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# Effect of melamine toxicity on *Tetrahymena thermophila* proliferation and metallothionein expression

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ABSTRACT

Melamine is a raw material in the chemical industry. Because of its high nitrogen content, melamine has been utilized by unscrupulous businessmen as a food additive to enhance the indices of protein content in food and feed testing. Tetrahymena has long been used as an excellent model organism in toxicological studies. The purpose of the present study was to determine the effect of melamine on Tetrahymena. In the present study, the effects of melamine on the proliferation and mating rate of Tetrahymena were examined by microscopic counting of the cell numbers. The comet assay and DAPI nuclear staining were performed to analyze the changes in the Tetrahymena genome. Flow cytometric analysis was conducted to detect apoptosis. Furthermore, RT-PCR was performed to determine the changes in the expression of the metallothionein gene in Tetrahymena that underwent stress treatment with varying concentrations of melamine. The results indicated that melamine affected the proliferation and sexual reproduction of Tetrahymena. High melamine concentrations damaged the Tetrahymena genome to a certain extent and induced apoptosis in the organism. Expression of the metallothionein gene was upregulated in Tetrahymena exposed to melamine stress to ameliorate melamine-induced damage. These results indicated that melamine displayed significant toxicity to Tetrahymena cells.



## 1. Introduction

Melamine is a nitrogen-containing heterocyclic organic compound belonging to the triazine family. Melamine is an important industrial chemical that has been widely used in the plastics, decoration, paint and paper industries (Chu and Wang, 2013). Melamine is non-edible, and according to national regulations, it is forbidden to add melamine to food products. However, due to the limitations of the methods employed by the food and feed industries for the determination of protein content, unscrupulous businessmen have violated the national regulations and have utilized melamine as food and feed additives to enhance the indices of protein content in food and feed testing. Melamine-containing food and feed have caused great harm to the health of infants and pets (Chu and Wang, 2013; Qiao et al., 2010; Rai et al., 2014).

In animals, the median lethal dose ($LD_{50}$) of melamine is rather high. Therefore, it is generally believed that melamine is only slightly toxic or non-toxic (Skinner et al., 2010). The $LD_{50}$ values for melamine ranged from 3.1 g/kg to 6.4 g/kg in rats and from 3.2 g/kg to 7.0 g/kg in mice (Melnick et al., 1984). In rabbits, melamine exhibited acute dermal toxicity only when the exposure dose exceeds 1 g/kg (Hau et al., 2009). However, a large number of studies have shown that melamine crystals did not dissolve easily and can only be eliminated from the body very slowly. Therefore, melamine readily accumulated in the body and may cause chronic toxicity (Okumura et al., 1992). Feeding monkeys with melamine at a dose of 700 mg/kg/day through nasogastric tubes resulted in renal toxicity, pericarditis and enhanced hematopoiesis (Early et al., 2013). In addition, cats and chickens fed with diets containing high doses of melamine may suffer from kidney damage (Brand et al., 2012; Puschner et al., 2007). Studies have confirmed that the simultaneous ingestion of melamine and cyanuric acid induced a series of reactions in the body, resulting in crystallization and stone formation in kidney cells, renal tubular obstruction and eventually kidney failure (Skinner et al., 2010). A study conducted by Ke et al. has shown that the formation of urinary calculi in infants was related to the consumption of melamine-containing milk powder. However, melamine did not cause any increase in the level of oxidative DNA damage (Ke et al., 2010).

A study performed by Zhang et al. has shown that melamine, as a category III carcinogen, failed to exhibit mutagenic effects on eukaryotic and prokaryotic cells. However, melamine caused DNA damage in sperm cells and induced sperm deformity (Zhang et al.,

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2011). Lu et al. have detected apoptotic cells in the renal cortex and the medulla in rats fed with a diet containing melamine and cyanuric acid (Lu et al., 2012). Guo et al. reported that melamine induced apoptosis in the rat kidney epithelial cell line NRK-52e, which was achieved through the generation of intracellular reactive oxygen species and the activation of the p38 mitogen-activated protein kinase (MAPK) signaling pathways (Guo et al., 2012a, 2012b). Han et al. studied the toxicity of melamine in the PC12 rat neuronal cell line and found that melamine inhibits cell proliferation and induces cellular oxidative damage and apoptosis (Han et al., 2011). Chang et al. performed a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and found that melamine induces apoptosis in mouse testicular cells (Chang et al., 2014). Kuo et al. demonstrated that melamine activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)/cyclooxygenase-2 (COX-2)/prostaglandin E2 (PGE2) signaling pathway in macrophages and human embryonic kidney cells and stimulates the generation of reactive oxygen species (Kuo et al., 2013). Hsieh et al. revealed that melamine induces damage and apoptosis in the HK-2 human renal proximal tubular cell line via transforming growth factor *beta* (TGF-β) and oxidative stress (Hsieh et al., 2012).

*Tetrahymena* (Protozoa, Ciliophora, Oligochaeta, Hymenostomatida) is a free-living unicellular eukaryote which can act as a representative for the study on unicellular organisms (Lynn and Doerder, 2012). *Tetrahymena* has been proven to be a very valuable biological model in molecular biology and genetics. In addition, the behavior of individual *Tetrahymena* is closely related to pollutants in the environment. Therefore, *Tetrahymena* has long been used as an important experimental organism in toxicological studies (Sauvant et al., 1999). A study performed by Wang et al. has shown that melamine inhibited the growth of *Tetrahymena*, induces morphological changes and damages in *Tetrahymena* cells, and interferes with the expression of numerous genes (Wang et al., 2011). However, the mechanisms by which melamine induces cellular damage in *Tetrahymena* are currently unclear. The present study used *Tetrahymena* as a research model. Through the investigation of the effects of melamine on the proliferation and sexual reproduction of *Tetrahymena* and the effects of melamine on DNA damage, apoptosis and the expression of the metallothionein gene MTT1 in *Tetrahymena*, the damaging effects of melamine on cells and the mechanisms of action of melamine were successfully explored.

## 2. Materials and methods

### 2.1. Culture of Tetrahymena

*Tetrahymena thermophila* strains B2086 and Cu428 were generously provided by Professor Wei Wang (Shanxi University, China). *Tetrahymena* was inoculated into Super proteose peptone (SPP) medium (prepared by dissolving 15 g tryptone, 5 g yeast extract and 1 g glucose in 1000 mL double distilled water) and was cultured at 30°C for 72 h. Prior to the experiments, the *Tetrahymena* culture was centrifuged at 3500 rpm for 20 min. The supernatant was discarded, and the cell pellets were resuspended in 10 mM Tris-HCl (pH 7.4) at 1/10 of the original culture volume.

### 2.2. Processing of melamine

Analytical-grade melamine was purchased from Shanghai General Reagent Factory (Shanghai, China). Melamine decomposes under high temperature and high pressure. Therefore, to eliminate microorganism contamination in melamine, the drug was exposed to ultraviolet germicidal irradiation for 1 h prior to use.

### 2.3. The effect of melamine on Tetrahymena proliferation

SPP medium was prepared and dispensed into 5 test tubes; each tube contained 5 mL of medium. After autoclaving, the 5 tubes of medium were mixed with 0, 0.005, 0.010, 0.015 and 0.020 g of melamine, for final melamine concentrations of 0, 1, 2, 3 and 4 g/L, respectively. Amphotericin was then added to each tube of medium to a final concentration of 20 g/L. *Tetrahymena thermophila* strain B2086 was suspended at a density of $2 \times 10^5$ cells/mL, and 100 μL of the cell suspension was inoculated into each tube of the culture medium. *Tetrahymena* cells were cultured for 20 h at 30 °C with constant shaking (220 rpm). *Tetrahymena* cells were then counted under a microscope using a hemocytometer, and the half maximal inhibitory concentration ($IC_{50}$) was calculated. The experiments were repeated three times.

### 2.4. The effect of melamine on Tetrahymena sexual reproduction

SPP medium (1% proteose peptone, 0.2% glucose, 0.1% yeast extract, 0.003% EDTA ferric sodium salt) was prepared and dispensed into 5 test tubes; each tube contained 5 mL of medium. After autoclaving, the 5 tubes of medium were mixed with 0, 0.005, 0.010, 0.015 and 0.020 g of melamine, for final melamine concentrations of 0, 1, 2, 3 and 4 g/L, respectively. Amphotericin was then added to each tube of medium to a final concentration of 20 g/L. *Tetrahymena thermophila* strain Cu428 was suspended at a concentration of $2 \times 10^5$ cells/mL, and 50 μL of the cell suspension was inoculated into each tube of the culture medium. The Cu428 *Tetrahymena* cells were cultured at 30°C with constant shaking (220 rpm) for 25–30 h until the density of *Tetrahymena* in each tube reached $2 \times 10^5$ cells/ml. Another 5 tubes of medium were prepared following the same procedure described above and were then inoculated with the *Tetrahymena thermophila* strain B2086. *Tetrahymena* cells were centrifuged at 3000 rpm for 3 min (centrifugal radius: 18 cm). The supernatant was discarded. Cell pellets were washed with 10 mM Tris-HCl (pH 7.4) and resuspended. The cells were then starved for 24 h at 30 °C. Subsequently, the two *Tetrahymena thermophila* strains B2086 and Cu428 were mixed at a 1:1 ratio to achieve a total *Tetrahymena thermophila* density of $2 \times 10^5$ cells/ml. After 6 h, the number of mating *Tetrahymena* was counted. All experiments were repeated three times.

Experimental data were expressed as $x \pm s$. Statistical analysis was performed using SPSS 11.5 software. Multiple group comparisons were conducted using the homogeneity of variance tests and one-way analysis of variance (ANOVA). Furthermore, pairwise comparisons between the groups were also conducted. If the variances were homogeneous, the Student–Newman–Keuls (SNK) test was performed. If the variances were not homogeneous, the Games-Howell test was performed. *P* values less than 0.05 were considered statistically significant.

### 2.5. The single-cell gel electrophoresis assay (comet assay)

The single-cell gel electrophoresis (SCGE) assay, also known as the comet assay, is a method developed to evaluate DNA damage and repair in nucleated cells at the single-cell level. The comet assay allows for the direct observation of DNA damage within individual cells. The basic steps of the comet assay were as follows: suspensions of single cells were prepared, and the cells were then embedded in agarose on microscope slides; cells were lysed to release the DNA; single-stranded DNA was obtained using alkaline solution and subjected to electrophoresis under alkaline conditions; the alkali was neutralized, the DNA was fluorescently stained by EB, and the comets were visualized; the number of comet cells was counted, and the length of the comet tails or the Oliver tail moment was measured.

The DNA was observed and imaged under a fluorescence microscope (Fig. 1). Under 515–560 nm excitation light, cellular DNA exhibited red fluorescence (emission wavelength 590 nm). Unbroken DNA remained at its original location and formed a bright circle. Damaged DNA developed single-strand breaks. The resulting DNA fragments migrated toward the anode and generated a spindle-shaped or a radial pattern, resembling a comet with a tail. The slides were numbered, imaged and analyzed using the double-blind method. Overall, at least 50 images of DNA comets were analyzed per slide, and at least 100 comet images were analyzed per treatment. Based on the ratio of comet tail length to nuclear diameter, the severity of DNA damage was divided into five grades: grade 0, no damage; grade 1, mild damage (5%–20%); grade 2, moderate damage (20%–40%); grade 3, severe damage (40%–95%); and grade 4, extremely severe damage (>95%). The percentage of tailed cells in each group was determined, and the rate of cell damage and the arbitrary units were calculated. The arbitrary unit is a special unit utilized to evaluate the extent of DNA strand breaks. Different grades of DNA damage were converted into numbers, and the overall level of DNA damage was obtained. The arbitrary unit was calculated based on the following equation: an arbitrary unit = 0 × the number of cells containing grade 0 DNA damage + 1 × the number of cells containing grade 1 DNA damage + 2 × the number of cells containing grade 2 DNA damage + 3 × the number of cells containing grade 3 DNA damage + 4 × the number of cells containing grade 4 DNA damage. Differences between the groups were analyzed using the t-test. *P* values less than 0.01 were considered statistically significant.

### 2.6. Examination of apoptosis using flow cytometric analysis

SPP medium was prepared and dispensed into 4 test tubes; each tube contained 5 mL of medium. After autoclaving, the 4 tubes of medium were mixed with 0, 0.005, 0.010 and 0.020 g of melamine, for final concentrations of 0, 1, 2 and 4 g/L, respectively. Amphotericin was then added to each tube of medium to a final concentration of 20 g/L. *Tetrahymena thermophila* strain B2086 was suspended at a concentration of $2 \times 10^2$ cells/μL, and 100 μL of the cell suspension was inoculated into each tube of the culture medium. *Tetrahymena* cells were then cultured for 20 h at 30°C with constant shaking (220 rpm). A total of 5 μL of fluorescein isothiocyanate (FITC)-Annexin V (250 μg/mL) was added to 490 μL cells, mixed thoroughly and incubated for 10 minutes in an ice bath in the dark. The cells were then washed 3 times with phosphate-buffered saline (PBS) and analyzed on a flow cytometer. A 488-nm argon-ion laser served as the light source. FITC emits fluorescence after the