spin resonance technology and spin trap methods. Therefore allicin can be used as an effective dietary supplement to prevent the DNA damage induced by glycidamide.

The ROS level is also an indication of genotoxicity. ROS is much more reactive than molecular oxygen and can cause severe damage to nucleic acids, cell membranes, and proteins at the same time (Farr and Kogoma 1991). ROS is also known to attack other cellular components, such as lipids, leaving behind reactive species that in turn can couple to DNA bases (Rogers et al. 2000). Besides, ROS is able to activate nuclear factor kappa B (NF-κB) and its controlled cytokine (Sayed and Morcos 2007; Sayed 2008; Morcos et al. 2008). In the present study, we found that glycidamide

treatment induced significant increase in intracellular generation and formation of ROS, which increased by 50.2% in the level of ROS, and was greater than the same dose of acrylamide (46.6%). Our results show these findings are consistent with the study of Xie et al. (2008) that allicin can scavenge oxygen free radicals and hydroxyl radicals. Free radicals seem to trigger the accumulation of leukocytes in the tissue involved, and thus cause further injury through activated neutrophils. Glycidamide can easily react with glutathione and produce oxidative stress (Mei et al. 2008).

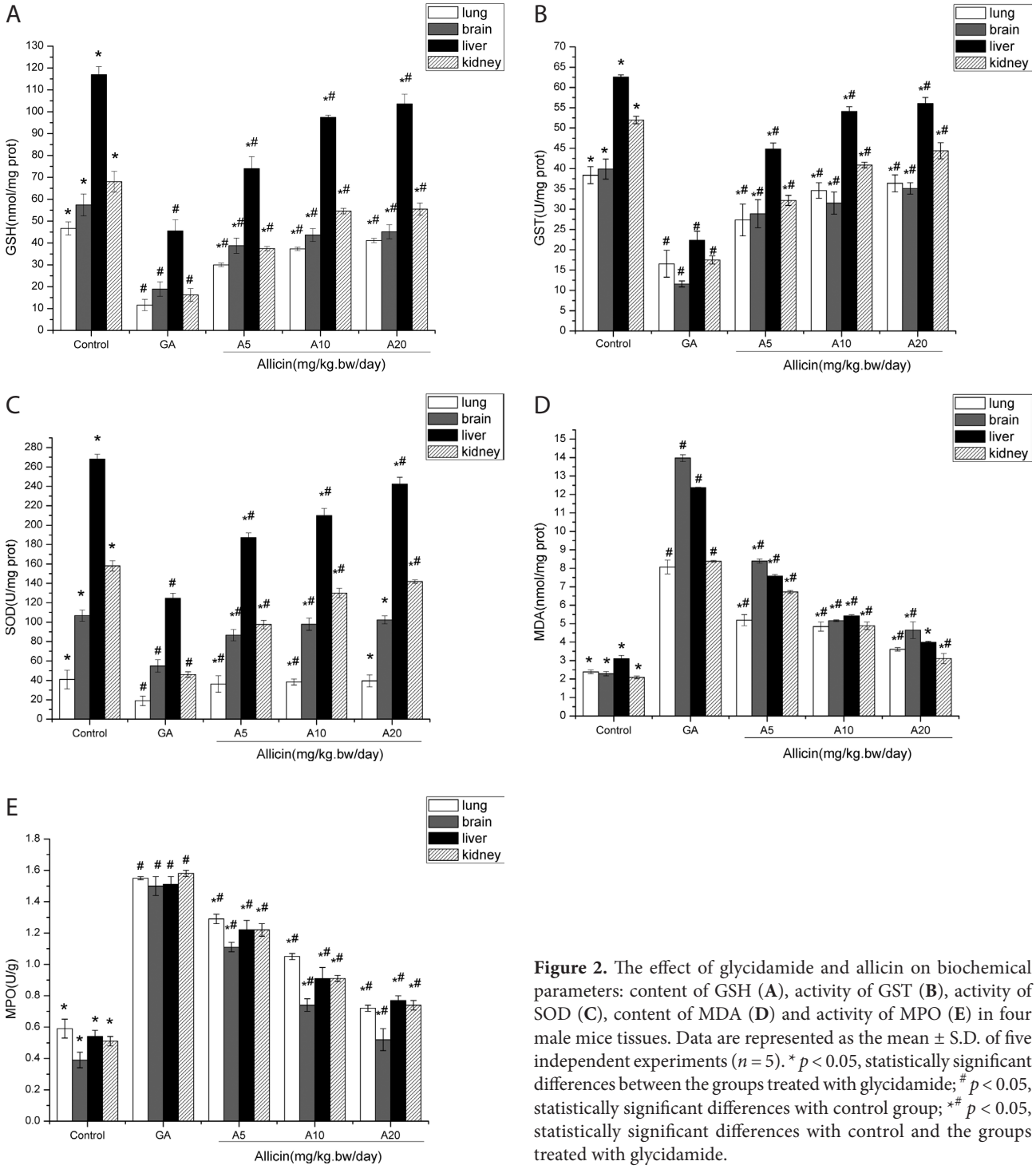

Figure 2. The effect of glycidamide and allicin on biochemical parameters: content of GSH (**A**), activity of GST (**B**), activity of SOD (**C**), content of MDA (**D**) and activity of MPO (**E**) in four male mice tissues. Data are represented as the mean ± S.D. of five independent experiments ($n = 5$). * $p < 0.05$, statistically significant differences between the groups treated with glycidamide; $^{\#}$ $p < 0.05$, statistically significant differences with control group; $^{*\#}$ $p < 0.05$, statistically significant differences with control and the groups treated with glycidamide.

Oxidative stress has been proven to be involved in mutation, chromosome aberration, tumour promotion, and cancer development, thereby making it an important mechanism of indirect genotoxicity (Speit et al. 2002). Compared with the toxicity of acrylamide in our previous study (Zhang et al. 2012, 2013), glycidamide is likewise more toxic than acrylamide, as assessed by the intracellular GSH depletion and the loss of hepatocyte viability. Our study showed that the decreased degree in GSH levels in liver and kidney induced by glycidamide were greater than that induced by acrylamide. Similarly, Kurebayashi and Ohno (2006) reported that both acrylamide and glycidamide induced the concentration- and time-dependent GSH depletion of hepatocytes. Glycidamide could deplete the GSH content at a rate that was 1.5 times faster than that of acrylamide.

Ghanayem et al. (2005) showed that glycidamide can cause the cell oxidation stress to increase sharply in the human body, thereby leading to DNA damage, mainly in the form of DNA breakage and deletion. The enhanced oxidative stress was associated with GSH depletion, and the GSH content reflected the level of protection against of oxidative stress. Xie et al. (2008) found that the addition of tea polyphenols and diallyl trisulfide can remarkably increase the GSH *S*-transferase activity and the GSH content. The primary role of GSTs is to detoxify xenobiotics by catalyzing the nucleophilic attack by GSH on electrophilic carbon, sulfur, or nitrogen atoms of said nonpolar xenobiotic substrates, thereby preventing their interaction with crucial cellular proteins and nucleic acids (Hayes et al. 2005). GSTs are also capable of binding nonsubstrate ligands, with important cell signaling implications. Several GST isozymes from various classes have been shown to inhibit the function of a kinase involved in the MAPK pathway that regulates cell proliferation and death, preventing the kinase from carrying out its role in facilitating the signaling cascade (Laborde 2010). Our findings suggested that a single dose of glycidamide given to mice by injection i.p. after 14 day caused depletion of glutathione S-transferase (GST) in the kidney, liver, brain and lung compared with the control groups (Fig. 2, $p < 0.05$). At the same time, we found that glycidamide has more toxic effects than isodose acrylamide on GST, which caused greater reduction of the GST levels. Administration of allicin significantly elevated the levels of GSH and GST compared with glycidamide-treated groups.

The role of allicin to reduce lipid peroxidation

Oxidative stress is characterised by an increased lipid peroxidation and/or altered non-enzymatic and enzymatic antioxidant systems. Therefore, the mechanisms of antioxidant defence (SOD activity) and the presence of lipid peroxides (MDA content and MPO content) were investigated for the glycidamide-induced tissue injury in mice. The SOD activity reflects the antioxidant enzyme activity when cells are attacked by free radicals, especially the superoxide anion radical. Therefore, the activity of this enzyme has been used to assess the level of oxidative stress in cells (Liu et al. 2012). In our study, the addition of allicin at a concentration of 20 mg/kg b.w./day significantly increased the SOD activity in mice as compared with the glycidamide group.

The levels of MDA, an end product of lipid peroxidation, were significantly decreased with the addition of allicin in the present study, as compared with the glycidamide groups. The results agreed with the study of Şener et al. (2000), which reported that an aqueous garlic extract significantly inhibits the increase of MDA, thereby allowing the MDA concentration to return back to the baseline levels. Thus, garlic extract can be protective against distant organ damage by preserving cellular integrity. On the other hand, it is reported that MPO which is an endogenous lysosomal enzyme that removes H_2O_2 and catalyzes the formation of toxic hypochlorous acid increased significantly due to acrylamide toxicity (Sawayama et al. 2008). Meanwhile our date indicated that allicin treatment prevented increased MPO activity in glycidamide-induced toxicity mice. Accordingly, allicin blocks neutrophils infiltration into the injured tissues. As a result of decreased neutrophil infiltration tissues produced less free radical. It has been shown that the protective effect of allicin against tissue damage is partly mediated by the inhibition of inflammatory responses. MPO and its oxidative products play a key role in the lipid peroxidation in liver damage.

The protective effects against glycidamide-induced kidney damage

Both the BUN and creatinine levels are important reliable markers for kidney damage induced by glycidamide. BUN is an indication of renal health. If Glomerular Filtration Rate (GFR) and blood volume decrease hypovolemia then BUN will increase. Other factors responsible for its increment are fever, increased catabolism, high protein diet and gastrointestinal bleeding (Le et al. 2007).

Serum creatinine is an easily-measured by-product of muscle metabolism that is excreted unchanged by the kidneys. Creatinine is removed from the blood chiefly by the kidneys, primarily by glomerular filtration but also *via* proximal tubular secretion. There is little or no tubular reabsorption of creatinine. If the filtration in the kidney is deficient, creatinine blood levels rise. Therefore, creatinine levels in blood and urine may be used to calculate the creatinine clearance (CrCl), which correlates with the GFR. Blood creatinine levels may also be used alone to calculate the estimated GFR (eGFR) (Allen 2012). The results showed

that glycidamide could significantly increase the BUN and creatinine levels as compared with the control groups, which indicated the increased damage and/or necrosis of kidney, and the damage caused by glycidamide was greater than that caused by acrylamide. While treated with allicin, the BUN and creatinine levels was decreased according to the concentration of allicin, which showed obvious inhibitory effect of allicin on renal function injury caused by glycidamide.

The protective effects against glycidamide-induced liver damage

The AST and ALT levels are two important reliable markers of liver function. Glycidamide could significantly increase the AST and ALT levels as compared with the control groups, which indicated the increased permeability as well as the damage and/or necrosis of hepatocytes (Goldberg and Watts 1965). The treatment of acrylamide and glycidamide, which changed much the levels of AST and ALT in the serum, or directly have great influence on the function of liver. So we can infer that the liver is one of the toxic target organs of acrylamide and glycidamide, and it has a very close relationship to the toxicity mechanism of acrylamide and glycidamide.

Conclusion

In the present study, allicin is demonstrated for the first time to be effective in preventing glycidamide-induced tissue damage and DNA damage *in vitro* and *in vivo*. After intraperitoneal injection of glycidamide, we can find the biochemical parameters in serum and four tissues have the similar tendency with acrylamide treatment. Allicin can prevent the accumulation of 8-OHdG, ROS, BUN, creatinine, LDH, AST, ALT, MDA, and MPO as well as promote the SOD and GSH, GST activities in the kidneys, livers, brains and lungs of male and female mice. We also compared the experimental data of glycidamide with acrylamide previously, which showed that glycidamide have damaged DNA and tissues, and has much greater toxicity than acrylamide. These findings are consistent with the extent of the DNA damage that can be induced by glycidamide. So we can infer that the potential protective effects of oral administration of allicin on acrylamide-induced toxicity may not only play a role on the conversion process of acrylamide to glycidamide, but also are useful to decrease the damage induced by glycidamide directly. However, the detailed protection mechanism of allicin also needs further validation. Thus, allicin, or even garlic extract, is a possible dietary supplement for the chemoprevention of glycidamide genotoxicity.

Acknowledgements. This work was supported by the Fund of National Basic Research Program of China ("973" Program, 2012CB720805), National High Technology Research and Development Program of China ("863" Project, 2011AA 100806), China Postdoctoral Science Foundation Special Funded Project (201104527). Accordingly, the authors gratefully acknowledge the funds supports.

References

Allen P. J. (2012): Creatine metabolism and psychiatric disorders: Does creatine supplementation have therapeutic value. Neurosci. Biobehav. Rev. **36,** 1442–1462 http://dx.doi.org/10.1016/j.neubiorev.2012.03.005

Besaratinia A., Pfeifer G. P. (2005): DNA adduction and mutagenic properties of acrylamide. Mutat. Res. **580,** 31–40 http://dx.doi.org/10.1016/j.mrgentox.2004.10.011

Besaratinia A., Pfeifer G. P. (2007): A review of mechanisms of acrylamide carcinogenicity. Carcinogenesis **28,** 519–528 http://dx.doi.org/10.1093/carcin/bgm006

Chen H. J., Chu G. H., Cao Z. J., Liang J. Y., Lu J. Z., Zhou G. J. (2010): Magnetic bead-based approach to monitoring of cigarette smoke-induced DNA oxidation damage and screening of natural protective compounds. Talanta **80,** 1216–1221 http://dx.doi.org/10.1016/j.talanta.2009.09.012

Dearfield K. L., Douglas G. R., Ehling U. H., Moore M. M., Sega G. A., Brusick D. J. (1995): Acrylamide: a review of its genotoxicity and an assessment of heritable genetic risk. Mutat. Res. **330,** 71–99 http://dx.doi.org/10.1016/0027-5107(95)00037-J

Dearfield K. L., Abernathy C. O., Ottley M. S., Brantner J. H., Hayes P. F. (1998): Acrylamide: its metabolism, developmental and reproductive effects, genotoxicity, and carcinogenicity. Mutat. Res. **195,** 45–77 http://dx.doi.org/10.1016/0165-1110(88)90015-2

Farr S. B., Kogoma T. (1991):Oxidative stress responses in Escherichia coli and Salmonella typhimurium. Microbiol. Mol. Biol. R. **55,** 561–585

Fearon I. M., Faux S. P. (2009): Oxidative stress and cardiovascular disease: Novel tools give (free) radical insight. J. Mol. Cell. Cardiol. **47,** 372–381 http://dx.doi.org/10.1016/j.yjmcc.2009.05.013

Ghanayem B. I., Witt K. L., Kissling G. E., Tice R. R., Recio L. (2005): Absence of acrylamide-induced genotoxicity in CYP2E1-null mice: Evidence consistent with a glycidamide-mediated effect. Mutat. Res. **578,** 284–297 http://dx.doi.org/10.1016/j.mrfmmm.2005.05.004

Goldberg D. M., Watts C. (1965): Serum enzyme changes as evidence of liver reaction to oral alcohol. Gastroenterology **49,** 256–317

Haase H., Hieke N., Plum L. M., Gruhlke M. C. H., Slusarenko A. J., Rink L. (2012): Impact of allicin on macrophage activity. Food Chem. **134,** 141–148 http://dx.doi.org/10.1016/j.foodchem.2012.02.080

Han J., Lawson L., Han G., Han P. (1995): Spectrophotometric method for quantitative determination of allicin and total garlic thiosulfinates. Anal. Biochem. **225,** 157–160

http://dx.doi.org/10.1006/abio.1995.1124
Hayes J. D., Flanagan J. U., Jowsey I. R. (2005): Glutathione transferases. Annu. Rev. Pharmacol. Toxicol. **45,** 51–88
http://dx.doi.org/10.1146/annurev.pharmtox.45.120403.095857
International Agency for Research on Cancer. (1994): Some Industrial Chemicals. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 389–433
Koyama N., Sakamoto H., Sakuraba M., Koizumi T., Takashima Y., Hayashi M., Matsufuji H., Yamagata K., Masuda S., Kinae N., Honma M. (2006): Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells. Mutat. Res. **603,** 151–158
http://dx.doi.org/10.1016/j.mrgentox.2005.11.006
Kumar P., Prasad Y., Patra A. K., Ranjan R., Swarup D., Patra R. C., Pal S. (2009): Ascorbic acid, garlic extract and taurine alleviate cadmium-induced oxidative stress in freshwater catfish (Clarias batrachus). Sci. Total. Environ. **407,** 5024–5030
http://dx.doi.org/10.1016/j.scitotenv.2009.05.030
Kurebayashi H., Ohno Y. (2006): Metabolism of acrylamide to glycidamide and their cytotoxicity in isolated rat hepatocytes: protective effects of GSH precursors. Arch. Toxicol. **80,** 820–828
http://dx.doi.org/10.1007/s00204-006-0109-x
Laborde E. (2010): Glutathione transferases as mediators of signaling pathways involved in cell proliferation and cell death. Cell. Death Differ. **17,** 1373–1380
http://dx.doi.org/10.1038/cdd.2010.80
Le T., Bhushan V., Rao D. A. (2007): First Aid for the USMLE Step 1. McGraw-Hill Medical. Lee Y. L., Yen M. T., Man J. L. (2007): Antioxidant properties of various extracts from Hypsizigus marmoreus. Food Chem. **104,** 1–9
http://dx.doi.org/10.1016/j.foodchem.2006.10.063
Liu C. M., Sun Y. Z., Sun J. M., Ma J. Q., Cheng C. (2012): Protective role of quercetin against lead-induced inflammatory response in rat kidney through the ROS-mediated MAPKs and NF-kappa B pathway. Biochim. Biophys. Acta **1820,** 1693–1703
http://dx.doi.org/10.1016/j.bbagen.2012.06.011
Mei N., Hu J. X., Churchwell M. I., Guo L., Moore M. M., Doerge D. R., Chen T. (2008): Genotoxic effects of acrylamide and glycidamide in mouse lymphoma cells. Food Chem. Toxicol. **46,** 628–636
http://dx.doi.org/10.1016/j.fct.2007.09.093
Morcos M., Schlotterer A., Sayed A. A. R., Kukudov G., Oikomonou D., Ibrahim Y., Pfisterer F., Schneider J., Bozorgmehr F., Rudofsky G. jr., et al. (2008): Rosiglitazone reduces angiotensin II and advanced glycation end product-dependent sustained nuclear factor-κB activation in cultured human proximal tubular epithelial cells. Horm. Metab. Res. **40,** 752–759
http://dx.doi.org/10.1055/s-0028-1082039
Muriel P. (2009): Role of free radicals in liver diseases. Hepatol. Int. **3,** 526–536
http://dx.doi.org/10.1007/s12072-009-9158-6
Puppel N., Tjaden Z., Fueller F., Marko D. (2005): DNA strand breaking capacity of acrylamide and glycidamide in mammalian cells. Mutat. Res. **580,** 71–80
http://dx.doi.org/10.1016/j.mrgentox.2004.11.009
Rabinkov A., Miron T., Konstantinovski L., Wilchek M., Mirelman D., Weiner L. (1998): The mode of action of allicin: trapping of radicals and interaction with thiol containing proteins. Biochim. Biophys. Acta **1379,** 233–244
http://dx.doi.org/10.1016/S0304-4165(97)00104-9
Rabinkov A., Miron T., Mirelman D., Wilchek M., Glozman S., Yavin E., Weiner L. (2000): S-Allylmercaptoglutathione: the reaction product of allicin with glutathione possesses SH-modifying and antioxidant properties. Biochim. Biophys. Acta **1499,** 144–153
http://dx.doi.org/10.1016/S0167-4889(00)00119-1
Rice J. M. (2005): The carcinogenicity of acrylamide. Mutat. Res. **580,** 3–20
http://dx.doi.org/10.1016/j.mrgentox.2004.09.008
Rogers A. E., Kim D. W., Russo J., Sovak M. A., Sonenshein G. E., Zanieski G., Nonet G., Romieu-Mourez R., Lau A. W., Hafer L. J., Yaswen P., Stampfer M. (2000): Activation of NF-kappaB/Rel occurs early during neoplastic transformation of mammary cells. Carcinogenesis **21,** 871–879
http://dx.doi.org/10.1093/carcin/21.5.871
Rüdiger H. W., Kratochvil E., Pilger A. (2006): Reply to the letter by Vijayalaxmi et al. Mutat. Res. **603,** 107–109
http://dx.doi.org/10.1016/j.mrgentox.2005.11.005
Sawayama Y., Miyazaki Y., Ando K., Horio K., Tsutsumi C., Imanishi D., Tsushima H., Imaizumi Y., Hata T., Fukushima T., et al. (2008): Expression of myeloperoxidase enhances the chemosensitivity of leukemia cells through the generation of reactive oxygen species and the nitration of protein. Leukemia **22,** 956–964
http://dx.doi.org/10.1038/leu.2008.8
Sayed A. A., Morcos M. (2007): Thymoquinone decreases AGE-induced NF-kappaB activation in proximal tubular epithelial cells. Phytother. Res. **21,** 898–899
http://dx.doi.org/10.1002/ptr.2177
Sayed A. A. R. (2008): Thymoquinone protects renal tubular cells against tubular injury. Cell. Biochem. Funct. **26,** 374–380
http://dx.doi.org/10.1002/cbf.1454
Şener G., Şatiroglu H., Kabasakal L., Arbak S., Öner S., Ercan F., Keyer-Uysal M. (2000): The protective effect of melatonin on cisplatin nephrotoxicity. Fundam. Clin. Pharmacol. **14,** 553–560
http://dx.doi.org/10.1111/j.1472-8206.2000.tb00440.x
Shin J. H., Ryu J. H., Kang M. J., Hwang C. R., Han J., Kang D. (2013). Short-term heating reduces the anti-inflammatory effects of fresh raw garlic extracts on the LPS-induced production of NO and pro-inflammatory cytokines by downregulating allicin activity in RAW 264.7 macrophages. Food Chem. Toxicol. **58,** 545–551
http://dx.doi.org/10.1016/j.fct.2013.04.002
Singh R., Sram R. J., Binkova B., Kalina I., Popov T. A., Georgieva T., Garte S., Taioli E., Farmer P. B. (2007): The relationship between biomarkers of oxidative DNA damage, polycyclic aromatic hydrocarbon DNA adducts, antioxidant status and genetic susceptibility following exposure to environmental air pollution in humans. Mutat. Res. **620,** 83–92
http://dx.doi.org/10.1016/j.mrfmmm.2007.02.025
Sirius Chung M. L., Eric Lee K. Y., Jetty Lee C. Y. (2013): Profiling of oxidized lipid products of marine fish under acute oxidative stress. Food Chem. Toxicol. **53,** 205–213
http://dx.doi.org/10.1016/j.fct.2012.11.047

Speit G., Dennog C., Radermacher P., Rothfuss A. (2002): Genotoxicity of hyperbaric oxygen. Mutat. Res. **512,** 111–119 http://dx.doi.org/10.1016/S1383-5742(02)00045-5

Taubert D., Glöckner R., Müller D., Schömig E. (2006): The garlic ingredient diallyl sulfide inhibits cytochrome P450 2E1 dependent bioactivation of acrylamide to glycidamide. Toxicol. Lett. **164,** 1–5 http://dx.doi.org/10.1016/j.toxlet.2005.11.004

Toyokuni S., Okamoto K., Yodoi J., Hiai H. (1995): Persistent oxidative stress in cancer. FEBS Letters **358,** 1–3 http://dx.doi.org/10.1016/0014-5793(94)01368-B

Xie Q. Y., Liu Y., Sun H. F., Liu Y. F., Ding X. F., Fu D. P., Liu K. X., Du X. M., Jia G. (2008): Inhibition of acrylamide toxicity in mice by three dietary constituents. J. Agric. Food Chem. **56,** 6054–6060 http://dx.doi.org/10.1021/jf0730542

Zhang L. L., Zhang H. J., Miao Y. T., Wu S. J., Ye H. Q., Yuan Y. (2012): Protective effect of allicin against acrylamide-induced hepatocyte damage in vitro and in vivo. Food Chem. Toxicol. **50,** 3306–3312 http://dx.doi.org/10.1016/j.fct.2012.05.060

Zhang L. L., Wang E. T., Chen F., Yan H. Y., Yuan Y. (2013): Potential protective effects of oral administration of allicin on acrylamide-induced toxicity in male mice. Food Funct. **4,** 1229–1236 http://dx.doi.org/10.1039/c3fo60057b

Received: April 1, 2014
Final version accepted: November 7, 2014
First published online: March 2, 2015