

## The Toxic Effect of Melamine on the Kidney of Male Rats as Revealed by Biochemical and Histopathological Investigations

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**Abstract:** This study aimed to evaluate the toxic effect of four different melamine doses (5000, 10000, 15000 and 20000 ppm) supplemented orally in the diet for 28 days on male rats. The appearance, anatomy, serum electrolytes, kidney functions (creatinine, urea and uric acid), serum melamine concentration, total body weight, food intake, food efficiency ratio (FER), body weight gain (BWG), percentage of body weight gain (BWG %), water consumed and histopathological examinations of three organs (kidney, ureter and urinary bladder) were investigated. The melamine supplemented rats turned yellow and showed different degrees of toxicity, hypertrophy and congestion, particularly the kidneys and the ureters as a result of melamine toxicity. Serum Na and Cl levels were decreased, whereas serum K, P and Ca levels were increased compared to the negative control. Kidney functions showed elevation of the mean values of serum creatinine, urea and uric acid. The histopathological examination of the three organs under study showed adverse pathological signs according to the melamine dose.

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### 1. Introduction:

Melamine, or 1,3,5-triazine-2,4,6-triamine, is a small, nitrogen-rich molecule. It was considered non-toxic based on early laboratory animal studies until significant morbidity and mortality related to crystalluria, nephrolithiasis and nephrotoxicity have resulted from pet food contamination (Bischoff, 2011).

It is harmful if swallowed, inhaled or absorbed through the skin and chronic exposure may cause cancer or reproductive damage. It is eye, skin and respiratory irritant (Weise, 2007). Direct contact results in skin irritation and eye irritation, and inhalation causes respiratory tract irritation. Oral ingestion affects the digestive tract, presenting as nausea, vomiting, and diarrhea (Jeong *et al.*, 2006). The mechanism of carcinogenesis was most likely secondary to epithelial hyperplasia caused by mechanical irritation (Hau *et al.*, 2009). Melamine is minimally, if at all, metabolized in monogastrics, but could be partially metabolized in the rumen of cattle and small ruminants. Melamine does not accumulate over time in the animal body. Renal elimination of unchanged melamine is approximately 90% complete within 24 hours (Qin *et al.*, 2010).

The combination of melamine and cyanuric acid in diet does lead to acute renal failure in cats (Puschner *et al.*, 2007) and rats (Dobson *et al.*, 2008). Dalal and Goldfarb (2011) reviewed the toxicology, epidemiology, and pathology due to melamine contamination of foodstuffs due to melamine

contamination only or in combination with cyanuric acid.

In 2008, melamine contamination of baby milk-based products was detected in China. Chinese authorities detected melamine concentrations between 2.5 and 2563 ppm in 13 commercial brands of milk powder and trace contamination in nine others (Bhalla *et al.*, 2009). Gonzalez *et al.* (2009) discovered renal failure in piglets in Spain between 2003 and 2006. They found that the kidneys contained melamine, cyanuric acid and relatively high concentrations of ammeline and ammelide.

Lv *et al.* (2010) found that melamine concentrations in the kidney were higher than concentrations in the skeletal muscle or liver of lambs, and concentrations decreased below 20 ppb 4 days after cessation of exposure. Addition of cyanuric acid to the diet did not affect melamine deposition. On the other hand, Shen *et al.* (2010) stated that melamine is excreted by dairy cattle into milk, particularly in high-producing cattle, though milk yield and composition are otherwise unaffected. Melamine can be detected in milk within 8 hours of exposure and remains detectable until 4 days after cessation of exposure. Approximately 0.3% of a melamine dose was excreted in milk in dairy goats, and milk melamine concentrations remained above the level of concern (1.0 µg/mL) until 3 days after cessation of dosing (Baynes *et al.*, 2010).

Kim *et al.* (2010) using rat models, investigated the renal crystal formation following the

ingestion of a melamine–cyanuric acid mixture (M+CA, 1:1) to gain insight into the M+CA-induced renal toxicity. They found that M+CA did not induce toxicity in precision-cut kidney slices, suggesting that M+CA does not have a direct nephrotoxicity. In addition, Puschner and Reimschuessel (2011) reported that both melamine and cyanuric acid were relatively nontoxic when given individually, but they caused crystal formation in renal tubules when given together. Moreover, Camacho *et al.* (2011) stated that co-exposure of rats to melamine and cyanuric acid leads to alterations in the expression of the genes encoding kidney injury molecule 1 (KIM-1), metalloproteinase inhibitor 1 (TIMP1), clusterin, osteopontin, and neutrophil gelatinase-associated lipocalin/lipocalin 2 (NGAL), which have been proposed as urinary biomarkers for nephrotoxicity.

Wen *et al.* (2010) reported that the basic treatment regimens for crystalluria and urolithiasis related to melamine ingestion include fluid therapy and supportive care in both veterinary and pediatric patients. Low urinary pH is associated with crystal formation in infants so, alkalization of the urine was used to maintain urine pH between 6.0 and 7.8 in affected children. Sodium bicarbonate or potassium citrate was added to intravenous fluids for this purpose (Gao *et al.*, 2010 and Wen *et al.*, 2010). Antispasmodic drugs such as anisodamine or atropine were given to facilitate excretion of uroliths in children, and pain management was instituted (Bhalla *et al.*, 2009 and Wen *et al.*, 2010). Most children recovered with this conservative management (Gao *et al.*, 2010 and Wen *et al.*, 2010). However, hemodialysis was required in some patients, as was surgical intervention (Wen *et al.*, 2010). Most children recovered fully, but 12% were found to have renal abnormalities 6 months after treatment (Liu *et al.*, 2010).

In the current study, the biochemical and histopathological changes resulted from the toxic effect of different melamine doses (for 28 days) on male rats were studied.

## 2. Materials and Methods

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 as follows: i. G1 were supplemented with 5,000 ppm, ii. G2 were supplemented with 10,000 ppm, iii. G3 were supplemented with 15,000 ppm and iv. 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 that lasted 28 days. Necropsies were performed on all animals at the end of the study. All rats were dissected and the target organs for histopathological investigations were kept in 10% formalin.

### Biochemical analysis

Serum electrolytes (Na, K, Cl, Ca and P) and kidney functions indices (creatinine, urea and uric acid) 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.

Serum melamine concentration was estimated using GC-MSD 5975 series from Agilent Technologies, USA according to the instruction of the suppliers.

### Body weight gain, food efficiency and consumed water

The water consumed was calculated for each group. Body weight per cage was recorded once per week. The mean body weight of each group was calculated by dividing the total weight of all surviving animals by the number of surviving animals in the group. Food intake per cage was recorded once per week. Weight gain (g), body weight gain ratio (BWG%) and food efficiency ratio (FER) were calculated as follows:

Weight Gain= Final weight (g) - Initial weight (g)

BWG%=Final weight-initial weight/initial weight X 100

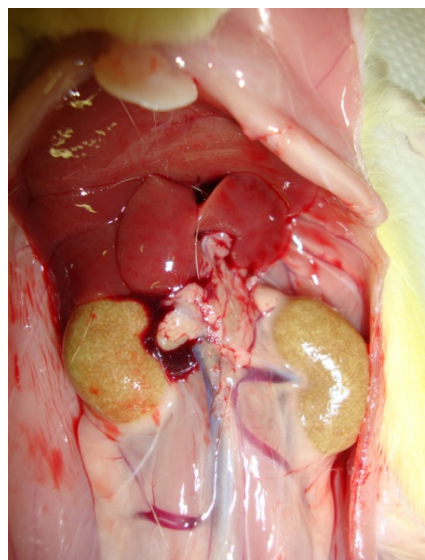
FER= Weight (g)/food intake (g).

### Histopathological investigations

The target organs (kidney, ureter, urinary bladder and testis) 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.

**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****Effect of melamine supplementation on the morphology and anatomy****A****B**

**Figure (1):** Effect of melamine supplementation for 28 days on the morphology and anatomy of rats; **A;** melamine fed rat for 28 days (yellow colored, dose 20,000 ppm), **B;** Dissected melamine fed rat as in A.

**Serum electrolytes**

Table (1), shows the effect of melamine supplementation for 28 days on serum electrolytes; Na, K, CA, CL, and P. The mean values of Na in all

melamine supplemented groups (G1, G2, G3 and G4) were non significantly lower than that of the negative control ( $138.20 \pm 1.39$ ,  $136.60 \pm 1.88$ ,  $137.80 \pm 1.95$ ,  $136.40 \pm 1.43$  and  $141 \pm 0.82$  mmol/L, respectively).

**Table (1):** Effect of melamine supplementation for 28 days on serum electrolytes (Na, K, CL, CA and P).

| Parameter    | Treatments<br>Statistics | (-)ve Control<br>(basal diet) | G1<br>5,000 ppm<br>melamine | G2<br>10,000 ppm<br>melamine | G3<br>15,000 ppm<br>melamine | G4<br>20,000 ppm<br>melamine |
|--------------|--------------------------|-------------------------------|-----------------------------|------------------------------|------------------------------|------------------------------|
|              |                          | Na<br>mmol/L                  | Mean±SE                     | 141±0.82                     | 138.20±1.39                  | 136.60±1.88                  |
|              | T-test                   |                               | 1.15 NS                     | 2.08 NS                      | 0.91 NS                      | 1.93 NS                      |
| K<br>mmol/L  | Mean±SE                  | 4.84±0.12                     | 5.56±0.21                   | 5.12±0.17                    | 5.26±0.25                    | 5.54±0.34                    |
|              | T-test                   |                               | -2.29*                      | -2.74*                       | -1.36*                       | -1.73*                       |
| Cl<br>mmol/L | Mean±SE                  | 97.21±0.47                    | 92.20±1.98                  | 84.60±1.96                   | 89.00±1.94                   | 88.80±2.22                   |
|              | T-test                   |                               | 2.21*                       | 6.10***                      | 3.80**                       | 3.41**                       |
| Ca<br>mmol/L | Mean±SE                  | 2.57±0.03                     | 2.63±0.04                   | 2.70±0.15                    | 2.88±0.09                    | 2.93±0.16                    |
|              | T-test                   |                               | -0.95 NS                    | -2.03 *                      | -3.39**                      | -0.30 **                     |
| P<br>mmol/L  | Mean±SE                  | 2.72±0.08                     | 2.84±0.05                   | 2.93±0.88                    | 3.31±0.23                    | 4.76±0.67                    |
|              | T-test                   |                               | -3.70*                      | -3.67*                       | -1.94**                      | -3.27**                      |

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 K in all melamine supplemented groups (G1, G2, G3 and G4) were significantly higher at 1% ( $P < 0.01$ ), than that of the negative control ( $5.56 \pm 0.21$ ,  $5.12 \pm 0.17$ ,  $5.26 \pm 0.25$ ,  $5.54 \pm 0.34$  and  $4.84 \pm 0.12$  mmol/L, respectively).

The mean values for Cl in all melamine supplemented groups (G1, G2, G3 and G4) were lower than that of the negative control ( $92.20 \pm 1.98$ ,  $84.60 \pm 1.96$ ,  $89.00 \pm 1.94$ ,  $88.80 \pm 2.22$ ,  $90.00 \pm 1.14$  and  $97.21 \pm 0.47$  mmol/L, respectively). The differences were significant at 5% ( $P < 0.05$ ) in G1, high significant at 1% ( $P < 0.01$ ) in G3 and G4 and very high significant at 0.1% ( $P < 0.001$ ) in G2.

The mean values of serum Ca in all melamine supplemented groups (G1, G2, G3 and G4) were higher than that of the negative control ( $2.63 \pm 0.04$ ,  $2.70 \pm 0.11$ ,  $2.88 \pm 0.09$ ,  $2.93 \pm 0.16$  and  $2.57 \pm 0.03$  mmol/L, respectively). Differences were significant at 5% ( $P < 0.05$ ) in G1 and G2 and highly significant at 1% ( $P < 0.01$ ) G3 and G4, when compared to the negative control.

The mean values of serum P in all melamine supplemented groups (G1, G2, G3 and G4) were higher than that of the negative control ( $2.84 \pm 0.05$  and  $2.93 \pm 0.88$ ,  $3.31 \pm 0.23$ ,  $4.76 \pm 0.67$  and  $2.72 \pm 0.08$  mmol/L, respectively). Differences were non significant in G1, significant at 5% ( $P < 0.05$ ) in G2 and highly significant at 1% ( $P < 0.01$ ) in G3 and G4.

#### Kidney Functions

Table (2), shows the effect of melamine supplementation for 28 days on kidney function indices; creatinine, urea and uric acid. As shown, the mean values of serum creatinine in all melamine supplemented groups (G1, G2, G3 and G4) were higher than that of the negative control ( $141.80 \pm 11.57$ ,  $189.60 \pm 20.80$ ,  $221.80 \pm 22.33$ ,  $313.80 \pm 47.21$  and  $43.01 \pm 2.51$  umol/L, respectively). Creatinine levels were proportionally increased with the increase in the melamine dose. However, differences were very high significant at 0.1% ( $P < 0.001$ ).

**Table (2): Effect of melamine supplementation for 28 days on serum creatinine, urea and uric acid.**

| Parameters        | Treatments | (-)ve Control (basal diet) | G1 5,000ppm melamine | G2 10,000 ppm melamine | G3 15,000 ppm melamine | G4 20,000 ppm melamine |
|-------------------|------------|----------------------------|----------------------|------------------------|------------------------|------------------------|
|                   | Statistics |                            |                      |                        |                        |                        |
| Creatinine umol/L | Mean±SE    | 43.01±2.51                 | 141.80±11.57         | 189.60±20.80           | 221.80±22.33           | 313.80±47.21           |
|                   | T-test     |                            | -9.16***             | -7.46***               | -8.36***               | -5.53***               |
| Urea umol/L       | Mean±SE    | 43.32±1.55                 | 45.80±9.97           | 48.20±1.93             | 51.80±3.24             | 56.00±1.51             |
|                   | T-test     |                            | -0.84*               | -5.51**                | -2.21***               | -11.25***              |
| Uric acid mg/dl   | Mean±SE    | 1.23±0.05                  | 1.30±0.07            | 1.42±0.93              | 1.50±0.24              | 1.61±0.16              |
|                   | T-test     |                            | -1.04 NS             | -1.51*                 | -2.21**                | -4.58**                |

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 same table shows that the mean values of serum urea in all melamine supplemented group (G1, G2, G3 and G4) were higher than that of the negative control ( $45.80 \pm 9.97$ ,  $48.20 \pm 1.93$ ,  $51.80 \pm 3.24$ ,  $56.00 \pm 1.51$  and  $43.32 \pm 1.55$  umol/L, respectively). Differences were significant at 5% ( $P < 0.05$ ) in G1 and highly significant at 1% ( $P < 0.01$ ) in G2 and very high significant at 0.1% ( $P < 0.001$ ) in G3 and G4.

The mean values of serum uric acid in all melamine supplemented group (G1, G2, G3 and G4) were higher than that of the negative control ( $1.30 \pm 0.07$ ,  $1.42 \pm 0.93$ ,  $1.50 \pm 0.24$ ,  $1.61 \pm 0.16$  and

$1.23 \pm 0.05$  mg/dl, respectively). Differences were significant at 5% ( $P < 0.05$ ) in G1 and G2 and highly significant at 1% ( $P < 0.01$ ) in G3 and G4, when compared to the negative control.

#### Serum melamine concentration

Table (3), show mean values of melamine content in serum after 28 days of melamine supplementation in all groups (G1, G2, G3 and G4). They were  $6.70 \pm 1.08$ ,  $7.48 \pm 1.96$ ,  $13.60 \pm 6.35$  and  $19.13 \pm 8.21$  mg/ml and the negative control was zero. However, differences were very high significant when compared to the negative control.

**Table (3): Melamine concentration in the serum.**

| Parameters     | Treatments | (-)ve Control (basal diet) | G1 5,000 ppm melamine | G2 10,000 ppm melamine | G3 15,000 ppm melamine | G4 20,000 ppm melamine |
|----------------|------------|----------------------------|-----------------------|------------------------|------------------------|------------------------|
|                | Statistics |                            |                       |                        |                        |                        |
| Melamine mg/kg | Mean±SE    | 0.00±0.00                  | 6.70±1.08             | 7.48±1.96              | 13.60±6.35             | 19.13±8.21             |
|                | T-test     |                            | -6.19***              | -3.80***               | -2.14***               | -2.32***               |

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.

**Total body weight**

Table (4), shows the effect of melamine supplementation for 28 days on the total body weight in rats. As shown, the mean values of the first weight (after one week) of all melamine supplemented

groups (G1, G2, G3 and G4) were non significantly lower than that of the negative control. (215.56±12.03, 220.82±6.42, 227.02±7.81, 216.33±8.24 and 228.75±12.98 g, respectively).

**Table (4): Effect of melamine supplementation for 28 days on total body weight.**

| Weight (g)             | Treatments | Control (basal diet) | G1 5,000 ppm melamine | G2 10,000 ppm melamine | G3 15,000 ppm melamine | G4 20,000 ppm melamine |
|------------------------|------------|----------------------|-----------------------|------------------------|------------------------|------------------------|
|                        | Statistics |                      |                       |                        |                        |                        |
| 1 <sup>st</sup> weight | Mean±SE    | 228.75±12.98         | 215.56±12.03          | 220.82±6.42            | 227.02±7.81            | 216.33±8.24            |
|                        | T-test     |                      | 1.64 NS               | 0.76 NS                | 0.15 NS                | 1.03 NS                |
| 2 <sup>nd</sup> weight | Mean±SE    | 263.06±13.59         | 249.93±10.82          | 253.88±6.09            | 255.51±6.81            | 250.62±8.99            |
|                        | T-test     |                      | 1.75 NS               | 0.76 NS                | 0.69 NS                | 0.95 NS                |
| 3 <sup>rd</sup> weight | Mean±SE    | 288.87±11.58         | 273.56±10.35          | 253.88±6.09            | 286.85±8.39            | 275.33±5.76            |
|                        | T-test     |                      | 1.31 NS               | 3.16**                 | 0.22 NS                | 1.17 NS                |
| 4 <sup>th</sup> weight | Mean±SE    | 310.45±13.74         | 267.13±5.95           | 277.13±5.04            | 287.79±7.99            | 275.93±4.32            |
|                        | T-test     |                      | 3.39**                | 2.08 NS                | 1.75 NS                | 3.12**                 |

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 the second weight (after two weeks) of G1, G2, G3 and G4 were non significantly lower than that of the negative control (249.93±10.82, 253.88±6.09, 255.51±6.81, 250.62±8.99 and 263.06±13.59 g, respectively).

The mean values of the third weight (after three weeks) G1, G3 and G4 were lower than that of the negative control (273.56±10.35, 253.88±6.09, 286.85±8.39, 275.33±5.76 and 288.87±11.58 g, respectively). However, the paired T-test did not show any significant differences in all groups except for G2 that was high significant at 1% ( $P < 0.01$ ) when compared to the negative control.

The mean values of the fourth weight (after four weeks and at the end of the experiment) of all melamine supplemented groups (G1, G2, G3 and G4)

were lower than that of the negative control (267.13±5.95, 277.13±5.04, 287.79±7.99, 275.93±4.32 and 310.45±13.74 g, respectively). However, differences were not significant in G2 and G3 and highly significance at 1% ( $P < 0.01$ ) in G1 and G4 when compared to the negative control.

**Food intake**

Table (5), show the effect of melamine supplementation for 28 days on food intake in rats under study. As shown, the mean values of the food intake in the first week of G1 were non significantly lower than that of the negative control (147.80±13.42, and 151.24±7.95 g, respectively), whereas that of G2, G3 and G4 was higher than that of the negative control (160.70±11.55, 155.35±9.10, 155.90±9.06 and 151.26±7.93 g, respectively).

**Table (5): Effect of melamine supplementation for 28 days on food intake.**

| Food Intake g | Treatments | (-)ve Control (basal diet) | G1 5,000 ppm melamine | G2 10,000 ppm melamine | G3 15,000 ppm melamine | G4 20,000 ppm melamine |
|---------------|------------|----------------------------|-----------------------|------------------------|------------------------|------------------------|
|               | Statistics |                            |                       |                        |                        |                        |
| 1st week      | Mean±SE    | 151.26±7.93                | 147.80±13.42          | 160.70±11.55           | 155.35±9.10            | 155.90±9.06            |
|               | T-test     |                            | 0.35                  | -0.90                  | -0.44                  | -0.53                  |
| 2nd week      | Mean±SE    | 116.15±10.62               | 160.88±3.51           | 172.07±5.62            | 187.18±2.98            | 197.85±12.00           |
|               | T-test     |                            | -4.54***              | -6.27***               | -8.31***               | -4.65***               |
| 3rd week      | Mean±SE    | 116.47±10.60               | 137.88±7.95           | 159.42±3.75            | 160.95±4.86            | 169.85±9.55            |
|               | T-test     |                            | -1.79***              | -4.67***               | -5.33***               | -6.09 NS               |
| 4th week      | Mean±SE    | 133.43±5.42                | 105.26±5.99           | 72.43±4.80             | 109.70±10.87           | 79.76±10.56            |
|               | T-test     |                            | 3.30**                | 6.85***                | 1.78 NS                | 3.67**                 |

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 food intake of all groups (G1, G2, G3 and G4) in the second week were higher than that of the negative control (160.88±3.51, 172.07±5.62, 187.18±2.98, 197.85±12.00 and 116.15±10.62 g, respectively). However, differences were very high significant at 0.1% ( $P < 0.001$ ) in all groups, when compared to the negative control.

The mean values of food intake of all groups (G1, G2, G3 and G4) in the third week were higher than that of the negative control (137.88±7.95, 159.42±3.75, 160.95±4.86, 169.85±9.55 and 116.47±10.60 g, respectively). However, differences were very high significant at 0.1% ( $P < 0.001$ ) in G1, G2 and G3 and non significant in G4.

The mean values of food intake of all groups (G1, G2, G3 and G4) in the fourth week were lower than that of the negative control (105.26±5.99,

72.43±4.80, 109.70±10.87, 79.76±10.56 and 133.43±5.42 g, respectively). However, differences were non significant in G3, highly significant at 1% ( $P < 0.01$ ) in G1 and G4 and very high significant at 0.1% ( $P < 0.001$ ) in G2, when compared to the negative control.

#### Food efficiency ratio, body weight gain and percentage of body weight gain

Table (6), show the effect of melamine supplementation for 28 days on food efficiency ratio (FER) and body weight gain body (g) weight gain % (BWG %) in rats under study. As shown, the mean value of weight gain of G1, G2, G3 and G4 were less than that of the negative control (49.77±9.23, 56.30±3.41, 60.77±1.79, 59.59±8.95 and 78.14±5.35 g, respectively), the differences were high significant at ( $P < 0.01$ ) in G1, G2, G3 and G4.

**Table (6): Effect of melamine supplementation for 28 days on food efficiency ratio (FER) and body weight gain body (g) weight gain % (BWG %) in rats under study.**

| Parameters         | Treatments | (-)ve Control<br>(basal diet) | G1<br>5,000 ppm<br>melamine | G2<br>10,000 ppm<br>melamine | G3<br>15,000 ppm<br>melamine | G4<br>20,000 ppm<br>melamine |
|--------------------|------------|-------------------------------|-----------------------------|------------------------------|------------------------------|------------------------------|
|                    | Statistics |                               |                             |                              |                              |                              |
| Weight Gain<br>(g) | Mean±SE    | 78.14±5.35                    | 49.77±9.23                  | 56.30±3.41                   | 60.77±1.79                   | 59.59±8.95                   |
|                    | T-test     |                               | 3.25**                      | 3.30**                       | 4.22**                       | 3.97**                       |
| BWG%               | Mean±SE    | 34.85±3.57                    | 24.17±5.51                  | 25.68±2.04                   | 26.88±1.20                   | 28.18±4.82                   |
|                    | T-test     |                               | 2.90**                      | 3.12**                       | 3.04**                       | 1.76 NS                      |
| FER<br>G           | Mean±SE    | 0.106±.009                    | 0.054±.012                  | 0.079±.018                   | 0.068±.006                   | 0.079±.020                   |
|                    | T-test     |                               | 3.90*                       | 1.65 NS                      | 5.77***                      | 1.50 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.

The same table shows the mean values of BWG% of all groups (G1, G2, G3, G4) were less than that of the negative control (24.17±5.51, 25.68±2.04, 26.88±1.20, 28.18±4.82, and 34.85±3.57%, respectively). The differences were non significant in G4, high significant at 1% ( $P < 0.01$ ) in G1, G2 and G3, when compared to negative control.

Concerning the FER, the mean FER values in all groups (G1, G2, G3 and G4) were less than that of the negative control (0.054±0.012, 0.079±.018, 0.068±.006, 0.079±.020 and 0.106±.009 g, respectively). However, differences were non significant in G2 and G4, significant in G1 at ( $P < 0.05$ ) and very high significant ( $P < 0.001$ ) in G3, when compared to negative control.

#### Water consumption

Table (7), shows the effect of melamine supplementation for 28 days on the amount of water consumed in rats under study. As shown, the mean values of water consumed in the 1<sup>st</sup> week of G1 were higher than that of the negative control (202.14±8.58 and 192.83±8.90 ml, respectively). Whereas the mean values of G2, G3 and G4 were lower than that of the negative control (184.28±6.49, 182.85±8.92, 174.28±7.51 and 192.83±8.90 ml, respectively).

However, differences were non significant in G1, G2 and G3 when compared to the negative control, whereas differences in G4 were highly significant at 1% ( $P < 0.01$ ).

The mean values of the water consumed in the second week in G2 and G4 were lower than that of the negative control (180.00±8.99, 174.28±11.92, and 185.14±9.46 ml, respectively), whereas that of G3 was equal to that of the negative control (187.14±8.370 and 185.14±9.46 ml, respectively). Moreover, the mean values of the water consumed in the second week in G1 were higher than that of the negative control (195.71±13.77 and 185.14±9.46 ml, respectively). However, the paired T-test did not show any significant differences between all groups and the negative control.

The mean values of the water consumed in the third week in G3 were lower than that of the negative control (194.28±7.19 and 196.71±2.95 ml, respectively), whereas that of all other groups (G1, G2 and G4) were higher than that of the negative control (208.57±9.36, 198.57±1.42, 210.00±2.97, 214.28±7.19 and 196.71±2.95 ml, respectively). However, differences were non significant in G1, G2

and G3 and highly significant at 1% ( $P < 0.01$ ) in G4, when compared to the negative control.

The mean values of water consumed in the fourth week of G2, G4 were lower than that of the negative control ( $166.66 \pm 7.76$ ,  $176.66 \pm 6.42$  and  $206.61 \pm 11.57$  ml, respectively), whereas that of G1 and G3 were higher than that of the negative control

( $166.66 \pm 7.76$ ,  $210.00 \pm 6.17$  and  $206.61 \pm 11.57$  ml, respectively). However, differences were non significant in G3, highly significant at 1% ( $P < 0.01$ ) in G2 and G4, and very high significant at 0.1% ( $P < 0.001$ ) in G1, when compared to the negative control.

**Table (7): Effect of melamine supplementation for 28 days on the amount of water consumed.**

| water consumed ml    | Treatments | (-)ve Control (basal diet) | G1 5,000 ppm melamine | G2 10,000 ppm melamine | G3 15,000 ppm melamine | G4 20,000 ppm melamine |
|----------------------|------------|----------------------------|-----------------------|------------------------|------------------------|------------------------|
|                      | Statistics |                            |                       |                        |                        |                        |
| 1 <sup>st</sup> week | Mean±SE    | 192.83±8.90                | 202.14±8.58           | 184.28±6.49            | 182.85±8.92            | 174.28±7.51            |
|                      | T-test     |                            | -1.11 NS              | 1.27 NS                | 0.95 NS                | 3.35**                 |
| 2 <sup>nd</sup> week | Mean±SE    | 185.14±9.46                | 195.71±13.77          | 180.00±8.99            | 187.14±8.370           | 174.28±11.92           |
|                      | T-test     |                            | -0.82 NS              | 0.65 NS                | 0.00 NS                | 1.32 NS                |
| 3 <sup>rd</sup> week | Mean±SE    | 196.71±2.95                | 208.57±9.36           | 198.57±1.42            | 194.28±7.19            | 210.00±2.97            |
|                      | T-test     |                            | -1.59 NS              | -1.00 NS               | 0.19 NS                | -2.70**                |
| 4 <sup>th</sup> week | Mean±SE    | 206.61±11.57               | 273.33±10.83          | 166.66±7.76            | 210.00±6.17            | 176.66±6.42            |
|                      | T-test     |                            | -8.09***              | 3.69**                 | -0.16 NS               | 3.35**                 |

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.

### Histopathological studies

The target organs (kidney, ureter and urinary bladder) tissues 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.

#### Kidney

The histological examination of kidney sections of control animals (Figure 2, A) shows normal renal tissues and normal uriniferous tubules and glomeruli in untreated rat group.

The rat kidneys of groups which were regularly supplemented with 5,000 and 10,000 ppm melamine, respectively, showed tissues with tubular epithelial damage, capillary proliferation (Figure 2, B & C). Kidney of rats which were regularly supplied by 15,000 ppm shows certain degenerated uriniferous tubules and dilatation of Bowman's capsule (Figure 2, D). On the other hand, the histological examination of kidney sections of highly concentrated doses animal groups which were regularly supplied by 20,000 ppm melamine, show highly degenerated renal tissues and accumulation of salt particles in uriniferous and collecting tubules and glomeruli (Figure 2, E).

Histological diagnosis of melamine-associated renal failure based on renal crystal characteristics. (A) Dilated distal rat tubule contains clusters of round green melamine crystals with radiating spokes and concentric striations (arrow). Surrounding proximal tubules appear unaffected. (B)

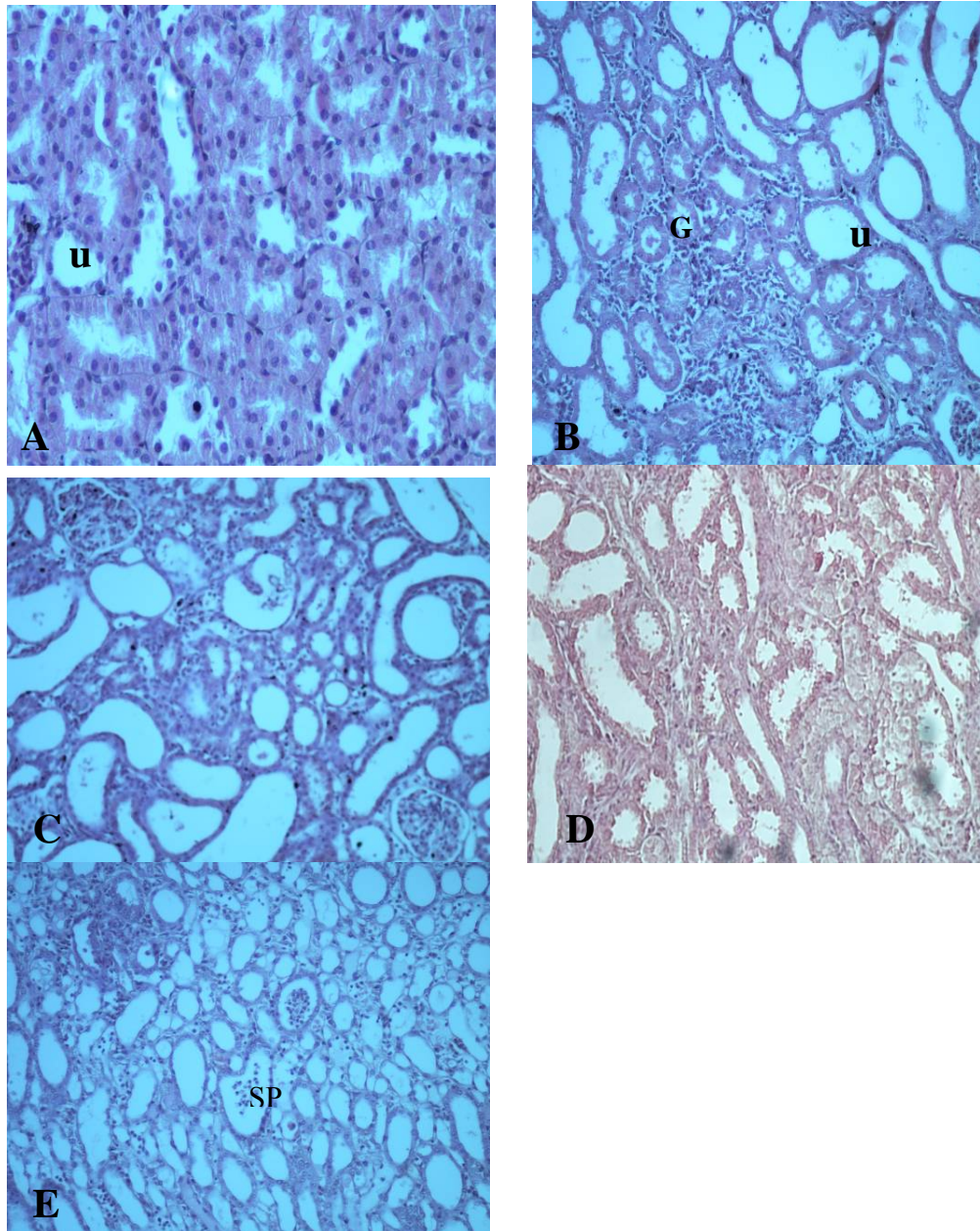
Dilated distal rat tubule contains fragmented or globular dense green melamine crystals (long arrows). Note attenuation of the lining epithelium with wide separation of nuclei (short arrow) and mitotic figure (arrowhead) indicative of tubular epithelial necrosis and regeneration as seen in Figure (2).

#### Ureter

The normal structure of the ureter of the rat is well represented in control group (Figure 3, A). The epithelium (E) is three to four cells deep with cuboidal or columnar basal cells, intermediate cells, and superficial squamous cells. The basal cells are attached by half desmosomes (D), or attachment plates, on their basal membranes to a basement membrane which separates the epithelium from the lamina propria (LP). Fine extracellular fibers, ca. 100 Å in diameters, are to be found in the connective tissue layer (CT) immediately below the basement membrane of this epithelium. The rat ureters of in Figure (3, B & C) which were regularly supplied by 5,000 and 10,000 ppm melamine, respectively, showed tissues with tubular epithelial damage. The plasma membranes of the basal and intermediate cells and the lateral and basal membranes of the squamous cells are deeply interdigitated, and melamine toxicity is associated with them. All the cells have a dense feltwork of tonofilaments which ramify throughout the cytoplasm (Figure 3, B & C). The histological architecture of ureter sections of the rats supplemented with moderately concentrated material

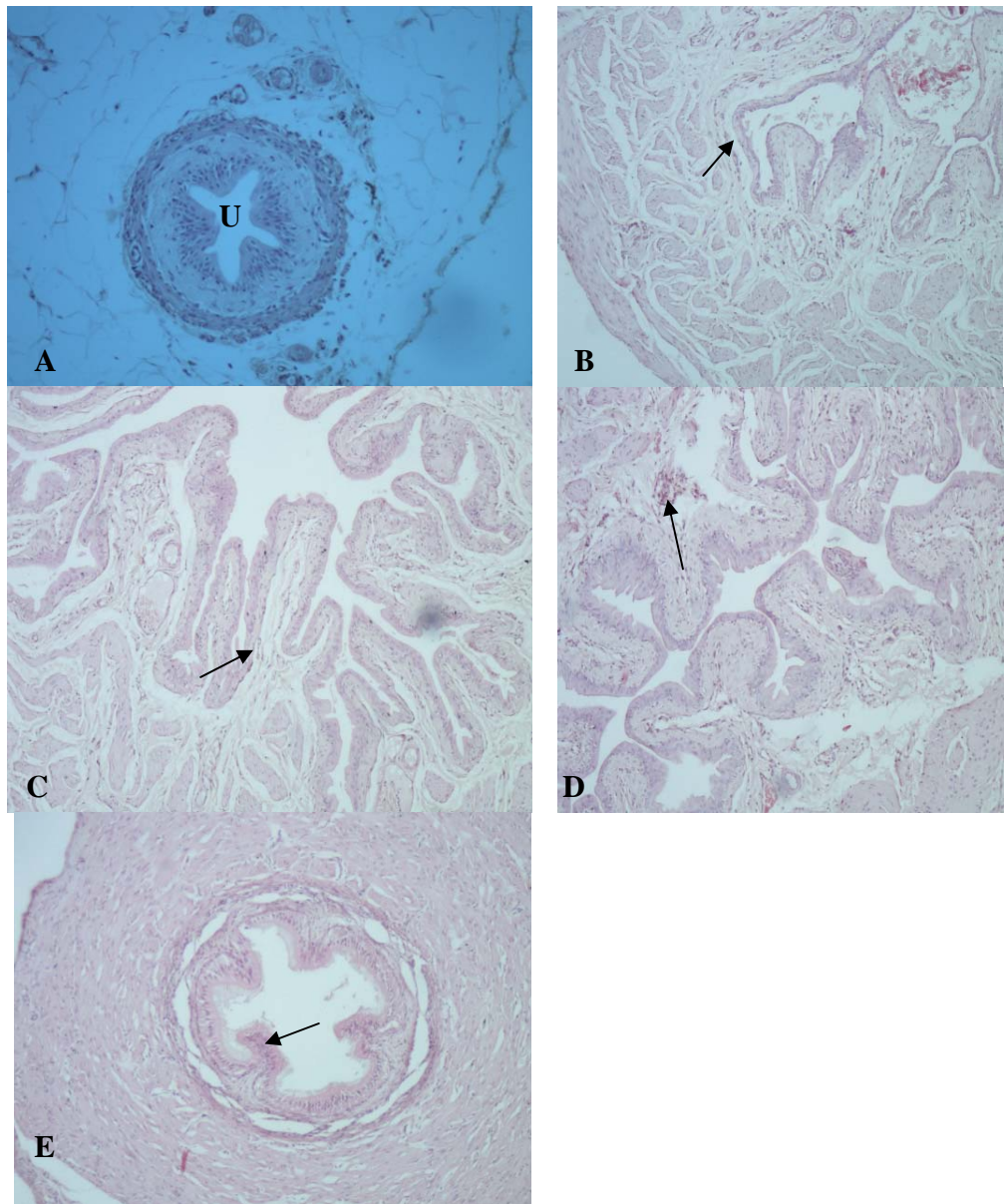
of melamine (15,000 and 20,000 ppm) showed as in legated blood vessels, an increase in modified muscle cells with accompanying increase in connective tissue

(particularly proximally to the ligature) occurs in the ureter (Figure 3, D & E).



**Figure (2):** **A;** renal tissues of control group showing normal renal structure with regulated nuclear arrangement of urineferous tubules (u), **B:** Renal tissues of 5,000 ppm melamine fed group, showing convoluted urineferous tubules (u) and glomeruli (G), **C:** renal tissues of 10,000 ppm melamine fed group, showing certain degenerated urineferous tubules, **D:** renal tissues of 15,000 ppm melamine fed group, showing dilatation of Bowman's capsule, **E:** renal tissues of 20,000 ppm melamine fed group showing highly degenerated renal tissues and accumulation of salt particles in uriniferous (SP) X 200 (H&E stains).



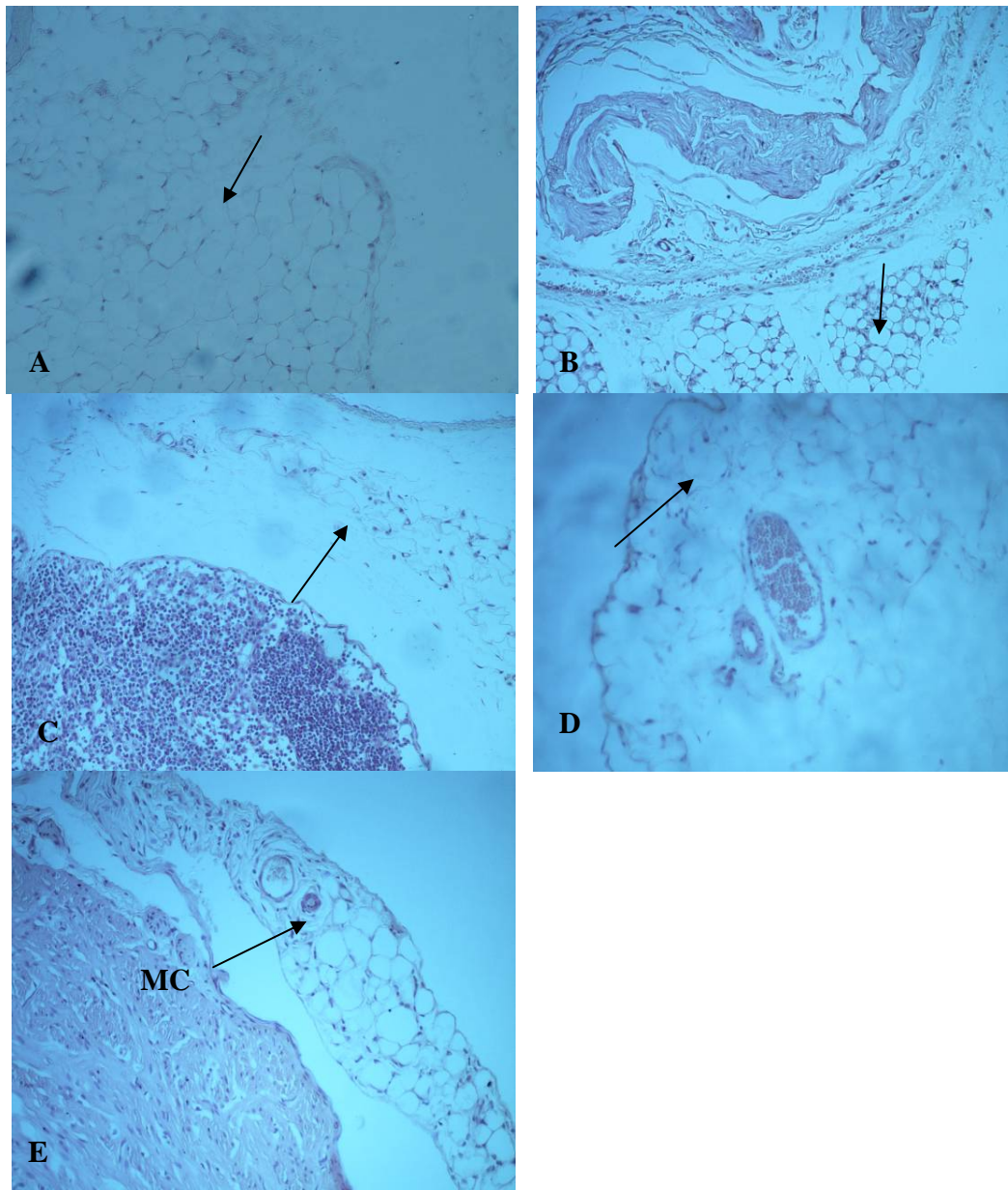


**Figure (3):** **A;** Ureter tissues of control group showing normal cuboidal or columnar basal cells, intermediate cells (U), and superficial squamous cells, **B;** ureter tissues of 5,000 ppm melamine fed group showing tissues with tubular epithelial damage (arrow), **C;** ureter tissues of 10,000 ppm melamine fed group showing plasma membranes of the basal and intermediate cells and the lateral and basal membranes of the squamous cells are deeply interdigitated (arrow), **D;** ureter tissues of 15,000 ppm melamine fed group, showing the cells have a dense feltwork of tonofilaments (arrow), **E;** ureter tissues of 20,000 ppm melamine fed group showing highly increase in modified muscle cells (arrow). X 200 (H&E stains).

#### Urinary bladder

Bladder tissues of controlled rats showed epithelium (E) is three to four cells deep with columnar basal cells, intermediate cells, and superficial squamous cells (Figure 4, A). The rat groups treated with melamine in a two-generation dose-response study in which 5,000 and 10,000 ppm in the diet had been identified as the no- high effect

level for primary bladder tissues with somewhat atretic tissues (Figure 4, B & C). Melamine crystals increase in the incidences of hyperplasia was found at the 15,000 and 20,000 ppm melamine doses (the high dietary levels tested). There were also compound-related increases in the incidence of transitional-cell papillomas (Figure 4, D & E).



**Figure (4):** **A;** Normal bladder tissues showing epithelium (arrow); three to four cells deep with columnar basal cells, **B;** bladder tissues of 5,000 ppm melamine fed group showing somewhat atretic tissues (arrow), **C;** bladder tissues of 10,000 ppm melamine fed group showing mild atretic tissues (arrow), **D;** bladder tissues of 15,000 ppm melamine group showing increase in the incidences of hyperplasia (arrow), **E;** bladder tissues of 20,000 ppm melamine group showing highly degenerated bladder tissues and accumulation of granules in bladder tissues (arrow). X 200 (H&E stains).

#### 4. Discussion

The toxic effect of oral administration of four melamine doses (5,000, 10,000, 15,000 and 20,000 ppm) for 28 days was investigated in male rats. Serum electrolytes, kidney functions indices, melamine concentration in the serum, as well as, the histopathology of kidney, ureters and urinary bladder were measured in order to investigate the toxic effect

of melamine on the kidney. Some other nutritional parameters were also tested.

The color of melamine supplemented rats turned yellow by increasing the time of melamine supplementation, compared to the normal white colored. In addition, the dissected melamine supplemented rats showed congestion of organs and yellowish color of kidneys, as a result of melamine toxicity. This result is consistent with that of Chen *et*

*al.* (2009) who noted that high doses of melamine supplementation causes yellow color in kidney due to the numerous melamine crystals of various sizes found mixed with the necrotic cell debris in both proximal and distal renal tubules. Slight to severe inflammatory cell infiltration was accompanied by renal tubular dilation and epithelial cell regeneration with interstitial fibrosis.

The mean values of Na in the melamine treated rats were non significantly lower than that of the negative control. This result is consistent with that of Jeong *et al.* (2006) and Chen *et al.* (2009), whereas the mean values of K in the melamine treated group were non significantly higher than that of the negative control. This result is consistent with other results (Puschner *et al.*, 2007) in cats. The mean values for Cl in the melamine supplemented group were significantly very low compared to the negative control. This result is consistent with that of Jeong *et al.* (2006) and Chen *et al.* (2009). The mean values of Ca in most treated groups were higher than that of the negative. Differences were non significant. This result is consistent with a similar one obtained by (Puschner *et al.*, 2007) in cats. The mean values of P in the melamine supplemented group were very high significantly more than that of the negative control. This result is consistent with that of Jeong *et al.* (2006), Puschner *et al.* (2007) and Chen *et al.* (2009).

The present results could be correlated with cell membrane damage which leads to disturbances in Na<sup>+</sup> and K<sup>+</sup> pumping and disorders in membrane permeability (Ganong, 1999 and El-Missiry *et al.*, 2001). The decrease in the mean values of serum sodium levels in the melamine fed rats for 28 days compared to that of the control is consistent with other findings observed by Jeong *et al.* (2006). They observed decrease in sodium levels after feeding the dogs and cats with commercial melamine contaminated diets for about one month. In contrast, serum potassium levels were significantly increased after treatment with melamine for 28 days compared to the control. This result is in agreement with other study that showed elevated serum potassium in melamine contaminated infants (Sun *et al.*, 2010).

The melamine toxicity resulted from supplementing the rats under experiment with melamine for 28 days, has greatly affected kidney function as revealed by the very high significant elevation of the mean values of serum creatinine, uric acid and urea in all treated groups than that of the negative control. The current results are consistent with that of Jeong *et al.* (2006), Tusing (2008), Kim *et al.* (2010) and Schnackenberg *et al.* (2012). Puschner *et al.* (2007) has got similar results in cats.

Melamine concentration in the serum was increased by the increase of the melamine dose. This

result is consistent with that of Wu *et al.* (2009) and Ding *et al.* (2012).

The current results showed that the effect of melamine supplementation for the first 14 days, on the total body weight was non significant when compared the negative control. Whereas, the third weight (after three weeks) showed non significant decrease in the body weight compared to the negative control. This result is in agreement with Chen *et al.* (2009). On the other hand, the mean values of body weight was affected greatly after four weeks that showed high significant differences at 1% ( $P < 0.01$ ), when compared to the negative control.

In spite of the non significant increase in the mean values of food intake the first week of melamine supplementation, they were very significantly increased in the second week. The non significant increase in the mean values of food intake in the third week was faced with very high significant decrease in the fourth week. This result is in agreement with that of Dobson *et al.* (2008) and Cianciolo *et al.* (2008). The reduction in food intake in the fourth week is due to the toxicity of melamine resulted from the longer period of melamine supplementation.

The mean values of weight gain (g), BWG% and FER were less than that of the negative control. However, differences for weight gain were very high significant at ( $P < 0.001$ ). This result is consistent with that of Dobson *et al.* (2008).

The high significant increase in the consumed water in the first and the third weeks was faced with non significant increase in the second week. In the fourth week, the mean values of the consumed water were very high significantly lower than that of the negative control.

Ren *et al.* (2012) and Gao *et al.* (2012) reported that, renal tubules in melamine supplemented rats were extended and the lining epithelium cell was degenerated accompanied by testicular atrophy that was recovered three months after the end of administration, so did the reversibility of renal tubular epithelium.

In the current study, renal tissues of rats supplemented with melamine showed various dramatic pathological changes and accumulation of salt particles in most glomeruli and melamine crystals, in kidney, ureter and urinary bladder according to the melamine dose. This result agrees with the current elevated kidney functions results and with that of Tusing (2008), Hau *et al.* (2009), Chen *et al.* (2009), Kim *et al.* (2010) and Schnackenberg *et al.* (2012), who stated that histological examination revealed that renal crystals that could be observed in kidneys of animals showing signs of nephrotoxicity. Urinary bladder tissues showed dramatic pathological



changes. 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.

It could be said that the toxic effect of melamine increases with increasing of the melamine dose. In addition, melamine supplementation affected kidney, ureter and urinary bladder, as revealed by the very high significant differences of the kidney functions and the adverse histological changes of these tissues.

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