## Letter to the Editor

# An Evaluation of Genotoxicity and Cytotoxicity of Melamine in Combination with Cyanuric Acid at Three Mass Ratios*

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**Melamine in combination with cyanuric acid has been considered to be more toxic than either melamine or cyanuric acid alone. The objective of this study was designed to evaluate the combined genotoxicity and cytotoxicity of melamine (M) and cyanuric acid (C) at three mass ratios (1:1, 1:2, 2:1). MC (1:1), MC (1:2), and MC (2:1) were evaluated for their potential genotoxic risk, at gene level by Ames test, and at chromosomal level by micronucleus test. In order to evaluate cytotoxicity in HEK-293 cells, the MTT assay was included. Western blot was also employed to investigate the renal injury molecule-1 (Kim-1) expression in HEK-293 cells exposed to MC. Neither genotoxicity at gene level nor at chromosomal level was observed for MC (1:1), MC (1:2), and MC (2:1). Based on MTT assay, three ratios of MC at 82.5 and 165 μg/mL slightly inhibited viability of HEK-293 cells ($P$<0.05). MC (1:1) at 41.25 and 82.50 μg/mL could elevate the Kim-1 expression in HEK-293 cells.**

Melamine is present as a trace contaminant in nitrogen supplements used in animal feeds or watered down milk, and has been found as a metabolite of the pesticide and the veterinary drug, cyromazine. Cyanuric acid is a derivative of melamine. Further investigation has determined that melamine and the tri-keto tautomer of cyanuric acid contain hydrogen bond donors and acceptors arranged in a complementary geometry. Upon mixing, large sheets of melamine cyanurate can form through hydrogen bonding involving the three sides of the molecules and different molar ratios of melamine and cyanuric acid, which may lead to distinct cocrystals[1]. At present, numerous researches on the joint toxicity of MC are focused on renal injury. Only a few of studies have reported the subchronic toxicity, neurotoxicity, and reproductive toxicity of MC[2]. However, the results on the combined genotoxicity and cytotoxicity of MC are scanty up to now.

Previous studies evaluated the combined effect of melamine and cyanuric acid on animals basically by co-administration at a 1:1 ratio[3-4]. The composition of expelled renal calculi from fifteen injured infants in milk formulas adulterated melamine incidents was analyzed. Results confirmed that the stones were composed of uric acid and melamine at a molar ratio ranging from 1.2:1 to 2.1:1[5]. However, cyanuric acid integrated with melamine through intermolecular hydrogen-bonding interaction is very similar to the binding mode between uric acid and melamine[1,6]. Given this mode of action, we reported a comparison among three mass ratios (1:1, 1:2, and 2:1) of melamine and cyanuric acid co-exposure scenarios to investigate whether melamine and cyanuric acid exhibited greater toxicity at other mass ratios. In this study, we used *in vivo* animal model and *in vitro* cell model to compare the combined genotoxicity and cytotoxicity of melamine and cyanuric acid at three mass ratios (1:1, 1:2, 2:1). At last, the joint action of melamine and cyanuric acid at 1:1 ratio to Kim-1 protein expression in human embryonic kidney cells was evaluated at a molecular level by Western blot to get a better knowledge about the mechanism of renal injuries caused by melamine-cyanuric acid.

An evaluation of mutagenicity of melamine in combination with cyanuric acid at three mass ratios was performed according to the plate incorporation procedure described by OECD Test Guideline 471.

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Salmonella typhimurium tester strains TA97, TA98, TA100, and TA102 used for the bacterial reverse mutation assay were procured from CCTCC (Wuhan, China). The *in vivo* micronucleus test was performed according to OECD Test Guideline 474, animal welfare regulations and bone marrow cell micronucleus test method (GB5193.5-2003, China). The possible clastogenic activity of melamine and cyanuric acid at three mass ratios was assessed with *in vivo* mouse bone marrow micronucleus test following double oral gavages 24 h apart. Each proportion of MC was tested at three doses approximating 1/2, 1/4, and 1/8 of the $LD_{50}$-values (based on pre acute toxicity test study). The MC mixtures were dissolved in corn oil and administered to the animals twice at 0 and 24 h by the oral route (gavage volume being 20 mL/kg b.w.). Concurrently, negative (Corn Oil, 20 mL/kg b.w.) and positive (CP, 40 mg/kg b.w.) controls were administered to separate group of animals under the similar test conditions.

Cytotoxicity experiments were carried out with HEK-293 cells essentially according to the method employed in previous studies[7]. The cells were exposed at various doses of freshly prepared MC compound (1:1, 2:1, 1:2) at different concentrations (2.578, 5.156, 10.313, 20.625, 41.25, 82.5, and 165 μg/mL) for 24 h. Based on results obtained in acute toxicity and cytotoxicity tests, melamine in combination with cyanuric acid at 1:1 mass radio was selected as test target due to its largest toxicity among the three ratios. All dosing solutions (82.5, 41.25, 20.625 μg/mL) were determined on the basis of MTT assay, and solvent control group was set at the same time. HEK-293 cells were exposed to different concentrations of MC (1:1) for 24 h and then examined for Kim-1 protein expression.

The results of the mutagenicity conducted with and without metabolic activation in four Salmonella tester strains are presented in Table 1. Prior to the main test, bacteria cytotoxicity and solubility of melamine and cyanuric acid mixtures were detected in the presence or absence of metabolic activation with the dosages ranging from 62.5 to 5 000 μg/plate at a common ratio of approximately 2. Based on the results of the preliminary experiment, precipitation of the test mixtures were observed at dosages >1 250 μg/plate with or without S9, and the precipitate was interfered with the scoring. For MC (1:1), bacterial toxicity was observed at 1 250 μg/plate in strains TA 98 and TA 100 with and without S9, and at dosages >625 μg/plate in strain TA102 with metabolic activation, whereas it was observed at 1 250 μg/plate in strains TA98, TA100 and TA102 with metabolic activation for MC (1:2) and MC (2:1).

The mutagenicity of MC (1:1), MC (2:1), and MC (1:2) were assessed at nontoxic concentrations ranging from 62.5 to 500 μg/plate. The bacteria grew normally in negative control. 2-Nitrofluorene for TA97, TA98, and TA 100 strain with S9, Dexon for TA97, TA98, and TA102 strain without S9 and sodium azide for TA100 strain without S9 as the positive controls showed much double number of colonies in comparison with those in the negative control, indicating that the test was valid. On the other hand, in four tested strains, according to the statistical analyses, neither a 2-fold change in the number of His revertant nor a dose-dependent response relationship was observed in MC (1:1), MC (2:1), and MC (1:2) groups. These findings demonstrated that melamine in combination with cyanuric acid at 1:1, 1:2, and 2:1 ratios had no mutagenic effect on TA97, TA98, TA100, and TA102 strains.

A summary of the data acquired with Giemsa staining techniques on the frequencies of MN-PCE and PCE/NCE observed in various treatment and control groups is presented in Table 2. Results for each MC-exposed male and female mice showed a tiny increase in the number of MN-PCE at 6 h after the last exposure, compared with corn oil exposed animals (negative control). However, the differences between melamine-cyanuric acid treated groups and negative control group had no statistical significance ($P$>0.05). Multiple comparisons showed that no significant difference in the induction of micronuclei was observed among the groups treated with different ratios and doses of melamine-cyanuric acid and corn oil. Under the condition of this study, the frequency of PCE-NCE in MC (1:1) treatment group was found to be little lower than that in the other two MC drugged groups. This result, on the other hand, reconfirmed that melamine and cyanuric acid at 1:1 proportion had greater toxicity. Evaluation of the PCE/NCE showed a pronounced cytotoxicity effect of CP (positive control) on the bone marrow erythrocytes at the dose of 40 mg/kg ($P$<0.05).

In the present study, the established genotoxicity tests (Ames and micronucleus test) had an equivalent result. Neither a 2-fold change in the number of His revertant nor a dose-dependent response relationship was observed in MC (1:1), MC (2:1), and MC (1:2) groups. Micronucleus rate at three mass ratios of MC treatment groups were all non-significantly increased compared with that in the

**Table 1.** Effects of MC (1:1), MC (2:1), and MC (1:2) on the Number of Spontaneous Revertant Colonies of Salmonella Typhimurium TA97, TA98, TA100, and TA102 with and without Metabolic Activator S9

| Substance | Concentration (μg/plate) | Number of $His^+$ Revertant Colonies/plate (mean±S.D.) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | TA 97 | | TA 98 | | TA 100 | | TA 102 | |
| | | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 |
| MC (1:1) | 500 | 180.8±13.5 | 190.4±21.3 | 46.7±7.4 | 51.3±6.4 | 185.3±17.3 | 205.7±20.1 | 292.0±28.5 | 298.2±31.5 |
| | 250 | 160.5±18.2 | 170.1±18.7 | 38.5±3.6 | 46.2±5.8 | 174.5±10.5 | 183.8±15.4 | 273.5±20.2 | 290.7±26.7 |
| | 125 | 176.4±9.5 | 204.7±16.4 | 42.9±5.2 | 34.6±5.2 | 158.8±14.2 | 170.5±9.6 | 286.2±18.6 | 302.5±21.6 |
| | 62.5 | 164.7±11.6 | 182.3±14.4 | 33.3±4.7 | 38.5±3.6 | 163.3±9.8 | 178.2±12.6 | 280.4±15.7 | 315.3±28.4 |
| MC (2:1) | 500 | 166.5±15.3 | 197.3±18.7 | 50.4±8.3 | 52.1±8.6 | 192.4±16.3 | 215.3±22.6 | 301.6±22.6 | 312.2±35.7 |
| | 250 | 184.3±20.0 | 158.5±16.4 | 35.2±6.2 | 45.5±7.2 | 170.8±9.4 | 200.6±16.8 | 289.5±27.3 | 285.7±31.5 |
| | 125 | 175.8±12.7 | 160.2±9.8 | 40.1±4.9 | 48.5±4.5 | 175.3±6.8 | 182.4±10.5 | 266.3±16.6 | 293.1±28.5 |
| | 62.5 | 170.3±8.6 | 175.5±11.7 | 38.7±7.2 | 36.4±54 | 162.1±10.5 | 192.3±8.4 | 278.2±19.2 | 299.5±23.1 |
| MC (1:2) | 500 | 158.4±16.4 | 175.2±25.2 | 47.3±9.4 | 48.4±5.7 | 180.4±20.1 | 198.5±16.7 | 296.5±25.6 | 286.5±44.2 |
| | 250 | 183.1±10.1 | 189.6±20.5 | 50.8±7.3 | 39.7±6.5 | 172.6±17.4 | 167.5±9.6 | 285.2±20.4 | 316.8±32.8 |
| | 125 | 178.6±8.8 | 194.1±17.5 | 44.5±5.8 | 42.6±3.8 | 176.4±10.6 | 180.2±11.2 | 302.8±18.1 | 292.3±27.4 |
| | 62.5 | 162.3±9.2 | 168.8±12.7 | 36.3±6.1 | 32.2±5.2 | 168.1±12.3 | 170.8±8.5 | 277.6±12.7 | 296.7±24.5 |
| Revertants | 0 | 156.7±15.4 | 161.5±13.6 | 30.8±6.3 | 28.2±7.2 | 152.5±6.3 | 170.6±14.5 | 258.6±24.0 | 265.2±32.5 |
| DMSO | – | 165.8±9.6 | 170.5±18.3 | 30.2±5.8 | 32.6±8.2 | 168.8±7.8 | 178.4±11.2 | 274.6±18.6 | 282.4±28.4 |
| Dexon | 50 | 2365.7±186.4 | – | 1028.5±56.4 | – | – | – | 752.8±40.2 | – |
| Sodium azide | 1.5 | – | – | – | – | 2695.2±95.4 | – | – | – |
| 2-Nitrofluorene | 10 | – | 1325.6±38.6 | – | 5764.3±876.5 | – | 2784.5±157.3 | – | – |

***Note.*** +S9: with metabolic activation; -S9: without metabolic activation. DMSO: negative control. Dexon: the positive controls for TA97, TA98, and TA102 strain without metabolic activation. Sodium azide: the positive controls for TA100 strain without metabolic activation. 2-Nitrofluorene: the positive controls for TA97, TA98, and TA 100 strain with metabolic activation. 2-aminoanthracene (40 μg/plate) for TA102 with S9 as the positive control, however, the revertant colonies in three plates were too numerous to be counted (the data not shown in the table).

negative control group. These results demonstrated that melamine combined with cyanuric acid was unlikely to have mutagenicity.

In MTT assay, some of the MC compounds were not very soluble, limiting the concentration that could be tested. The MC (1:1) compound tested results showed an inhibitory effect on HEK-293 cells moderately elevated in a dose dependent manner, as shown in Figure 1. Time-to-inhibition of cell proliferation was dependent on the toxin dose, while the significant decrease in cell viability was first observed when MC (1:1) or MC (1:2) concentrations were above 20.625 μg/mL and 41.25 μg/mL, respectively. However, MC (2:1) only at the highest concentration of 165 μg/mL exhibited a prominent inhibition to cells proliferation. Furthermore, by 24 h of exposure to high doses of MC (1:1), cell viability decreased to 81.71% (82.5 μg/mL) and 75.25% (165 μg/mL), compared with that in the solvent control group. These were far less cell viability in MC (1:2) and MC (2:1) treatment groups at the same concentration. This finding once again demonstrated that MC (1:1) could produce greater toxicity than MC (1:2) and MC (2:1). Melamine was confirmed to cause apoptosis of cells via excessive intracellular ROS and the activation of p38 MAPK pathway[8]. Graver oxidative damage could be induced by melamine-cyanuric acid mixture which seriously inhibits cells proliferation.

**Table 2.** Frequencies of Micronucleated Polychromatic Erythrocytes (MN-PCE) in the Bone Marrow of Kunming Mice Treated with Three Ratios of MC and Their Non-infected Controls

| Treatment | Dose (mg/kg) | | MN-PCE (per 1000PCE) | | PCE/NCE (per 200erythrocytes) | |
|---|---|---|---|---|---|---|
| | male | female | Male (*n*=5) | Female (*n*=5) | Male (*n*=5) | Female (*n*=5) |
| MC (1:1) | 137 | 200.5 | 5.6±2.07 | 6.0±2.92 | 1.04±0.12 | 1.04±0.14 |
| | 68.5 | 100.25 | 5.0±1.87 | 5.6±2.88 | 1.07±0.09 | 1.07±0.10 |
| | 34.25 | 50.125 | 4.2±1.30 | 4.4±1.14 | 1.06±0.07 | 1.09±0.12 |
| MC (2:1) | 200.5 | 273 | 5.4±1.82 | 5.8±2.17 | 1.06±0.14 | 1.12±0.14 |
| | 100.25 | 136.5 | 5.0±2.00 | 5.2±2.17 | 1.13±0.09 | 1.14±0.16 |
| | 50.125 | 68.25 | 3.8±1.48 | 4.4±2.07 | 1.17±0.17 | 1.13±0.12 |
| MC (1:2) | 172 | 294.5 | 5.2±2.49 | 5.8±2.68 | 1.04±0.14 | 1.09±0.12 |
| | 86 | 147.25 | 4.2±1.30 | 5.0±2.00 | 1.06±0.08 | 1.11±0.13 |
| | 43 | 73.625 | 3.6±1.14 | 4.2±1.64 | 1.11±0.11 | 1.11±0.10 |
| Corn Oil (Negative control) | 20 mL/kg | | 3.4±1.14 | 3.2±1.30 | 1.27±0.15 | 1.30±0.11 |
| CP (Positive control) | 40 | | 16.8±2.77[a] | 17.4±1.82[a] | 0.84±0.06[b] | 0.80±0.05[b] |

***Note.*** Data are shown as values±SD; *n*, number of treated mice; CP, Cyclophosphamide; a, Significantly higher than negative control (Corn Oil) at $P<0.05$; b, Significantly lower than negative control (Corn Oil) at $P<0.05$.

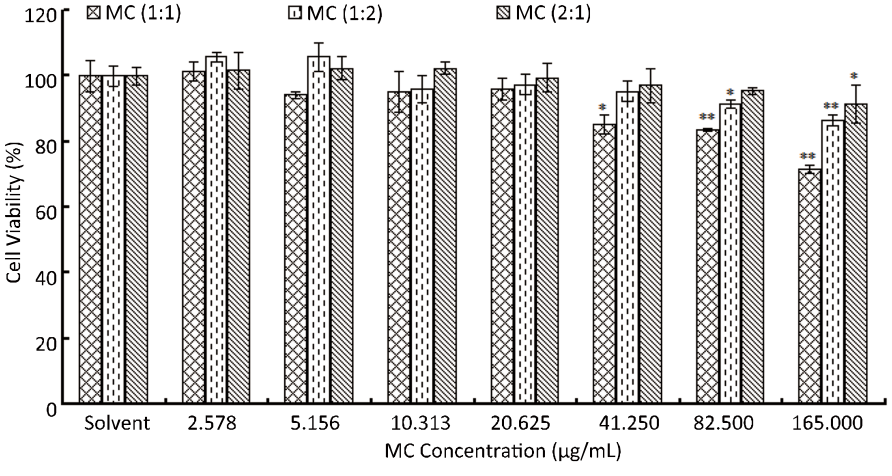


**Figure 1**. The impact of three ratios of melamine and cyanuric acid on HEK-293 cells after 24 incubation. Data are shown as mean values±SD. *, $P<0.05$; **, $P<0.01$; Significant negative impact on cells viability compared with the control group.

Kim-1 is an apoptotic-cell phagocytosis and scavenger receptor that is most highly unregulated in proximal tubular epithelium in acute and chronic kidney injury[9]. Western blotting showed that Kim-1 protein level increased but not in a concentration-dependent manner in HEK-293 cells after treatment with 20.625 μg/mL (1.13-fold), 41.25 μg/mL (1.71-fold), and 82.5 μg/mL (1.28-fold) of MC (1:1) (data not shown) (Figure 2B). Thereinto, the Kim-1 expression in the highest dose (82.5 μg/mL) group was less than in the 41.25 μg/mL dose group. The same results were obtained after three times repeated trials. In study of Kim-1 in human renal disease, researchers found that in severely damaged tubule cells, such as completely flattening or atrophic cells, it was not easy to detect Kim-1[10]. Therefore, combined with cytotoxicity experimental results, we surmised that the dose of 82.5 μg/mL generated a greater cytotoxicity, which led to more serious cell injuries or death before the production of Kim-1. The difference between these two treatment groups were all significant in comparison with that in the control group ($P$<0.05).

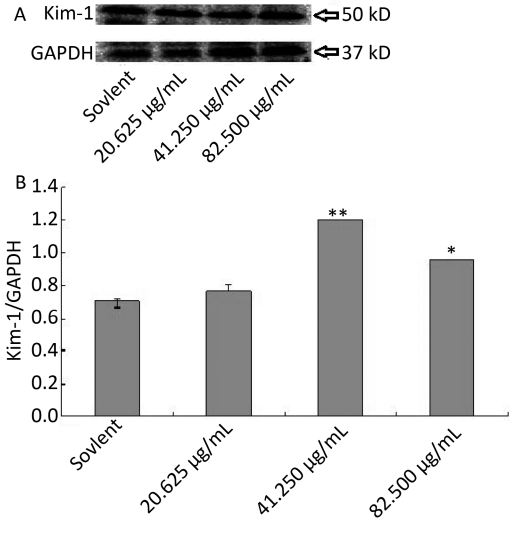


**Figure 2**. Kim-1 protein expression levels of HEK-293 cells identified by Western blot assay. (A) SDS-PAGE electrophoresis image. (B) The ratio of the gray scale value of Kim-1 and internal control GAPDH. Data are shown as mean values±SD. $^{*}$, $P$<0.05; $^{**}$, $P$<0.01; compared with the control group.

This study helps us make a comprehensive understanding of the combined toxicity of melamine-cyanuric acid. These results would be a supplement to be the combined toxic profile of MC and be useful in further assessing the safety of Melamine combined with cyanuric acid.

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