Full Length Research Paper

DNA damage by the cobalt (II) and zinc (II) complexes of tetraazamacrocyclic in *Tetrahymena thermophila*

Zhong-Dong Xu[1]*, Rong-Hua Shi[2], Wei Wang[1], Rui-Song Tao[1], Jian-Hong Bi[3] and Zhao-Jun Wei[4]*

[1]Department of Biology, Hefei Teachers College, Hefei, 230061, China.
[2]School of Life Science, University of Science and Technology of China, Hefei, 230027, China.
[3]Department of Chemistry, Hefei Teachers College, Hefei, 230061, China.
[4]Department of Biotechnology, Hefei University of Technology, Hefei 230009, China.

Using the single cell gel electrophoresis method, the tetraazamacrocycle Zn(II) complex (Zn(II)-L) and the tetraazamacrocycle Co(II) complex (Co(II)-L) were investigated focusing on their DNA damage to *Tetrahymena thermophila*. When the cells were treated with the 0.05, 0.25 and 0.50 mg/ml Zn(II)-L, the tail length increased significantly, with 10.83, 11.56 and 11.87 μm, respectively. With the dose of 0.5 mg/ml Zn(II)-L treatment, 45.5% cells distributed in grade 3. After treatment with the 0.05, 0.25 and 0.50 mg/ml Co(II)-L, the tail length of the cells also increased significantly, with the length of 15.64, 17.75 and 19.21 μm, respectively. When treated with 0.5 mg/ml Co(II)-L, 98.1% cells showed tail and 75.6% cells distributed in grade 3. The results indicated that Co(II)-L induced a relatively high level of DNA damage in comparison with the level of damage induced by Zn(II)-L.

Key words: Tetraazamacrocycle Zn(II) complex, tetraazamacrocycle Co(II) complex, *Tetrahymena thermophila*, DNA damage, the comet assay.

INTRODUCTION

Although essential metals carry out important biological functions because they act as cofactors for a wide variety of metalloproteins and enzymes, they are cytotoxic when they accumulate in excess of cellular needs (William et al., 2007). Cobalt is a relatively rare element of the earth's crust, which is essential to mammals in the form of cobalamin (vitamin B12). The adult human body contains approximately 1 mg of cobalt, 85% of which is in the form of vitamin B12. However, long-term exposure to cobalt metal may be toxicity. Cobalt toxicity includes cardiomyopathy, adverse pulmonary effects, carcino-

genicity and T-cell-mediated allergic contact dermatitis (Per, 2007). Zinc is an essential catalytic and structural element of many proteins and a signaling messenger that is released by active neurons at many central excitatory synapses. Excessive synaptic release of zinc followed by entry into vulnerable neurons contributes severe neuronal cell death. Bae et al. (2007) demonstrated that the zinc–citrate compound is an apoptotic inducer in malignant trophoblast cells.

Azamacrocyclic ligands include soft side chain-RCOO- (hard base) and rigid cyclic ligand (soft base) in their structures (Burkhard et al., 2000), which makes it easy for the ligands to bind to metal ions broadly without losing selectivity in the meantime. Especially the 12, 14-N substituted tetraazamacrocycles, with its specific inner caves and pH-selective acetyl group, show strong coordination effect to metal ions (Bi et al., 2002). In previous research, the N-carboxymethyl derivative of the tetraazamacrocyclic ligand LH4 (5,7,12,14-tetramethyl -1,4,8,11-tetraazamacrocyclo-tetradecane-N,N',N"N"'-tetraacetic acid) and its complexes with Co(II) and Zn(II)

had been synthesized and characterized by elemental analyses, UV, IR, 1H NMR spectra and X-ray powder diffraction (Bi et al., 2002, 2006). However, the cytotoxic potential of the tetraazamacrocycle Zn(II) complex (Zn(II)-L) and the tetraazamacrocycle Co(II) complex (Co(II)-L), especially the DNA damaging ability, is not reported yet.

The single cell gel electrophoresis method (SCGE), also called comet assay, is a rapid and sensitive tool to demonstrate the damaging effects of different compounds or physical treatments on DNA at individual cell level. Cells with damaged DNA display increased migration of DNA fragments from the nucleus, generating a "comet" shape (Ashby et al., 1995). When the technique is carried out at alkaline condition (pH > 13), the length of the migration indicates the amount of cells with DNA single-strand breaks, incomplete excision repair sites and alkali-labile sites. DNA damage is often estimated by the percent of damaged cells or the migration length of the tail (Tice et al., 2000). Comet assay has been widely used to measure the DNA damaging in human cells, fish cells (Collins, 2004) and plant cells (Koppen and Verschaeve, 1996). The fresh-water ciliate, *Tetrahymena thermophila*, is a highly evolved cell with a functional complexity that rivals that of metazoa. Moreover, it combines ease of growth with the availability of advanced genetic tools, which has made *T. thermophila* a key model for studies of cell and molecular biology (Lah et al., 2004).

In present study, we applied comet assay to detected the DNA damage ability of the tetraazamacrocycle Zn(II) complex (Zn(II)-L) and the tetraazamacrocycle Co(II) complex (Co(II)-L), using *T. thermophila* as a model. The toxicity to *T. thermophila* of these two complexes at different concentrations were determined and the effects of Co(II)-L and Zn(II)-L were compared.

MATERIALS AND METHODS

Chemicals

The N-carboxymethyl derivative of the tetraazamacrocyclic ligand LH4 (5,7,12,14-tetramethyl-1,4,8,11-tetraazamacrocyclo-tetradecane-N,N',N"N"'-tetraacetic acid) and its complexes with Co2+ and Zn2+ were synthesized in previous research (Bi et al., 2002, 2006), and characterized by elemental analyses, UV, IR, 1H NMR spectra and X-ray powder diffraction. The stability constants of mononuclear complexes of LH4 with the ions above were determined at 35 ± 0.1, 45 ± 0.1 and 55 ± 0.1^0C in 0.5 mo1 L-1 KNO$_3$ solution by means of potentiometric and computer fitting.

Tetrahymena thermophila cultures and metal treatment

T. thermophila was kindly provided by Institute of Hydrobiology, Chinese Academy of Sciences, and was grown axenically at 37℃ as previously described (Santovito et al., 2007). Treated cells were grown in the same medium supplemented with Zn(II)-L and Co(II)-L (0.05, 0.25, 0.5 mg/ml) for 2 h. Cell density was determined by counting cells in a Bürker chamber.

Single cell gel electrophoresis (comet assay)

The comet assay was performed according to the method of Mourón et al. (2001). Briefly, conventional slides were covered with a first layer of 180 µl of 0.5% normal agarose (GIBCO BRL). An amount of 75 µl of 0.5% low melting point agarose (GIBCO BRL) were mixed with approximately 10,000 cells suspended in 10 µl and layered onto the slides, which were then immediately covered with coverslips. After agarose solidification at 4℃ for 5 min, the coverslips were removed and the slides were were immersed in freshly lysing solution (2.5 M NaCl, 100 mM Na$_2$EDTA, 10 mM Tris, pH 10) overnight at 4℃ containing 1% Triton X-100 and 10% dimethylsulfoxide, added just before use. Two slides were prepared from each control and treatment group under dimmed light conditions. After lysis, the slides were placed on an horizontal gel electrophoresis unit filled with fresh electrophoretic buffer (300 mM NaOH, 1 mM Na$_2$EDTA, pH >13) and left there for DNA unwinding for 20 min and then were electrophoresed for 30 min at 1.25 V/cm (300 mA). These procedures were performed at 4℃ under dim light. After electrophoresis, the slides were washed with neutralizing buffer (0.4 M Tris, pH 7.5) and the cells were stained with ethidium bromide (EB) at the recommended dilution. Observations were made at 400× magnification using a fluorescent microscope (Olympus BX40, equipped with a 515 – 560 nm excitation filter) connected through a Sony 3CCD-IRIS Color Video Camera. The image for each individual cell was acquired immediately after opening the microscope shutter to the computer monitor, employing the Image Pro Plus 3.0 program. When possible, pictures of 100 randomly selected comets per slide were analyzed.

Statistical analysis

According to the ratio of the migration tail length to the head length of the cell, the cells were classified in five categories, ranging from 0 (no visible tail) to 5 (still a detectable head of the comet but most of the DNA in the tail): 0 degree (no damage, < 5%), 1 degree (low damage, 5 - 20%), 2 degree (middle damage, 20 - 40%), 3 degree (high damage, 40 - 95%) and 4 degree (severe damage, > 95%) (Mourón et al., 2001). 100 cells were observed per gel and comet tail length, tailing ratio, and cell damage ratio (percentage of injured cells among all cells) were calculated, separately. The effect of chemical treatment on frequency of tailed cells, grade of damaged cells and migration was analyzed by T-test method using SPSS13.0 software (SPSS 13.0 for Windows, Apache Software Foundation, SPSS Inc., USA).

RESULTS AND DISCUSSION

The comet assay images of *Tetrahymena* DNA damage

After treatment with different concentration of Zn(II)-L and Co(II)-L, the cells showed different length tails. One hundred comets on each slide were scored visually according to the relative length of the tail. The extent of DNA damage was analyzed and then scored by the same experienced person, using a specific pattern when moving along the slide. The method of the observation was barred in a blind way during which the observer had no knowledge of the identity of the slide. The typical images of the cells of *Tetrahymena* from grade 0 (undamaged), 1(soft damaged), 2(middle damaged), 3 (high damaged) to grade 4 (severely damaged) were selected and showed
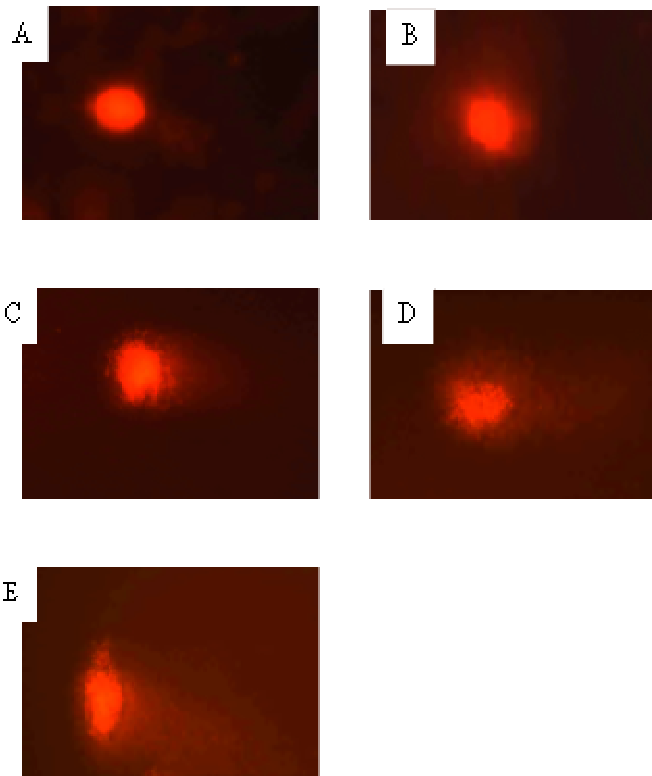
Figure 1. The typical images of the comets of *Tetrahymena* cells. A, grade 0 (undamaged); B, grade 1 (soft damaged); C, grade 2 (middle damaged); D, grade 3 (high damaged); E, grade 4 (severely damaged).
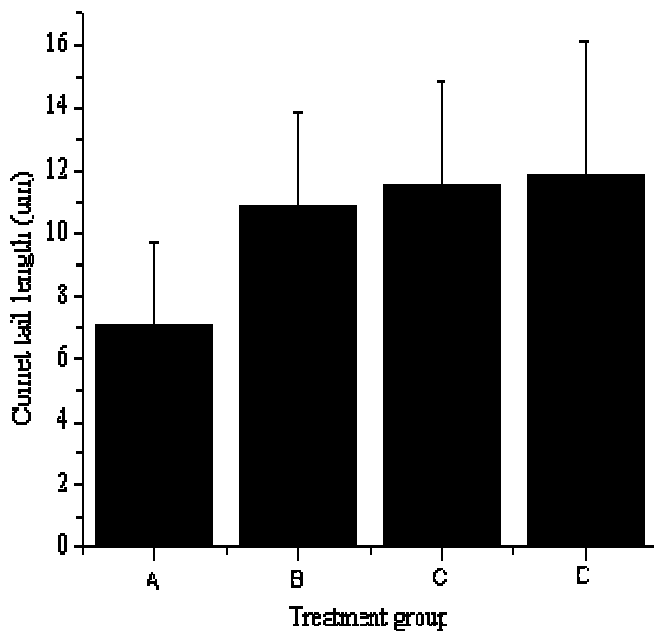
Figure 2. Effect of the Zn(II)-L treatment in the comet assay. A: control; B: 0.05 mg/ml Zn(II)-L ; C: 0.25 mg/ml Zn(II)-L; D: 0.5 mg/ml Zn(II)-L.

in Figure 1A to 1E.

Effect of Zn(II)-L treatment

Figure 2 shows the mean migration length and standard deviation. The tail length of the cells in the control group (untreated cells) measured about 7.07 µm. When the cells were treated with the 0.05, 0.25 and 0.50 mg/ml Zn(II)-L, the tail length increased significantly, to 10.83, 11.56 and 11.87 µm, respectively. In addition, the percentage of cells with tails showed high significant differences between the control group and those treated with the three concentrations of Zn(II)-L (Table 1). However, there were no significant differences of the percentage of cells with tails with the increasing dose of Zn(II)-L. In the control group, the cells with tails mainly distributed in grade 0 and 1. After treatment with Zn(II)-L, the cells in grade 3 increased significantly (Table 1). In the 0.5 mg/ml Zn(II)-L treatment, the cells in grade 3 was highest, with 45.5%. The above results demonstrated that after treatment with high concentrations of Zn(II)-L, the majority of cells showed more damage (grade 3 and 4) than cells from the control cultures.

Zn(II)-L can induce DNA damage in *T. thermophila*, which is similar to that observed in other zinc (II) complex. Quercetin zinc (II) complex successfully promotes the cleavage of plasmid DNA, producing single and double DNA strand breaks. The amount of conversion of supercoiled form of plasmid to the nicked circular form depends on the concentration of the complex as well as the duration of incubation of the complex with DNA (Jun et al., 2007). The zinc–citrate compound had a selective cytotoxic effect on choriocarcinoma cell line in dose- and time-dependent patterns, and is an apoptotic inducer in malignant trophoblast cells (Bae et al., 2007).
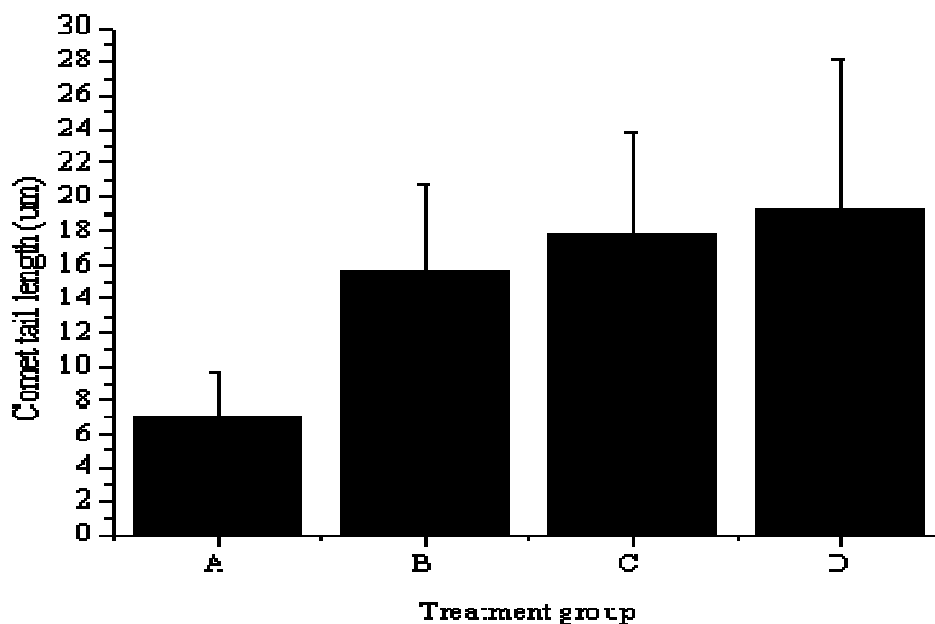
Effect of Co(II)-L treatment

After treatment with the 0.05, 0.25 and 0.50 mg/ml Co(II)-L, the tail length of the cells also increased significantly, to the length of 15.64, 17.75 and 19.21 µm, respectively (Figure 3), which were longer than those treated with the same dose of Zn(II)-L (Figure 2). The percentage of cells with tails and the grade of damage are presented in Table 2. The results showed that percentage of cells with tails increased significantly after the cells were treated with different concentration Co(II)-L. With the dose added, the cells in grade 3 increased. When treated with 0.5 mg/ml Co(II)-L, 98.1% cells showed tail, and 75.6% cells were distributed in grade 3 (Table 2), which is higher than those treated with Zn(II)-L (Table 1). These results indicated that Co(II)-L induced a relatively higher level of DNA damage in comparison with the level of damage induced by Zn(II)-L.

In present study, the results that the DNA damage

Table 1. Effects of Zn(II)-L on the mean migration length and the percentage of cells with tails.

Treatment	Percentage of different grade cells with tails (%)					Percentage of cells withtails (%)
	0	1	2	3	4	
Control	29.2	43.4	22.3	5.1	0	70.8
Zn(II)-L (0.05 mg/ml)	6.5	4.6	64.7	24.2	0	93.5[a]
Zn(II)-L (0.25 mg/ml)	5.2	9.1	44.6	39.6	1.5	94.8[a]
Zn(II)-L (0.5 mg/ml)	4.1	8.3	40.1	45.5	2.0	95.9[a]

[a]Significantly different from controls at $P < 0.001$.

Figure 3. Effect of the Co(II)-L treatment in the comet assay. A: control; B: 0.05 mg/ml Co(II)-L; C: 0.25 mg/ml Co(II)-L; D: 0.5 mg/ml Co(II)-L.

Table 2. Effects of Co(II)-L on the mean migration length and the percentage of cells with tails.

Treatment	Percentage of different grade cells with tails (%)					Percentage of cells with tails (%)
	0	1	2	3	4	
Control	29.2	43.4	22.3	5.1	0	70.8
Co(II)-L (0.05 mg/ml)	4.4	7.5	22.6	62.7	2.8	95.6[a]
Co(II)-L (0.25 mg/ml)	3.0	4.5	12.7	68.4	11.4	97.0[a]
Co(II)-L (0.5 mg/ml)	1.9	1.7	7.3	75.6	13.5	98.1[a]

[a]Significantly different from controls at $P < 0.001$.

ability of Co(II)-L indicate that cobalt is cytotoxic to *T. thermophila*. The data available in the literature indicate that cobalt is also cytotoxic to many other cell types, including neural cells (Wang et al., 2000; Olivieri et al., 2001), human MG-63 osteoblasts (Fleury et al., 2006) and can induce cell death by apoptosis and necrosis (Huk et al., 2004).

ACKNOWLEDGMENTS

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