INTRODUCTION
Cadmium is a heavy metal widely distributed in the environment. It is a serious industrial and environmental toxicant, known for its hepatotoxic, nephrotoxic and carcinogenic effect in humans and animals [1].

Industrial and agricultural use of cadmium leads to environmental contamination, a serious global concern as it enters food chain thereby undergoing bioaccumulation and endangering human health. For example, vegetables falling into the trade networks of few countries (Brassica oleracea, Abelmoschus esculentus, Beta vulgaris), can be dangerous for humans due to the high degree of contamination with heavy metals \( \text{(Cu}^{2+}, \text{Zn}^{2+}, \text{Cd}^{2+} \text{and Pb}^{2+}) \) [2]. The major industrial sources of cadmium include oil refining, smelting of metals such as copper and zinc, combustion of fossil fuel and nickel-cadmium battery manufacture and disposal. In humans, non-occupational exposure to cadmium predominantly results from smoking, air pollution and consumption of cadmium-contaminated sea foods and water [3].

It is found that cadmium is virtually absent in mammals by birth but over time it accumulates, especially in liver and kidneys, such that up to 75% of the total body burden is found in these organs [4]. Age-dependent toxicity of cadmium on the living beings has been reported earlier [5]. Metals are also considered to play a specific role in the intensification of the aging processes [6]. A decrease in life expectancy has been reported in animals exposed to water containing cadmium and lead [7]. Thus, animal's age can be considered an important factor in assessing the adverse effects of cadmium. Despite its inability to generate free radicals under physiological conditions, lipid peroxidation is considered as the primary mechanism for cadmium induced toxicity [8]. Cadmium exerts its toxic effects via oxidative damage to cellular organelles...
by inducing the generation of reactive oxygen species (ROS) which consist mainly of the superoxide anion radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical ('OH). Depletion of glutathione and other endogenous antioxidants may also contribute significantly to cadmium-induced oxidative stress [9].

Various chemopreventive agents, anti-oxidants and metal-chelating agents have been used in the treatment of oxidative stress-mediated diseases, including vitamins (C and E), carotenoids, and minerals such as selenium [10-12]. Recently, there has been a growing interest towards exploiting the biological activities of different medicinal herbs, due to their natural origin, cost effectiveness and fewer side effects. Among those, a protein isolated from the leaves of the herb Cajanus indicus [13], green tea catechins [14], naringenin [15], curcumin, resveratrol, melatonin and quercetin [16] have been studied to possess protective efficacy against cadmium-induced oxidative stress.

Garlic is an extensively studied herb (Allium sativum L.), which has been used since ancient times as a cure for many diseases. Two main classes of antioxidant components, namely flavonoids and sulfur-containing compounds (diallyl sulfide, trisulfide and allylcysteine) have been reported in garlic [12]. These are responsible for the hypolipidemic [17], hypercholesterolemic [18] activity and garlic effects against arsenic (metal) induced toxicity [12]. In general the protective efficacy of garlic has been mainly ascribed to its potent antioxidant action [19]. Hepatoprotective effects of garlic against cadmium-induced toxicity have been reported previously both in vivo and in vitro, however, the major mechanism at molecular level leading to its protective efficacy are still unclear [20], especially in the aspect of age. Most of these studies focused on the use of aged garlic extract (AGE) or other commercial products. In the present study, the anti-oxidant properties of fresh ethanol extract of garlic against cadmium induced oxidative stress in rats of different age groups have been studied.

MATERIALS AND METHODS

Chemicals

All the chemicals and reagents were obtained from Sigma-Aldrich (UK), Merck (Germany) and Ukrainian chemical plants.

Preparation of garlic extracts

The local Ukrainian varieties of garlic (Allium sativum L) were obtained from the local market in Kharkiv, Ukraine. Cold extraction of garlic was carried out as follows: fresh garlic bulbs were grounded to a fine paste using a mechanical grinder. 625 g of the paste was placed in a 1000 ml conical flask, covered with 220 ml of 80% ethanol, stoppered with cotton wool, and allowed to stay in the dark for 48 h at the room temperature (20-22°C). The ethanol extract was filtered off into pre-weighed evaporating dishes, while the residue in the flask was washed with 220 ml of 80% ethanol and added to the extracts in the evaporating dishes. The filtrates were then evaporated to a syrupy residue using a rotary evaporator at 40°C. The extracts were pooled together into an airtight container and refrigerated (at –4°C) until required for use. The yield of the dry plant substances equalled 90 mg/ml of extract.

Laboratory animals and experimental design

Male albino Wistar rats were obtained from the V.N. Karazin Kharkiv National University vivarium, (Kharkiv, Ukraine). The experimental protocol was approved by the Animal Ethics Committee of V.N. Karazin Kharkiv National University.

All experiments were conducted on young (3-4 month) and old (30 months) male rats. They were housed in polypropylene cages in an air conditioned room with temperature maintained at 22 ± 2°C, relative humidity of 50 ± 5% and 12 h alternating light and dark cycles. The rats were provided a standard diet and drinking water ad libitum throughout the study. Animals (old and young) were divided into 4 groups of 10 rats each and treated as below:

Group 1; normal animals

Group 2; cadmium as CdCl$_2$•2½H$_2$O (5 mg/kg) intraperitoneally, once in the last day of garlic extract intubation

Group 3; garlic (250 μl/kg, once daily) per os during 4 days

Group 4; cadmium (as in group 2) in combination with garlic (as above) in last day of extract intubation

The dose of cadmium chloride and garlic were decided on the basis of preliminary experiments conducted in our laboratory.

Animals were fasted overnight and sacrificed by cervical dislocation under diethyl ether anesthesia. Liver was removed and washed free of extraneous material with ice cold normal saline, followed by 0.15 M Tris buffer (pH 7.4), blotted and weighed. It was then homogenized in 0.3 M sucrose, 1 mM EDTA and 10 mM Tris buffer, pH 7.4, to a concentration of 4 g per 25 ml. The homogenate was centrifuged at 600g for 10 min at 4°C. The supernatant was further centrifuged at 600g for 15 min at 4°C, and sediment washed twice with 0.3 M sucrose and 10 mM Tris buffer, pH 7.4. The resulting mitochondrial pellets were re-suspended in 0.3 M sucrose and 10 mM Tris, pH 7.4. After a centrifugation to isolate mitochondria, the supernatant (post mitochondria fraction) was further centrifuged at 10,000g for 15 min at 4°C with the
addition of CaCl₂ to a final concentration of 10 mM and sediments washed twice with 0.15 M KCl. The resulting pellets were re-suspended in 10 mM Tris buffer with 0.15 M KCl, pH 7.4.

Blood was collected in centrifuge tubes for serum separation. The serum obtained after centrifugation (600g for 10 min at 4°C) was used for various serum biochemical assays.

**Biochemical assays**

*Serum biochemical markers;* activities of aspartate transaminase (AST) and alanine transaminase (ALT) were assayed spectrophotometrically using 1-chloro-2,4-dinitrobenzene as the substrate [27].

Protein level was estimated by the method of Lowry et al [28] using bovine serum albumin as standard.

**Statistical analysis**

Results were analyzed using a one-way analysis of variance (ANOVA). Comparison within groups was carried out using the unpaired Student’s t-test. Values were considered statistically significant at p < 0.05. All the data were expressed as mean ± SD of number of experiments (n = 10).

### RESULTS

**Effects of garlic on cadmium induced changes in membrane hepatic markers**

Table 1 shows the effect of garlic on cadmium induced changes in the serum biochemical markers indicative of liver damage. Cadmium exposure produced abnormal liver function as indicated by increased serum ALT and AST activities in both young and old rats compared to respective rats. Administration of garlic with cadmium significantly reduced (p < 0.05) the activity of these serum markers in cadmium exposed rats.

**Effects of garlic on cadmium induced changes in lipid peroxidation**

To evaluate the effect of garlic on membrane lipid damage induced by cadmium, lipid hydroperoxides levels were determined by measuring thiobarbituric acid reactive substance and are presented in Fig. 1. Exposure to cadmium led to a significant increase in serum hydroperoxides levels in the young rat while there was no change in hydroperoxides levels in old animals (Fig. 1). Hydroperoxides levels were significantly reduced in garlic treated young rats pre-exposed to cadmium compared to respective control. No effect of garlic on the other hand on above variables in cadmium exposed rats was noted in old rats (Fig. 1).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Rats</th>
<th>Normal control</th>
<th>Cadmium</th>
<th>Garlic</th>
<th>Cadmium + Garlic</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (Unit)</td>
<td>Young</td>
<td>0.45 ± 0.019</td>
<td>0.88 ± 0.043*</td>
<td>0.41 ± 0.03</td>
<td>0.63 ± 0.042***</td>
</tr>
<tr>
<td></td>
<td>Old</td>
<td>0.363 ± 0.027</td>
<td>0.848 ± 0.050*</td>
<td>0.35 ± 0.012</td>
<td>0.658 ± 0.041***</td>
</tr>
<tr>
<td>AST (Unit)</td>
<td>Young</td>
<td>0.64 ± 0.024</td>
<td>0.993 ± 0.015*</td>
<td>0.395 ± 0.025*</td>
<td>0.6 ± 0.035**</td>
</tr>
<tr>
<td></td>
<td>Old</td>
<td>0.577 ± 0.048</td>
<td>1.03 ± 0.028*</td>
<td>0.5 ± 0.03</td>
<td>0.855 ± 0.043***</td>
</tr>
</tbody>
</table>

p < 0.05 compared to *normal control or **cadmium exposed rats

http://www.oamsjournal.com

103
Effects of garlic on cadmium induced changes on protein oxidation indices

Figure 2 shows changes in the levels of protein carbonyl content in the serum of control and exposed groups. In contrast to the level of lipid hydroperoxides, protein carbonyl content changed with the administration of cadmium chloride in both the groups of animals studied. Significant elevations in the levels of protein carbonyl content were observed in exposed group as compared to the control group. However, significantly lowered levels of protein carbonyl content in the liver were observed only in the old animals exposed to cadmium in combination with garlic.

Effects of garlic on microsomal monooxygenase activity indicative of membrane damage

Figure 3 depicts the levels of the cytochrome P-450, which significantly reduces with the age in control animals. The administration of cadmium essentially altered the levels of cytochrome P-450 in young animals only, and no statistically significant changes were observed following concomitant oral administration of garlic in this age group of animals.

Effects of garlic on cadmium induced changes on non-enzymatic antioxidant (ceruloplasmin) status

Figure 4 shows the levels of ceruloplasmin in the serum of control and exposed groups. Cadmium induced toxicity leads to decreased levels of ceruloplasmin in the rats exposed to cadmium alone. However the altered levels were recovered following garlic administration in young rats. No such recovery was observed in old rats.

Effects of garlic on cadmium induced changes in enzymatic antioxidant status

Figures 5-7 illustrate the levels of enzymatic antioxidants, viz. GPx and GST in the serum and liver mitochondria of control and exposed rats of different age groups. GPx activity in control animals was lower in the serum of old rats in comparison to the young ones as evident by Fig.5. Enzyme activity in serum decreased significantly in all animals in response to the administration of cadmium chloride. No alteration in
the activity of GPx was observed following co-administration of garlic and cadmium chloride in young animals as compared to the old animals where significant recovery of the enzyme activity to the normal levels was observed.

Figure 6 depicts the activity of GPx in liver mitochondria for the young and old rats. In response to the administration of cadmium, activity of GPx declined and administration of garlic significantly recovered the enzyme activity in old rats in comparison to the young rats.

Figure 7 depicts the activity of GST in the liver microsomes of young and old rats. The administration of cadmium chloride significantly reduced the enzyme activity only in young rats, and no significant protective efficacy of garlic in young rats was observed.

**DISCUSSION**

Generation of ROS is currently being considered as the major mechanism involved in the toxic manifestations of cadmium. However, these effects (free radical generation) depends on the dose and the duration of exposure. Low level and long term exposure to cadmium is more often used than acute exposure. Although oxidative stress following single cadmium exposure has been studied in the past however the same after multiple exposures is less well-defined. Due to induced adaptation mechanisms following a chronic exposure to metal, changes in ROS-related gene expression are less significant during chronic exposure as compared to the acute exposure [29]. It has been reported that tissue lipid peroxidation increases significantly already after 6 to 12 h of intoxication with cadmium [30]. Hence in the present study biomarkers of cadmium induced oxidative stress after an acute exposure has been studied.

Aspartate and alanine transaminases are an important class of enzymes whose level increases in response to the toxic effect of heavy metals [31]. Administration of cadmium could cause cell lysis and release of cytoplasmic enzymes into the blood circulation, thereby leading to increased levels of these enzymes in the serum. This property is often used to assess the extent of cadmium-induced cellular damage. In the current study, elevated levels of AST and ALT were noted in response to cadmium induced toxicity. However, the level of enzymes significantly declined following concomitant administration of garlic extract (Table 1). The observed results suggest that garlic may have the potential to preserve the structural integrity of the tissues, and protects tissue against the toxic effects of cadmium in rats of different age groups. But it should be noted that administration of garlic unexpectedly significantly reduced AST activity in
young rats in comparison to the young control animals (Table 1). At the moment we do not know the causes of these phenomena, but we can already make some assumptions:

It is well known that both aminotransferases are highly concentrated in the liver; ALT is localized solely in the cellular cytoplasm, whereas AST is both cytosolic (20% of total activity) and mitochondrial (80% of total activity) [32]. It is possible that the peculiarities of the garlic effects on the mitochondrial membrane of young animals associated with slight (but significant) decrease in AST release from hepatocytes at this age. In principle, the garlic ability to influence the structure of mitochondrial membrane [33] and its characteristics in animals of different ages [34] is known, yet quite a few studies have been published on this subject. In view of controversial results, further investigation is required to characterize the active garlic hepatoprotective principle in animals of different ages.

The microsomal enzymes in liver were indicative of toxic effects of cadmium on liver membrane functions. Decreased content of microsomal cytochrome P-450 (Fig.3) was observed following acute exposure to cadmium in young rats only. The scientific evidences and observed results suggest that cadmium exerts dose- and duration-dependent membranotoxic effects in rats; however, the level of toxicity is age-dependent.

Few reports reveal the levels of lipid peroxidation in liver and other tissues of rats of different age. Both elevation and decline, as well as no changes were reported in the liver of old rats [35-37]. Present investigations revealed no changes in the levels of lipid hydroperoxides in control rats of different ages. Exposure to cadmium led to a significant increase in the level of lipid hydroperoxides only in the young rats’ liver; however, the levels of lipid hydroperoxides have decreased after treatment with garlic extract (Fig.1).

Scientific evidence suggests that toxic effect of metals might result in modification of proteins by a large number of reactions involving reactive oxygen species. Among these reactions, carbonylation has attracted a great deal of attention due to its irreversible and irreparable nature. Carbonylated proteins can escape degradation and form high-molecular-weight aggregates that accumulate due to adverse effect of environmental factors and age. Such carbonylated aggregates can become cytotoxic and have been associated with a large number of disorders, including Parkinson's disease, Alzheimer's disease, and cancer [38]. The present results depict (Fig.2) that the degree of oxidative protein damage (measured as carbonyl content in the serum) was significantly higher in rats of both age groups, exposed to cadmium alone. At the same time, oral administration of garlic in combination with cadmium significantly lowered the levels of protein carbonyl content in the liver of old rats only. It was reported that carbonyl content could be related to both oxidative and non-oxidative mechanisms [39]. Formation of significant amount of carbonylated proteins in the old animals can be attributed to non-oxidative mechanisms and protective efficacy of garlic might be able to overcome these processes.

Antioxidant system of the body consists of two mechanisms: nonenzymatic and enzymatic. A nonenzymatic mechanism involves antioxidants, scavengers of free radicals, transition metal ions, albumins, metallothioneins, and ceruloplasmin. On the other hand, an enzymatic mechanism involves enzymes such as superoxide dismutase, catalase, peroxidases and reductase. Thus, the balance between the pro-oxidant and antioxidant determines the extent of lipid peroxidation. Antioxidant enzymes form the second line of defense system which provides a broad range of primary and secondary defenses against oxidative stress. Primary antioxidant enzymes (superoxide dismutase, catalase) are mostly preventive; these enzymes can decompose ROS and prevent the damage to cellular constituents. Secondary defenses typically involve excision or repair of any lesions caused by ROS. In the case of ROS induced lipid peroxidation, secondary defense enzymes are involved in the removal of lipid hydroperoxides to terminate the autocatalytic chain of lipid peroxidation and protect membranes. GPx and GST which catalyze glutathione-dependent reduction of lipid hydroperoxides through their peroxidase activity are the major secondary defense enzymes against ROS-induced lipid peroxidation.

In the current study, the level of glutathione-dependent enzyme activity was significantly reduced in rats following cadmium administration (Figs.5-7). Renugadevi and Prabu [15] have reported the decreased levels of reduced glutathione and its dependent enzymes in cadmium-intoxicated rats in accordance with our present results. The activity of Se-dependent GPx in serum and liver mitochondria was essentially decreased in response to the cadmium induced toxicity and did not altered upon garlic administration in young rats. However in old rats administration of garlic extract led to a substantial increase in the enzyme activity (in serum and liver mitochondria, respectively). Meanwhile, results (Fig.7) also suggest significant reduction in GST activity in young rats only; less pronounced protective effect of garlic extract in this age group when compared with older animals was also observed. At the same time, garlic extract elevated the levels of ceruloplasmin in the serum of young rats, which has been reduced due to toxic effects of cadmium (Fig.4).

On the whole, these results demonstrate that young rats are more vulnerable to the used cadmium dose and

DOI 10.5455/oams.200812.or.014
exposure duration. These data are consistent with results of other researchers [5, 40] whose study suggested that mature and senescent animals were more resistant to cadmium induced hepatotoxicity compared with young rats. At the same time, Ando [41] showed that older rats were more sensitive to cadmium than younger ones. The causes of these phenomena require further study, but we can already make some assumptions:

First of all, probably we should suggest the possibility of various cadmium accumulation in rats’ tissues at different ages. Indeed, it is shown that the degree of absorption depended on the age of the animal: young mice retained 10%, while adult mice retained only 1% [42], and, according to Shimada et al [43], the remarkable resistance to cadmium induced hepatotoxicity in Wistar-Imamichi rats is associated, at least in part, with a lower tissue accumulation of the metal compared to other strains such as Fischer 344 rats.

According to Yamano et al [5], acute hepatotoxicity involves two pathways, one for the initial injury produced by direct effects of cadmium and the other for the subsequent injury produced by inflammation. And the attenuation of cadmium induced liver injury in senescent rats is caused also by an impairment in Kupffer cell activation, leading to a lower production of cytokine-induced neutrophil chemoattractant and less inflammatory liver injury. But the precise roles of the soluble mediators of inflammation warrant further investigation.

Another reason for lower sensitivity of old animals to cadmium may be different endogenous metallothionein levels compared with young rats. So, Thomas et al [44] showed that ontogenic variation in accumulation of metallothionein mRNA after cadmium treatment may be a factor in developmental variation in the acute lethality of cadmium in C57BL/6J mice. In the investigation of Wormser and Nir [45] cadmium induced metallothionein synthesis became elevated to 36-times compared with the original value in the youngest (3-month-old) rats and 90- and 74-times, respectively, in the 12- and 24-month-old groups. However, the authors of this paper observed paradoxical high mortality of 75% occurred in the aged (24-month-old) group following cadmium administration.

As it has been demonstrated, the early response to the exposure of cadmium includes the enhancement in lipid peroxidation and the delayed response includes induction of metallothionein [30, 46]. We assume that possible differences in the level of metallothionein are probably not the leading cause of the observed age-related differences in the animals response to acute cadmium administration.

In view of controversial results and interpretations in modern literature, the mechanisms involved in age differences in the animals’ response to the acute administration of cadmium need further study.

On the whole, these results demonstrate the ability of garlic extract to reduce the age dependent toxic manifestations of cadmium induced hepatotoxicity. Co-administration of garlic in old rats provided essential protection against cadmium toxicity. Significant depletion of the cadmium-dependent carbonyl proteins level and restored activity of antioxidant enzymes were also observed. In young rats, concomitant administration of garlic leads to less pronounced hepatoprotective effect compared to that in old rats as evident by higher levels of carbonyl proteins and incomplete recovery of cytochrome P-450 and antioxidant enzymes. On the other hand, elevated levels of non-enzymatic antioxidants in young rats were also observed.

Our study and numerous literature data showed that cadmium induced oxidative damage in rat liver is amenable to attenuation by moderate dose of garlic extracts. But, it was somewhat surprising that garlic ameliorated the toxic reactions of wide variety of some others toxic agents as well (CCl4, N-nitrosodimethylamine and acetaminophen) [47]. It seems unlikely that garlic is only an antioxidant in these situations.

It is known that cadmium generated oxidative stress is often accompanied by activation of redox sensitive transcription factors (e.g., NF-κB, AP-1 and Nrf2) and alteration of ROS-related gene expression [29]. In recent years much attention has been paid to one of these transcription factors. Nuclear factor-erythroid-2-related factor 2 (Nrf2), which plays a crucial role in the coordinated induction of those genes encoding many stress-responsive and cytoprotective enzymes and related proteins [48, 49]. Recently, several studies reported that Nrf2 is associated with aging and various diseases [50]. Its nuclear levels decline with age which in part explains the age-related loss of glutathione synthetic and other phase II enzymes [51]. For example, according to Tomobe et al [52], a higher level of oxidative stress in SAMP8 mice might be caused by a lower level of Nrf2. For the purposes of this paper it is important that it has been shown that garlic can significantly increase Nrf2 levels in cells [53, 54], providing evidence that this mechanism is involved in the induction of protective enzymes, and, in turn, to the increased survival of cadmium exposed cells.

In general, these results show that garlic extract has beneficial effects against cadmium induced toxicity, and these protective effects are likely to be mediated, at least in part, by the enhanced induction of the cytoprotective enzymes in Nrf2-dependent manner. But
the composition of these cytoprotective enzymes is probably different at different ages, and a more pronounced protective effect of garlic in old animals may be associated with reduced Nrf2 baseline in these animals. Of course, the mechanisms involved need further study.

Collectively, the results obtained support various scientific reports about the activation of the oxidative stress by cadmium in experimental animals. It also validates garlic as the potential protective agent against acute exposure to toxic metals but an organism's age could be an important factor in deciding its efficacy. Protective effect of garlic in young animals is associated more with maintaining an adequate level of non-enzymatic antioxidant defense, whereas in old animals this effect is associated with activation of the enzymatic antioxidant defense. However further investigations is needed for the establishment of the fact that hepatoprotective action of garlic is age dependent.

ACKNOWLEDGEMENTS

Authors are grateful to Dr. Yu. Nikitchenko (V.N. Karazin Kharkiv National University) for help on antioxidant enzymes studies. Authors are also grateful to the State Agency on Science, Innovations and Information of Ukraine and Department of Science and Technology (DST) for the Government of the Republic of India of Indo-Ukraine Fellowship.

COMPETING INTERESTS

This work presents no conflict of interest.

REFERENCES


49. Lewis KN, Mele J, Hayes JD, Buffenstein R. Nrf2, a guardian of healthspan and gatekeeper of species longevity. Integr Comp Biol 2010; 50:829-43.


