Biochemical and histopathological effects of melamine on liver, spleen, heart and testes in male rats

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Abstract: This study aimed to evaluate the toxic effect of different melamine doses (5000, 10000, 15000 and 20000 ppm), supplemented orally in the diet for 28 days to male rats, on biochemical parameters and the histopathology of liver, testes, spleen. The complete blood count (CBC), serum proteins, serum bilirubin, serum liver enzymes, sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of serum proteins, histopathological examinations of four organs (liver, spleen, heart and testes) were investigated. The CBC showed non significant changes in the melamine supplemented groups. Liver function enzymes were slightly affected. Serum protein was decreased and serum bilirubin was increased. The SDS-PAGE showed induction of two new high molecular weight bands and another low molecular weight band as a result of melamine supplementation. The histopathological examination of the four organs under study showed adverse pathological signs according to the melamine dose.
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1.Introduction

Melamine [$C_3N_3(NH_2)_3$] is a white color, crystalline, nitrogen rich organic base, slightly soluble in water, used in organic synthesis and in the manufacture of resins. Melamine was considered non-toxic, until significant morbidity and mortality related to crystalluria, nephrolithiasis and nephrotoxicity resulted from pet food and milk-based products contamination in China in 2008, children in Taiwan, Hong Kong and Macau (Hau *et al*., 2009 and Skinner *et al.,* 2010). Clinical signs of melamine poisoning in cats and dogs were inappetence, vomiting, polyuria, polydipsia and lethargy. Urine and kidneys from affected animals contained large golden-brown birefringent crystals consisting of melamine and co-contaminant cyanuric acid (Gossner *et al.,* 2009).

The European Union set a standard for acceptable human consumption (Tolerable Daily Intake) of melamine at 0.5 milligrams per kg of body mass. It was reduced to 0.2 mg per kg (Harrington, 2010). Canada declared a limit of 0.35 mg and the US FDA's limit was put at 0.63 mg, but was later reduced to 0.063 mg daily. The World Health Organization's food safety director estimated that the amount of melamine a person could stand per day without incurring a bigger health risk, the "tolerable daily intake" (TDI), was 0.2 mg per kg of body mass (Endreszl, 2008).

The Melamine Incident in China surfaced in September 2008 was one of the largest food adulterations for profit that affected about 300,000 Chinese infants and young children, with six reported deaths. The impacts were not limited in China but raised global food safety issues of milk and milk-containing products exported from China (Gossner *et al.,* 2009). More than 150 pet food products were identified as containing contaminated ingredients and were recalled. Analysis revealed that these products contained up to approximately 3200 ppm melamine and 600 ppm cyanuric acid (Cianciolo *et al*., 2008 and Skinner *et al*., 2010).

Melamine cyanurate is more toxic than either melamine or cyanuric acid alone (Babayan and Aleksandryan, 1985 and Puschner and Reimschuessel, 2011). Diagnosis of melamine poisoning is based on the presence of melamine and cyanuric acid in samples of urine or kidney from affected animals, or contaminated food or feed. Gas chromatograph/mass spectroscopy (GC/MS) and liquid chromatography/tandem MS are used to analyze for melamine and cyanuric acid (Cianciolo *et al*., 2008; Hon *et al*., 2011 and Puschner and Reimschuessel, 2011). Schnackenberg *et al*. (2012) found that the male rats were slightly more affected than female rats following dosing with the 120 and 180 ppm melamine. On the other hand, Ren *et al*. (2012) concluded that the rapid development and rapid regression, and melamine withdrawn plays a key role in the stone dissolution-discharge necessary for BEH regression.

On the other hand, Bai *et al.* (2010) stated that the highest tissue melamine concentrations in chickens fed melamine-containing diets were found in the kidneys, with lower concentrations in the liver and muscle. Whereas Jingbin *et al.* (2010) reported that, tissue residues were depleted 10 to 20 days after exposure ceased. Osborne *et al*. (2008) and Bhalla *et al*., (2009) stated that melamine and cyanuric acid crystallize; forming a lattice structure at the molecular level, at a pH of 5.8. On the other hand, melamine contained crystals recovered from kidneys and urine of cats that ingested melamine-contaminated food contained 70% cyanuric acid and 30% melamine based on infrared spectra results (Osborne *et al*., 2008 and Thompson *et al*., 2008).

Several analytical methods were developed to determine melamine in the serum and organs; after intravenous administration (Wu *et al.*, 2009), whereas Huang *et al.* (2012) developed a method for determination of melamine and related triazine by-products based on capillary electrochromatography-mass spectrometry using poly(divinyl benzene-alkene-vinylbenzyl trimethylammonium chloride) monolithic stationary phases. Liu *et al.* (2012) developed a new method for detection of melamine in a human renal uric acid stone by matrix-assisted laser desorption / ionization time-of-flight mass spectrometry (MALDI-TOF MS).

The toxic effect of different melamine doses (5000, 10000, 15000, and 20000 ppm), supplemented to male rats orally in the diet for 28 days was studied as revealed by biochemical parameters and histopathology of liver, testes, spleen and heart in male rats.

2.Materials and methods

The toxic effect of melamine was tested in male Albino Wister rats (*Rattus rattus*) of an average of 200-225 g were obtained from King Fahd Centre for Medical Research, King Abdulaziz University, KSA. Rats were housed five per cage and held for approximately two weeks before the study began for acclimatization. Cages, bedding, and glass water bottles (equipped with stainless steel sipper tubes) were replaced twice per week. Test diets, control diets, and tap water are available *ad libitum.* Conventional animal basal diet was obtained from Grain Silos & Flour Mills Organization–Makkah branch in Jeddah- Saudi Arabia and Melamine (99%) was purchased from Sigma-Aldrich Cat. No. M2659-5G. The rats were distributed into five groups; rats of the first group were fed normal basic diet as a (-)ve control and four melamine supplemented groups; G1 were supplemented with 5,000 ppm, G2 were supplemented with 10,000 ppm, G3 were supplemented with 15,000 ppm and G4 supplemented with 20,000 ppm. Test diets were prepared by mixing the suitable melamine weight according to the dose to the basal diet. The rats were observed twice daily for mortality and morbidity and were weighed every week and at the end of the study. Necropsies were performed on all animals at the end of the study (28 days). All rats were dissected, photographed and the target organs for histopathological investigations were kept in 10% formalin.

Complete blood counts (CBC)

The following CBC parameters were measured using Flex reagent cartridge, URCA method of Dimension Vista System, Siemens Health Care, Diagnostics Inc., Newark, DE 19714, USA, according to the instruction of the supplier: GRP (granulocytes), Hb (hemoglobin), HCT (hematocrit), LY (lymphocytes), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), MCV (mean corpuscular volume), MO (monocytes), MPV (mean platelet volume), PCT (platelet-Crit), PDW (platelet distribution width), PLT (platelet count), RBC (red blood cell), RDW (red cell distribution width), WBC (white blood cell).

Serum bilirubin (direct and total) and serum liver enzymes (ALT, AST, ALP and GGT) were measured also using Flex reagent cartridge, URCA method of Dimension Vista System, Siemens Health Care, Diagnostics Inc., Newark, DE 19714, USA, according to the instruction of the supplier. The Sodium dodecyl sulphate polyacrlamide gel electrophoresis (SDS-PAGE) was performed using a modified method from Laemmli (1970).

Histopathological investigations

The liver, spleen, heart and testes were dissected out and fixed in 10% formalin, dehydrated in gradual ethanol (50-99%), cleared in xylene, and embedded in paraffin. Sections were prepared and then stained with hematoxylin and eosin dye for microscopic investigation (Drury *et al.,* 1976). The stained sections were examined and photographed under a light microscope to detect histopathological changes.

Data analysis

All data were analyzed using the SPSS (Statistical Program for Sociology Scientists) Statistics Version 17.0 for computing the mean values, the standard errors (SE), and the test of significance (t-test).

3. Results

Complete blood count (CBC)

Data in Table (1), shows the effect of melamine supplementation on white blood cell count. The mean values of WBC in all melamine supplemented groups (G1, G2, G3 and G4) were non significantly lower than that of the negative control (17.36±0.64, 19.86±1.89, 16.62±0.77, 17.84±1.91

and 20.91±3.98 X10^ 3 / uL, respectively). The mean values of LY% in all treatments (G1, G2, G3 and G4) were lower than that of the negative control (78.58±2.37, 70.02±2.72, 79.28±0.33, 78.88±3.37 and 86.63±3.16 %, respectively). Whereas differences were non significant in G1 and G4; significant at 5% (P <0.05) in G3 and highly significant at 1% (P <0.01) in G2, when compared to negative control. The mean values of MO% in all treatments (G1, G2, G3 and G4) were higher than that of the negative control (14.32±1.36, 19.38±1.71, 14.52±0.90, 11.32±1.50 and 7.71±0.49%, respectively). The differences were significant at 5% (P <0.05) in G4, highly significant at 1% (P<0.001) in G1 and very high significant at 0.1% (P <0.0001) in G2 and G3, when compared to the negative control.

Table (1): Effect of melamine supplementation for 28 days on white blood cell (WBC).

Parameter	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine	G4 20,000 ppm melamine
WBC X10^ 3 / uL	Mean±SE	20.91±3.98	17.36±0.64	19.86±1.89	16.62±0.77	17.84±1.91
	T-test		-0.98 NS	-0.21 NS	-1.28 NS	-0.86 NS
LY %	Mean±SE	86.63±3.16	78.58±2.37	70.02±2.72	79.28±0.33	78.88±3.37
	T-test		1.63 NS	2.95**	2.42*	1.56 NS
MO %	Mean±SE	7.71±0.49	14.32±1.36	19.38±1.71	14.52±0.90	11.32±1.50
	T-test		-4.28**	-5.40***	-11.54***	-2.19*
GR %	Mean±SE	6.75±2.41	7.10±1.11	10.56±1.15	7.28±0.39	9.80±3.15
	T-test		-0.11 NS	-1.11 NS	-0.25 NS	-0.76 NS
LY X10^ 3 / Ul	Mean±SE	23.96±5.14	13.62±0.50	13.74±1.05	12.98±0.48	13.94±1.42
	T-test		2.02	2.06	2.20*	2.02
MO X10^ 3 / uL	Mean±SE	1.73±0.42	2.48±0.30	3.92±0.57	2.44±0.25	4.04±2.19
	T-test		-1.42	-2.41*	-3.29**	-0.95
GR X10^ 3 / uL	Mean±SE	1.92±0.95	1.94±0.21	2.16±0.38	3.22±0.10	3.84±2.25
	T-test		0.64	-0.19	0.80	-0.72

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P<0.05), ** means high significant at 1% (P<0.01), *** means very high significant at 0.1% (P<0.001) and NS means non significant.

The mean values of GR% in all the groups (G1, G2, G3 and G4) were non significantly higher than that of the negative control (7.10±1.11, 10.56±1.15, 7.28±0.39, 9.80±3.15 and 6.75±2.41%, respectively). The mean values of LY in all treatments (G1, G2, G3 and G4) were non significantly lower than that of the negative control (13.62±0.50, 13.74±1.05, 13.94±1.42 and 23.96±5.14X10^3/Ul, respectively). The mean values of MO in all treatments (G1, G2, G3 and G4) were higher than that of the negative control (2.48±0.30, 3.92±0.57, 2.44±0.25, 4.04±2.19 and 1.73±0.42X10^3/Ul, respectively). Differences were non significant in G1 and G4; significant at 5% (P <0.05), in G2 and highly significant at 1% (P <0.01) in G3. The mean values of GR in G1 and G3 were non significantly lower than that of the negative control (1.94±0.21, 2.16±0.38, 3.22±0.10, 3.84±2.25 and 1.92±0.95X10^3/Ul, respectively).

Table (2), shows the effect of melamine supplementation on red blood cell indices in rats. The mean value of RBC in G1, G2, G3 and G4 were higher than that of the negative control (7.90±0.004, 7.86±0.09, 7.75±0.01, 7.81±0.03 and 7.46±0.09 X10^ 6/uL, respectively). However, differences were highly significant at 1% (P <0.01) in G1, G2, G3 and G4, when compared to the negative control. The mean values of hemoglobin (Hgb) in G1, G2, G3 and G4 were higher than that of the negative control (15.40±0.61, 16.08±0.44, 15.26±0.37, 16.18±0.24 and 14.71±0.28 g/dl, respectively). The differences were non significant in G1 and G3, and significant at 5% (P <0.05) in G2, when compared to negative control.

The mean values of HCT% in G1, G2, G3 and G4 were lower than that of the negative control (41.80±0.26, 41.56±0.06, 41.90±0.35, 40.77±0.12 and 43.07±0.56%, respectively). The differences were non significant in G1 and G3; highly significant at 1% (P <0.01) in G4 and very high significant at 0.1% (P <0.001) in G2, when compared to the negative control.

Table (2): Effect of melamine supplementation on red blood cell indices (RBC).

Parameter	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine	G4 20,000 ppm melamine
RBC X 10^ 6/uL	Mean±SE	7.47±0.10	7.90±0.004	7.86±0.09	7.75±0.01	7.81±0.03
	T-test		-4.32**	-3.19**	-3.26**	-2.95**
Hgb g/dl	Mean±SE	14.71±0.28	15.40±0.61	16.08±0.44	15.26±0.37	16.18±0.24
	T-test		-1.48 NS	-2.30*	-1.13 NS	3.47**
HCT %	Mean±SE	43.07±0.56	41.80±0.26	41.56±0.06	41.90±0.35	40.77±0.12
	T-test		2.01 NS	-4.85***	1.733 NS	4.04**
MCV fl	Mean±SE	56.26±0.69	54.18±0.66	54.32±0.99	54.24±0.56	52.28±0.30
	T-test		1.55*	1.18*	2.35*	5.82***
MCH pg	Mean±SE	19.02±0.05	17.65±0.06	19.00±0.17	18.70±0.13	17.85±0.14
	T-test		16.74***	-3.50**	3.35**	6.69***
MCHC g/dl	Mean±SE	3.32±0.09	3.33±0.15	3.46±0.06	3.45±0.10	3.42±0.26
	T-test		-5.2***	-9.8***	-10.99***	-4.75***
RDW %	Mean±SE	1.54±0.37	1.55±0.69	1.84±3.09	1.57±0.44	2.11±0.65
	T-test		-0.02 NS	-0.90 NS	-0.87 NS	-8.45***

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P<0.05), ** means high significant at 1% (P<0.01), *** means very high significant at 0.1% (P<0.001) and NS means non significant.

The mean values of MCV in all treatments (G1, G2, G3 and G4) were lower than that of the negative control (54.18±0.66, 54.32±0.99, 54.24±0.56, 52.28±0.30 and 56.26±0.69 fl, respectively), however differences in G1, G2 and G3 were significant at 5% (P <0.05) and in G4 was very high significant at 0.1 (P <0.001), when compared to the negative control. The mean MCH values of G1, G2, G3 and G4 were lower than that of the negative control (17.65±0.06, 19.00±0.17, 18.70±0.13, 17.85±0.14 and 19.02±0.05 pg, respectively), whereas the MCH value in G2 was higher than that of the negative control (19.65±0.17 and 19.00±0.04 pg, respectively). Differences of G2 and G3 were highly significant at 1% (P <0.01) and very high significant at 0.1% (P <0.001) in G1 and G4, when compared to negative control. The mean MCHC values in all treatments (G1, G2, G3 and G4) were higher than that of the negative control (3.33±0.15, 3.46±0.06, 3.45±0.10, 3.42±0.26 and 3.32±0.09 g/dl, respectively), whereas differences were very high significant at 0.1% (P <0.001) in all treatments, when compared to the negative control. The mean values of RDW % in G1, G2, G3 and G4 were higher than that of the negative control (1.84±3.09, 1.55±0.69, 1.57±0.44, 2.11±0.65 and 1.54±0.37%, respectively). The differences were non significant in all treatment except for G4 which were very high significant at 0.1% (P <0.001), when compared to the negative control.

Table (3), illustrates the effect of melamine supplementation on platelet indices in rats under study. The mean values of platelet indices of all treatments (G1, G2, G3 and G4) were non significantly higher than that of the negative control (1.03±25.11, 1.04±52.77, 1.05±86.55, 1.17±66.70 and 1.01±17.31 X10^ 3 / uL, respectively). The mean values of MPV in all melamine supplemented groups were higher than that of the negative control (5.10±0.07, 5.22±0.08, 5.45±0.07, 6.42±1.01 and 5.01±0.06 fL, respectively). Differences were non significant in all treatments except for G3, which was very high significant at 0.1% (P <0.001), when compared to the negative control.

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P<0.05), ** means high significant at 1% (P<0.01), *** means very high significant at 0.1% (P<0.001) and NS means non significant.

The mean values of PCT% in G1, G2, G3 and G4 were higher than that of the negative control (0.51±0.02, 0.54±0.01, 0.55±0.04, 0.59±0.02 and 0.48±0.03%, respectively). Differences were highly significant at 1% (P <0.01) in G4 and non significant in all other groups, when compared to the negative control. The mean values of PDW% in all treatments (G1, G2, G3 and G4) were lower than that of the negative control (1.61±0.42, 1.58±0.30, 1.60±0.28, 1.63±0.38 and 1.72±0.48%, respectively), however, differences were non significant in G4; highly significant at 1% (P <0.01) in G1 and G2, and very

high significant at 0.1% (P <0.001) in G3, when compared to the negative control.

Serum proteins

Table (4), shows the effect of melamine supplementation for 28 days on serum protein (albumin and total protein) in rats under study. As shown, the mean values of albumin in all melamine supplemented groups were non significantly lower than that of the negative control (10.80±0.20, 10.60±0.24, 11.20±0.20, 10.60±0.40, and 11.18±0.4 g/L, respectively).

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P<0.05), ** means high significant at 1% (P<0.01), *** means very high significant at 0.1% (P<0.001) and NS means non significant.

The mean values of total protein (TP) in all melamine supplemented groups (G1, G2 and G4) were lower than that of negative control (62.60±1.12, 62.00±1.00, 61.60±2.01, 61.60±2.01 and 63.20±1.04 g/L, respectively). However, differences were non significant in all groups, except in G3 that was significant at 5% (P <0.05).

Serum bilirubin

Table (5), illustrate the effect of melamine supplementation for 28 days on serum bilirubin (total and direct bilirubin) in rats. As shown, the mean values of the total bilirubin in G1, G2, G3 and G4 were higher than that of the negative control. (2.60±0.24, 3.20±0.37, 2.98±0.45, 3.00±0.31 and 2.15±0.22 mmol/L, respectively). However, differences were non significant in G1 and G2, significant at 5% (P <0.01) in G3 and G4.

Table (3): Effect of melamine supplementation for 28 days on platelets indices (PLT).

Parameter	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine	G4 20,000 ppm melamine
PLT index X 10^ 3 / uL	Mean±SE	1.01±17.31	1.03±25.11	1.04±52.77	1.05±86.55	1.17±66.70
	T-test		0.72 NS	-0.15 NS	-0.24 NS	-1.51 NS
MPV fL	Mean±SE	5.01±0.06	5.10±0.07	5.22±0.08	5.45±0.07	6.42±1.01
	T-test		0.00 NS	-1.90 NS	-5.03***	-1.02 NS
PCT %	Mean±SE	0.48±0.03	0.51±0.02	0.54±0.01	0.55±0.04	0.59±0.02
	T-test		-1.21 NS	0.84 NS	-1.75 NS	-4.03**
PDW %	Mean±SE	1.72±0.48	1.61±0.42	1.58±0.30	1.60±0.28	1.63±0.38
	T-test		3.83**	2.81**	5.24***	1.145 NS

Table (4): Effect of melamine supplementation for 28 days on serum protein (albumin and total protein), in rats under study.

Parameters	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine G3	G4 20,000 ppm melamine
ALB g/L	Mean±SE	11.18±0.20	10.80±0.20	10.60±0.24	11.00±0.20	10.60±0.40
	T-test		1.63 NS	1.50 NS	1.01 NS	1.50 NS
TP g/L	Mean±SE	63.20±1.04	62.60±1.12	62.00±1.00	61.60±2.01	62.80±1.24
	T-test		0.24 NS	1.53 NS	2.76*	0.09 NS

Table (5): Effect of melamine supplementation for 28 days on serum bilirubin (total and direct bilirubin), in rats under study.

Parameters	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine	G4 20,000 ppm melamine
Total bilirubin mmol/L	Mean±SE	2.15±0.22	2.60±0.24	3.20±0.37	2.98±0.45	3.00±0.31
	T-test		-1.75 NS	-1.86 NS	-2.17*	-2.13*
Direct bilirubin mmol/L	Mean±SE	7.17±0.37	38.44±4.19	81.22±10.07	56.00±4.78	66.30±8.36
	T-test		-7.57***	-7.19***	-9.91***	-6.93***

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P<0.05), ** means high significant at 1% (P<0.01), *** means very high significant at 0.1% (P<0.001) and NS means non significant.

The mean values of direct bilirubin in G1, G2, G3 and G4 were higher than that of the negative control (38.44±4.19, 81.22±10.07, 56.00±4.78, 66.30±8.36 and 7.17±0.37 mmol/L, respectively). However, the differences were very high significant at 0.1% (P <0.001), when compared to the negative control.

Serum liver enzymes

Table (6), shows the effect of melamine supplementation for 28 days on serum liver enzymes (ALT, AST, ALP, GGT) in rats under study. The mean values of ALP in all groups (G1, G2, G3 and G4) were lower than that of the negative control (147.80±20.75, 100.40±15.06, 114.80±17.93, 116.00±12.13 and 152.21±13.08 U/L, respectively. However, the paired T-test did not show any significant differences in G1, G3 and G4, whereas the value of G2 was significant at 5% (P <0.05), when compared to that of the negative control. The mean values of AST in all groups (G1, G2, G3 and G4) were lower than that of the negative control (80.60±16.75, 106.00±23.83, 84.00±8.27, 76.60±9.37, and 107.22±3.38 U/L, respectively. However, the paired sample T-test did not show any significant in G1 and G2, whereas differences in G3 and G4 were they were significant at 5% (P <0.05), when compared to the negative control. Similarly for ALT, the mean values of all groups (G1, G2, G3 and G4) were lower than that of the negative control (56.00±10.45, 31.40±5.93, 29.40±3.04 and 69.61±3.95 U/L, respectively). However, differences were non significant in G1, and very high significant at 0.1% (P <0.001) in G2, G3 and G4, when compared to the negative control. The mean values of Gamma-glutamyl Transpeptidase (GGT) in all supplemented groups (G1, G2, G3 and G4) were non significantly higher than that of the negative control (3.08±0.03, 3.10±0.03, 3.12±0.03, 3.16±0.02 and 3.07±0.03 U/L, respectively).

Table (6): Effect of melamine supplementation for 28 days on serum liver enzymes (ALT, AST, ALP, GGT), in rats under study.

Parameters	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine G3	G4 20,000 ppm melamine
ALB g/L	Mean±SE	11.18±0.20	10.80±0.20	10.60±0.24	11.00±0.20	10.60±0.40
	T-test		1.63 NS	1.50 NS	1.01 NS	1.50 NS
TP g/L	Mean±SE	63.20±1.04	62.60±1.12	62.00±1.00	61.60±2.01	62.80±1.24
	T-test		0.24 NS	1.53 NS	2.76*	0.09 NS

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P<0.05), ** means high significant at 1% (P<0.01), *** means very high significant at 0.1% (P<0.001) and NS means non significant.

SDS-PAGE of serum proteins

Figure (1), shows the serum protein electrophoretic profile of the four groups supplemented with melamine compared to protein marker and the negative control. Five major protein bands of a molecular weight of about 200, 180, 60, 29, 26 kDa and one minor band of a molecular weight of about 24 kDa, were appeared common in all treatments. Two new high molecular weight (at about 160 and 140 kDa, respectively) bands appeared in the high doses of melamine fed groups (15,000 and 20,000 ppm). Another band of a molecular weight about 22 kDa appeared in all melamine fed rats.

Histopathology

Liver

The rat liver of G1 and G2 groups which were regularly supplied with 5,000 and 10,000 ppm melamine, respectively showed massive fatty changes, necrosis, and broad infiltration of the lymphocytes (Figure 2, B & C), comparable to those of the control group (Figure 2, A).

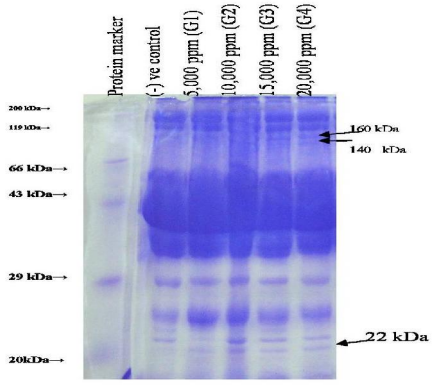

Figure (1): SDS-PAGE of serum proteins. Lanes were arranged as follows: M: protein markers, (-)ve control, G1: fed on 5000 ppm melamine, G2: fed on 10,000 ppm melamine, G3: fed on 15,000 ppm melamine, G4: fed on 20,000 melamine.

The histological architecture of liver sections of the rats supplemented with moderate doses of melamine (15,000 and 20,000 ppm) showed more or less abnormal patterns, with a mild degree of necrosis and slightly lymphocyte infiltration, almost

comparable to those of the control group (Figure 2, D & E).

Spleen

The spleen of the control group contains hematopoietic and lymphoid elements, is a primary site of extramedullary hematopoiesis, and removes degenerate and aged red blood cells as well as particulate materials and circulating bacteria from the blood supply. Lesions of this important component of the immune system may center on the red pulp, the white pulp or involve both compartments (Figure 3, A). The appearance of pigment in the red pulp (Figure 3, B & C) is a common background lesion in 5,000 and 10,000 ppm melamine treated rats. Pigment, can be ceroid/lipofuscin or hemosiderin, is usually harbored in macrophages and may be present in the marginal zone in addition to being in the red pulp. In both rat groups, hemosiderin is more prominent in females. Iron stains, e.g., Perl's Prussian blue, can demonstrate the iron in hemosiderin-containing macrophages. After rats were supplemented with high doses (15,000 and 2,0000 ppm), they showed large amounts of pigment present as in this example. It can usually be detected at low magnification in the red pulp. Higher magnification of Figure (3, D & E) shows brown granular hemosiderin pigment predominantly within macrophages in the red pulp.

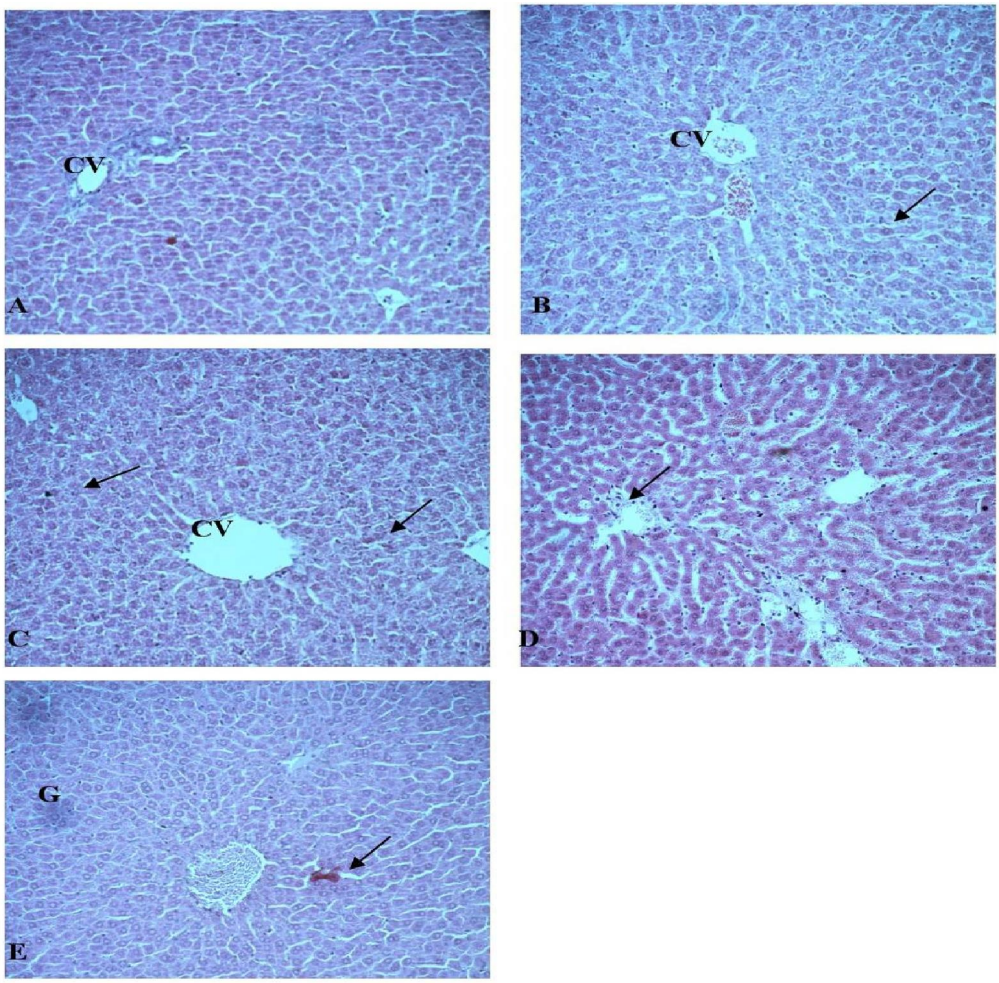

Figure (2): A; Normal hepatic tissues showing hepatic strands of cells around the central vein (CV) leaving blood sinusoids (S), **B;** hepatic tissues of 5,000 ppm melamine fed group showing cellular necrosis around the central veins (arrow), **C;** hepatic tissues of 10,000 ppm melamine fed group showing mild cellular necrosis, **D;** hepatic tissues of 15,000 ppm melamine fed group showing highly degree of necrosis and accumulation of melamine salt particles (arrow), **E;** hepatic tissues of 20,000 ppm melamine showing highly degenerated hepatic tissues and accumulation of granules in hepatic tissues (G). X 200 (H&E stains).

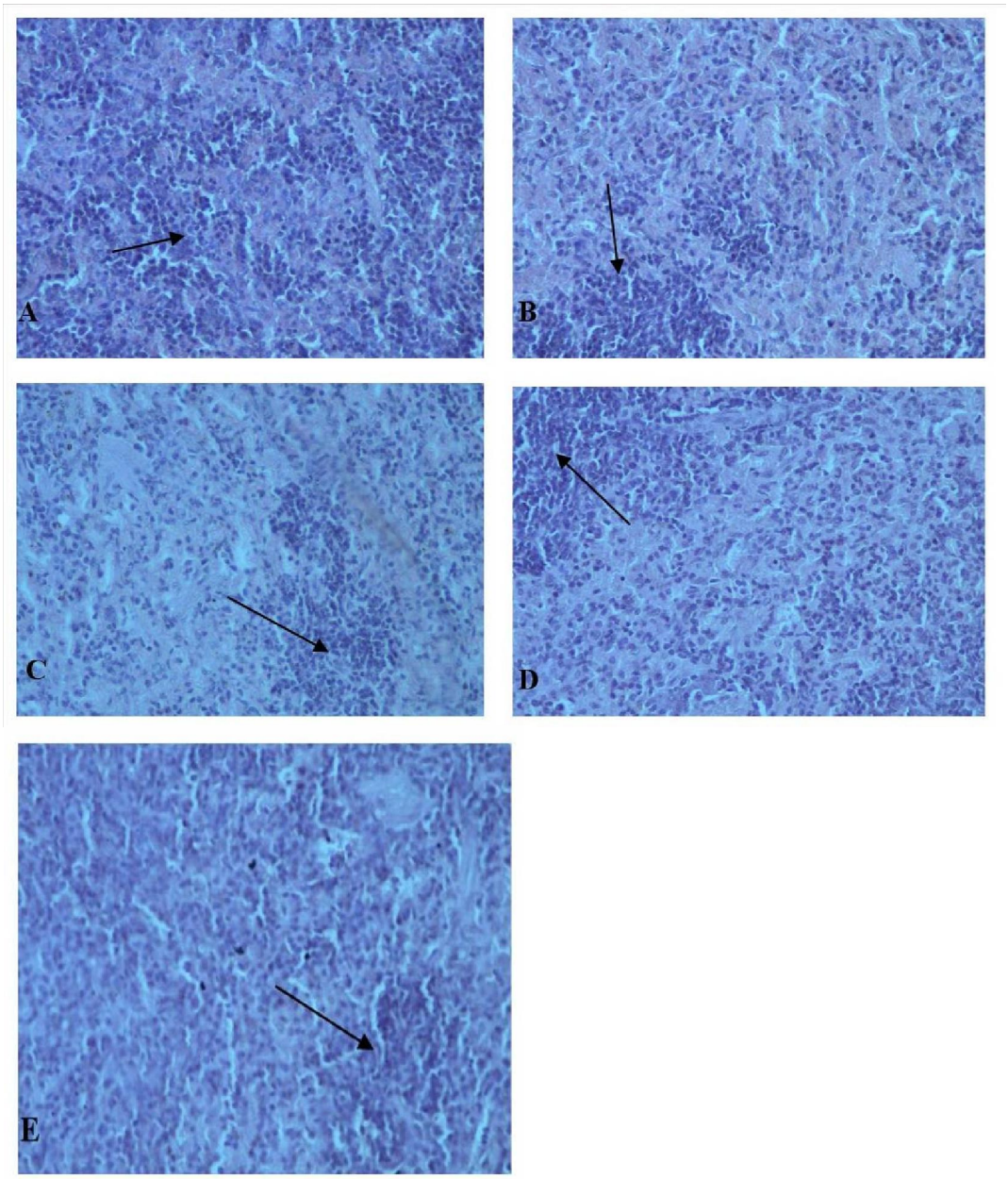

Figure (3): A; Normal spleen tissues showing hematopoietic and lymphoid elements (arrow), **B;** spleen tissues of 5,000 ppm melamine fed group showing the appearance of pigment in the red pulp (arrow), **C;** spleen tissues of 10,000 ppm melamine fed group showing hemosiderin-containing macrophages (arrow), **D;** spleen tissues of 15,000 ppm melamine showing increases in the incidences of hyperplasia (arrow), **E;** spleen tissues of 20,000 ppm melamine showing highly granular hemosiderin pigment in spleen tissues (arrow). X 200 (H&E stains).

Heart

The normal structure of cardiac muscles is shown in control group (Figure 9, A).

Hyalinization in the cardiac muscles appeared in the low melamine doses which were supplemented 5,000 and 10,000 ppm (Figure 4, B & C, respectively) groups, but minimal cardiac muscles damage in the melamine-supplemented rats that received 10,000 ppm melamine (Figure 4, C). In addition, heart tissues of 15,000 ppm melamine supplemented rats, showed pathological changes such as congestion and slight infiltration, focal parenchymatous degeneration of cardiomyocytes and the presence of single basophils was observed (Figure 4, D), but the heart tissue of the rats given 20,000 ppm (Figure 4, E) indicated maximal hyalinization of

muscle fibers, with focal cellular infiltration or necrosis of muscle fibers.

Testes

The light microscopy examination of the testis of the control rats had normal structure, completely enveloped by a thick capsule, tunica albuginea, which is composed mainly of dense collagenous fibrous connective tissue. The structural components of the testis are the seminiferous tubules (ST) and interstitial tissues (IT). The seminiferous tubules are two types of cells, the Sertoli cells, resting on the thin basal lamina (basement membrane) and the spermatogenic cells (SC). These cells are many layers, namely, the spermatogonia (S), primary spermatocytes (PS); secondary spermatocytes (SS); spermatoids (SP) and finally mature spermatozoa (Z), (Figure 5 A).

In the low doses of melamine supplemented groups (5,000 and 10,000 ppm melamine, respectively), showed cellular changes. The seminiferous tubules had thickened in basement membrane together with focal areas of vacuolar degenerative changes appeared in the cytoplasm of the spermatogenic epithelium (Figure 5, B & C).

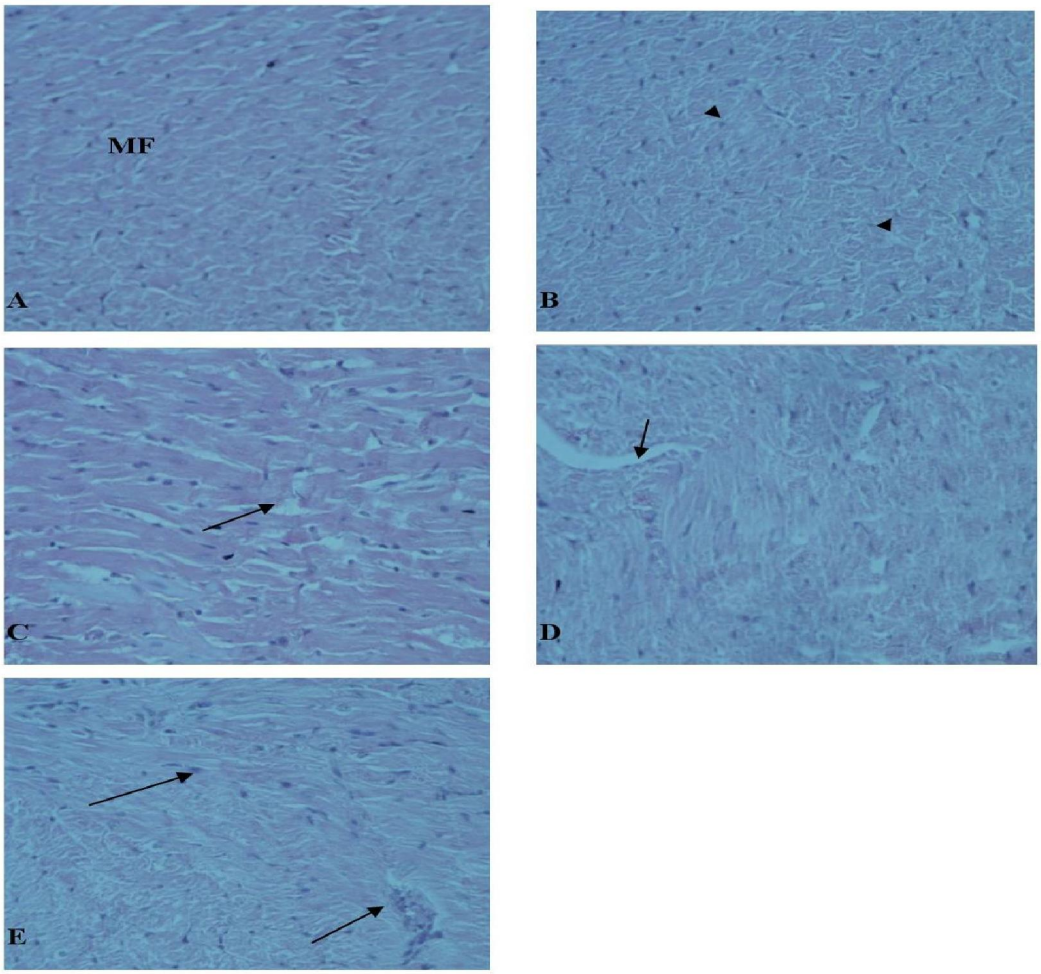

Figure (4): A; Normal cardiac tissues showing normal structure of cardiac muscles consists of muscle fibers (MF), **B;** cardiac tissues of 5,000 ppm melamine fed group showing increased hyalinization (arrow heads), **C;** cardiac tissues of 10,000 ppm melamine fed group showing minimal cardiac muscles damage (arrow), **D;** cardiac tissues of 15,000 ppm melamine fed group showing degeneration of cardiomyocytes (arrow), **E;** cardiac tissues of 20,000 ppm melamine showing necrosis of muscle fibers (arrow). X 200 (H&E stains).

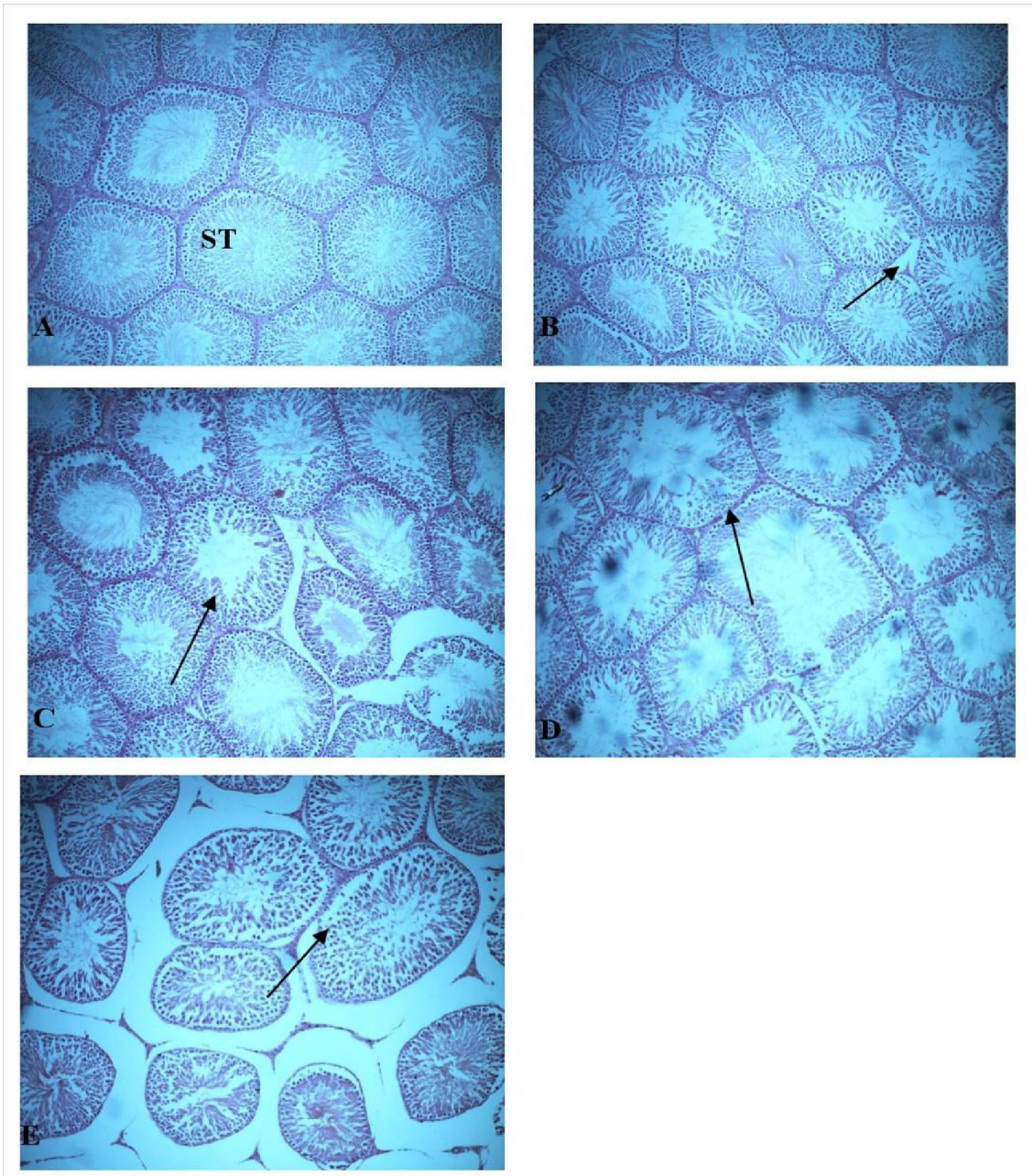

Figure (5): A; Testicular tissues of control group showing structural components of the testis which are the seminiferous tubules (ST), **B;** testicular tissues of 5,000 ppm melamine fed group showing the seminiferous tubules had thickened in basement membrane together (arrow), **C;** testicular tissues of 10,000 ppm melamine fed group showing vacuolar degenerative changes appeared in the cytoplasm of the spermatogenic epithelium (arrow), **D;** testicular tissues of 15,000 ppm melamine fed group showing the form of degenerative changes (arrow) and **E;** testicular tissues of 20,000 ppm melamine fed group showing highly degenerated testicular tissues and few fragmented sperms in the lumen (arrow). X 200 (H&E stains).

After rats had been supplemented with higher doses (15,000 and 20,000 ppm) of melamine, they showed more exaggerated features of focal areas of spermatogenesis, arrest at the spermatid level, in the form of degenerative changes in the germinal cells together with few fragmented sperms in the lumen and acquired a thick, irregular basement membrane (Figure 5, D & E).

4. Discussion

The present investigation shows the effect of an oral supplementation of four melamine doses (5,000,

10,000, 15,000 and 20000 ppm) in the diet on the biochemical indices and histopathology of liver, spleen, heart and testes in male rats.

The CBC analysis showed that mean values of WBC were non significantly lower than that of the negative control. On the other hand, the mean values of RBC were slightly higher than that of the negative control. In addition, the mean values of platelet indices were non significantly higher than that of the negative control. This result agrees with that of Chen *et al.* (2009), who noticed that rats fed with higher melamine doses diets experienced only small changes in hematological parameters.

The serum protein (albumin and total protein) were non significantly increased compared to the negative control. This result is in concordant with similar results of Puschner *et al.* (2007) and Chen *et al.* (2009), who noted that a significant decline in albumin levels were detected.

The serum bilirubin (total and direct bilirubin) showed significant increase in the mean values than that of the negative control. This was expected due to the toxicity of melamine and the yellowish color appeared in the melamine treated rats. In contrast, Puschner *et al.* (2007) found that bilirubin in cats supplemented with melamine was not affected.

The non significant decrease in liver enzymes activity (ALT, AST, ALP, GGT), revealed that melamine supplementation for 28 days have not greatly affected the studied liver enzymes in studied male rats. The current liver enzymes results are consistent with those of Jeong *et al.* (2006) and Chen *et al*., 2009), who found that no significant change occurred in the levels of aspartate aminotransferase, alanine aminotransferase, or other examined enzymatic parameters. Furthermore, Bai *et al.* (2010) stated that the highest tissue melamine concentrations in chickens fed melamine-containing diets were found in the kidneys, with lower concentrations in the liver and muscle. It could be said that in spite of the high toxic effect of melamine on kidney as revealed by the very high significant differences of the kidney functions, it did not greatly affect the liver.

On the other hand, (Lv *et al.*, 2010) stated that melamine concentrations in the kidney were higher than concentrations in the skeletal muscle or liver of lambs. This result is supported by other investigations in rats (Wu *et al.*, 2009 and Ding *et al.*, 2012).

The SDS-PAGE showed induction of two new high molecular weight bands (at about 160 and 140 kDa, respectively) and a low molecular weight protein band (ca 22 kDa), as a result of melamine supplementation for 28 days. These bands may be induced due to the expression of antitoxicity genes. The current results are supporting to Camacho *et al.* (2011) who noted that histopathology and clinical chemistry examination indicated marked toxicity only in the animals exposed to two high combined doses of melamine and cyanuric acid and consistent with these observations, quantitative real-time polymerase chain reaction analysis of kidney tissue indicated increased expression of all genes analyzed relative to the control in both male and female rats fed daily with 229 or 694 ppm melamine and cyanuric acid.

In spite of the dramatic pathological changes of liver tissues and accumulation of hepatic granules and melamine crystals in most tissue, this is not reflected on the liver enzymes. This may be due to the chelating of enzymes by cyanuric acid that resulted from the contamination of melamine. The non significant changes in liver enzymes result is consistent with that of Lv *et al.* (2010), who reported that melamine concentrations in the kidney were higher than concentrations in the skeletal muscle or liver of lambs; and Bai *et al.* (2010) who stated that the highest tissue melamine concentrations in chickens fed melamine-containing diets were found in the kidneys, with lower concentrations in the liver and muscle. On the other hand, Chen *et al*. (2009) found that liver weight in male rat was not affected as a result of melamine toxicity.

Liver, heart, testes and spleen tissues showed dramatic pathological changes. Testes showed decrease in the number of spermatogonia. The cardiac tissues showed pathological changes and damage. Spleen showed vascular obstruction, hemorrhage and accumulation of melamine crystals. This result is consistent with Gao *et al.* (2012) who reported pathological changes in the heart, testes, spleen and urinary bladder of rats as a result of long term melamine supplementation. On the other hand, Chen *et al.* (2009) found that testes weight in male rats was increased as a result of melamine toxicity.

Based on the current results, it could be said that the toxic effect of melamine increases with increasing the melamine dose, as revealed from the histopathological investigations and SDS-PAGE of serum proteins. In addition, melamine supplementation affected other organs beside the kidney as revealed from the biochemical and histopathological investigations.

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