Full Length Research Paper

Protective effect of garlic oil against liver injury in experimental animals

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Food derived antioxidants have a strong potential effect on the long term as chemopreventive agents in disease states involving oxidative stress, such as hepatitis. This study was done to clarify the potential effect of garlic oil in protecting the liver from lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats. Sixty male albino rats were used in the study. They were divided into four groups (15 rats in each) as follow: Group I (control group) received normal saline, group II (garlic group) received garlic oil orally, group III (DGa1N/LPS intoxicated) received normal saline orally for 15 days and then injected intraperitoneally by DGa1N/LPS for induction of hepatitis and group IV (garlic pretreated) received garlic oil (200 mg/kg body weight/day) for 15 days and then injected by DGa1N/LPS.DGalN/LPS induced hepatic damage in rats manifested as significant increase in the mean levels of serum liver enzymes and production of oxidative stress manifested as significant increase in urinary F_2-isoprostane (lipid peroxidation parameter), AOPP (protein oxidation parameter) and urinary 8-hydroxyguanosine (parameter of oxidative DNA damage). The damage of liver tissue is also confirmed by the histopathological examination. Pretreatment with garlic oil significantly ameliorated the toxic effect of DGa1N/LPS on the liver.

Key words: Hepatitis, galactosamine, oxidative stress, garlic oil.

INTRODUCTION

Hepatitis infection is a major cause of chronic liver diseases which infect more than 170 million persons worldwide, often leading to cirrhosis, hepatic failure and hepatocellular carcinoma (Zuo et al., 2007). D-galactosamine induced experimental model system in rats is recognized to be much like viral hepatitis in humans from both morphological and functional points of view (Najmi et al., 2005). Galactosamine-induced dismutase; is generally attributed to the formation of the

highly reactive hydroxyl radical (OH$^{.}$) which leads to severe oxidative damage of the liver cells' components like lipids, proteins and DNA (Mckillop and Schrum, 2005). A potentially mutagenic DNA base, 8-hydroxyguanosine (8-OH-guanine or 8- oxo guanine) is repaired, released from the cell, and eventually excreted via the urine as the base (8-OH- guanine) or the nucleoside, 8- hydroxyl - 2- deoxyguanosine (8 – OH – dG, 8 – oxo – dG). The urinary content of 8 – OH – dG represents an average rate of oxidative damage to guanine in the form of the free nucleotide (dGTP) and in DNA (Svoboda et al., 2008).

The peroxidation of endogenous lipids has been shown to be a major factor in the cytotoxic action of galactosamine. F_2 isoprostanes, a class of prostanoids produced by non enzymatic free radical-catalyzed peroxidation of arachidonic acid, is an important advance in assessing lipid peroxidation *in vivo* (Awad et al., 1996; Vimal and Dvaki, 2004; Subash et al., 2010). Advanced

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Abbreviations: ALT, Alanine amino transferase; **AST,** aspartate amino transferase; **γGT,** gamma glutamyl **ELISA,** enzyme linked immunosorbent assay; **AOPP,** Advanced oxidation protein products; transferase; **ALP,** alkaline phosphatase; **SOD,** superoxide oxidative damage.

oxidation protein products (AOPP) are a reliable marker to estimate the degree of oxidant-mediated protein damage (Witko-Sarsat et al., 1996). Food derived antioxidants have a strong potential for long term use as chemopreventive agents in disease states involving oxidative stress, such as hepatitis and alcohol induced liver diseases (Mckim et al., 2002). Garlic (*Allium sativum*) besides being used as food, has been used as medicinal plant for over 4000 years for a variety of ailments including headache, bites, intestinal worms and tumors (Block, 1985). Several studies investigated the effect of garlic oil on different liver diseases such as CCl_4 and alcohol-induced liver injury in the experimental animals, but in this study, we aimed to evaluate the potent effect of garlic oil in protecting the liver from lipopolysaccharide–induced hepatitis in D-galactosamine sensitized rats.

MATERIALS AND METHODS

Materials

Sixty male albino rats (Sprague Dawely Strain) weighing 180-200 g were obtained from the animal house of National Research Center (NRC), Giza, Egypt. D-Galactosamine hydrochloride (DGa1N), lipopolysaccharide (LPS) and 8- hydroxyguanosine standard were obtained from Sigma - Aldrich Company - St - Louis, MO, USA. Garlic oil was obtained from local market.

Methods

Induction of hepatitis

Liver damage was induced by intraperitoneal (i.p) injection given 24 h before sacrifice of animals, with D-galactosamine (105 mg/180 g b.w.) and lipopolysaccharide (42 µg / 180 g b.w.) dissolved in saline with pH adjusted at 7.4. Blood samples were withdrawn to check the induction of hepatitis (He et al., 2001a).

Experimental design

Sixty male albino rats were housed individually in stainless steel cages in a controlled environment and were fed standard diet and water was available *ad libitum*. The experiment was carried out in accordance with the national regulations of animal welfare and Institutional Animal Ethical Committee (IAEC), National Research Center. Rats were divided into four groups (15 rats in each) as follow:

(i)   Group I (control group): received normal saline (1 ml/Kg b.w./day) orally.
(ii) Group II (garlic group): received garlic oil (GO) (200 mg/Kg b.w./day) orally for 15 days (Wu et al., 2001).
(iii) Group III (DGa1N/LPS intoxicated group): received normal saline (1 ml/Kg b.w./day) orally for 15 days then injected by DGa1N/LPS for induction of hepatitis.
(iv) Group IV (garlic pretreated group): received garlic oil (200 mg/Kg b.w./day) orally for 15 days and then injected by DGa1N/LPS. After the experimental period, 24 h urine was collected from each animal for estimation of urinary

8-hydroxyguanosine and F_2 isoprostane. Then, fasting blood samples were withdrawn from the retro-orbital venous plexus under light anaesthesia by diethyl ether (Madway et al., 1969). Blood samples were collected and serum was separated for estimation of the other biochemical parameters.

Biochemical assays

Serum alanine amino transferase (ALT) and aspartate amino transferase (AST) activities were determined using commercial kit purchased from BioMed Diagnostics based on the method described by Reitman and Frankel (1957). Serum gamma glutamyl transferase (γGT) was estimated according to Whitefield et al. (1973). Serum alkaline phosphatase (ALP) was determined according to Kind and King (1954). Blood superoxide dismutase (SOD) activity was measured according to Sun et al. (1988). Advanced oxidation protein products (AOPP) was estimated by immunodiagnostic kit according to Deschamps-Latscha et al. (2005). Urinary F_2 isoprostane was estimated by enzyme immunoassay (ELISA) by a kit derived from Cayman Ann Arbor, USA according to Montuschi et al. (2004). Protocol for urinary 8-OHdG analysis was modified from the method described by Kim et al. (2001). 8-OHdG was extracted from 1 ml urine. The eluents were dried under ultra – pure N_2 stream and reconstituted in 5 ml deionized water for injection in HPLC.

HPLC condition

HPLC column for 8-OHdG was C_{18} (250 × 4.6, particle size 5 µ). The mobile   phase consists of acetonitrite / methanol / phosphate buffer (25/10/965). Phosphate buffer was prepared by dissolving 8.8 g of potassium dihydrogen Phosphate in1000 ml deionized water and pH was adjusted at 3.5. The buffer then   filtered 2 times before used at a flow rate of 1 ml/min and used electrochemical detector with cell potential 600 mv.

Histopathological studies

The livers of different groups were removed and fixed in 10% formal saline. Paraffin sections of 5 µm thick were stained with haematoxylin and eosin (Drury and Walligton, 1980). Protein stains (Mazia et al., 1953) were also performed. All sections were investigated by the light microscope. Further sections were stained for DNA (Feulgen and Rosenbeck, 1942) and DNA analysis was performed by lecia Qwin 500 image cytomery in the pathology department , National Research Center. For each section (100 to 120 cells) were randomly measured. The threshold values were defined by measuring control cells. The results are presented as histograms and tables which demonstrate the percentage of the diploid cells (2C), the triploid cells (3C), the tetraploid cells (4C) and the aneuploid cells (> 5C). The DNA histogram is classified according to Danque et al. (1993) (Figure 5).
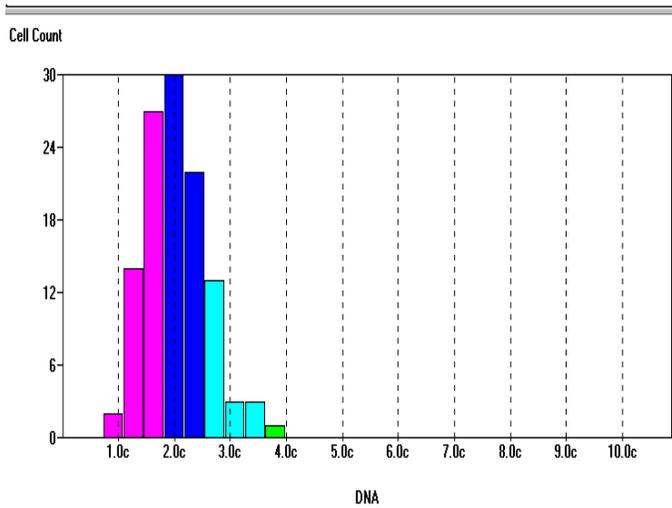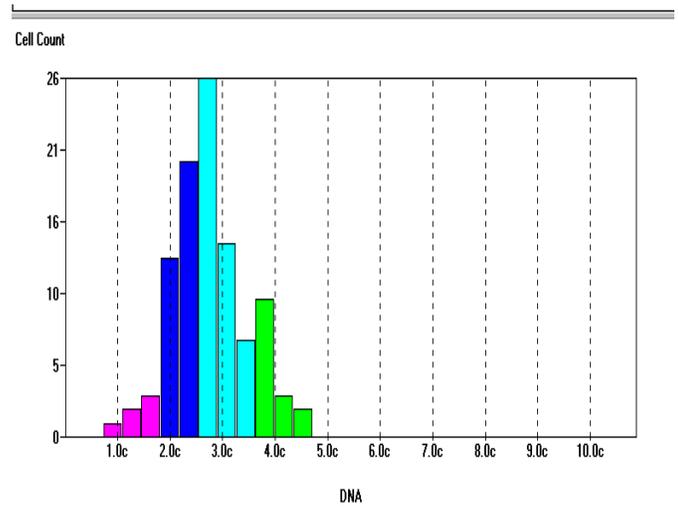
RESULTS

Biochemical assessment

Assessment of Ga1N/LPS–induced hepatitis

The rats intoxicated with DGa1N/LPS (Group 3) developed a state of hepatic damage as shown from the significant increase in the mean serum levels of ALT, AST and γGT compared to the normal control group
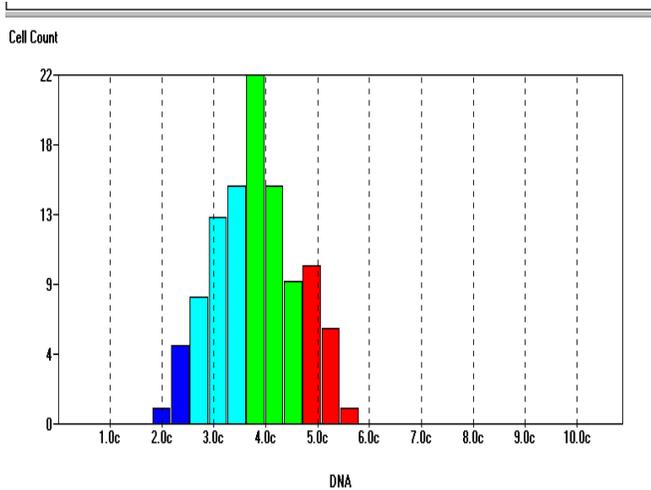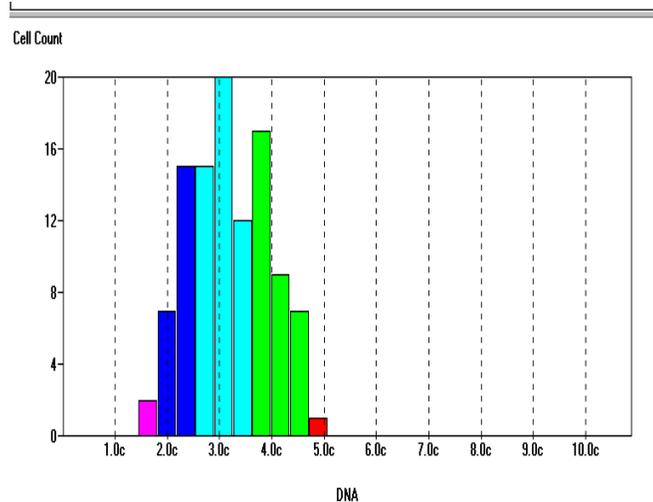
Histogram 1

Histogram 2

Histogram 3

Histogram 4

Figure 5. DNA analysis.

(Group 1) (Table 1).

Assessment of oxidative stress state induced by DGalN/LPS

The rats intoxicated with DGa1N/LPS (Group 3) developed a state of oxidative stress as noticed by the significant increase in lipid peroxidation measured as urinary F_2 isoprostane, protein oxidation measured as AOPP and DNA damage measured as urinary 8-hydroxyguanosine, together with significant decrease in

blood SOD activity compared to the normal control group (Group 1) (Table 2).

Assessment of the protective effect of garlic oil supplementation

There was no significant change in the level of either the liver enzymes or the oxidative stress parameters in the groups of rats that received garlic oil alone (Tables 1 and 2) compared with Group 1. These findings revealed that the safety of garlic oil supplementation. The pretreatment

Table 1. Serum liver enzymes in the different studied groups.

Groups	Control group	Garlic group	DGa1N/LPS intoxicated group	Garlic pretreated group
ALT (U/L)	27.30 ± 1.15[(b)]	24.00 ± 0.49[(b)]	83.00 ± 5.36[(a)]	56.30 ± 4.45[(a),(b)]
AST (U/L)	112.30 ± 0.75[(b)]	100.00 ± 1.23[(b)]	167.10 ± 3.39[(a)]	132.00 ± 4.43[(a),(b)]
γ GT (U/L)	21.52 ± 0.80[(b)]	21.82 ± 0.88[(b)]	36.54 ± 1.04[(a)]	34.270 ± 0.67[(a)]

Values are given as mean ± SE.; a: significant difference at P< 0.05 compared to the control group; b: significant difference at P< 0.05 compared to the DGa1N intoxicated group; Number of rats per group n = 15.

Table 2. Oxidant / antioxidant parameters in the different studied groups.

Groups	Control group	Garlic group	DGa1N/LPS Intoxicated group	Garlic pretreated group
Isoprostane ng/mg creatinine	2.00 ±0.18	1.23 ± 0.15[(a),(b)]	2.55 ± 0.20	1.32± 0.18[(a),(b)]
AOPP (µmol/L)	13.03 ± 0.82[(b)]	13.61± 0.86[(b)]	29.50 ± 2.28[(a)]	13.38 ± 0.77[(b)]
8-OHdG ng/mg creatinine	4.46 ± 0.14[(b)]	5.75 ± 0.17[(a),(b)]	19.18 ± 0.53[(a)]	9.84 ± 0.22[(a),(b)]
SOD (U/gHb)	68.53 ± 5.02 [(b)]	64.26 ± 4.47[(b)]	45.97 ± 3.76[(a)]	52.66 ± 2.02[(a)]

Values are given as mean ± SE. a: significant difference at p< 0.05 compared to the control group; b: significant difference at p< 0.05 compared to the DGa1N intoxicated group; Number of rats per group n = 15.

**Table 3.** Correlation between oxidative stress parameters and liver enzymes.

		AOPP	8-OHdG	F_2-isoprostane	SOD
ALT	r	0.682**	0.876**	-0.032	-0.621**
	p	0.001	0.001	0.843	0.001
AST	r	0.615**	0.605**	-0.019	-0.305
	p	0.001	0.001	0.908	0.056
GGT	r	0.525**	0.819**	0.167	-0.475**
	p	0.001	0.001	0.304	0.002

** Correlation is significant at the 0.01 level (2 tailed) histological results.

with garlic oil to the rats intoxicated with DGa1N/LPS showed a significant protection against DGa1N/LPS intoxication. This is manifested by the significant decrease in the mean serum levels of ALT, AST, γGT, and AOPP, together with the decrease in urinary F_2-isoprostane, 8-hydroxyguanosine in the pretreated group (group 4) compared to the intoxicated group (group 3) although their levels are still higher than those of the control group (Table 1 and 2). Also, the pretreatment with garlic oil significantly improved the blood SOD activity to be near that of the control group. The correlation analysis for the above studied parameters revealed significant positive correlation between the liver enzymes (ALT, AST and γGT) and the oxidant/antioxidant parameters (AOPP, 8- OHdG and F_2 isoprostane), together with significant negative correlation between the liver enzymes and SOD activity (Table 3).

Histological results

The results of this study revealed the normal structure of the liver tissue (Figure 1a); it also revealed that treatment of animals with garlic oil only was safe as the architecture of liver tissue in this group of animals was comparable to that of the control group except for very slight dilatation of blood sinusoids that denotes increased blood flow in these vessels (Figure 1b). Treatment of the animals with D-galactosamine and lipopolysaccharide caused marked damage of liver tissue in the form of severe dilatation and congestion of portal vein with fibrosis in the portal area, focal areas of necrosis of variable sizes and marked dilatation and congestion of blood sinusoids with diffuse cellular infiltration (Figure 2). Using garlic oil as a protective agent with D-galactosamine and lipopolysaccharide revealed a good protective effect of garlic observed as decrease in fibrosis and cellular infiltrate with no dilatation or congestion in the portal
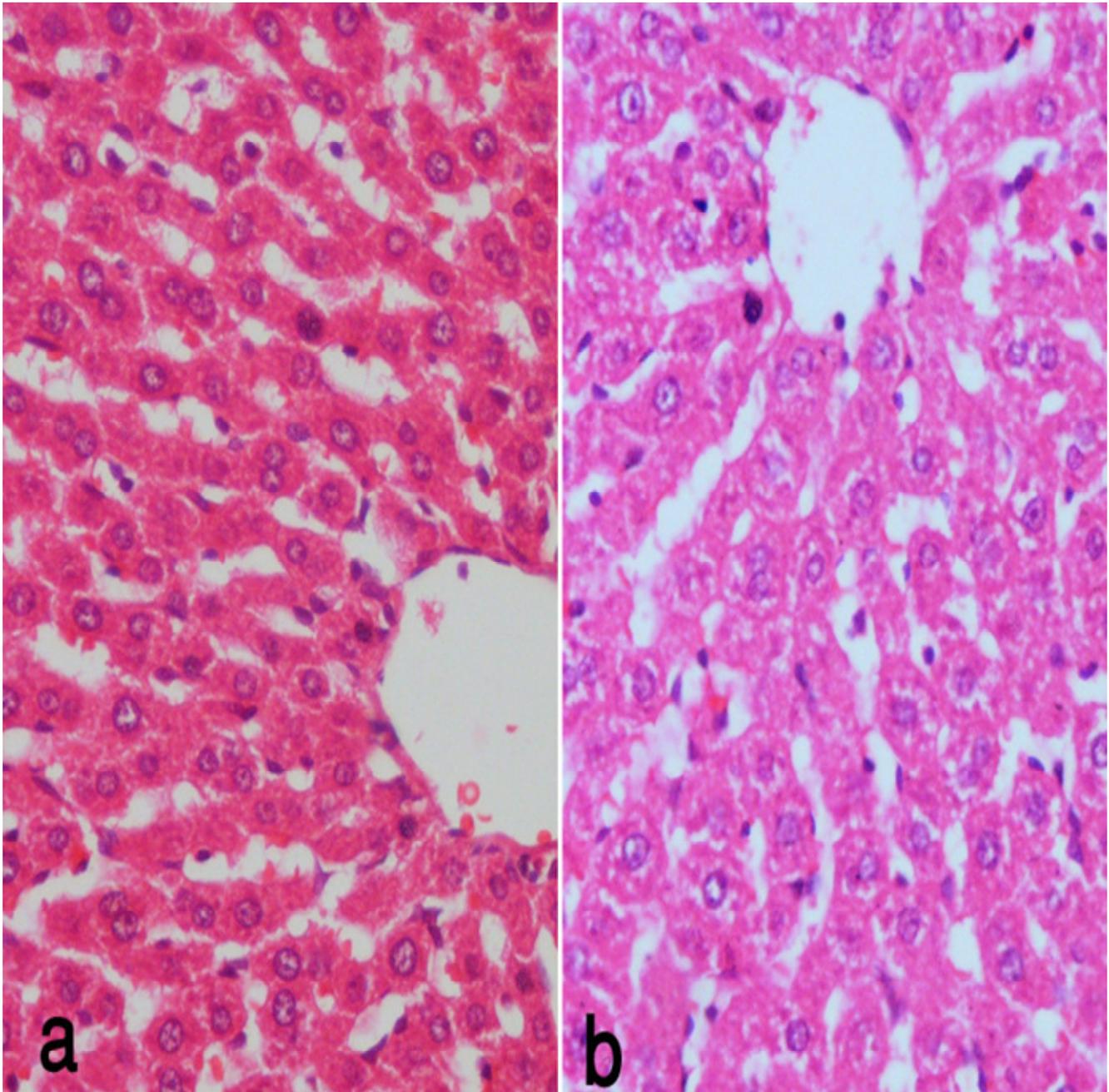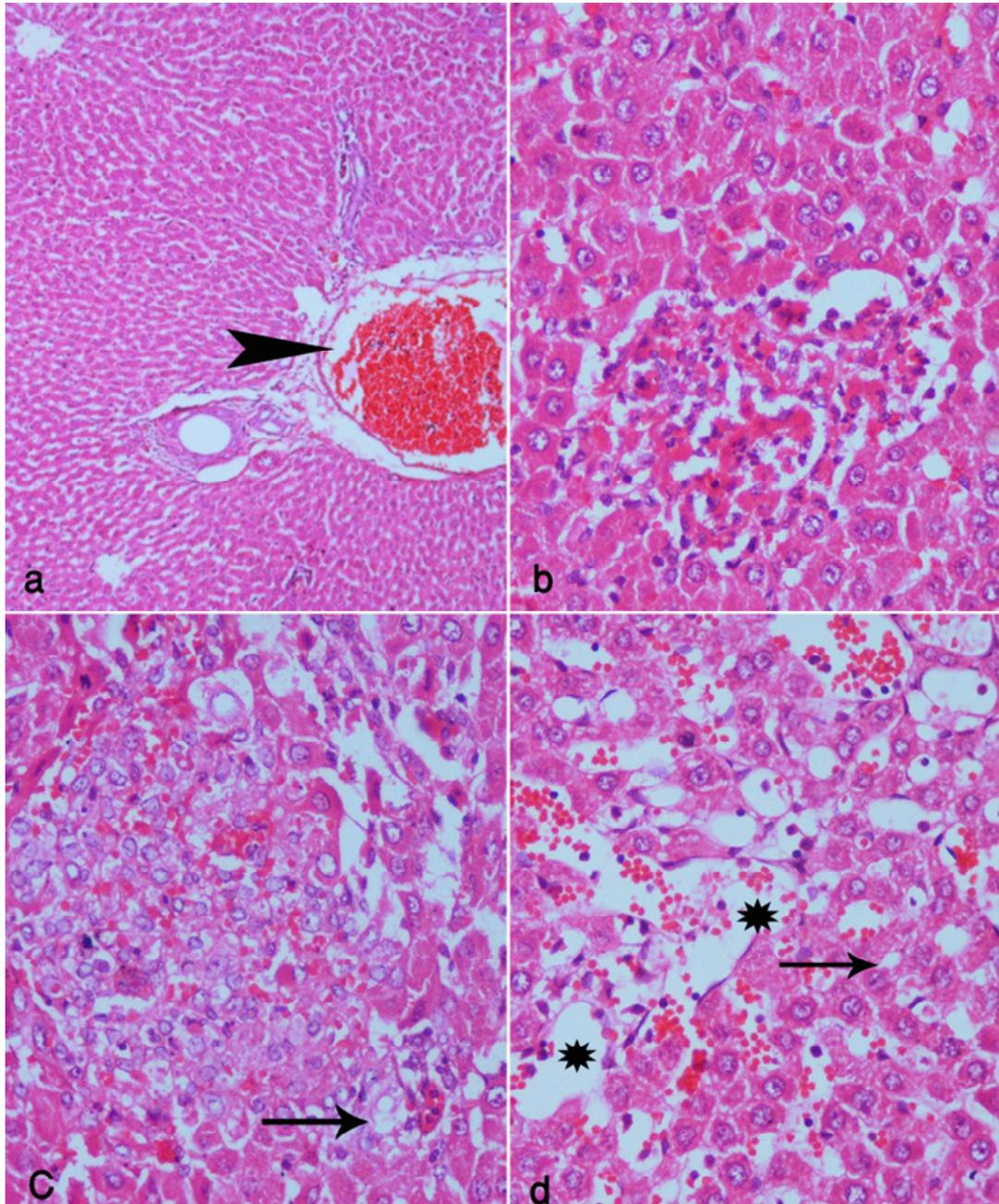
Figure 1. (a) is a photomicrograph of a section of liver tissue from a control rat showing the normal architecture of liver tissue. (b) is a photomicrograph of a section of liver tissue from rat received garlic showing very mild dilatation of blood sinusoids in between hepatocytes without congestion. Hepatocytes appear normal **(**Hx. and E. × 200).

vein was noticed. A decrease in the size of focal areas of necrosis and in congestion and dilatation of blood sinusoids was also shown (Figure 3).

Histochemical results

Staining sections from different groups with bromophenol

blue stain revealed the normal content of protein in cytoplasm of hepatocytes (Figure 4a) and that garlic had a good effect on the protein content of hepatocytes (Figure 4b), while D-galactosamine and lipopolysaccharide caused a dramatic decrease in the protein content of these cells (Figure 4c). Using garlic oil as a protective agent with D-galactosamine and lipopolysaccharide showed a marked preservative effect on protein content of these cells (Figure 4d).
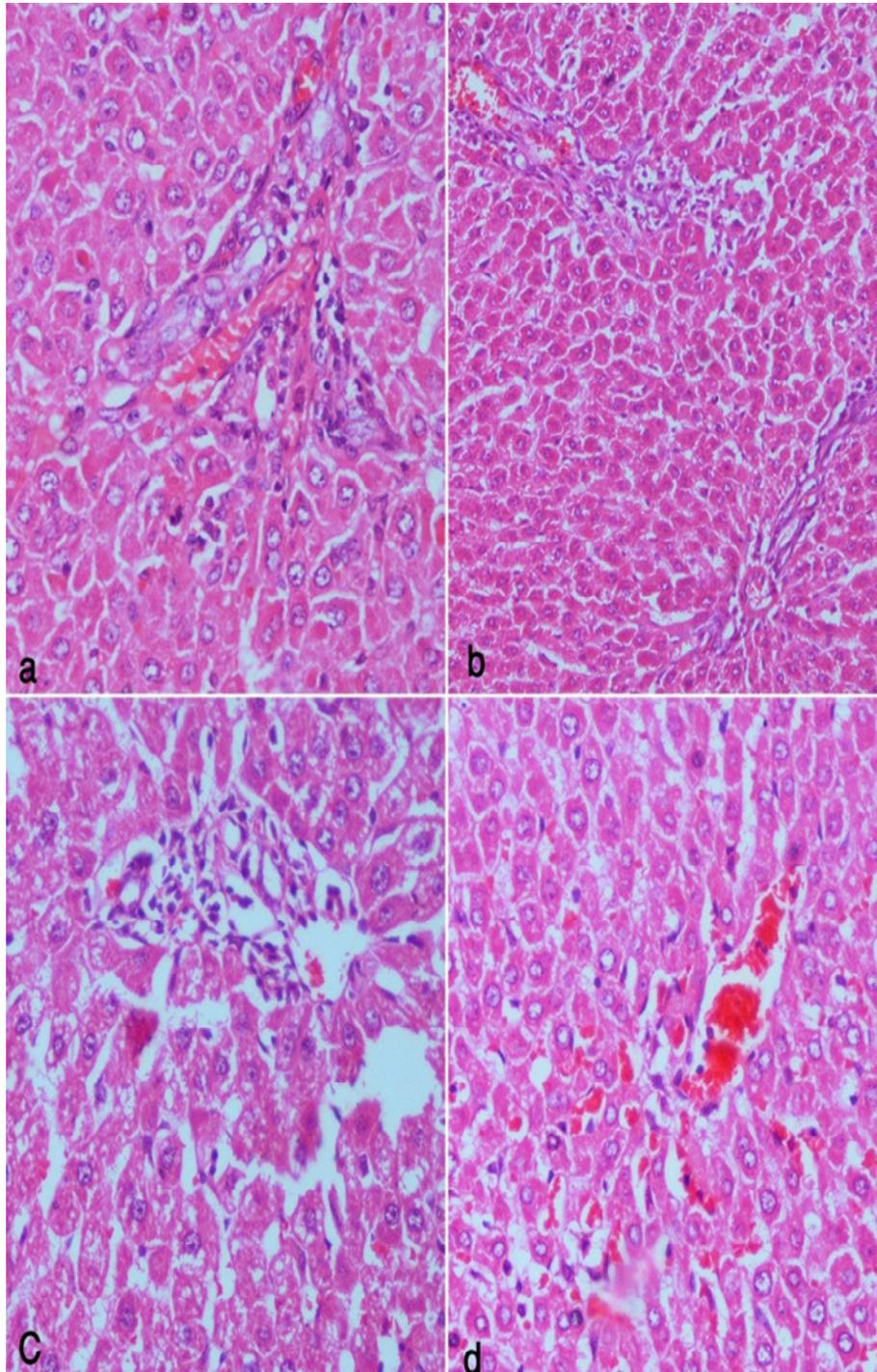
Figure 2. This is a photomicrograph of liver tissue from rat received D-galactosamine + Lipopolysaccharides showing the damaging effects of these drugs on liver tissue in the form of severe dilatation and congestion of portal vein with fibrosis around in the portal area (arrow head)in (a), focal areas of necrosis of variable sizes with cellular infiltration in (b) and (c), vacuolar degeneration in some hepatocytes (arrow) and marked dilatation and congestion of blood sinusoids with diffuse cellular infiltration (star) in (d) (Hx. and E. × 50 for (a) and200).

DNA analysis

In the present study, the Qwine 500 image analyzer was used to evaluate the DNA content. The image analysis system automatically express the DNA content of each individual cell measured then gave the percentage of each cell out of the total number of cells examined. Also, it classifies the cells into 4 groups; diploid (2C), proliferating cells (3C), tetraploid (4C) and aneuploid cells (>5C).

Figure 3. This is a photomicrograph of liver tissue from rat received garlic and then D-galactosamine and lipopolysaccharide showing marked improvement in the damaging effect caused by D-galactosamine. (a) and (b) show observable decrease in fibrosis and cellular infiltrate with no dilatation or congestion in portal vein. (c) shows noticeable decrease in the size of focal areas of necrosis. (d) shows very mild congestion and dilatation of blood sinusoids. [Hx.and E. × 100 for (a,c and d) and 50 for (b)].

The proliferating cells were further classified according to Lee et al. (1999) into: (< 10%) low proliferating index, (10 to 20%) medium proliferating index and (> 20%) high proliferating index. Normal distribution of DNA content in
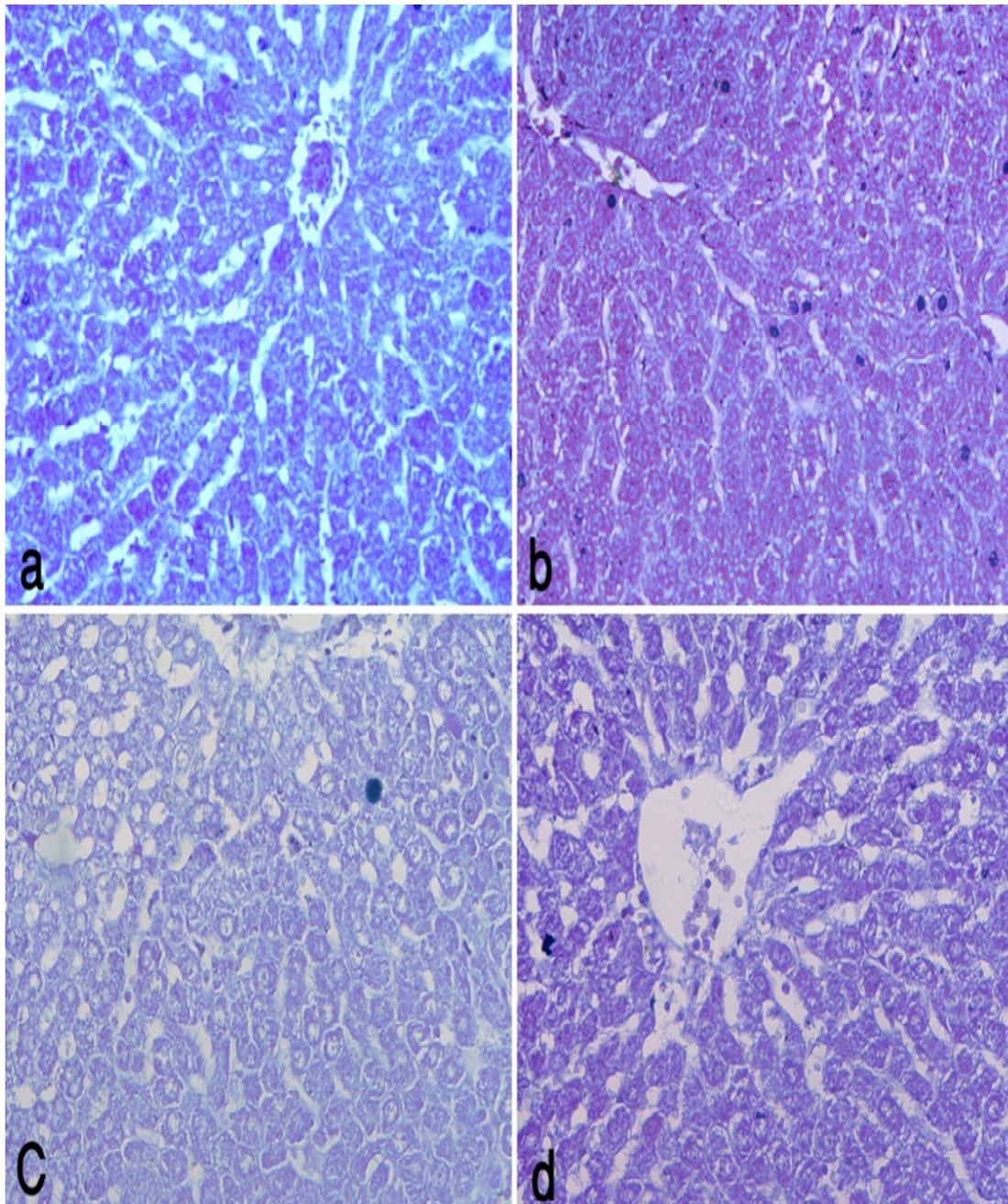
Figure 4. Photomicrograph of a section of liver tissue showing the protein content in cytoplasm of hepatocytes. (a) Normal (b) Shows the protein content in hepatocytes of a rat that received garlic only. (C) Shows a noticeable decrease in the density of the stain that denotes a decrease in the protein content in cells of a rat that received D-galactosamine and lipopolysaccharide. (d) Shows marked improvement in the protein content in hepatocytes of rat that received garlic and then D-galactosamine with lipopolysaccharide (Bromophenol blue × 100). Histochemical results.

the liver of control group showed that 17.39% of the examined cells contained DNA (< 1.5C), 62.60% of the examined cells contained diploid DNA value (2C), 18.26% of the examined cells contained (3C) DNA value (medium proliferating index and 1.73% of the examined cells at (4C) area (Histogram 1 and Table 4). The group treated with garlic oil only showed that 2.97% of the examined cells contained DNA (< 1.5C), 34.65% of examined cells contained DNA (2C), 42.57% of the examined cells contained DNA (3C) which means increase in proliferating cells as compared with control group and 19.80% of examined cells contained (4C)

Table 4. DNA ploidy in the different studied groups.

Range	Control				Garlic				DGaIN/LPS intoxicated				Garlic pretreated			
	Total cells	% Cells	DNA index	Std. dev.	Total cells	% Cells	DNA index	Std. dev.	Total cells	% cells	DNA index	Std. dev.	Total cells	% Cells	DNA index	Std. dev.
All	115	100.0	1.000	0.567	101	100.0	1.355	0.716	105	100.0	1.87	0.799	105	100.0	1.56	0.755
5cER	0	0.0	-	-	0	0.0	-	-	10	9.52	2.56	0.212	0	0.0	-	-
< 1.5c	20	17.39	0.631	0.169	3	2.97	0.575	0.104	0	0.0	-	-	0	0.0	-	-
1.5c-2.5c	72	62.60	0.974	0.280	35	34.65	1.055	0.225	6	5.71	1.13	0.113	21	20.0	1.05	0.236
2.5c-3.5c	21	18.26	1.362	0.276	43	42.57	1.404	0.250	29	27.61	1.51	0.270	44	41.905	1.45	0.272
3.5c-4.5c	2	1.73	1.836	0.308	20	19.80	1.894	0.269	48	45.71	1.93	0.273	38	36.19	1.93	0.307
> 4.5c	0	0.0	-	-	0	0.	-	-	22	20.95	2.43	0.309	2	1.905	2.33	0.076

(Histogram 2 and Table 4).

Treatment of the rats with D-galactosamine with lipopolysaccharide showed that 5.71% of the examined cells contained diploid DNA value (2C), 27.61% of the examined cells contained (3C) DNA value, 45.71% of the examined cells at (4C) area and 20.95% of the examined cells contained (5C) DNA value (Histogram 3 and Table 4). The group pretreated with garlic oil before D-galactosamine and lipopolysaccharide showed that 20.0% of the examined cells contained diploid DNA value (2C), 41.9% of the examined cells contained (3C) DNA value (high proliferating index), 36.19% of the examined cells at (4C) area and 1.9% of the examined cells contained (5C) DNA value (Histogram 4 and Table 4). These results indicate that treatment with D-galactosamine and lipopolysaccharide caused decrease DNA values (hypoploidy), while pretreatment with garlic oil showed increase of DNA values and percentage of proliferating cells with high proliferating index.

Statistical analysis

Statistical analysis of the results was carried out using the standard computer program SPSS (V. 904, Echo soft corporation USA 1998). Normally, distributed results were compared using student "t" test. Differences among groups were evaluated using one way ANOVA. Results were expressed as mean ± SE. "P" values less than 0.05 were considered to be significant. Correlations between various variables were done using Spearman rank correlation equation. A probability value (p value) less than 0.05 was considered statistically significant.

DISCUSSION

Viral infection, alcohol and drug toxicity may elicit an interaction with the liver cells that may lead to hepatic damage (Chang et al., 2005). In the current study, DGa1N / LPS intoxication resulted in a significant increase in the serum level of the liver enzymes (AST, ALT and γGT) compared to the control group. This is in agreement with Najmi et al. (2005) who explained that elevation of transaminases could be taken as an index of liver damage and this may be due to the fact that DGa1N / LPS administration disrupts plasma membrane permeability causing leakage of the enzymes from the cells. These findings go in agreement with the results of the histopathological study which revealed multiple but small vacuoles in the hepatocytes, suggesting the primary stage of steatosis, focal areas of necrosis of variable sizes and diffuse cellular infiltration.

In our study, administration of DGa1N/LPS caused also significant increase in the lipid peroxidation parameter (urinary F_2-isoprostane), protein oxidation parameter (AOPP) and oxidative DNA damage (urinary8- hydroxyguanosine), this is together with decreased activity of the antioxidant enzyme (SOD), reflecting an oxidative stress state. Marked decrease in the protein

content in hepatocytes as well as hapoploidy was also proved. Bacterial endotoxin such as lipopolysaccharide (LPS) is among the agents that cause immunological stimulation of Kupffer cells (Villaverda et al., 1997). Activation of Kupffer cells contributes to liver injuries by releasing cytotoxic agents, inflammatory cytokines and reactive oxygen species (ROS), this may lead to severe oxidative damage of the liver cells (Wang et al., 2005) and the cellular components like cell membrane, lipids, proteins and DNA (Mckillop and Schrum, 2005).

Hino et al. (1994) also reported that DGa1N/LPS intoxication increases the neutrophil infiltration into the liver cells with increased release of reactive oxygen species from the activated neutrophils. Similar to our results, significant increase in the oxidative stress parameters due to D-galactosamine/LPS intoxication was obtained by EL-Beshbishy (2008). Also, Zhou et al. (2008) indicated that treatment with DGa1N deceased the antioxidant enzymes activity of glutathione reductase, catalase and superoxide dismutase. Selective inhibition of antioxidant enzyme activities by DGa1N/LPS might by justified by the suggestion of Decker and Keppler (1974) that DGa1N can selectively block hepatic transcription and indirectly blocks hepatic protein synthesis.

In this study, oral pre-treatment with garlic oil effectively protected the liver from the toxicity of DGa1N / LPS by decreasing the oxidation process proved by decreasing the levels of urinary F_2-isoprostane, 8-hydroxyguanosine and serum AOPP together with increasing the serum activity of SOD. Concomitantly, it partly prevented liver enzymes from elevation indicating the protection of the cell membrane from free radicals attack. The histopathological examination confirmed these results showing the improvement in the signs of fibrosis and cellular infiltration together with marked increase in the protein content of hepatocytes' cytoplasm as well as noticeable increase in DNA content and in the percentage of proliferating cells. There is a possibility that orally administered garlic oil exerts a preventive effect on liver injury progression in DGa1N/LPS treated rats through its indirect antioxidant action to maintain antioxidant defense system in addition to its direct antioxidant action to scavenge ROS and to inhibit lipid peroxidation (El-Beshbishy et al., 2008).

The hepatoprotective property of garlic may be attributed to the presence of organosulfur compounds (such as diallyle disulfide and diallyle sulfide), which have antioxidant and detoxifying properties. This detoxifying effect is explained by the induction of phase II antioxidant enzymes (Munday and Munday, 2004). Moreover, He et al. (2008) indicated that the enzyme activity of SOD in 100 g of garlic ranges from 20000 to 30000 units much more than that of another SOD abundant plant. Also, garlic contains certain compounds such as germanium and selenium that play an important role in normalizing the oxygen utilization in the cells (Hussein et al., 2007). Also, the results of this study proved significant statistical

correlation between the levels of liver function enzymes on one hand and the oxidant / antioxidant parameters on the other hand which are in line with the above mentioned mechanisms.

Conclusion

Garlic oil seems to be a highly promising compound in protecting the hepatic tissue against oxidative damage and in preventing hepatic dysfunction due to DGa1N / LPS – induced hepatitis in rats.

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