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# The Effect of Ascorbic Acid and Garlic Administration on Lead-Induced Apoptosis in Rat Offspring's Eye Retina

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## ABSTRACT

**Introduction**: Lead toxicity induces retinal cell apoptosis. Vitamin C and garlic may decrease lead-induced apoptosis. This study was undertaken to investigate vitamin C and garlic protective effects on lead-induced apoptosis in eye retina. **Methods**: Pregnant Wistar rats (n = 72) were divided randomly into 9 groups: (L) treated rats with lead acetate in drinking water and (L+AA) with leaded water and vitamin C intraperitoneally;(L+G), the rats received leaded-water and garlic juice via gavage; (L+AA+G) treated rats with leaded water, ascorbic acid, and garlic juice, (AA) with ascorbic acid, and (G) with garlic juice; (AA+G) treated rats with vitamin C and garlic juice and (Sh) with tap water plus normal hydrogen chloride (HCl) and glucose; normal (N). After 21-day lactation, blood lead level (BLL) in rats was measured, and then their offspring and the rat offspring's eyes were removed and processed for using TUNEL method. TUNEL positive cells in the eye retina were counted and all groups were compared. **Results:** BLL increased in L group compared to the control groups and decreased significantly in L + G, L + AA, and L+ AA + G groups compared to L group ($P<0.05$). TUNELL positive cell number in eye retina significantly increased in L group compared to control groups ($P<0.05$) and decreased in L+ G, L+ AA, and L+AA + G groups compared to L group ($P<0.05$). **Conclusion:** Garlic juice and ascorbic acid administration during pregnancy and lactation may protect lead-induced apoptosis in rat offspring's eye retina. *Iran. Biomed. J. 17 (4): 206-213, 2013*

*Keyword*: Lead, Garlic, Ascorbic acid, Apoptosis, Retina

## INTRODUCTION

Lead as a heavy metal, non-biodegradable, and environmentally pollutant enters environment via gasoline, cosmetics, recycling of old products, and manufacturing processes widely [1-3]. Naturally, there is no any lead in biological systems, but it might affect any biological system. Therefore, it is possible that it accumulates within various organs [4]. Ninety percent of lead accumulates in the bones of the body and has a half-life of more than twenty years. Bone releases lead during periods of increased bone turnover in women's lives, such as pregnancy, lactation, and menopause [1]. It is well known that lead can cross the placenta during pregnancy and can cause intrauterine fetal death, preterm delivery, low birth weight, and abnormalities on brain and eyes [1, 2]. Lead toxicity may have an effect on the eye lens and retina by loss of lens transparency [3] and induce retinal cell apoptosis [5]. Apoptosis may happen with low levels of lead in the developmental phase of retina *in vitro* and *in vivo* conditions [6, 7]. Rods assist in seeing in dim light, and the other types of retinal cells, cones, are responsible for color and spatial vision. High levels of lead exposure result in an increase in lysosomal inclusions in the retinal pigment epithelium, swollen photoreceptor mitochondria, photoreceptor disorientation, and necrosis [8]. Antioxidants such as vitamin C may decrease injurious activity of reactive oxygen species (ROS), especially on the newly formed neurons [9, 10] and may clear ROS and free radicals [11, 12] to protect lead toxicity. In addition, vitamin C may decrease reaction between lead and critical biomolecule, modification of genomic protection through suppression of intracellular ROS, and apoptosis inhabitation [13]. It has been reported that vitamin C acts as a chelating agent for lead, and its effect is almost equal to EDTA [14]. On the other hand, herbs have been used in primary health care for centuries before the advent of modern medicine [15].

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Today, medicinal plants are used for treatment of many diseases instead of synthetic materials [16]. Some of these plants such as garlic (*Allium sativum*), due to ingredients, including sulfydryl, diallyl sulpide, and Alexin has antitoxic, antimutagenic, and anticarcinogenic properties, is used to improve metal toxicity [4, 15, 17, 18]. It has been indicated that the water-soluble organosulfur and Cysteine S-allyl compounds of garlic extract have strong antioxidant potential that can cause free radical scavenging in lead poisoning comparable with the vitamin C antioxidant properties [19, 20]. In this regard, the present study was designed to investigate the effect of ascorbic acid and garlic administration on lead-induced apoptosis in rat offspring's eye retina.

## MATERIALS AND METHODS

All experiments and procedures involving animals were carried out according to National Institutes of Health and Department of Health Education and Welfare Guideline and approved by the Institutional Animal Care Committee of Mashhad University of Medical Sciences (Mashhad, Iran).

***Animals.*** For this study, 96 Wistar rats (72 females + 24 males) with 250-300 g body weight were used. The animals were maintained at the animal house under controlled conditions (12 h light and dark cycle, 21°C and 50% relative humidity) with laboratory chow and water provided *ad libitum*. After adaption, the animals were mated in the special matting cages (3 female + 1 male) overnight. The day on which spermatozoa were found in vaginal smear was considered as gestational day ($GD_0$).

***Study design and experimental groups.*** The pregnant rats were randomly divided into 9 groups as follows (8 rats in each group): 1) lead-exposed (L) group, the animals treated with lead acetate (1500 ppm) in drinking water starting at GD0. The lead exposure regimen was chosen based on a previous study [21]; 2) lead + ascorbic acid (L + AA) group, pregnant rats treated with leaded water (1500 ppm) and vitamin C (500 mg/kg/day) via i.p. injection; 3) lead + garlic juice (L + G) group, the animals received leaded-water (1500 ppm) and also fresh garlic juice (1 ml /100 g/ body weight) by a gavage once a day; 4) lead + ascorbic acid + garlic (L + AA + G) group, the animals treated with leaded water (1500 ppm) and ascorbic acid (500 mg/kg) via i.p. injection and fresh garlic juice (1 ml /100 g/ body weight) by a gavage once a day [21]; 5) ascorbic acid (AA) group, the animals treated with ascorbic acid (500 mg/kg) via i.p. injection once a day; 6) garlic (G) group, the animals treated with fresh garlic juice (1 ml/ 100 g/ body weight) by a gavage once a day; 7) ascorbic acid + garlic (AA + G) group, the animals treated with ascorbic acid (500 mg/kg) via i.p. injection and fresh garlic juice (1 ml/100 g/ body weight) by a gavage once a day; 8) sham (Sh) group, the animals treated with tap water plus 0.4 ml/l normal hydrochloric acid and 0.5 mg/l glucose and 9) normal group, the animals administrated with tap water and normal diet. All treatments were continued during pregnancy and lactation, postnatal day 21 (P21).

***Preparation of leaded water.*** To prepare 1500 ppm leaded water, 30 g lead acetate (Merck, Germany), 8 cc normal hydrochloric acid (to avoid lead precipitation, Merck, Germany) and 10 g glucose (for favorite taste) were dissolved in 20 L tap water [21].

***Preparation of garlic juice.*** To prepare garlic juice, fresh garlic bulbs, identified by botanists in Ferdowsi University of Mashhad, Mashhad, Iran, were collected from a natural habitat around Mashhad during June to August 2012. A voucher number was deposited (FUMH: 39493), then garlic bulbs were separated, peeled, and washed with distilled water. After drying in a shed, the clean garlic bulbs were crushed with an electric grinder, and the extract was decanted carefully through muslin cloth [21]. In order to use fresh garlic juice, 500 g fresh garlic was used, and 250 ml garlic juice was extracted. The concentration of the juice was calculated as:

$$\text{Gram equivalent} = \frac{\text{weight of the fresh garlic}}{\text{weight of the juice}}$$

***Blood lead level (BLL) measurement.*** At first, tail vein blood sampling in all the animals in each group was performed before any intervention. Then at the end of experiments, adult rats (mothers) and their offspring (P21) were deeply anesthetized with chloroform, and the blood samples were taken transcardinally. To measure lead level in whole blood samples, a Perkin-Elmer Model 3030 atomic absorption spectrophotometer with a Perkin-Elmer Heat Graphite Atomizer 400 graphite furnace and hydride system MHS-10 were used together with hydrochloric acid (hallow-cathode lamp) and electrode discharge lamp for metal measurement of even low levels. Blood was diluted 1:10 with Triton X-100 with the addition of a matrix modifier containing ammonium phosphate monobasic and magnesium nitrate. All specimens were run in batches, which included standard methods for calibration. BLL was measured in each animal group before and after interventions in mothers (rats) as well as their offspring at the end of the experiment (P21).

***Histological methods.*** At the end of the experiment, the young rat pups were deeply anesthetized, and their eyes were removed carefully, washed in normal saline, and fixed in normalized fixative containing 10% formaldehyde in 0.01 M PBS at room temperature overnight. After fixation, the specimens were dehydrated with an ascending ethanol series, cleared with xylene, and embedded in paraffin [22]. The blocks were cut into 5-µm serial sections, and 5 sections were randomly chosen and mounted on poly-L-lysine coated slides for TUNEL technique.

***Apoptotic cell detection.*** For apoptosis analysis, TUNEL immunohistochemical technique was performed. In this method, DNA fragmentations in apoptotic cell nuclei were revealed using TUNEL reaction by means of TUNEL Kit (Roche, Germany). For doing this method, tissue sections were deparaffinized with the xylene, rehydrated through descending concentrations of ethanol, and rinsed in 0.1 M PBS for 10 min and then treated with 20 µg/ml proteinase K (Roche, Germany) at room temperature for 20 min. The specimens were treated with 3% $H_2O_2$ in methanol for 10 min to inactivate endogenous peroxidase. After washing with PBS, specimens were incubated in the labeling reaction mixture containing terminal deoxynucleotidyl transferase and the deoxynucleotide mixture at 4°C overnight. After incubation, all the sections were rinsed in PBS and incubated with horseradish peroxidase (1:500) at room temperature for 30 min. After incubation, the sections were washed extensively with PBS for 3 min and treated with diaminobenzidine (Sigma, USA) solution (30 mg diaminobenzidine and 200 µl $H_2O_2$/100 ml PBS) in dark at room temperature for 15 min. After being washed under running water, all the sections were counterstained with haematoxylin for 1 min. Finally, the sections were dehydrated in increasing graded ethanol, cleared in xylene, and mounted with coverslip. In this method, apoptotic nuclei were identified by the presence of dark brown staining. For positive control, the tissue sample was treated with DNase I (Roche, Germany), and negative control sections were incubated with the labeling mix lacking the TdT enzyme.

***Quantification of apoptotic cells.*** The sections were scanned and photographed using a light microscope with a 100× objective lens (UPlan FI, Japan), and images were transferred to a computer using a high resolution camera (BX51, Japan). Morphmeterical methods were used to count TUNEL positive cells per unit area in eye retina. The numbers of TUNEL positive cells were counted using a 10,000 $\mu m^2$ counting frame. The mean number of TUNEL positive cells per unit area (NA) in eye retina was calculated using the following formula [23]:

$$N_A = \frac{\Sigma \overline{Q}}{a/f. \Sigma^P}$$

In this formula, "$\Sigma\overline{Q}$" is the sum of counted particles appeared in sections, "a/f" is the area associated with each frame, and "$\Sigma^P$" is the sum of frame associated points hitting space.

***Statistical analysis.*** The acquired data from BLL measurements and the cells counting methods were reported as mean ± SE. The measurement of BLL was carried out in each animal group before and after interventions and compared by using paired sample *t*-test. In addition, BLL was compared among all groups before and after interventions as well as among their P21 offspring by using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test using SPSS 11.5 software.

## RESULTS

***Blood lead level in rats and their offspring.*** Comparison of BLL before and after intervention in adult rats and their offspring was performed. There was no significant difference among all groups before interventions (Fig. 1). However after interventions, comparison of BLL between pretreatment and post-treatment in each group showed a significant increase at post-treatment in four groups, including L, L + G, L + AA, and L + AA + G ($P<0.05$, Fig. 1). In addition, comparison of BLL at post-treatment among all groups showed a significant increase in four groups, including L ($P<0.001$), L + G and L + AA ($P<0.01$), and L + AA + G ($P<0.05$) compared to normal and sham groups (Figs. 1 and 2). Although BLL in the L + G and L + AA ($P<0.05$) as well as L + G + AA ($P<0.01$) groups were reduced compared to the L group (Figs. 1 and 2), there was no significant difference between treated groups (L + AA, L + G, and L + AA + G) to each other ($P>0.05$, Figs. 1 and 2).

***Apoptosis cell density.*** Apoptosis is a gene-regulated phenomenon and characterized by distinct morphological features, including chromatin condensation, nuclear shrinkage, and oligonucleosomal DNA fragmentation, that can be detected by means of TUNEL method in which apoptotic nuclei are identified by the presence of dark brown staining. There was no significant difference of apoptotic cell numbers in bipolar and multipolar retinal layers in comparison to all groups. However, there was a significant increase of apoptotic cells in photoreceptor retinal layer in L group as compared with control and

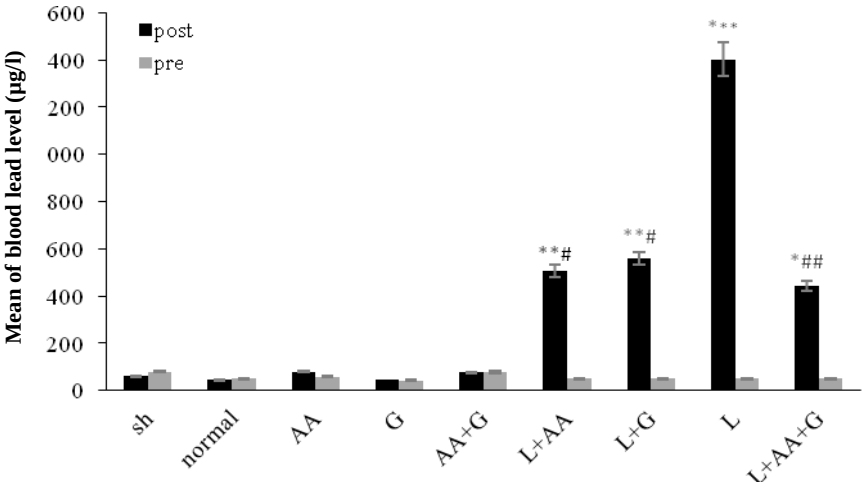


**Fig. 1.** Comparisons of blood lead levels (BLL) in rats (mothers) at pretreatment and post-treatments in each group as well as in different groups (mean ± SE). At post-treatment, BLL increased significantly in lead-exposed, L + AA, L + G, and L + AA + G groups compared to pretreatment ($P$<0.05). BLL increased significantly in L, L + AA, L + G, and L + AA + G groups compared to normal and SH, AA, G, AA + G groups (***$P$<0.001, **$P$<0.01, and *$P$<0.05) and decreased significantly in L + AA, L + G, and L + AA + G groups compared to L group ($^{\#}P$<0.05, $^{\#\#}P$<0.01). sh, sham; AA, ascorbic acid; G, garlic; AA + G, ascorbic acid + garlic; L + AA, lead + ascorbic acid; L + G, lead + garlic; L, lead; L + AA + G, lead + ascorbic acid + garlic

sham groups ($P$<0.01, Figs. 3 and 4). Comparison of apoptotic cells in L + AA, L + G, and L + G + AA groups with L group showed a significant decrease in apoptotic cells ($P$<0.001, Figs. 3 and 4). Our results also showed a significant difference between the three treatment groups (L + AA, L + G, and L + G + AA) and the control groups, and there was no significant difference between the three mentioned groups to each other (Figs. 3 and 4). Consequently, garlic, vitamin C and vitamin C together garlic reduce apoptotic cells in the photoreceptor layer in retina.

## DISCUSSION

This study was performed to investigate the garlic and vitamin C effects on lead-induced apoptosis in rat newborn's retinal cells.

Apoptotic cells are characterized by specific morphological and biochemical changes, including nuclear chromatin condensation, cytoplasm condensation, membrane blebbing, and nuclear DNA fragmentation. The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellular, such as toxins, hormones,

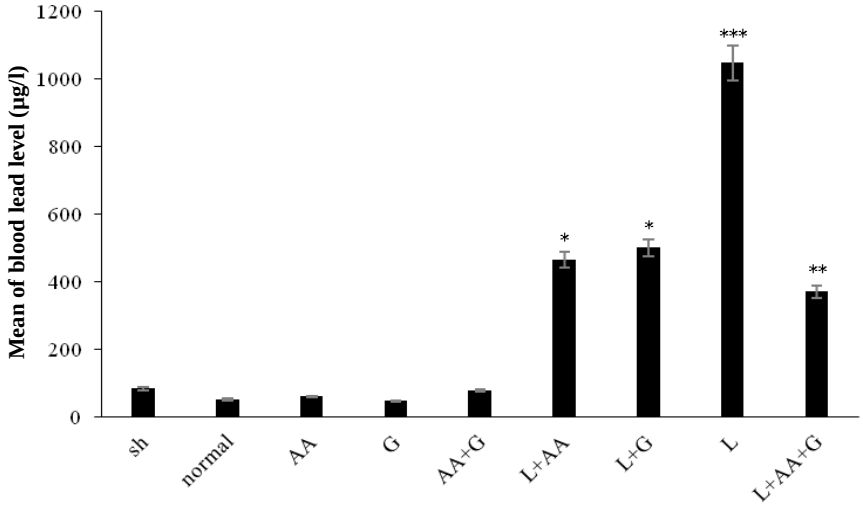


**Fig. 2.** Comparison of blood lead levels (BLL) in rat offspring after intervention in different groups (mean ± SE). BLL increased significantly in L group compared to normal, SH, AA, G, and AA+ G groups (***$P$<0.001) and decreased in L + AA, L + G, and L + AA + G groups compared to L group significantly (*$P$<0.05 and **$P$<0.01). sh, sham; AA, ascorbic acid; G, garlic; AA + G, ascorbic acid + garlic; L + AA, lead + ascorbic acid; L + G, lead + garlic; L, lead; L + AA + G, lead + ascorbic acid + garlic

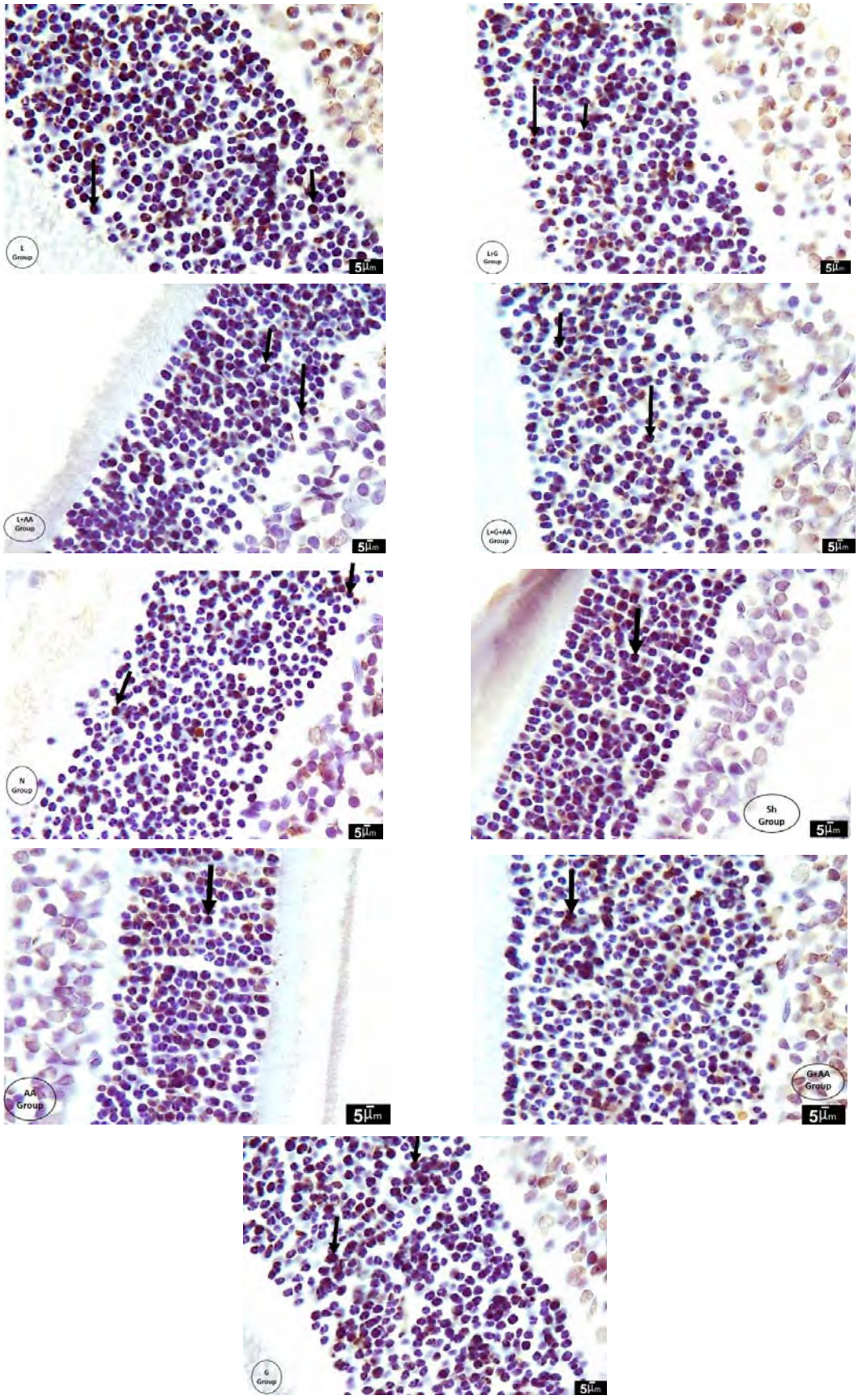


**Fig. 3.** Photomicrographs showing the TUNEL positive cells in the photoreceptor layer of retina in different groups of rat offspring at P21. Arrows show apoptotic cell (magnification 100×, scale bar = 5 µ).

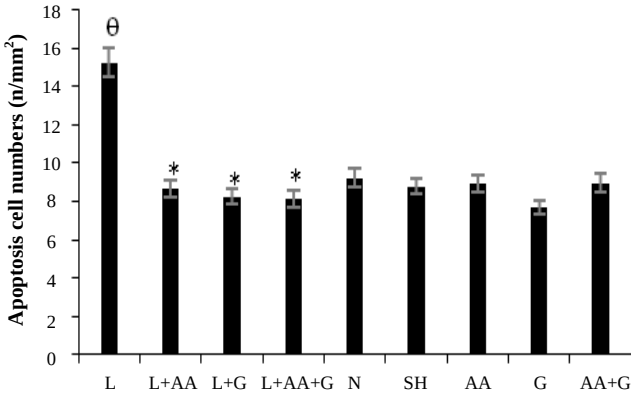


**Fig. 4.** Comparison of TUNEL positive cell (apoptotic cell) numbers pre unit area in rat offspring's retina photoreceptor layer. θ shows comparison between L and control groups ($P$<0.05) and * shows comparison among L + G, L + AA, L + AA + G, and L groups ($P$<0.05). L, lead; L + AA, lead + ascorbic acid; L + G, lead + garlic; L + AA + G, lead + ascorbic acid + garlic; N, normal; SH, sham; AA, ascorbic acid; G, garlic; AA + G, ascorbic acid + garlic

growth factors, nitric oxide or intracellular. These signals may affect apoptosis positively or negatively. In addition, apoptosis in retinal cells depends on cell type, stage of maturation, and inductive factors of apoptosis. A previous study has shown that the lead exposure induces cell apoptosis in rods and bipolar retinal cells [5]. Our results also revealed that lead exposure during pregnancy and lactation induces and increases apoptosis in rat offspring's retinal photoreceptor layer ($P$<0.05, Fig. 4).

Although the mechanism of lead-induced toxicity has not fully understood, it has been reported that the lead toxicity derives from its ability to cause oxidative stress by inducing the generation of ROS, reducing the antioxidant defense system of cells via depletion of inhibiting sulfhydryl-dependent enzymes, and/or increasing susceptibility of cells to oxidative stress [24]. Other suggested molecular mechanisms for lead-induced toxicity are interfering with $Ca^{2+}$-dependent enzyme, alteration of cGMP phosphodiesterase activity, and mitochondrial alterations, that play critical roles in apoptosis processes [6].

Based on the lead-induced toxicity mechanisms, we used vitamin C as a known antioxidant, and garlic juice as an antioxidant candidate to protect lead-induced toxicity during pregnancy and lactation on rat's eye retina. Vitamin C, a water-soluble vitamin, is necessary for growth and repair of tissues in all parts of the body, and as a highly effective antioxidant protects vital molecules from damage by free radicals and ROS [12, 25]. Vitamin C is used to prevent and treat heart diseases, lead poisoning, cancer, the common cold, stroke, and osteoarthritis [26, 27].

The present study shows that vitamin C consumption during pregnancy and lactation reduces BLL and photoreceptor cell apoptosis. Other scientists showed that blood lead concentration was very low in vitamin C supplement consumers [28]. Further published results also showed that the consumption of 500 mg vitamin C for two weeks reduced BLL significantly and also caused a significant increase of urinary lead excretion [29]. Simon and Hudes [30] reached the conclusion that high serum levels of ascorbic acid is related to lower lead serum levels. It has been reported that vitamin C, as an antioxidant, protects tissues of the eye against photo peroxide [31] and cataract induced by selenite due to oxidative stress on the lens [32]. The present study also reveals that garlic extract administration during pregnancy and lactation may protect the lead-induced toxicity in rat offspring's retina. On the other hand, consuming garlic, vitamin C, and both of them together had a great effect on reduction of BLL in rats and their offspring ($P$<0.05, Fig. 2).

Previous investigations regarding the effect of garlic on the tissue lead level and with different doses of garlic showed significantly lower lead concentration in tissues and blood, which confirm our results. For expressing the role of garlic in BLL decrease, it is suggested that garlic has biologically active compounds (s-allyl cystein, s-allyl mercaptocystein, etc), which prevent gastrointestinal absorption and increase urinary excretions of lead [4]. Other scientists who studied the effects of garlic and vitamin C on the teratogenic effects of cypermethrin in Wistar rats concluded a significant reduction in the maternal anomalies [16].

In the present study, garlic and vitamin C reduced the number of apoptotic cell on photoreceptor layer, and there was a significant difference between L group plus L + AA, L + G, and L + AA + G groups ($P$<0.05, Fig. 4). The efficiency of garlic was perhaps due to the presence of sulfur-containing amino acids, such as S-allyl cystine, S-allyl mercaptocystein, and free carboxyl (C = 0) as well as amine ($-NH_2$) groups [33-35]. These biologically active compounds might have chelated lead and enhanced its excretion from the body, resulting in reduced lead accumulation in tissues and blood. It can be suggested that the ameliorative potential of garlic juice is perhaps due to its combined effects both on metal absorption and on excretion from the body. Allicin and Alexin are major components of garlic organosulfur, and their antioxidant properties in neutralizing several types of ROS have been confirmed [19].

In summary, this study indicates that lead exposure during pregnancy and lactation can cause apoptosis in photoreceptor retinal layer in rat offspring. Moreover, the fresh garlic juice as well as ascorbic acid showed preventive and beneficial effects in lead-induced apoptosis in eye retina.

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