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In Vivo Toxicity Study of Malachite Green In Mice: Estimation of Hepatotoxicity, Oxidative Stress And Genotoxicity

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Abstract: Malachite green (MG) is a green-colored synthetic triphenylmethane dye of industrial and medicinal uses as well as it is used as a food additive. MG enters the food chain and causes several cytotoxic, mutagenic and carcinogenic effects on mammalian body. However, few information are available about the mechanism of these toxic effects. Our study aimed to evaluate the hepato - toxic effects and genotoxicity of MG and to estimate the role of oxidative stress. Serum biochemical and hepatic antioxidant parameters were detected after MG oral administration in mice. Also, comet test and histopathological examinations were carried out on liver tissue. In this study mice were divided into three groups (20 animals/group) , First group was orally administered normal saline and kept as the control, second group was orally administered 2.5 mg/Kg body weight (1/20 LD50), while the third group was orally administered 5 mg/Kg body weight (1/10 LD50). Samples were taken after 14 and 28 consecutive days. The results revealed that MG increased serum liver enzymes and decreased total protein besides DNA damages in hepatic tissue after 28 days. There was hydropic degeneration of hepatocytes, dysplasia, congestion of central vein with focal necrosis. Oxidative stress may have role.

Keywords: Malachite green, Hepatotoxicity, Oxidative Stress, Genotoxicity, Mice.

Introduction

Malachite green (MG) is a triarylaminmethane water soluble dyeof multiple uses. It is commonly used as a food additive and a food coloring agent. It has fungicidal, antiseptic,anthelminthic effects and used as an medical disinfectant and effective treatment to proliferative kidney disease of salmonid fish, moreover it is used as a dye in silk, wool, jute, leather, cotton, paper and acrylic industries[1].

Although MG has been prohibited by several agencies in different countries it still used due to its efficacy, its relative low cost and high availability[2].

Leucomalachite green (LMG) is the reduced form of MG rapidly formed after MG absorption and has high tissue persistence even at the high temperature of cooking[3] and slow elimination rate which reaches 10 months[4].

MG enters the food chain and causes several cytotoxic, mutagenic, and carcinogenic effects on mammalian body .However, few information are available about the mechanism of these toxic effects, the generation of free radicals and oxidative stress induction may be accused[5,6]. In addition to the previous toxic effects, MG might induce genotoxicity particularly through chromosomal breaks[7].

The repeated oral dosing with MG in rabbit causes renal changes[8]. In rats and mice, it decreases food intake, growth and fertility rates. Also, it induces damage to liver, spleen, kidney and heart and produces lesions in skin, eyes, lungs and bones along with its teratogenic effects[9]. [10]Long-term (28 days) administration of MG in rats produced prominent weight loss and changes in serum levels of urea and aspartate aminotransferase.

MG has mutagenic effects in rats and mice; and also causes significant developmental abnormalities in pregnant New Zealand white rabbits[11]. LMG in the thyroid gland results in tumors in thyroid follicle cells of rats[12].

Administration of MG for 6 weeks resulted in mild circulatory disturbance that noticed as congestion of blood vessels with hepatic sinusoidal dilatation especially after 2 weeks exposure and hydropic degeneration of the hepatic cells that was detected all over the experiment[13]**.** Hepatic histopathological changes, characterized by deviated nuclei on sinusoidal surface and scattered acidophil bodies in hepatic cells with abnormal shaped nuclei and mild lobular inflammation, were seen after oral administration of MG for 7 and 14 days in mice. These effects increased at the 21 and 28 days[14]**.**

Malachite green exhibits a highly cytotoxic action, lipid peroxidation and the formation of dangerous free radicals on all mammalian cells[15]. However, Information on genotoxicity, bioaccumulation and reproductive genotoxicity is still incomplete and the acceptable daily intake (ADI) limits have not been set [15]**.**

Our work aimed to investigate the possible toxic effects of MG on liver, the genotoxicty induced by MG oral administration in mice and to detect the role of oxidative stress in these toxicities.

Materials and Methods

Chemicals

Malachite green was purchased from Loba Chemie company (India). kits used for antioxidant parameters were obtained from Cayman Chemical Co. (Ann Arbor, Michigan, USA), kits used for serum biochemical parameters were bought from Biodiagnostics Co. (Cairo, Egypt) ,while comet assay kits were obtained from Sigma-Aldrich Chemical Company (St Louis, Missouri, USA). All chemicals were of the highest analytical grade.

Animals

Sixty healthy male mice of (8 weeks old with an average weight of 20-25 g) were obtained from the laboratory animals resource center, faculty of veterinary medicine, Suez Canal University, Ismailia, Egypt and kept to acclimatize for one week before the beginning of the experiment. Animals were maintained under standard conditions of temperature, humidity and light, standard food and water were allowed *ad libitum*. The study was approved by the Research Ethical Committee of the Faculty of Veterinary Medicine,Suez Canal University, Ismailia, Egypt.

Animal Treatments

After the acclimatization animals were divided into 3 groups (20animals/group). First group was orally administered normal saline and kept as control, second group (low dose group) was orally administered 2.5 mg/Kg body weight (1/20 LD50, [16] while, the third group (high dose group) was orally administered 5mg/Kg body weight (1/10 LD50, [16]). The study was conducted for 14 and 28 consecutive days. After 24 h of the last dose, blood samples were collected from the medial canthus of the eye in non-heparinized tubes and left to clot at room temperature then centrifuged at 1200g (3000 r/min) for 15 min. Sera were then collected and kept at – 20 °C for biochemical analysis.

Mice were sacrificed then livers were rapidly excised from each animal and washed with normal saline solution (0.9% NaCl in distilled water). A part of liver samples were kept in 1.5 mL Eppendorf tubes at –80 °C for evaluation of antioxidants biomarkers and single cell gel electrophoresis / comet assay for genotoxicity assessment and the other part was reserved in 10% buffered formalin for histopathological examination.

Serum biochemical analysis of liver enzymes and protein

Serum used to estimate Alkaline phosphatase (ALP) according to[17], alanine aminotransferase (ALT) and aspartate aminotransferase (AST) according to[18], while total protein was measured according to[19].

Liver tissue antioxidant status

Liver tissue used to estimate, superoxide dismutase (SOD) according to[20], Catalase according to the method of[21], glutathione reductase (GSH) according to the method of[22] while, glutathione peroxidase (GPx) was estimated according to[23].

Single cell gel electrophoresis (SCGE) / comet assay for genotoxicity assessment

The method is according to [24]. In summary, place a piece of liver tissue in 1 mL cold Hank's Balanced Salt Solution (HBSS) containing 20 mM EDTA with 10% Dimethylsulfoxide (DMSO). Mince into large pieces, let settle, aspirate mincing solution, add new fresh mincing solution, mince into finer pieces, remove and mix 5 µl of the cell suspension with 75 µl Low Melting Point Agarose (LMPA).

Liver histopathological examination

Liver sections taken immediately from each animal and fixed in 10% buffered formalin then embedded in paraffin. Sections (4–5 µm thick) were stained with hematoxylin and eosin (H-E) then examined microscopically for the pathological findings of hepatic changes.

Statistical analysis

Data were analyzed using SPSS version 17.0 (SPSS Inc., Chicago, Illinois). One way ANOVA was done followed by Duncan's post hoc to determine the significant differences among means in this study. These differences were analyzed at the 5% probability level (P value of ≤ 0.05 was considered statistically significant). All data were expressed as means ±SE.

Results

Serum biochemical parameters

There were no-significant differences between control and both MG treated groups in ALP, ALT, AST and total protein after 14 days (Table 1 & Fig.1). After 28 days there was significant increase of ALP, AST, and ALT in addition to significant decrease of total protein in low dose group compared with the control group while, in high dose group there was significant increase of ALP and no significant difference in AST, ALT, and total protein when compared with the control group (Table 2 & Fig.1).

Table 1: Serum biochemical parameters after 14 days administration of malachite green

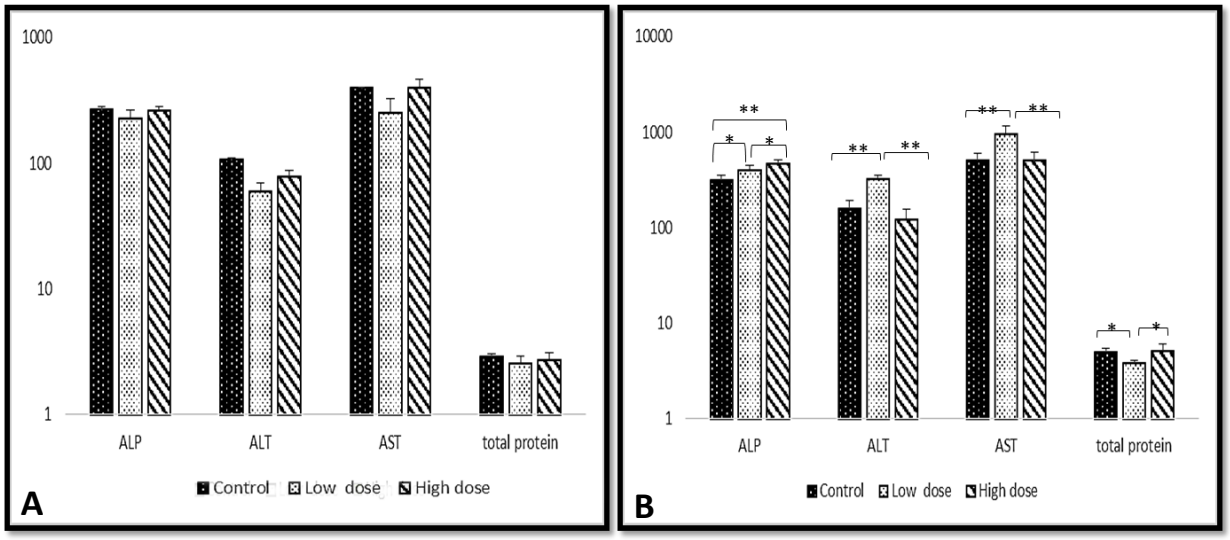
Parameter/ Group	Control	Low dose	High dose
ALP U/L	$265.80^{c} \pm 14.69$	$226.30^{c} \pm 36.05$	$260.20^{c} \pm 18.37$
ALT u/ml	$105.53^{b} \pm 3.76$	$58.87^{b} \pm 10.46$	$78.30^{b} \pm 9.92$
AST u/ml	$4.67^{b} \pm 6.52$	$249.57^{b} \pm 76.26$	$399.57^{b} \pm 62.74$
total protein	$2.91^{b} \pm 0.10$	$2.53^{b} \pm 0.39$	$2.73^{b} \pm 0.35$

Note: Data are expressed as means ± SE. Values having different alphabetic superscripts within the same raw are significantly different ($P \leq 0.05$).

Table 2: Serum biochemical parameters after 28 days administration of malachite green

Parameter/ Group	Control	Low dose	High dose
ALP U/L	315.20^{a} ± 35.92	402.00^{ab} ± 41.44	466.40^{bc} ± 39.65
ALT u/ml	158.93^{b} ± 31.63	319.37^{a} ± 29.81	122.67b ± 33.59
AST u/ml	506.07^{b} ± 91.69	953.90^{a} ± 200.13	499.53b ± 108.96
total protein	4.99^{a} ± 0.44	3.78^{ab} ± 0.35	5.12a ± 0.87

Note: Data are expressed as means ± SE. Values having different alphabetic superscripts within the same row are significantly different ($P \leq 0.05$).



***=significant, **= highly significant, others=non-significant**

Figure 1: Effect of MG low and high doses on Serum biochemical parameters .(A) effect of MG low and high doses after 14 days administration, (B) effect of MG low and high doses after 28 days administration.

Liver tissue antioxidant parameters

Oral administration of MG caused significant decrease of all antioxidant parameters (SOD, Catalse, GSH and GPx) in all treated groups all over the experimental period compared with the control group (Tables 3,4 & Fig.2).

Table 3: Antioxidant parameters in hepatic tissue after 14 days administration of malachite green

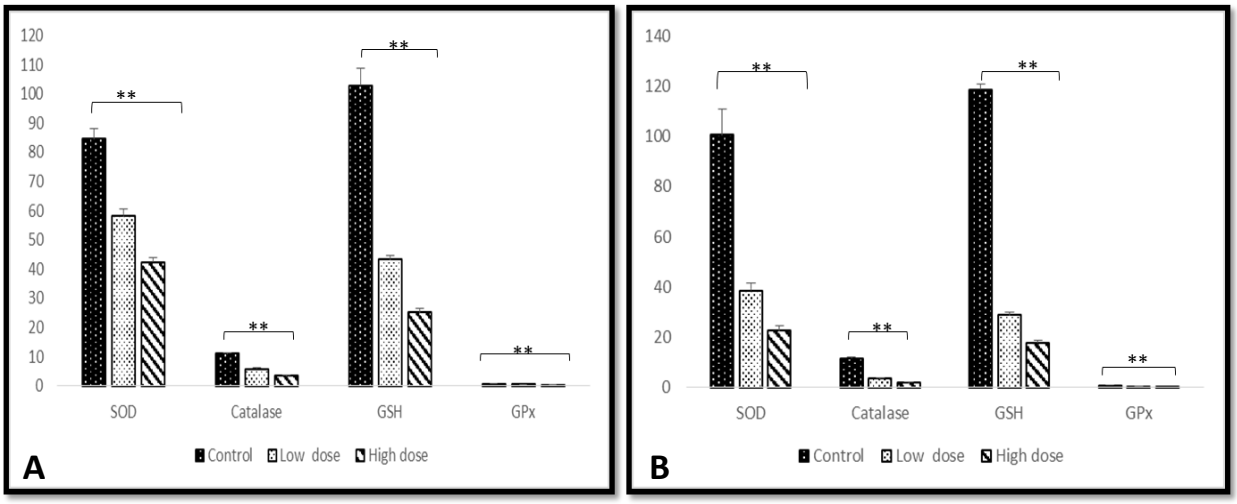
Parameter/ Group	Control	Low dose	High dose
SOD U/ g tissue	84.63^{a} ± 3.67	58.10^{b} ± 2.55	42.30^{c} ± 1.51
Catalase U/ g tissue	10.97^{a} ± 0.41	5.80^{b} ± 0.35	3.50^{c} ± 0.12
GSH mg/g tissue	102.87^{b} ± 5.79	43.23^{c} ± 1.36	25.17^{d} ± 1.20
GPx mol /g tissue	0.71^{ab} ± 0.03	0.54^{b} ± 0.02	0.32^{c} ± 0.01

Note: Data are expressed as means ±SE. Values having different alphabetic superscripts within the same raw are significantly different ($P \leq 0.05$).

Table 4: Antioxidant parameters in hepatic tissue after 28 days administration of malachite green .

Parameter/ Group	Control	Low dose	High dose
SOD U/ g tissue	100.70^{a} ± 10.16	38.57^{b} ± 3.08	22.50^{c} ± 2.05
Catalase U/ g tissue	11.50^{a} ± 0.46	3.53^{b} ± 0.19	1.93^{c} ± 0.15
GSH mg/g tissue	118.77^{a} ± 2.09	28.80^{b} ± 1.36	17.73^{c} ± 1.22
GPx mol /g tissue	0.83^{a} ± 0.06	0.32^{b} ± 0.02	0.11^{c} ± 0.01

Note: Data are expressed as means ± SE. Values having different alphabetic superscripts within the same raw are significantly different ($P \leq 0.05$).



***=significant, **= highly significant, others=non-significant**

Figure 2: Effect of MG low and high doses on liver antioxidant parameters .(A) effect of MG low and high doses after 14 days administration, (B) effect of MG low and high doses after 28 days administration.

Single cell gel electrophoresis (SCGE) / comet assay for genotoxicity assessment

After 14 days oral administration of MG there were no significant differences between low dose, high dose , and the control group in Comet%, head diameter, DNA % in head, tail length and the tail moment, although there was significant increase in tail DNA % between the three groups (Table 5). Moreover, after 28 days administration of MG there was significant difference between the two treated groups and the control group characterized by increase in tail length ,DNA% in tail and the tail moment and decrease in DNA % in head, however there was no significant difference between the two treated groups in Comet% and head diameter when compared with the control group (Table 6).these data concluded that 28 days administration of MG by both low and high doses caused DNA damages and genotoxicity indicated by the presence of significant increase of tail length , DNA % in Tail , and tail moment. (Fig. 3,4)

Table 5: Comet assay parameters on liver tissue after 14 days administration of malachite green.

Parameter/ Group	Control	Low dose	High dose
Comet%	$13.73^{a} \pm 0.80$	$17.87^{a} \pm 1.79$	$15.33^{a} \pm 1.25$
head diameter (px)	$65.60^{a} \pm 7.30$	$57.60^{a} \pm 1.97$	$69.83^{a} \pm 3.68$
DNA % in head	$83.80^{a} \pm 4.40$	$85.07^{a} \pm 1.17$	$82.43^{a} \pm 0.64$
Tail length	$8.50^{a} \pm 1.50$	$10.07^{a} \pm 1.62$	$9.87^{a} \pm 0.73$
DNA % in tail	$11.04^{a} \pm 0.73$	$14.90^{b} \pm 1.20$	$17.53^{c} \pm 0.64$
Tail moment	$1.21^{a} \pm 0.10$	$1.92^{a} \pm 0.44$	$2.00^{a} \pm 0.29$

Note: Data are expressed as means ± SE . Values having different alphabetic superscripts within the same raw are significantly different ($P \leq 0.05$).

Table 6: Comet assay parameters on liver tissue after 28 days administration malachite green.

Parameter/ Group	Control	Low dose	High dose
Comet%	$17.10^{a} \pm 2.06$	$20.77^{a} \pm 1.94$	$19.27^{a} \pm 2.40$
Head diameter (px)	$70.63^{a} \pm 9.39$	$71.53^{a} \pm 10.65$	$80.37^{a} \pm 4.32$
DNA % in head	$82.77^{a} \pm 0.37$	$76.40^{b} \pm 0.81$	$84.03^{c} \pm 1.33$
Tail length	$7.82^{a} \pm 0.99$	$15.32^{b} \pm 3.09$	$18.50^{c} \pm 0.97$
DNA % in tail	$17.17^{a} \pm 0.33$	$21.37^{b} \pm 2.34$	$27.40^{c} \pm 0.87$
Tail moment	$2.24^{a} \pm 0.47$	$3.28^{b} \pm 0.39$	$6.23^{c} \pm 0.61$

Note: Data are expressed as means ± SE . Values having different alphabetic superscripts within the same row are significantly different ($P \leq 0.05$).

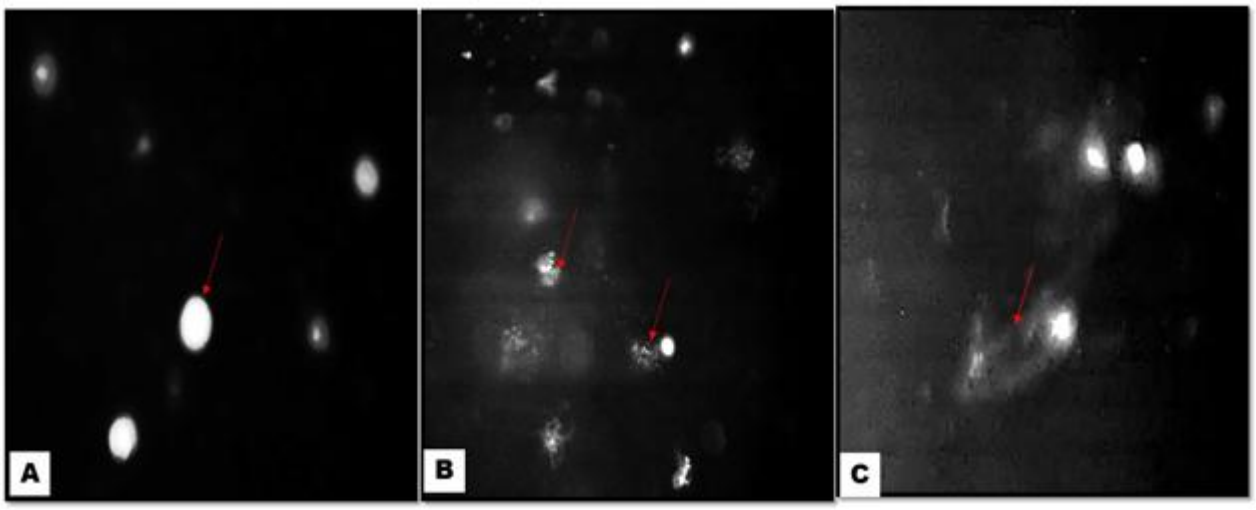
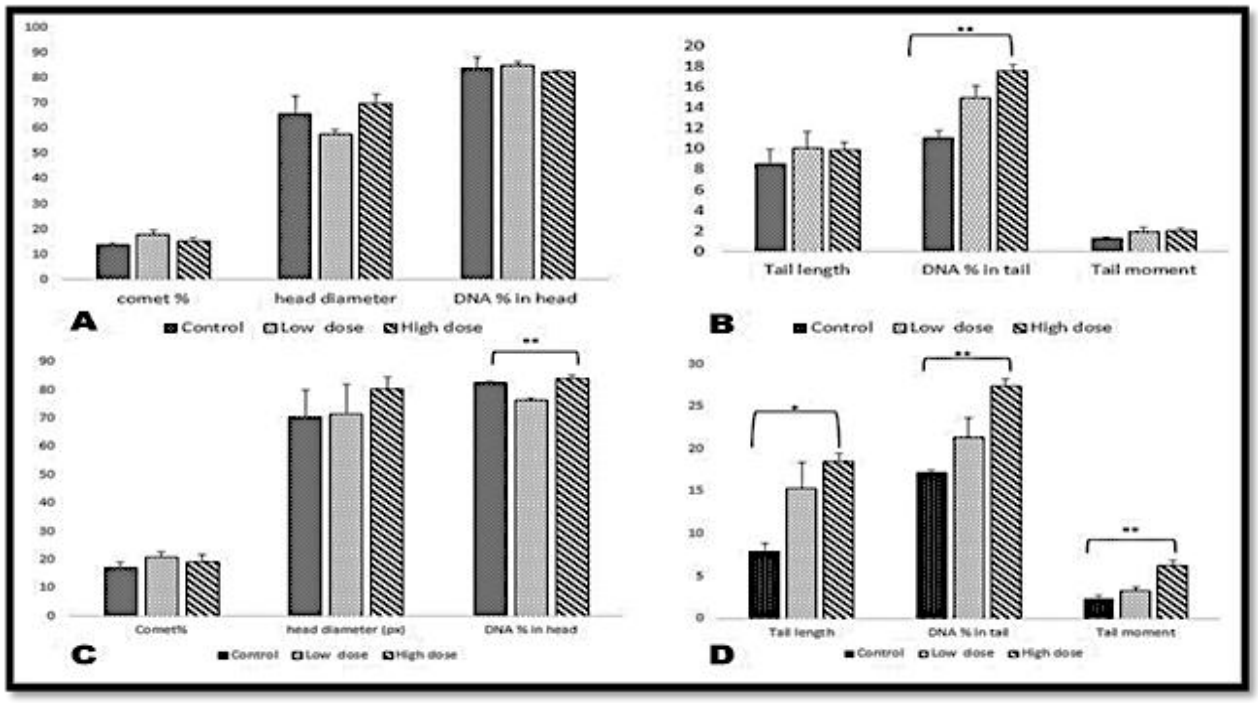


Figure 3:- Evaluation of DNA damage by comet assay in liver. (A) indicate intact nucleus (referred by red arrow) of control group, (B) indicate moderate degree of DNA damage with short DNA tail (referred by red arrow) after 28 days treatment with MG low dose , (C) sever degree of DNA damage with long DNA tail (referred by red arrow)l after 28 days treatment by MG high dose.



***=significant, **= highly significant, others=non-significant**

Figure 4:- Evaluation of DNA damage by comet assay in liver. (A &B) are comet parameters after 14 days treatment with MG using low and high doses ,while (C &D) are comet parameters after 28 days treatment with MG using low and high doses.

Histopathological Results

In control groups there was preserved hepatic lobular architecture with hepatocytes arranged in cell plates with sinusoidal spaces in-between, cells showed abundant eosinophilic cytoplasm and central nucleus.

After 14 days of MG administration the liver tissue showed preserved architecture with hepatocytes arranged in thin and thick cell plates. There was moderate hydropic degeneration of hepatocytes (fragmentation and clearing of cytoplasm), with mild focal necrosis and congested vessels in both doses. After 28 days of the MG administration in a high dose livers demonstrated partially preserved architecture with hepatocytes arranged in

thick cell plates with partially obliterated sinusoids. There was moderate focal lymphocytic infiltrate with scattered cells showing dysplastic large nuclei and moderately congested central vein. In case of the low dose group the changes were more evident as the liver tissue showed partially distorted lobular architecture with hepatocytes arranged in thick cell plates. There is marked diffuse hydropic degeneration of hepatocytes (fragmentation and clearing of cytoplasm), focal dysplastic large nuclei along with few necrotic and apoptotic cells. (Fig.5).

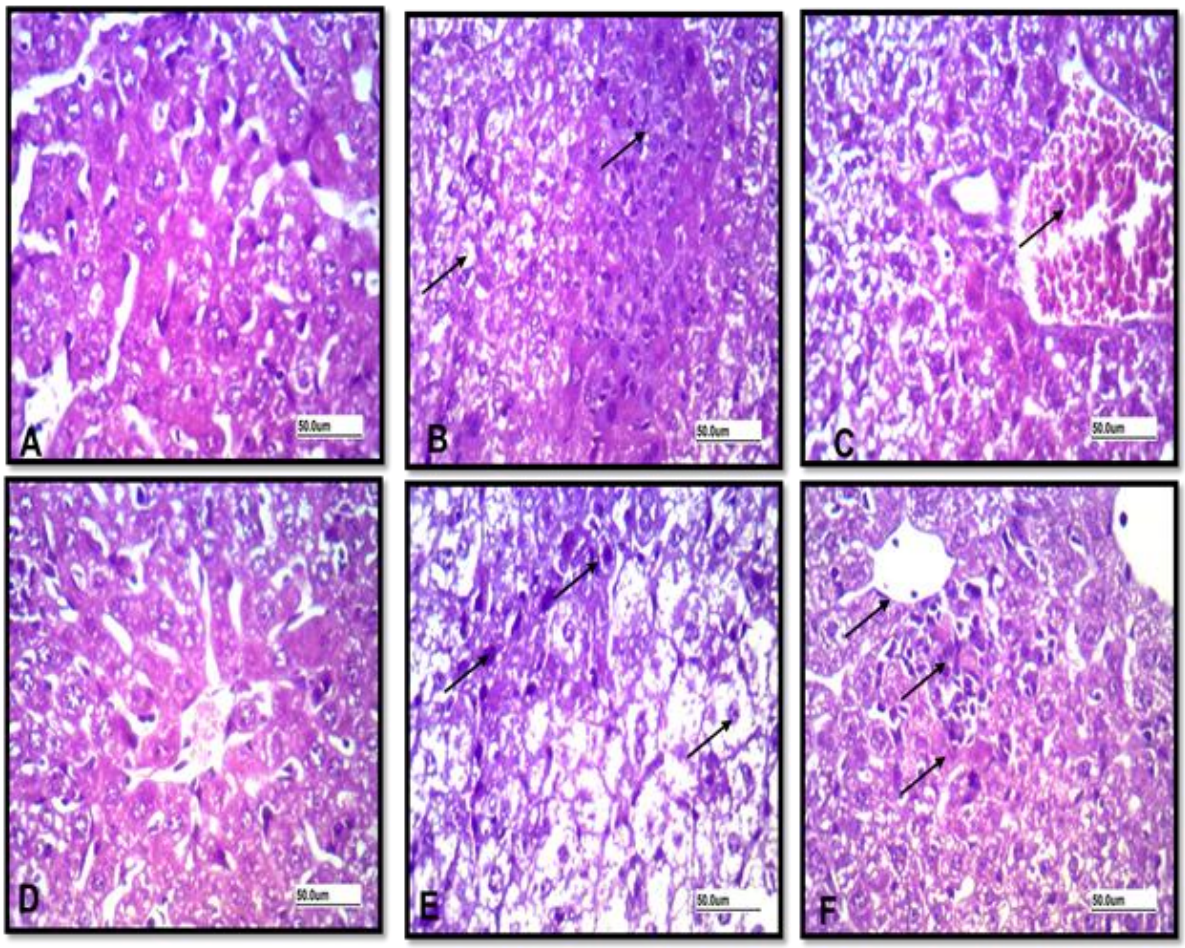


Figure 5:- Hepatic sections (H&E X400). After 14 days (A: Control, B: MG 2.5 mg/kg.b.wt. - intoxicated, C: MG 5 mg/kg.b.wt. - intoxicated) and 28 days (D: Control, E: MG 2.5 mg/kg.b.wt. - intoxicated, F: MG 5 mg/kg.b.wt. - intoxicated) treated rats with malachite green.

Discussion

Treatment with MG for 28 days resulted in significant increase of ALT ,AST, and ALP in addition to significant decrease of total protein, the same results were observed by[14]. This increase of liver enzymes may be attributed to the hepatocellular damage resulting from chemical-toxicity, where these enzymes levels showed an intimate relationship to cell necrosis and/or increased cell membrane permeability that leads to discharge of the enzyme to blood stream and increase of its level in serum[25]. While the decrease of total protein may be due to the cellular damage caused by toxins[26] or due to the increase in messenger RNA degradation by the effect of reactive oxygen species (ROS) produced by the MG toxicity[27]. Administration of MG for 14 days revealed non-significant changes between control and treated groups in serum liver enzymes and total protein.

MG low and high doses for 14 and 28 consecutive days caused significant decrease of all estimated antioxidant parameters (SOD, Catalse, GSH and GPx) the same results were observed by Jayanta et al.[28]. This reduction may be attributed to the oxidative stress status and the reactive oxygen species (ROS) induced by MG in hepatic tissue, and the important role of these parameters in scavengering free radicals so in case of MG toxicity the antioxidant parameters stores are substantially depleted by large amount of ROS produced and this

depletion is crucial determinant for liver necrosis and cell death in oxidative stress conditions[5,29-30-31]. Harmful effects caused by ROS occur as a result of an imbalance between the formation and inactivation of these species leading to abnormalities in cellular physiology and different pathological conditions[32]. The ROS cause oxidative damage to several biomolecules including DNA, lipids, proteins, and lipoproteins[33].

After 14 days oral administration of MG there were no significant differences between the low dose, high dose , and the control group in Comet%, head diameter, DNA % in head, tail length and the tail moment, although there was significant increase in tail DNA % between the three groups. Moreover, after 28 days administration of MG there was significant difference between the two treated groups and the control group characterized by increase in tail length ,DNA% in tail and the tail moment and decrease in DNA % in head, however there was no significant difference between the two treated groups in Comet% and head diameter when compared with the control group .these data indicated the genotoxicity induced by MG in both doses markedly after 28 days treatment These results were in the same line with[34], who observed a dose-dependent increase in DNA damage in the form of 'tail moment' by MG, which is indicative of induction of DNA strand breaks. Also[7] reported that, MG induced genotoxicity particularly through chromosomal breaks. This may be attributed to the oxidative stress and DNA oxidative damage as a result of production of high levels of free radicals[14]. The loss of hepatic architecture and hydropic degeneration as well as lymphocytic infiltration and focal necrosis in animals intoxicated with MG were noticed in the present study. The histopathological results also confirmed the hepatotoxicity. Our histopathological findings were matching with those of[13,14].

Conclusion

Our study concluded that MG administration using the both tested doses for 28 days revealed sever toxicity which indicated by the increase of liver enzymes, decrease in total protein, decrease of antioxidant parameters in hepatic tissue, and increased DNA damages in liver tissue, on contrary these effects not appears in the 14 days administration .For the previous reasons, it was important to pay great attention to the selection of malachite green to be used, take into account the recommended dosages and exposure periods.

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