CHAPTER V

EXPERIMENT III

EFFECTS OF DIETARY MELAMINE, UREA-FORMALDEHYDE OR THEIR MIXTURES ON PERFORMANCE, EGG QUALITY, MICROSCOPIC AND HISTOPATHOLOGICAL CHANGES IN LAYING HEN TISSUES

5.1 Introduction

Melamine (1,3,5-triazine-2,4,6-triamine) is a nitrogen-containing compound used in the manufacture of amino resins, plastics, in the production of melamine-formaldehyde resins used in the manufacture of laminates, plastics and coatings, including food contact materials such as kitchenware. In addition, melamine are used as flame retardants; it is also used as a fertilizer (Anderson, 1995; Weil and Choudhary, 1995). Melamine is reacted with other starting substances, mainly formaldehyde and urea. Because of this nitrogen enrichment, the use of melamine as a non-protein nitrogen source for cattle but hydrolysis is slowly and less complete than urea (Newton and Utley, 1978). Recently, melamine contamination in foods and feeds are widespread around the world. Melamine might cause urolithiasis and bladder cancer. In 2004 and 2007, pet food containing a wheat gluten exported from China was responsible for kidney failure and dead in domesticated cats and dogs in the United States and Europe (Brown et al., 2007; Reimschuessel et al., 2008). In September 2008, renal crystals and associated renal failure were found among infants and children in China due to the consumption of the milk powder containing melamine. Moreover, egg powders were found to contain melamine at concentrations ranging from 0.1 to 4.0 μg/g, and fresh eggs were found to contain melamine at concentrations ranging from 2.9 to 4.7 μg/g in China.

Melamine is often combined with formaldehyde to produce melamine formaldehyde which is a number of amino resin family and used a wide variety of products that are still in use today (Deppe, 1982; El-Sayed, 2006). Both melamine and formaldehyde or urea-formaldehyde (UF) are known human health threats and melamine formaldehyde releases monomers of both (Ishiwata et al., 1986; Bradley et al., 2005). Many consumer products containing formaldehydes based resins release formaldehyde vapor, leading to consumer dissatisfaction and health related complaints. Urea-formaldehyde resin is the more important type of pelleting binders in both animal and aqua feeds. Chicken diets are often pelleted to improve gains or feed utilization (Lanson and Smyth, 1955; Proudfoot and Hulan, 1980). Stilborn et al. (1991) reported that urea-formaldehyde resin pellet binder can be used in poultry diets with no adverse effects on performance and blood parameters.

Previous studies showed that melamine induced crystalluria and mortality in sheep to high doses of melamine 100 g daily for 11 days (Clark, 1966). Moreover, weight losses and mortalities of sheep fed with diets containing either 9.8 or 19.6 g of melamine (MacKenzie, 1966). Research studies in domestic animals have been reported graded levels of melamine (0.50-3.00%) in poultry diets showed melamine reduced body weight gain, feed intake and high mortality. Feed conversion ratio were less efficiency (Brand et al., 2009; Ledoux et al., 2009; Lu et al., 2009a). Moreover, the kidney weight was higher when fed $\geq$ 1.00% melamine and showed pale and enlarged kidney in turkeys fed 2.00-3.00% melamine. The bile of turkeys that died contained crystals that were either microscopic (< 2 micron) in size or were large white crystals visible to the naked eye. Gross lesion in necropsied and microscopic examination revealed crystals and having enlarged pale kidneys and gallbladder containing opaque. Lu et al. (2009) reported that Cherry Valley ducks fed $\leq$ 1,000 mg/kg of diet showed no difference in weight gain. The kidney was found to accumulate the highest concentration of melamine. Gao et al. (2010) showed that melamine (1-100 ppm of diets) in laying ducks had no adverse effect on laying performance. Renal lesions and melamine residue in egg were in correlation with increasing levels of dietary melamine. Similar reported by Bai et al. (2010) found that hen fed with melamine (8.6-140.9 ppm of BW per day) contaminated diets for 34 days showed no effects on the survival, body weight gain, or egg production. The kidneys were found crystals in one of three kidneys of hens treated with melamine either doses. The distribution of melamine in hen tissues and that the highest melamine residue was in the kidney, followed by liver, muscle, ovary, uterus, and duodenum. Chen et al. (2010) studied the disposition profile of melamine in laying hens, with special focus on the transfer of melamine to eggs. Laying hens were given 0-100 ppm of feed and found melamine concentrations in eggs were 0.16-1.48 mg/kg egg. There was no evidence for a further accumulation of melamine in the egg yolk following long-term exposure and the calculated transfer rates vary between 1.5 and 3.2%. Few studies on the risk assessment of melamine or UF or equal mixture of both in domestic animals have been reported. More information on the effects of melamine or UF on domestic animals is needed because of the link between animals and public health. In this study, we investigated the effects of melamine or UF or their equal mixture of both in laying hen by evaluating the biochemical and histopathological changes caused by graded dietary levels of melamine or UF or their equal mixture of both.

5.2 Materials and methods

5.2.1 Animals, diets and experimental design

5.2.1.1 Melamine and urea-formaldehyde (UF)

Melamine (purity $\geq$ 99.5%) was obtained from Tianjin BASF Chemical Company, Tianjin, China and UF resin (water content $\leq$ 2%, pH 7.0-7.5) was obtained from Luyuan Adhesive Material Co. LTD. Guangdong, China. Melamine and UF was diluted with ground corn to a concentration of 10% and appropriate amounts of this diluted material were then mixed in basal diets (Table 5.1 and 5.2) to prepare treatment diets with graded levels of melamine or UF or equal mixture of melamine and UF.

5.2.1.2 Birds, feeding, and management

The experimental procedures were reviewed and approved by the Adviser Committee of the Animal Science Department, Khon Kaen University. The study was conducted as a completely randomized design to evaluate the effects of graded levels of melamine or UF or mixture of melamine and UF (1:1) in laying hens. Thirteen treatment diets were formulated to contain four graded levels (0.25, 0.50, 0.75 and 1.00%) of melamine or UF or their equal mixtures and no added for negative control. The negative control (Table 5.1) was formulated according to the NRC (1994) and contained 16.05% CP, 2982.35 AME/kg, 1.22% calcium, 0.39% available phosphorus, 0.88% methionine+cystine, 0.76% lysine and 0.57% threonine, the feeding trial contained of 112d (4x28d period) for 12-18 weeks. After this developer period (12-16 wk), the hens were initially fed the diets contain four graded levels (0.25, 0.50, 0.75 and 1.00%) of melamine or UF or their equal mixture and no added for negative control. The negative control (Table 5.2) was formulated according to the NRC (1994) and contained 17.46% CP, 2886.88 AME/kg, 4.19% calcium, 0.35% available phosphorus, 1.00% methionine+cystine, 0.84% lysine and 0.62% threonine, the feeding trial contained of 112d (4x28d period). Both feed and water were provided for *ad libitum* consumption.

Three hundred and twelve ISA Brown laying hens (12 and 37 weeks old) were randomly divided into thirteen groups in a completely randomized design experiment. Hens were allotted to treatments so that the mean BW for each treatment was similar. There were 4 replications for each group, and each replicate was composed of a battery of three cages with two hens in one cage (30x40 cm) in an open house system. A regimen of 17h light and 7h darkness was provided throughout the experiment.

5.2.2 Data collection and sampling procedures

5.2.2.1 Performance

Hen weight, feed consumption, feed conversion ratio and survival rate on 12 and 18 weeks of age. Egg production, egg weight and survival rate were recorded daily during experiment (19-34 and 37-52 wk). Feed consumption and feed conversion ratio (kg feed/kg egg) data were recorded every 28 days (period). Egg mass were calculated from egg production and egg weight.

5.2.2.2 Egg quality

At the end of every 28 day laying cycle for three consecutive days, internal and external egg qualities were analyzed. The specific gravity of a whole egg was measured by Archimedes's method with an instrument designed by the measurement of the egg weight in air (W_a) and in water (W_w). The specific gravity was calculated by the formula [Specific gravity = $W_a/(W_a-W_w)$] (Wells, 1968) at the same. Haugh unit was calculated with following formula where the H_A is albumen height and W_E is egg weight (Haugh unit = 100log H_{A} + 7.57- 1.7$W_E^{0.37}$) (Card and Nesheim, 1972).The shell thickness were measured from the three different parts of shell in each (equator, top and truncated edge) egg using a micrometer and was averaged and recorded as shell thickness. The values of egg yolk colour were measured by a colour scale fan (The Roche Yolk Colour Fan, 1993).

5.2.2.3 Microscopic characterization of crystals in kidney, liver, spleen and ovary

At the end experiment (34 and 52 weeks old), one chick was randomly selected from each replicate cage and euthanized by cervical dislocation, then samples of the liver, kidney, ovary and spleen were obtained and kept at -20°C until analyzed by using an inverted microscope fitted with a digital video camera.

5.2.2.4 Histological examination

At the end experimental (34 and 52 weeks old), one chick was randomly selected from each replicate cage and euthanized by cervical dislocation. After sacrifice, liver, kidney, spleen and ovary of each animal were taken for histopathological examination. Organ samples were fixed in 10% buffered neutral formalin, embedded in paraffin, cut on a microtome in slices 4-5 μm thick and stained with hematoxylin and eosine. Wet-mount sections of organs were immediately evaluated via light microscopy for appearance of crystals.

5.2.2.5 Statistical analysis

Data were analyzed using the one-way analysis of variance (ANOVA) and means were compared by Duncan's multiple-range test (SAS, 1995). Treatment means were tested for linear and quadratic effects of graded levels of either melamine or UF or their mixtures. Single degree of freedom contracts were made among treatment means. The pen was the experimental unit for all measurements.

Differences were considered significant when $P<0.05$. A nonlinear regression model was used among treatments.

Table 5.1 Composition and nutrient contents in the basal diet (12-16 wk)

Ingredients	Content (%)
Corn (7.5% CP)	60.90
Soybean meal (44% CP)	17.00
Fish meal (58% CP)	4.00
Rice bran (12% CP)	14.00
Dicalcium phosphate (P18)	1.40
Salt	0.45
Limestone	1.20
DL-Methionine	0.33
L-lysine	0.12
Choline chloride 50%	0.10
Premix[a]	0.50
Calculated chemical analysis	
ME, kcal/kg	2,982.35
Crude protein (%)	16.05
Ether extract (%)	3.43
Crude fiber (%)	5.85
Calcium (%)	1.22
Available phosphorus (%)	0.39
Methionine (%)	0.63
Methionine+cystine	0.88
Lysine (%)	0.76
Threonine (%)	0.57

[a]Provided the following per kilogram of feed: vitamin A, 14,440 IU; vitamin D_3, 2,220 IU; vitamin E, 22.2 IU; vitamin K, 3.3 mg ; vitamin B_1, 2.2 mg; vitamin B_2, 6.7 mg ; nicotinic acid, 38.9 mg; pantothenic acid, 15.6 mg; vitamin B_6, 6.7 mg; vitamin B_{12}, 0.028 mg; folic acid, 1.1 mg; manganese, 50 mg; iodine, 0.333 mg; zinc, 88.9 mg; iron, 66.7 mg; copper, 8.9 mg; selenium, 0.111 mg.

Table 5.2 Composition and nutrient contents in the basal diet (18-52 wk)

Ingredients	Content (%)
Corn	52.00
Fullfat soybean	3.50
Soybean meal	19.80
Fish meal	4.00
Rice bran	5.00
Dicalcium phosphate (P18)	1.25
Rice bran oil	3.00
Salt	0.30
Limestone	10.00
DL-Methionine	0.33
L-lysine	0.12
Choline chloride 50%	0.20
Premix[a]	0.50
Calculated chemical analysis	
ME, kcal/kg	2,886.88
Crude protein (%)	17.46
Ether extract (%)	6.77
Crude fiber (%)	3.80
Calcium (%)	4.19
Available phosphorus (%)	0.35
Methionine (%)	0.63
Methionine+cystine	1.00
Lysine (%)	0.84
Threonine (%)	0.62

[a]Provided the following per kilogram of feed: vitamin A, 14,440 IU; vitamin D_3, 2,220 IU; vitamin E, 22.2 IU; vitamin K, 3.3 mg ; vitamin B_1, 2.2 mg; vitamin B_2, 6.7 mg ; nicotinic acid, 38.9 mg; pantothenic acid, 15.6 mg; vitamin B_6, 6.7 mg; vitamin B_{12}, 0.028 mg; folic acid, 1.1 mg; manganese, 50 mg; iodine, 0.333 mg; zinc, 88.9 mg; iron, 66.7 mg; copper, 8.9 mg; selenium, 0.111 mg.

5.3 Results

5.3.1 Nutritive values of dietary

Table 5.3 shows the analyzed nutritive composition of the diets used in the experiment. Apparently, the diets at 12-18 wk had contents of moisture, crude protein, ether extract, calcium, phosphorus, ash, crude fiber and gross energy. The analyzed nutritive compositions of the diets were reasonable agreed with the calculated values.

Table 5.3 Analyzed nutritive composition of the experimental diets, during 12-18wk and 18-52 wk of age

Nutritive values (%)	12-18wk	18-52 wk
Moisture	9.34	9.91
Crude protein	16.64	17.80
Ether extract	5.53	4.36
Calcium	1.31	3.70
Phosphorous	1.59	0.70
Ash	5.79	9.79
Fiber	3.96	3.70
Gross energy (kcal/kg)	4,754	4,310

5.3.2 Production parameters

Dietary treatment had a significant ($P<0.05$) effect on BWG on developer period (12-18 wks). Hens fed melamine diets showed less BWG than those fed UF and their equal mixtures, with the greatest decreased in hens fed 1.00% melamine. However, FI, FCR and survival rate and visible signs of illness showed no adverse effects on graded levels of melamine, UF or their equal mixtures added the diets (Table 5.4). There were no effects on FI and survival rate of hens during 19-34 wks testing period. Dietary treatments showed significant ($P<0.05$) effect on egg production (EP), egg weight (EW), FCR, egg mass (EM) and number of egg. All hens fed melamine diets demonstrated less production parameters (Table 5.5). It was found that performance was linearly decreasing when levels of melamine or UF or their equal mixtures supplementation was increased, with the greatest decreased in hens fed 1.00% melamine. There was no significant difference (P>0.05) in production parameters between hens fed diets containing UF or their equal mixtures.

Table 5.6 described the effects of dietary melamine, UF or their equal mixtures on egg performance (34-52 wks). There were no difference (P>0.05) in FI and survival rate among controls and hens fed all graded levels of melamine, UF or their equal mixtures. EP decreased significantly (P<0.05) in hens fed ≥ 0.50% melamine, with the greatest decrease in EP observed in hens fed 1.00% melamine. There was no significant difference (P>0.05) in EP between hens fed diets containing UF or their equal mixtures compared with controls. Significant linear (P<0.05) effect on EP as the levels of melamine in the diets increased. FCR was significant less efficient (P<0.05) for hens fed either melamine, UF or their equal mixtures compared with controls. FCR showed less efficient linearly (P<0.05) with increasing levels of melamine and showed the greatest decreased efficiency when fed 1.00% melamine, while no such effect was observed for hens fed UF or their equal mixtures. Significant linear (P<0.05) effect on EM as the levels of melamine or UF in the diets increased. However, no significant difference (P>0.05) in EM between hens fed diets containing their equal mixtures compared with controls. Number of egg decreased linear significant (P<0.05) for hens fed either melamine or UF compared with controls.

Table 5.4 The effects of dietary melamine (M) or urea-formaldehyde (UF) or their equal mixtures on performance parameters (12-18 wk)

Treatment		Feed intake	BWG	FCR	Survival
Product	Concentration (%)	(g/d)	(g/d) 12-18 wk		
Basal	0	71.77	10.65[a]	6.763	100.0
M	0.25	71.46	10.57[abc]	6.801	100.0
M	0.50	71.79	10.28[abc]	6.992	100.0
M	0.75	71.85	9.98[bc]	7.218	100.0
M	1.00	71.52	9.91[c]	7.223	99.0
UF	0.25	71.64	10.63[ab]	6.758	100.0
UF	0.50	71.25	10.57[ab]	6.744	100.0
UF	0.75	71.76	10.35[abc]	6.949	100.0
UF	1.00	71.61	10.20[abc]	7.031	99.0
M+UF	0.25	71.61	10.54[abc]	6.801	100.0
M+UF	0.50	72.29	10.45[abc]	6.927	100.0
M+UF	0.75	72.23	10.23[abc]	7.072	100.0
M+UF	1.00	72.41	10.01[abc]	7.246	100.0
Pooled SEM		1.611	0.195	0.268	0.409
		----------Probability----------			
Product (P)		NS	NS	NS	NS
Concentration (C)		NS	**	NS	NS
P x C		NS	NS	NS	NS
Contrast					
Control vs other		NS	NS	NS	NS
L^1		NS	NS	NS	NS
Q^1		NS	NS	NS	NS
L^2		NS	NS	NS	NS
Q^2		NS	NS	NS	NS
L^3		NS	NS	NS	NS
Q^3		NS	NS	NS	NS

[a-d] Means within a column with no common superscript differ significant (P<0.05).

L^1=Linear for M., Q^1=Quadratic for M, L^2=Linear for UF, Q^2=Quadratic for UF.

L^3=Linear for M + UF, Q^3=Quadratic for M + UF.

NS=Not significant; * P<0.05; ** P<0.01.

Table 5.5 The effects of dietary melamine (M) or urea-formaldehyde (UF) or their equal mixtures on egg production parameters (19-34 wk)

Treatment		FI	Egg	Egg	FCR	Survival	Egg	Number
Product	Concentration (%)	(g/h/d)	production (%)	weight (g)	g egg / g feed	(%)	mass	of egg (egg/28d)
Basal	0	103.46	77.44^{a}	50.50^{a}	2.049^{d}	100.00	39.99^{a}	21.68^{a}
M	0.25	103.52	74.47^{b}	49.37^{bcd}	2.097^{abcd}	98.96	37.76^{c}	20.85^{b}
M	0.50	103.36	72.81^{c}	48.98^{bcd}	2.111^{abc}	98.96	36.70^{de}	20.39^{c}
M	0.75	102.89	71.58^{cd}	48.78^{cde}	2.111^{abc}	100.00	35.99^{ef}	20.04^{cd}
M	1.00	103.11	69.07^{e}	48.05^{e}	2.147^{a}	100.00	34.19^{g}	19.34^{e}
UF	0.25	103.39	76.46^{a}	49.59^{bc}	2.085^{bcd}	100.00	38.84^{b}	21.41^{a}
UF	0.50	102.83	74.53^{b}	49.14^{bcd}	2.093^{bcd}	100.00	37.64^{cd}	20.87^{b}
UF	0.75	103.56	72.74^{c}	48.95^{bcd}	2.116^{abc}	98.96	36.58^{e}	20.37^{c}
UF	1.00	103.53	70.89^{c}	48.67^{de}	2.127^{ab}	98.96	35.46^{f}	19.85^{d}
M+UF	0.25	102.49	77.19^{a}	49.69^{b}	2.063^{cd}	100.00	39.28^{ab}	21.61^{a}
M+UF	0.50	103.46	76.01^{a}	49.29^{bcd}	2.099^{abcd}	98.96	38.46^{bc}	21.28^{a}
M+UF	0.75	103.25	74.49^{b}	49.20^{bcd}	2.099^{abcd}	98.96	37.63^{cd}	20.86^{b}
M+UF	1.00	103.53	72.59^{c}	48.80^{cde}	2.122^{ab}	98.96	36.37^{ef}	20.33^{c}
Pooled SEM		0.604	0.479	0.261	0.016	0.867	0.334	0.134
		--			Probability	--		
Product (P)		NS	**	**	*	NS	**	**
Concentration (C)		NS	**	**	*	NS	**	**
P x C		NS	NS	NS	NS	NS	NS	NS
Contrast								
Control vs other		NS	**	**	*	NS	**	**
L^1		NS	**	**	NS	NS	**	**
Q^1		NS	NS	NS	NS	NS	NS	NS
L^2		NS	**	**	**	NS	**	**
Q^2		NS	NS	NS	NS	NS	NS	NS
L^3		NS	**	**	NS	NS	**	**
Q^3		NS	NS	NS	NS	NS	NS	NS

$^{a-g}$ Means within a column with no common superscript differ significant (*P*<0.05).

L^1=Linear for M., Q^1=Quadratic for M, L^2=Linear for UF, Q^2=Quadratic for UF.

L^3=Linear for M + UF, Q^3=Quadratic for M + UF.

NS=Not significant; * *P*<0.05; ** *P*<0.01.

Table 5.6 The effects of dietary melamine (M) or urea-formaldehyde (UF) or their equal mixtures on egg production parameters (37-52 wk)

Treatment		FI	Egg	Egg	FCR	Survival	Egg mass	Number
Product	Concentration (%)	(g/h/d)	production (%)	weight (g)		(%)		of egg (egg/28d)
Basal	0	115.47	92.44^{a}	59.54^{a}	1.939^{c}	100.00	55.06^{a}	25.88^{a}
M	0.25	116.26	89.41^{abc}	58.02^{ab}	1.970^{bc}	100.00	52.80^{bc}	25.04^{abc}
M	0.50	116.19	87.20^{bcd}	58.36^{cd}	1.991^{abc}	95.96	50.90^{cde}	24.42^{bcd}
M	0.75	117.22	84.85^{d}	58.12^{bcd}	2.018^{ab}	97.92	49.33^{e}	23.76^{d}
M	1.00	117.28	79.98^{e}	57.11^{d}	2.054^{a}	97.92	45.71^{f}	22.39^{e}
UF	0.25	116.24	90.01^{ab}	58.76^{ab}	1.979^{bc}	100.00	52.88^{bc}	25.20^{ab}
UF	0.50	116.85	86.91^{bcd}	58.72^{ab}	1.990^{abc}	100.00	51.04^{bcde}	24.33^{bcd}
UF	0.75	116.62	85.86^{cd}	58.55^{abc}	1.993^{abc}	98.96	50.27^{de}	24.04^{cd}
UF	1.00	116.37	85.38^{d}	57.60^{cd}	2.020^{ab}	98.96	49.20^{e}	23.91^{d}
M+UF	0.25	115.95	90.37^{ab}	58.86^{ab}	1.970^{bc}	100.00	53.20^{ab}	25.30^{ab}
M+UF	0.50	117.79	88.45^{bcd}	58.82^{ab}	2.003^{abc}	98.96	52.05^{bcd}	24.77^{bcd}
M+UF	0.75	117.22	87.98^{bcd}	58.73^{ab}	1.996^{abc}	98.96	51.67^{bcd}	24.63^{bcd}
M+UF	1.00	116.05	87.83^{bcd}	58.05^{bcd}	1.999^{abc}	100.00	50.98^{bcde}	24.59^{bcd}
Pooled SEM		0.973	1.129	0.335	0.021	2.312	0.689	0.316
			-----------------	-----------------	Probability	-----------------	-----------------	
Product (P)		NS	**	NS	NS	NS	**	**
Concentration (C)		NS	**	**	*	NS	**	**
P x C		NS	NS	NS	NS	NS	*	NS
Contrast								
Control vs other		NS	*	*	**	NS	**	**
L^1		NS	**	**	**	NS	**	**
Q^1		NS	NS	NS	NS	NS	NS	NS
L^2		NS	**	**	NS	NS	**	**
Q^2		NS	NS	NS	NS	NS	NS	NS
L^3		NS	**	**	NS	NS	**	**
Q^3		NS	NS	**	NS	NS	NS	NS

$^{a-f}$ Means within a column with no common superscript differ significant (P<0.05).

L^1=Linear for M., Q^1=Quadratic for M, L^2=Linear for UF, Q^2=Quadratic for UF.

L^3=Linear for M + UF, Q^3=Quadratic for M + UF.

NS=Not significant; * P<0.05; ** P<0.01.

5.3.3 Egg quality

Table 5.7 described the effects of dietary melamine, UF or their equal mixtures on egg quality (specific gravity, egg yolk weight, Haugh Units; HU, yolk color score and egg shell thickness). At 19-34 wk, there was no difference (P>0.05) in specific gravity, yolk color score and egg shell thickness among controls and hens fed all graded levels of melamine, UF or their equal mixtures. There were significant difference decreased (P<0.05) in egg yolk weight and HU for hens fed melamine, UF or their equal mixtures $\geq$ 0.75% in the diets compared with controls. At 37 to 52 weeks of age (Table 5.8), significant difference in specific gravity, egg yolk weight and HU, except yolk color score and egg shell thickness. Hens fed melamine 1.00% showed the greatest decreased egg quality when compared among treatments. There was no significant difference in egg quality between the diets contained UF and their equal mixture of melamine and UF.

The production of commercial laying hens is shown more value added with quality of egg evaluation. The productive index (PI) parameters calculated from EP, survival rate, EW, HU, egg shell thickness, FCR and days for the trial were evaluation. Table 5.9 described the effects of dietary melamine, UF or their equal mixtures on PI (19-34 and 37-52 wk). There were significant difference (P<0.05) for all both periods, with the greatest decrease in PI observed in hens fed 1.00% melamine, while no such effects were observed for hens fed UF or their equal mixtures.

Table 5.7 The effects of dietary melamine (M) or urea-formaldehyde (UF) or their equal mixtures on egg quality parameters (19-34 wk)

Treatment		Specific gravity	Egg yolk weight (g)	Haugh Units	Yolk color score	Egg shell thickness (mm.)
Product	Concentration (%)					
Basal	0	1.197	14.20^{a}	89.94^{a}	10.57	0.356
M	0.25	1.196	13.97^{ab}	88.21^{ab}	10.56	0.354
M	0.50	1.196	13.77^{ab}	87.60^{abc}	10.54	0.355
M	0.75	1.194	13.71^{b}	86.72^{bc}	10.55	0.353
M	1.00	1.193	13.13^{c}	85.59^{c}	10.55	0.353
UF	0.25	1.198	13.85^{ab}	88.65^{ab}	10.57	0.356
UF	0.50	1.197	13.87^{ab}	88.43^{ab}	10.55	0.355
UF	0.75	1.197	13.88^{ab}	87.13^{bc}	10.55	0.354
UF	1.00	1.193	13.66^{b}	85.89^{bc}	10.55	0.354
M+UF	0.25	1.197	14.05^{ab}	87.86^{abc}	10.55	0.356
M+UF	0.50	1.196	13.96^{ab}	87.64^{abc}	10.55	0.355
M+UF	0.75	1.197	13.78^{ab}	87.01^{bc}	10.55	0.355
M+UF	1.00	1.196	13.70^{b}	86.76^{bc}	10.55	0.354
Pooled SEM		0.002	0.139	0.675	0.045	0.001
		-----------------------------------Probability-----------------------------				
Product (P)		NS	*	*	NS	NS
Concentration (C)		NS	*	*	NS	NS
P x C		NS			NS	NS
Contrast						
Control vs other		NS	*	*	NS	NS
L^1		NS	**	**	NS	NS
Q^1		NS	NS	NS	NS	NS
L^2		NS	NS	*	NS	NS
Q^2		NS	NS	NS	NS	NS
L^3		NS	NS	NS	NS	NS
Q^3		NS	**	**	NS	NS

$^{a-c}$ Means within a column with no common superscript differ significant ($P<0.05$).

L^1=Linear for M., Q^1=Quadratic for M, L^2=Linear for UF, Q^2=Quadratic for UF.

L^3=Linear for M + UF, Q^3=Quadratic for M + UF.

NS=Not significant; * $P<0.05$; ** $P<0.01$.

Table 5.8 The effects of dietary melamine (M) or urea-formaldehyde (UF) or their equal mixtures on egg quality parameters (37-52 wk)

Treatment		Specific gravity	Egg yolk weight (g)	Haugh Units	Yolk color score	Egg shell thickness (mm.)
Product	Concentration (%)					
Basal	0	1.225^{a}	15.52^{a}	83.69^{a}	10.55	0.363
M	0.25	1.221^{ab}	15.41^{a}	82.70^{abc}	10.49	0.361
M	0.50	1.218^{abc}	15.06^{ab}	81.72^{bcd}	10.48	0.359
M	0.75	1.211^{bc}	15.03^{ab}	81.18^{cd}	10.47	0.357
M	1.00	1.208^{c}	14.52^{c}	80.74^{d}	10.52	0.357
UF	0.25	1.219^{abc}	15.43^{a}	83.27^{ab}	10.47	0.361
UF	0.50	1.218^{abc}	15.11^{ab}	82.70^{abc}	10.55	0.359
UF	0.75	1.216^{abc}	15.08^{ab}	82.38^{abcd}	10.56	0.361
UF	1.00	1.213^{abc}	14.68^{bc}	81.66^{bcd}	10.50	0.360
M+UF	0.25	1.218^{abc}	15.16^{ab}	83.22^{ab}	10.56	0.361
M+UF	0.50	1.215^{abc}	15.13^{ab}	82.71^{abc}	10.56	0.359
M+UF	0.75	1.217^{abc}	15.06^{ab}	81.99^{abcd}	10.53	0.360
M+UF	1.00	1.212^{abc}	15.03^{ab}	81.43^{cd}	10.54	0.360
Pooled SEM		0.004	0.161	0.522	0.068	0.002
		----------------	----------------	Probability	----------------	----------------
Product (P)		NS	NS	*	NS	NS
Concentration (C)		*	**	**	NS	NS
P x C		NS	NS	NS	NS	NS
Contrast						
Control vs other		*	*	*	NS	NS
L^1		*	*	NS	NS	NS
Q^1		NS	NS	NS	NS	NS
L^2		NS	**	**	NS	NS
Q^2		NS	NS	NS	NS	NS
L^3		NS	NS	**	NS	NS
Q^3		NS	NS	NS	NS	NS

$^{a\text{-}d}$ Means within a column with no common superscript differ significant (P<0.05).

L^1=Linear for M., Q^1=Quadratic for M, L^2=Linear for UF, Q^2=Quadratic for UF.

L^3=Linear for M + UF, Q^3=Quadratic for M + UF.

NS=Not significant; * P<0.05; ** P<0.01.

Table 5.9 The effects of dietary melamine (M) or urea-formaldehyde (UF) or their equal mixtures on productive index of laying hen (19-34 and 37-52 wk)

Treatment		Productive index*	Productive index*
Product	Concentration (%)	19-34 wk	37-52 wk
Basal	0	545.57[a]	770.18[a]
M	0.25	484.16[def]	714.25[bcd]
M	0.50	464.51[egf]	662.24[ef]
M	0.75	453.64[gh]	618.87[g]
M	1.00	413.92[i]	561.19[h]
UF	0.25	512.71[bc]	717.43[bc]
UF	0.50	485.86[de]	678.48[cde]
UF	0.75	458.85[fgh]	661.56[ef]
UF	1.00	435.68[hi]	632.41[fg]
M+UF	0.25	519.44[b]	724.70[b]
M+UF	0.50	490.85[cd]	681.76[cde]
M+UF	0.75	477.36[defg]	675.69[de]
M+UF	1.00	453.22[gh]	667.32[ef]
Pooled SEM		8.242	13.061
		----------Probability----------	
Product (P)		*	*
Concentration (C)		*	*
P x C		NS	NS
Contrast			
Control vs other		*	*
L^1		**	**
Q^1		NS	NS
L^2		**	**
Q^2		NS	NS
L^3		**	**
Q^3		NS	NS

[a-i] Means within a column with no common superscript differ significant (*P*<0.05).

*Productive index = (egg production x survival x egg weight x Haugh Units x egg shell thickness) / (FCR x 112 x 100)

L^1=Linear for M., Q^1=Quadratic for M, L^2=Linear for UF, Q^2=Quadratic for UF.

L^3=Linear for M + UF, Q^3=Quadratic for M + UF.

NS=Not significant; * *P*<0.05; ** *P*<0.01.

5.3.4 Microscopic examination of crystal in various tissues

Figure 5.1 and 5.2 showed melamine "crystals" were detected in the liver, spleen and kidney and Figure 5.3, melamine in egg of laying hens (34 and 52 wk of age) subjected to graded levels of melamine from 0.25 to 1.00% in the diets. Compared with the controls, obvious golden-brown or pale yellow crystals with "spoke wheel" appearance were seen in the tissues. The crystals in the tissues of laying hens fed with 0.50% melamine in their diets were < 2 micron and some were large enough to be visible to the naked eye. The liver, kidney and spleen tissues examined under microscopy revealed "golden-brown crystals" have a globular to flattened shape with fine linear radiations. For the birds fed control diets, there were no obvious changes in the tissues structure and golden-brown crystals in the liver, kidney and spleen tissues (Figure A1, A2, A3). The amount and the size of golden-brown crystals of melamine and also tissue lesions become more score and evident with increasing dietary melamine concentration (Figure B1-B3,C1-C3,D1-D3,E1-E3).

5.3.5 Histological examination of crystal in various tissues

No pathological change was observed in the tissue organs of laying hens fed with negative control treatment. Histological lesions were observed in the liver, kidney, spleen and ovary fed with graded levels of melamine (0, 0.25, 0.50, 0.75 and 1.00% melamine in the diets) are presented in Figure 5.4. Compared with control group, there were no obvious changes in the kidney, liver and spleen structure of broilers fed ≤0.50% of melamine (Figure 5.4). The kidney, liver and spleen lesions became more severe with increasing dietary melamine dosages. Liver tissues showed hepatocyte swelling, congestion, cell membrane less prominent, red blood cells distension and sinusoidal dilatation. Spleen tissues showed congestion, white blood cell infiltration. Photomicrograph showed brown melamine crystals deposited in the interstitial tissue surrounding the renal tubules of laying hens fed 1.00% melamine contaminated in diet. Melamine crystals were not found in the livers and spleens of any laying hens. Figure 5.5 showed photomicrograph ooplasm of oocyte in the egg tissues of laying hens, with 1.00% melamine in the diet. The oocyte was spherical in shape. The ooplasm exhibits a heterogeneous and appearance compared to a homogeneous and lightly stained as of the laying hens fed control treatment diet.

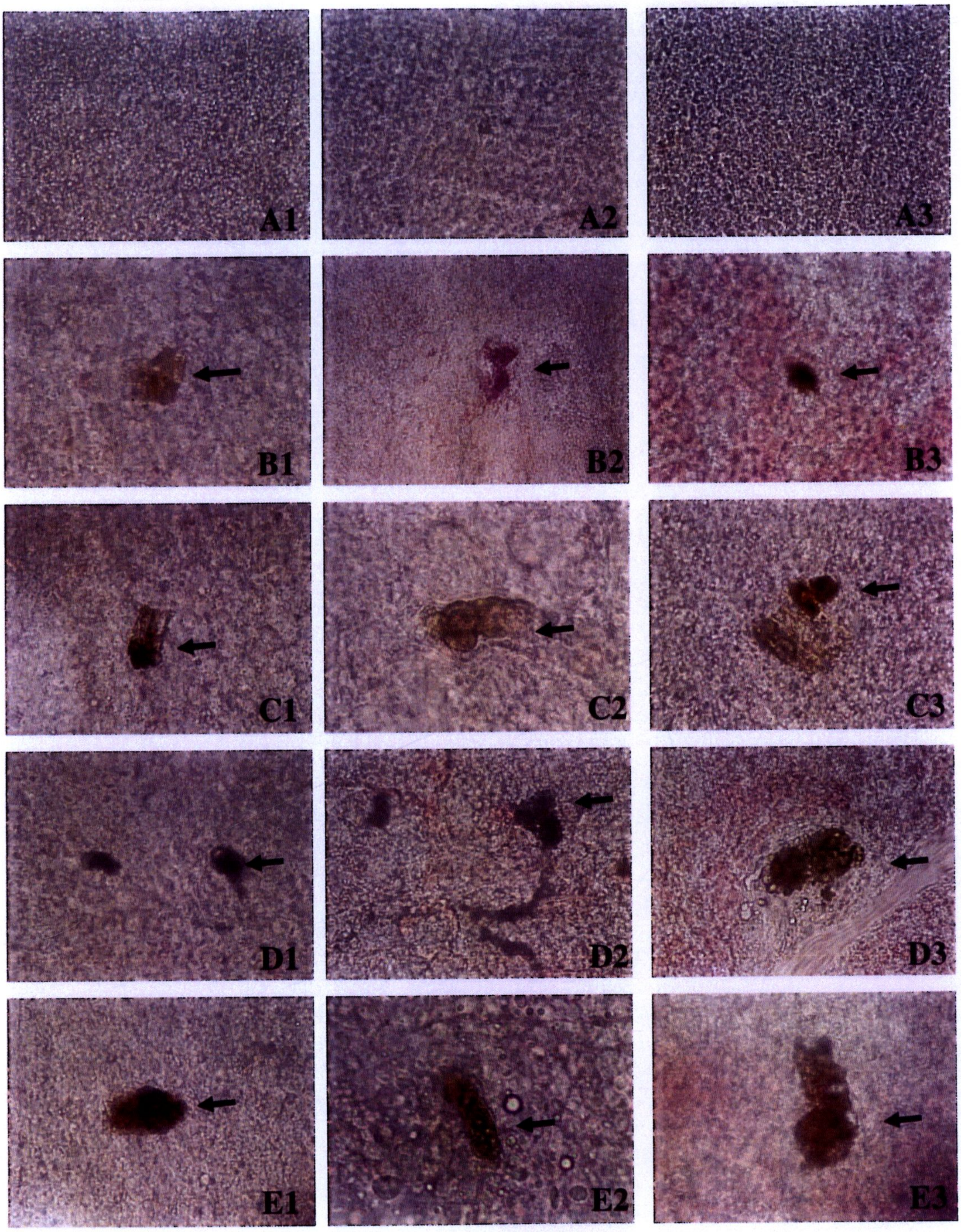

Figure 5.1 A microscopic view of the graded levels of dietary melamine supplementation in laying hen diets for 34 weeks in the tissues of the liver (A1-E1), kidney (A2-E2) and spleen (A3-E3) with magnification 400-1000X under inverted microscopy: (A) control; (B) 0.25% melamine in the diet; (C) 0.50% melamine in the diet; (D) 0.75% melamine in the diet; (E) 1.00% melamine in the diet

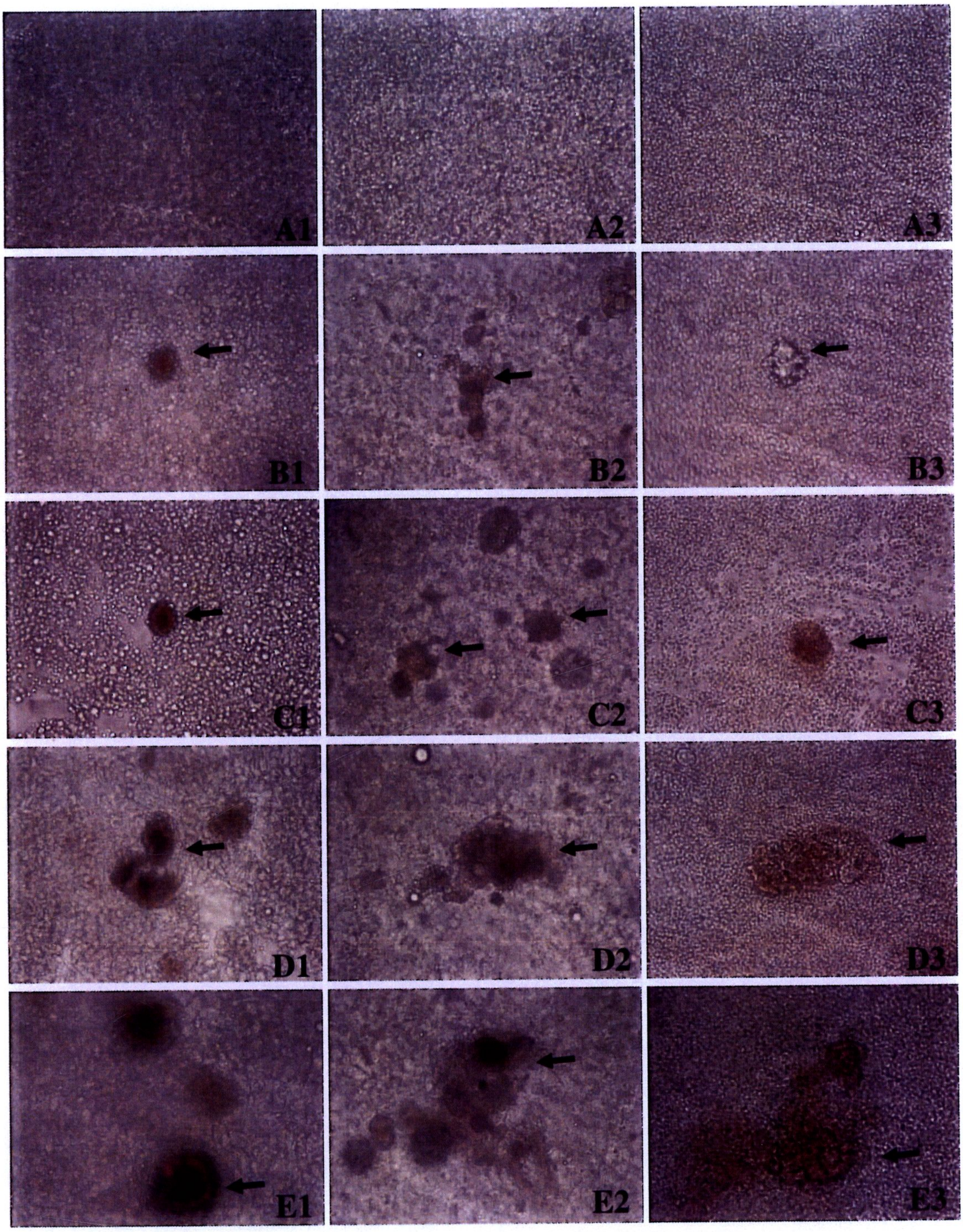

Figure 5.2 A microscopic view of the graded levels of dietary melamine supplementation in laying hen diets for 52 weeks in the tissues of the liver (A1-E1), kidney (A2-E2) and spleen (A3-E3) with magnification 400-1000X under inverted microscopy: (A) control; (B) 0.25% melamine in the diet; (C) 0.50% melamine in the diet; (D) 0.75% melamine in the diet; (E) 1.00% melamine in the diet

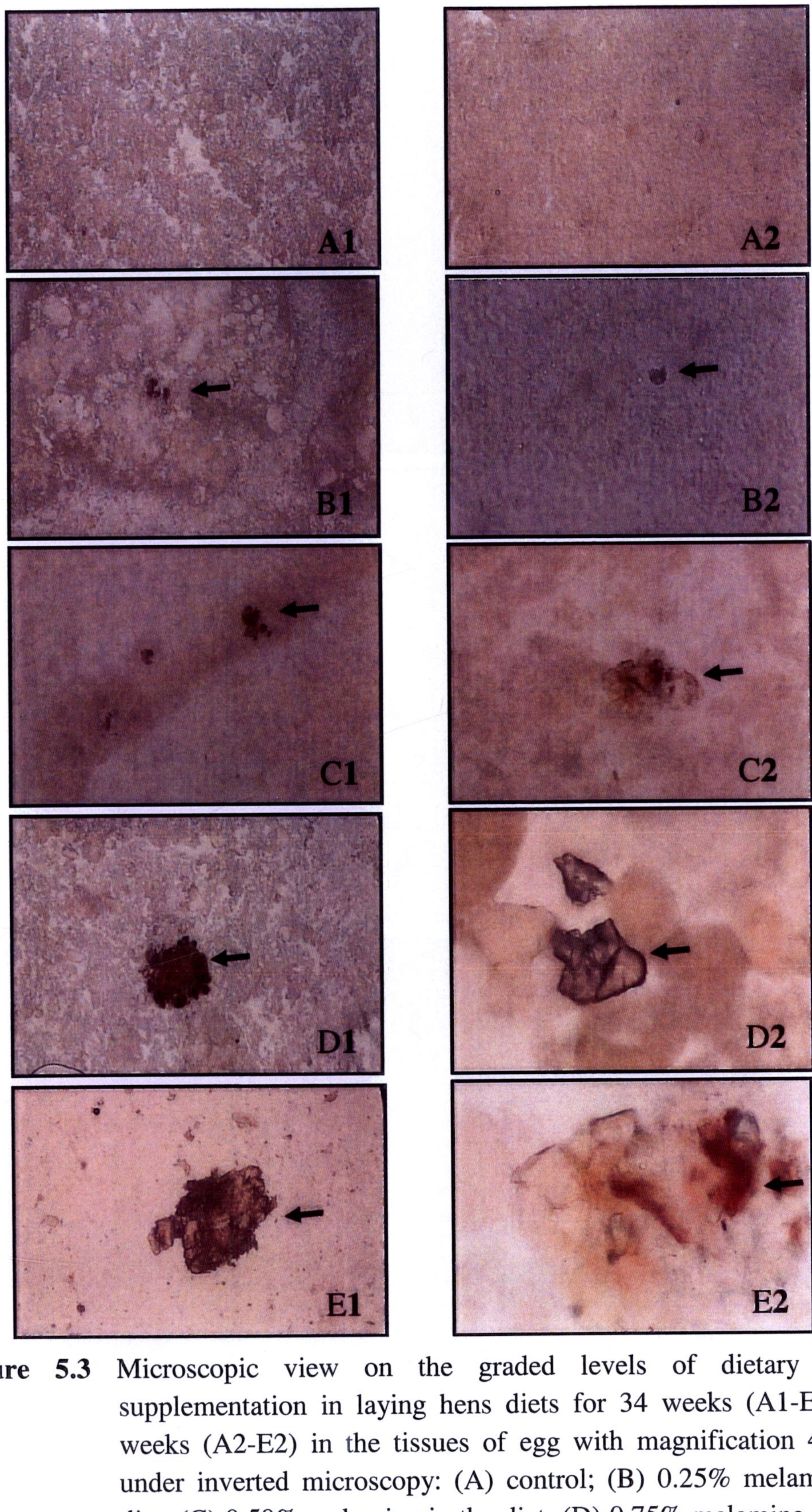

Figure 5.3 Microscopic view on the graded levels of dietary melamine supplementation in laying hens diets for 34 weeks (A1-E1) and 52 weeks (A2-E2) in the tissues of egg with magnification 400-1000X under inverted microscopy: (A) control; (B) 0.25% melamine in the diet; (C) 0.50% melamine in the diet; (D) 0.75% melamine in the diet; (E) 1.00% melamine in the diet

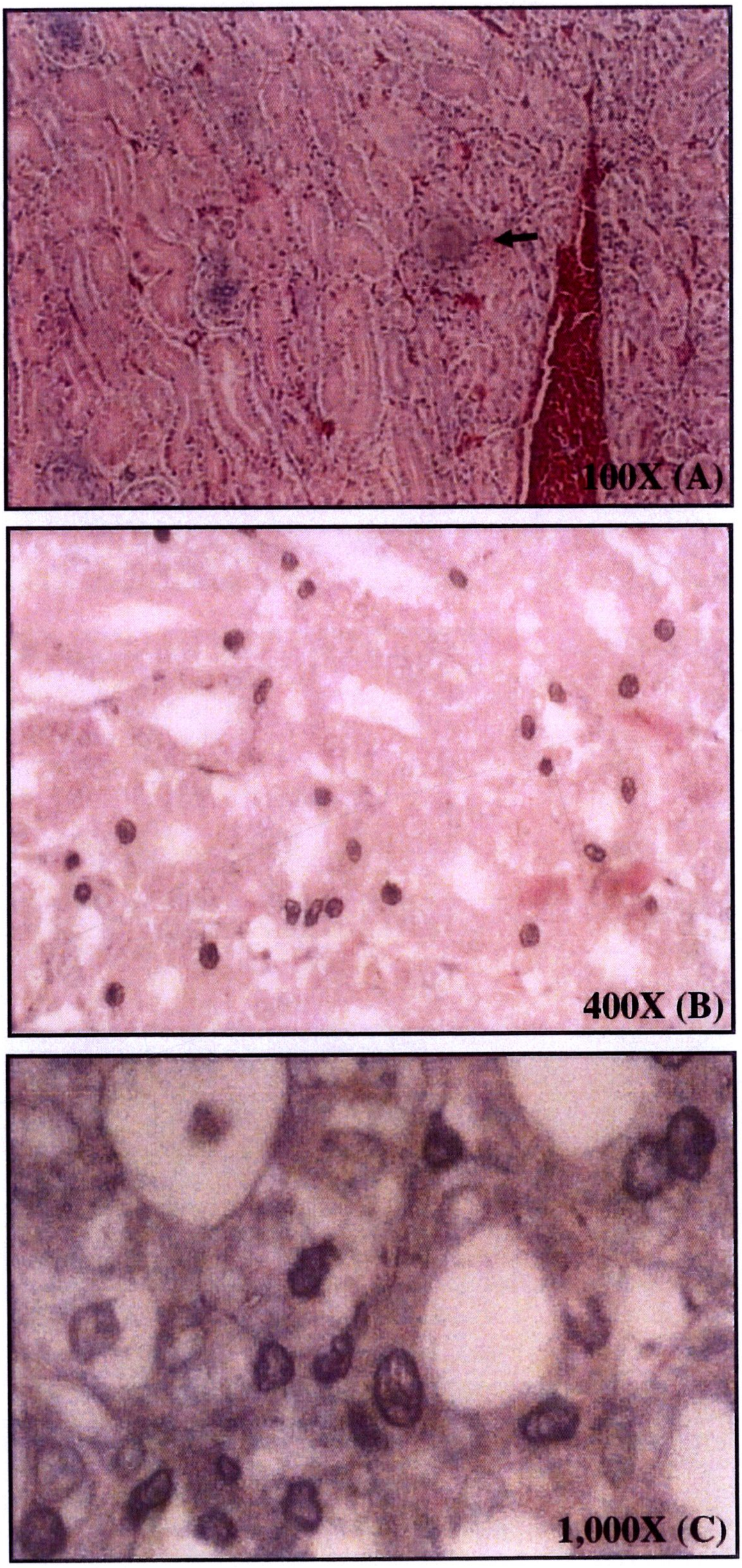

Figure 5.4 A histological view of melamine level 1.00% supplementation in laying hens diets in the tissues of kidney with magnification 100X(A), 400X(B) and 1,000X (C) under inverted microscopy

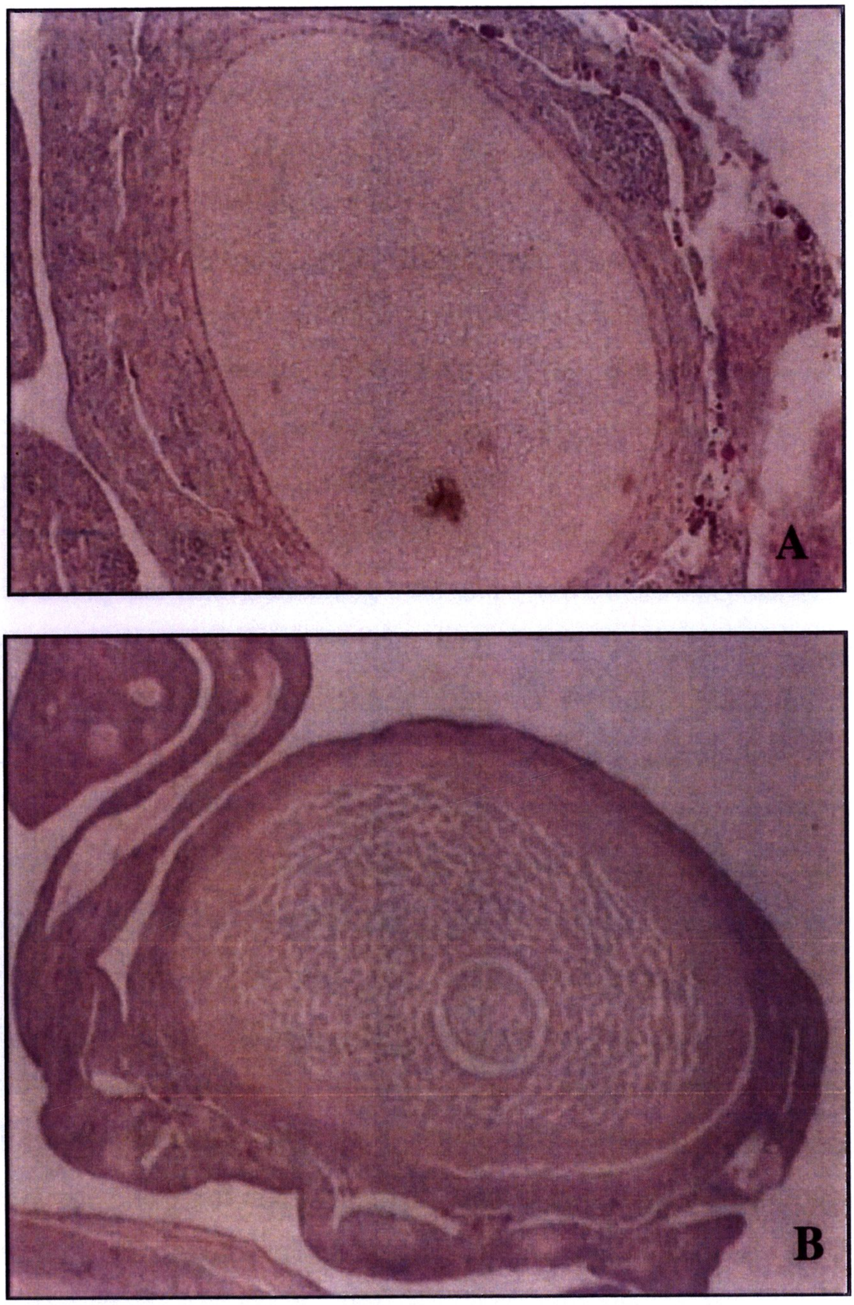

Figure 5.5 A histological view of melamine supplementation in laying hens diets in the tissues of egg under inverted microscopy; (A) control and (B) 1.00% melamine in the diet

5.4 Discussion

5.4.1 Production parameters and egg quality

At 18 wk, BWG of hens in cages was significantly and greatest decreased in hens fed 1.00% melamine. However, FI, FCR and survival rate were not significant for overall period. Similar results were reported by Gao et al. (2010) in laying ducks fed graded levels (0-100 ppm) of melamine in diets. They found that diets containing different levels of melamine had no obvious adverse effects on the laying performance of ducks. In addition, Lu et al. (2009) reported in broilers fed graded levels (2-1,000 ppm) of melamine in diets. However, previous studies in other animals revealed a decreased of feed intake in rats (Ogasawara et al., 1995) and fish (Liu et al., 2009) fed diets containing 3.00 and 1.00% melamine, respectively. In the present study, the decrease of BWG at level 1.00% melamine in laying hens was significant. Thus, it was indicated that dietary melamine as $\geq$0.75% of diet was tolerable, but higher melamine levels may cause toxicity in laying hens.

The results of this study demonstrated hens (19-34 and 37-52 wks) fed with melamine showed linear decrease effect on EP, egg weight, egg mass, and number of egg as the levels of melamine in the diets increased with the greatest decreased in EP, egg weight, egg mass, and number of egg observed in hens fed 1.00% melamine. Feed efficiency or FCR showed less efficient as the levels of melamine increased in the diets and also showed the greatest decreased efficiency when fed 1.00% melamine. The results agree with a early studies by Ledoux et al. (2009) fed graded levels of melamine (0.50-3.00%) in young broilers from hatch to 14 days showed no effect in FI among controls and chicks fed 0.50 or 1.00% melamine, and BWG decreased when fed $\geq$ 1.00% melamine with the greatest decrease in BWG observed in birds fed $\geq$ 2.00% melamine. Similar previous study also reported by Brand et al. (2009) in young turkey poults fed with graded levels of melamine (0.50-3.00%) from hatch to 21 days indicated that FI was reduced in poults fed diets containing 1.50% melamine, whereas BWG was reduced in birds fed $\geq$ 1.00% melamine when compared with controls. However, Bai et al. (2010) reported in laying hens fed with 8.6-140.9 ppm melamine for 34 days. There were no effects on the survival, BWG, or EP and also no pathological changes were observed in the kidney of hens. In addition, Gao et al. (2010) fed six dietary of melamine (0-100 ppm) in laying ducks for 21 days. No visible signs of illness or mortality were observed and no adverse effects on egg weight, EP, feed intake, and FCR (P>0.05).

Egg specific gravity has been used extensively as a measure of shell strength (Holder and Bradford, 1979). Because the egg contents (yolk and albumen) maintain a constant specific gravity, any difference in egg specific gravity relates to the amount of calcium deposition (shell thickness). In the current study, eggs from hens fed melamine or UF or their mixtures had consistently the lowest specific gravity (Table 5.3). Therefore, it is possible that calcium absorption was impaired due to melamine is rapidly absorbed from the intestine and attains maximal plasma concentrations in 1 h following a single oral dose in rats (Mast et al., 1983; Sugita and Maekawa, 1991). Moreover, melamine eliminated essentially unchanged by the kidney (Worzalla et al., 1974; Mast et al., 1983). Yolk color for overall this experiment was no significant difference among the treatments. The main contributing factor for yolk color is the diet (Leeson and Summers, 1991), and although the hens were all fed the same diet, except melamine and UF, in which fine white crystalline powder.

5.4.2 Microscopic and histological examination of crystal in various tissues

The carryover of melamine from animal feed to animal-derived products is an important subject for risk assessment purposes. The liver, kidney and spleen tissues examined under microscopy revealed "golden-brown crystals" have a globular to flattened shape with fine linear radiations. For the birds fed control diets, there were no obvious changes in the tissues structure and golden-brown crystals in the liver, kidney and spleen tissues. The amount and the size of golden-brown crystals of melamine and also tissue lesions become more score and evident with increasing dietary melamine dosage. A study conducted by Andersen et al. (2008) indicated that fish dosed with 299-471 ppm/day of melamine for 3 days contained melamine levels ranging from 81 to 210 ppm in catfish, from 34 to 80 ppm in trout, from 0.02 to 177 ppm in tilapia and from 58 to 94 ppm in salmon. In the study of Bermudez et al., (2008), broiler chickens were fed feed containing 0-3.00% melamine from 1 to 21 days of age. For the 3.00% dose group, melamine concentrations in pectoral muscle, liver and kidney were found to be 600, 700, and 1,000 ppm, respectively. In the present study, Histological lesions were observed in the kidney, liver and spleen fed with graded levels of melamine compared with control group, there were no obvious changes in the kidney, liver and spleen structure of broilers fed $\leq$ 0.50% of melamine. The kidney, liver and spleen lesions became more severe with increasing dietary melamine dosage. Liver tissues showed hepatocyte swelling, congestion, cell membrane less prominent, red blood cell distension and sinusoidal dilatation. Spleen tissues showed congestion, white blood cell infiltration. Kidneys tissues showed brown melamine crystals deposited in the interstitial tissue surrounding the renal tubules of laying hens fed 1.00% melamine contaminated in diet. Melamine crystals were not found in the livers and spleens of any laying hens. The similar results were also reported by Gao et al. (2010) who supplemented 0 to 100 mg of melamine/kg of duck diet. Their results showed that histological lesions were observed in the kidneys of ducks subjected to $\geq$25 mg/kg melamine. Histological lesions in liver tissues from birds fed melamine supplemented diets were less prominent than those observed in kidneys. Fatty degeneration and liver fibrosis were occasionally observed in the livers of ducks fed diets containing 25 mg of melamine/kg of feed. Piecemeal necrosis and inflammatory cells were present within the adjacent interstitium in some livers of the ducks fed 50 mg of melamine/kg of feed. Liver cell vaculoation, inflammatory cell infiltration, and fatty degeneration were found in some liver of the ducks fed with 100 mg of melamine/kg of feed. Bai et al. (2010) reported that melamine supplement at 17.4 to140.9 mg/kg of body weight (BW) for 34 days, showed histopathology revealed dilated renal tubules and small blood vessel expansion in hens. Furthermore, crystals were found in one of three kidneys of the melamine treated hens levels 62.6 and 140.9 mg/kg of BW per day. Addition, Reimschuessel et al. (2008) described that chickens fed melamine could develop spherulite crystals containing uric acid, a normal excretion product of chickens. Furthermore, previous studies have shown that pigs, fish, cats and rats fed melamine and cyanuric acid in a 1:1 ratio develop renal crystals composed of melamine-cyanurate (Puschner et al., 2007; Dobson et al., 2008; Reimschuessel et al., 2008). However, no changes in renal functions were observed in cats and fish treated with either melamine or cyanuric acid alone at doses of 181 or 400 mg/kg of BW (Reimschuessel et al., 2008).

5.5 Conclusion

The results of this study indicated that over the developer period (12-18 wk), melamine concentration $\geq$ 0.75% depressed BWG also affect on egg performance and quality of egg (19-34 wk). Moreover, at laying hens period 37 to 52 wk, melamine concentration $\geq$ 0.75% depressed egg performance and quality of egg and showed decreased PI. Renal lesions were correlated with increasing levels of dietary melamine. Melamine residue in eggs increased with dietary melamine supplemental level increased, which allowed for rapid decreased after melamine was removed from the feed.