Cytotoxicity Assessment of Melamine Using the Ciliated Protozoan
*Tetrahymena pyriformis*

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**Abstract:** Melamine was once used as a non-protein nitrogen (NPN) source for cattle, and unethically added to food productions in order to increase the apparent protein content. In this study, the cytotoxicological impact of melamine was determined using a unicellular eukaryote bioassay model, the ciliated protozoan *Tetrahymena pyriformis* (*T. pyriformis*). The results showed a concentration-dependent inhibitory effect of melamine on the cell proliferation rate of *T. pyriformis* populations, and the IC₅₀ value was estimated to be 0.78g · L⁻¹. Deformed cells were also observed. Our experiment demonstrated that *T. pyriformis* bioassay, with advantages such as less time-consuming, lower cost and simpler operations, provides a good alternative *in vitro* method to animal experiments for the risk assessment of potentially toxic substances.

**Keywords:** *Tetrahymena pyriformis*; cytotoxicity; melamine

1 Introduction

Melamine, IUPAC name 1,3,5-triazine-2,4,6-triamine, is an organic base with the chemical formula C₃H₆N₆ and contain 66% nitrogen by mass. It is used to make fertilizers and, in combination with formaldehyde, for the production of melamine resin. In a 1958 patent, melamine was used as a non-protein nitrogen (NPN) source for cattle (Colby and Mesler Jr, 1958). However, Newton and Utley (1978) pointed out that the hydrolysis of melamine in cattle was less complete than that of other nitrogen sources and was unabsorbable for ruminants. In 1999, the United States Environmental Protection Agency (U.S. EPA) proposed to remove melamine for the tolerance expression (Environmental Protection Agency, 1999). The Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA) also provided a test method for analyzing melamine in animal tissues (Environment Protection Agency, 1999). However, in Lipschitz’s study, melamine in low doses was given orally to rats, rabbits and dogs with no significant toxic effects observed (Lipschitz and Stokey, 1945). Until recently, melamine was reported to have an oral LD₉₀ of >3000mg · kg⁻¹ based on rat data, and a dermal LD₉₀ of >1000mg · kg⁻¹ for rabbits (Laboratory, 2008). In 2008, melamine was illegally added to infant formula milk powders to increase the nitrogen contents and thus the apparent protein contents in standard tests such as Kjeldahl and Dumas tests, which estimates protein levels by measuring the nitrogen contents. This event had caused at least 290,000 infants in China to be hospitalized, mainly treated for kidney calculi or renal failure. The toxicity assessment of melamine is therefore important and urgent. To our knowledge, *in vitro* test has not been performed to assess the cellular toxicity of melamine. In this study, we demonstrated the cytotoxicity of melamine with a unicellular eukaryote bioassay.
model, *Tetrahymena pyriformis* (*T. pyriformis*).

*T. pyriformis* is a ciliated protozoa which are eukaryotic life-forms. It is ubiquitous in the aquatic environment and can be easily cultured axenically in a small volume of a complex culture medium. Its physiology and biochemistry are well known and is characterized by a short generation time, approximately 4h but variable in different culture conditions (Sauvant *et al.*, 1999). Therefore, the toxic impacts of test substances can be readily studied through several generations in a short period (Bonnet *et al.*, 2003). Because many structural features of its organelles are sensitive markers of the physiological state of *T. pyriformis*, it can be used as a simple bioassay model to study the potential toxicity of many substances (Sauvant *et al.*, 1999). In Erlanger Ciliaten test, *T. pyriformis* even showed a similar sensitivity as human cell cultures (Beermann *et al.*, 1999).

The aims of this study were (1) to assess the toxicity of melamine *in vitro* with the unicellular bioassay model, *T. pyriformis*; (2) to demonstrate that melamine cannot be utilized by eukaryotic cells as a non-protein nitrogen source; and (3) to show that *T. pyriformis* bioassays can be used as an effective method for the risk assessment of potentially toxic substances.

2 Materials and methods

2.1 Chemicals

Melamine, sucrose and D-Glucose (analytical grade) were obtained from Beijing Chemical Reagent Company, Beijing, China. Tryptone and yeast extract were purchased from Oxoid (Hampshire, England). Formaldehyde solution (analytical grade) was obtained from Shantou Xilong Chemical Factory, Guangdong, China.

2.2 Cell culture

The *T. pyriformis*, granted by associate professor Xu Fang in Shanghai Jiao Tong University, was grown axenically at 25°C in a liquid culture medium contained 1% (w/v) Tryptone, 0.1% (w/v) yeast extract, and 0.2% (w/v) D-Glucose for 24h to maintain the cells in exponential growth phase.

0, 0.001, 0.005, 0.01, 0.05, and 0.1g of melamine were added to a set of seven conical flasks containing 20mL of liquid medium, and the final concentration of melamine in each conical flask was 0, 0.05, 0.25, 0.5, 2.5, and 5g ·L⁻¹ respectively. Another two parallel sets of test culture medium were prepared. All the flasks were pasteurized at 103kPa, 121°C, for 20min.

2.3 Population growth rate test

Test cultures were prepared by inoculating 200µL from stock culture solution to each conical flask (time 0=T₀). These flasks were all incubated at 25°C in darkness without shaking. 184µL of test culture solution was withdrawn from the flask and was mixed with 16µL of formaldehyde solution to fix the cells, and then cell counts were performed microscopically by a hemocytometer.

Cell count was performed firstly at time T₀ to acquire initial cell concentration (D₀) which was 1×10⁶ cells ·mL⁻¹ in this experiment, and was then performed continuously every four or five hours successively for 52h. For each concentration of melamine, the recorded result was the average cell count of three parallel samples.

The growth dynamics of the populations was evaluated by plotting time versus Dₙ/D₀ ratios, where Dₙ and D₀ are cell concentration at time T₀+x hours and time T₀, respectively. The populations were characterized by their generation time (GT) which is the time required for the Dₙ/D₀ ratio to increase from 1 to 2 (Bonnet *et al.*, 2003).

The presence of toxic compounds could cause a decrease in cell growth with an increase in GT. Although the concentration of toxic compounds varied, the generation times of the control population (GTC) and those of the substance-treated populations (GTT) were determined. For each concentration, the relative generation time (RGT), expressed as percentage, was calculated using the equation: RGT(%) = [(GTT–GTC)/GTC]×100 (Bonnet *et al.*, 2003).

The relative toxicity of melamine was quantified
by determining the half maximal inhibitory concentration (IC50), which is the concentration required to induce a 50% increase in RGT (i.e. GTT is prolonged to 1.5 GTC). If the toxicity of melamine is concentration dependent, RGT values could be calculated by monadic linear regression analysis, and the data are fitted to the following model: RGT (%) = aX + b (Bonnet et al., 2003), where X represents the concentration of melamine. In the calculation of IC50 value, RGT(%) is 50, and X is the concentration of melamine under which GTC is prolonged to 1.5 GTT.

3 Results

3.1 Effects of melamine on the Tetrahymena pyriformis population growth rate

Fig.1 shows the trend of D0 with time for different concentrations of melamine. The presence of melamine causes a concentration-dependent decrease in population growth; i.e. the plot of log (D/D0) against time (Fig.2) yields the linear equation which provides an evaluation of GT of each test culture population.

![Fig.1 The time course of T. pyriformis growth dynamics](image1)

![Fig.2 The evaluation of generation time](image2)

When the cell density doubles, D/D0=2 and log (D/D0)=0.301. According the control culture (y = 0.1144+0.0325x), which dose not contain melamine; the GTC could be deduced at 5.7h for the experiment duration (52h). All of the test cultures were processed in the same manner to obtain GTT for each of them. Then the RGT was calculated using the ration: RGT (%)=[(GTT–GTC)/GTC]×100 (Bonnet et al., 2003). The plot of RGT versus concentration is presented in Fig.3. The IC50 value is determined mathematically from the linear regression equation Y=85.368x−16.7340. When RGT(%)=50, concentration is 0.78g·L⁻¹.

![Fig.3 The determination of IC50](image3)

Our experiments showed a concentration-dependent inhibitory effect of melamine on T. pyriformis. With the increase of melamine concentration, the growth of T. pyriformis was inhibited and the generation time of T. pyriformis was prolonged accordingly. When the concentration was 0.25g·L⁻¹, melamine noticeably inhibited the growth of T. pyriformis at the twentieth hour of cell culture. When the concentration of melamine reached 2.5g·L⁻¹, the growth of T. pyriformis had been significantly inhibited.

3.2 Effects of melamine on the Tetrahymena pyriformis cell morphology

We also observed deformed cells in the culture medium with the presence of melamine.

The normal pear-like shape of T. pyriformis was shown in Fig.4A. When the concentration of melamine was 0.05g·L⁻¹, the cell shape was not visibly affected (figures not shown). However, when
the concentration of melamine was 0.25g·L⁻¹, deformed cell appeared, with a cytomorphosis rate of approximately 10%, as shown in Fig.4B. When the concentration of melamine was 2.5g·L⁻¹, the cytomorphosis rate reached to approximately 90%, and the previous pare-like shape of T. pyriformis could not be maintained (Fig.4C), with many cells even being ruptured.

4 Discussion

In this study, we determined the cytotoxicity of melamine with a eukaryote unicellular model, the ciliated protozoa T. pyriformis. Experiments demonstrated the concentration-dependent inhibitory effect of melamine on T. pyriformis population growth.

Like other types of eukaryotic cells, T. pyriformis has an intracellular metabolism system which demands sufficient nitrogen sources and carbon sources to maintain the growth and proliferation of cells. However, the addition of nitrogen-rich melamine to culture medium did not induce a rapid increase of cell population during the defined period, i.e., the generation time of T. pyriformis did not decrease in the presence of melamine. Contrarily, the generation time was prolonged and its increase rate was concentration-dependent with melamine. The result showed that nitrogen element in melamine could not be utilized by T. pyriformis as a nitrogen source. Melamine is a trimer of cyanamide, with a 1,3,5-triazine skeleton. This structure may tend to behave as an imide (Ellie et al., 1940). Unlike utilizable organic nitrogen sources, such as amino acid, amide and amine, melamine is slightly soluble in water and cannot be decomposed easily. Although nitrogen gas can be released when melamine is burned or charred, most types of eukaryotes cannot utilize this kind of nitrogen source. Once melamine is absorbed by cells, probably via osmosis or endocytosis, it cannot be completely utilized or excreted through metabolism. Furthermore, the accumulation of a high dose of melamine may influence the normal function of cells and thus inhibit the growth of T. pyriformis. Although melamine in a low dose may not cause visual effects, long term exposure to a high dose of melamine can be harmful to eukaryotic cells.

The direct cytotoxicity of melamine to eukaryotic cell has not been previously evaluated. However, relationship between calculus formation in the urinary bladder of rats administered with melamine has been evaluated (Okumura et al., 1992). It was confirmed that a 3.0% dose level of melamine induced tumors in both the urinary bladder and the urethra (Okumura et al., 1992). An evaluation of a melamine dendrimer in cell culture showed that a substantial decrease in viability could be observed at 0.1g·L⁻¹ (Neerman et al., 2004). A melamine-linked nitrofurantoin also showed pronounced activity against parasites in mice (Stewart et al., 2004). Our results further demonstrate the chronic toxicity of melamine directly to eukaryotic cells. With T. pyriformis, this study confirms the inhibitory effect of melamine on the growth of cells.
It is proved that melamine cannot be used as a non-nitrogen source for eukaryotes, and its cytotoxicity will be accumulated in cells.

The less axenic culture requirements, experimental simplicity, sensitivity and rapidity demonstrate that *T. pyriformis* is an ideal model for in vitro bioassay, and *Tetrahymena pyriformis* bioassays can be well used for the risk assessment of potentially toxic chemicals.

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**References**


三聚氰胺对纤毛类原生动植物梨形四膜虫的细胞毒性

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**摘要:** 三聚氰胺曾被用为牛类的非蛋白类氮源, 并被非法掺入食品中从而提高其观蛋白含量。实验室用一种单细胞真核生物模型——纤毛类原动物梨形四膜虫 (*Tetrahymena pyriformis*) 研究了三聚氰胺的细胞毒性。结果显示, 三聚氰胺对梨形四膜虫的生长率具有抑制作用, 并且这种抑制作用与三聚氰胺浓度成正相关性, 其 IC₅₀ 值为 0.78g·L⁻¹。细胞同时发生变形。实验证明梨形四膜虫生物测定法相对于动物实验具有耗时短、成本低、操作简便的优势, 在潜在毒性物质的风险性评估实验中可成为一种优良的替代模型。

**关键词:** 梨形四膜虫; 细胞毒性; 三聚氰胺

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