Sensitivity of Different Cytotoxic Responses of Vero Cells Exposed to Organic Chemical Pollutants and their Reliability in the Bio-toxicity Test of Trace Chemical Pollutants[1]

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Objective To find a sensitive cytotoxic response to reflect the bio-toxicity of trace organic pollutants, the sensitivity and reliability of morphological change and proliferation inhibition of Vero cells exposed to 2, 4, 6-trichlorophenol (TCP) and the leachate from products related to drinking water (PRDW) were compared, and the mechanism of the morphological change in Vero cells exposed to chemical pollutants was studied. **Methods** Vero cells were treated by different concentration of TCP and the leachate from PRDW. Methylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide (MTT) assay was carried out for proliferation inhibition. Bioluminescence method was carried out as another method to test the toxicity of TCP. Flow Cytometry assay was used to test cell Apoptosis and damage of cell-membrane. **Results** 0.25 mg/L TCP had an effect on cell morphology, and the proportion of morphologically changed cells increased with increasing TCP concentration. At low TCP concentrations, inhibition of cell proliferation did not seem to correlate to TCP concentration, and was negative when TCP concentration was <1.0 mg/L. After exposure to leachate from PRDW extracted at different temperatures, the percentage of morphologically changed cells increased with extracting temperature, but the inhibition of cell proliferation failed to reflect the correlation between extracting temperature and proliferation inhibition of Vero cells. Although the Sensitivity of bioluminescence method seems to be similar to morphological change in Vero cells, the bacterial in this method is not homologous enough with human body cells to reflect the toxicity to human body. These imply cell morphological change is a more sensitive and reliable method to reflect bio-toxicity of organic pollutants than proliferation inhibition. Flow cytometry analysis and cell rejuvenation experiments indicated cell membrane damage, which results in cell morphological change, was an early and sensitive cytotoxic response comparing with necrosis. **Conclusion** These results indicated that the cell membrane toxicity represented by morphological changes is a more sensitive and reliable method to indicate the composite bio-toxicity of trace chemicals than proliferation inhibition, inhibition on bioluminescence and necrosis. Nevertheless, the quantification of morphological change should be studied further.

Key words: Vero cell; Morphological change; Membrane damage; Bio-toxicity test; Drinking water

INTRODUCTION

Chemicals from industrial processes inevitably seep into the environment during their production, transportation, and utilization. They can cause pollution of water, air, and soil. Drinking water refers to people's drinking water and domestic water, and it is so closely related to human health and quality of life that its importance is not less than food. 80% of the diseases and 50% of child deaths are related to the bad quality of drinking water in the world. Up to 50 kinds of diseases, such as digestive disease, infectious disease, skin disease, diabete, cancer, stone, *etc*., are caused by poor drinking water quality. It is reported that there are 2 221 types of detectable chemical pollutants in drinking water, some of which are carcinogenic, teratogenetic, or mutagenic[1-4]. Establishing validated assessment methods for the safety of drinking water is important because of the great variety and very low concentration of such chemicals in drinking water.

Traditional methods of using aquatic organisms have low sensitivity and long testing times. They therefore cannot be used to rapidly analyze the bio-toxicity of trace compounds. Photogenic bacteria are not sufficiently homologous to the human body to be used to assess the toxic effect of pollutants on the human body (even though this method has high sensitivity). Ames testing can be used only to assess carcinogenicity-mutagenicity-teratogenicity rather than composite toxicity, which reflects the composite effect of several types of trace chemicals to the

[1]This research was supported by the National Natural Science Foundation in China (No. 20677043 and 40871217).

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0895-3988/2010
CN 11-2816/Q

organism. Currently, evaluation of water safety focuses on testing the content of hundreds of chemicals in water, but such methods are complex and constrained by technological barriers. The risk to human health remains because of adducts and the synergistic effect of chemicals even though the concentration of every type of chemical is permissible. Therefore, establishing a broad-spectrum, reliable, sensitive, and easy method to assess the composite toxicity of trace chemicals and drinking water is important.

Vero cells come from the kidney of the African green monkey (*Cercopithecus aethiops*). They are homologous with human body cells and are readily cultured. As stated in the British Standard 6920-2.5:2000, healthy Vero cells have a triangular shape, but tend to "round-off" if poisoned by non-metallic products for use in contact with water[5]. Testing the composite toxicity of chemicals using Vero cells is therefore possible[6]. Romero *et al*. used Vero cells to test the toxicity of $HgCl_2$ and its response concentration[7]. The amount and viability of surviving cells after exposure to poisonous conditions can be analyzed by methylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide (MTT) colorimetry[8]. This method has been widely used to test the biological safety of foreign substances in medicines and food[9]. The bioluminescence method is based on the measurement of the reduction in light emission of the luminescent bacteria *Vibrio fischeri* after contact with a toxicant. It has been widely used as a reproducible and sensitive screening method to determine overall toxicity from different types of samples. The three toxicity test methods mentioned above are sensitive, but there is no overall study comparing their sensitivity and reliability in testing trace chemical pollutants.

In this experiment, 2, 4, 6-trichlorophenol (TCP) is selected as a representative material on refractory organic pollutants (POPs) and endocrine disrupting chemicals (EDCs) and a typical precedence-controlled organic pollutant[10-11], which is the current research focus, to be taken as one subject of study. Sensitivity of the above two cytotoxic responses of Vero cells and the Photogenic bacteria after exposure to TCP were studied. And we selected extract from products related to drinking water (PRDW) as the other subject of study to assess the reliability of the above two methods in testing the composite toxicity. TCP is a single substance and extract from PRDW is a mixture, and both of them are research hotspots in the field of environment and health. The mechanism of morphological change of Vero cells after exposure to organic chemical pollution was preliminarily investigated with flow cytometry, a quantitative method to assess individual cells[12-14]. These experiments could provide useful aids for finding a more sensitive and reliable method to evaluate composite toxicity of trace chemicals in the environment, and for identifying molecular markers of morphological change of Vero cells to quantify cell deformation.

MATERIALS AND METHODS

Cell Culture

The Vero cell line (CCL-81) was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). The cell line was cultured in Eagle's minimum essential medium (MEM, Gibco, Grand Island, NY, USA), supplemented with 7% or 2% (v/v) fetal calf serum (FCS, Gibco), penicillin (100 IU/mL) and streptomycin (100 g/mL). If cells grew too fast, MEM with 7% (v/v) fetal calf serum was used for subculture, and MEM with 2% (v/v) fetal calf serum was used for maintenance of cell growth after complete attachment of cells. Cells were inoculated in a 100-mm culture dish at a density of 3×10^4 cells/mL. They were incubated at 37 ℃ in an incubator humidified with 5% CO_2.

Photogenic Bacterium Culture

Vibrio qinghaiensis sp.-*Q67* was bought from East China Normal University. The culture medium consists of 13.6 mg KH_2PO_4, 35.8 mg $Na_2HPO_4{\cdot}12H_2O$, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.61 g $MgCl_2{\cdot}6H_2O$, 33.0 mg $CaCl_2$, 1.34 g $NaHCO_3$, 1.54 g NaCl, 5.0 g yeast extract, 5.0 g tryptone, 3.0 g glycerin, and 1 000 mL distilled water.

Before each test, the bacterium was inoculated from a stock culture, which is maintained on *Q67* culture medium agar at 4 ℃, to a fresh agar and cultured at (22±1) ℃ for 24 h. The cells were further grown in liquid culture medium by shaking (120 r/min) at (22±1) ℃ for 18 h and harvested by centrifuge at 3 000 rpm for 10 min. The pellet was resuspended with test medium and centrifuge twice. The bacterial suspension was made by adding test medium until the final relative light unit (RLU) reach 1.2×10^7. The suspension was kept at (22±1) ℃ for 30 min before the test.

Preparation of Leachate from the PRDW

According to the fifth part of International Biological Assessment for Medical Appliance (GB/T 16886.5-2003), the leaching ratio was set to 1.5 cm^2/mL. A water supply pipe of polyvinyl chloride (PVC) (ISO9001:2000 certification, Zhejiang Zhongcai Pipe Technology Company Limited, Zhejiang, China), and a single-use plastic cup made

from polypropylene (PP) (Xintianli brand, Zhejiang, China) were soaked separately in water for 24 h in thermotanks at 4 ℃, 37 ℃, and 60 ℃.

Cell Exposure to TCP/ PRDW

When cells were at the exponential growth phase, the monolayer was washed once in phosphate-buffered saline (PBS). After cell dissociation using 0.25% trypsin-EDTA (Gibco), exponential cells were inoculated at 3×10^3, 1×10^4, and 1×10^5 cells/mL into 24-well tissue culture plates (Corning, NY, USA), and the new medium containing experimental solutions of TCP at 0.25, 0.5, 1, 5, 10, 20, and 50 mg/L were added to it. After 12 h or 24 h exposure, the following experiments were carried out. Stock solutions of TCP (Sigma-Aldrich, St. Louis, MO, USA) were prepared by ultrasonication with sodium dodecyl sulfate (SDS); the SDS concentration in the medium was not >0.001%/PRDW.

Morphological Observation

The overall appearance of monolayer cultures of Vero cells was examined by phase contrast microscopy (IX71; Olympus, Tokyo, Japan) after culturing. Images were taken in white light by microscopy (×40).

Determination of Inhibition of Cell Proliferation (MTT Colorimetric Assay)

After culturing in different conditions, Vero cells were incubated for 4 h with 20 μL MTT solution. The medium was then replaced with acidified isopropanol and shaken for 10 min or left to stand for 40 min. Plates were transferred to a microplate reader (Model 550, Bio-Rad, Philadelphia, PA, USA) to measure the absorbance of the solution at 570 nm (reference wavelength, 650 nm)[15-17]. A solution of MTT (MTT Power, Beijing Boda Taike Biology and Genetics Company Limited, Beijing, China) was prepared in PBS to a final concentration of 0.5 mg/mL. The solution was sterilized by filtration and stored at 4 ℃ in a darkened environment. Preservation time did not exceed two weeks. The 5 different test concentrations in two parallels were arranged and MTT test was repeated three times.

Determination of Bioluminescence Inhibition on Q67

Toxicity tests of TCP on bacterial Q67 were performed on the Veritas™ luminometer with a 96-well microplate (Turner BioSystems Inc., USA). For an individual TCP, an appropriate dilution factor was selected as 0.4, 0.5, 0.8, 2, 4, 5, 10, 20 mg/L. To construct the CRC of TCP, the 8 different test concentrations in three parallels and 8 controls (milli-Q water) in a 96-well microplate were arranged and the microplate test was repeated three times.

The procedure in detail was as follows: 100 μL of milli-Q water was added to 10 wells of the first row in a microplate as controls, 8 toxicant concentration series were added to 8 wells of the second row and milli-Q water was added to make a total volume of 100 μL. In the same way as for the second row, various test solutions were added to the 8 wells of the third and fourth rows. Then 100 μL bacterial suspensions were added into each well to make the final test volume 200 μL. The Relative light unit (RLU) measurements of Q67 in various wells in the test microplate were then determined using the VeritasTM luminometer after 15 min exposure to the toxicants at (22 ±1) ℃.

Cell Apoptosis and Damage of Cell-membrane Assay (Annexin-V-fluorescein isothiocyanate-propidium Iodide (Annexin-V-FITC/PI)-bivariate Flow Cytometry)

Apoptosis, necrosis or damage of the cell membrane can be analyzed by flow cytometry after cell staining with annexin-V-FITC/PI. In the early stage of apoptosis, a change that occurs at the cell surface is translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer, whereby PS becomes exposed at the external surface of the cell[18]. Annexin-V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS. This protein can therefore be used as a sensitive probe for PS exposure upon the cell membrane. PS translocation to the external cell surface is not unique to apoptosis, but also occurs during cell necrosis. The difference between these two forms of cell is that during the initial stages of apoptosis, the cell membrane remains intact, whereas at the moment necrosis occurs, the cell membrane loses its integrity and becomes "leaky". Measurement of binding of annexin-V to the cell surface is therefore indicative of apoptosis, and must be done in conjunction with a dye exclusion test to establish integrity of the cell membrane[19].

PI is a type of DNA dye that cannot pass through the integrated plasma membrane. The intact plasma membrane can exclude the cationic fluorochrome PI. PI can stain only nuclei in cells with a disrupted membrane whether cells are in the stage of apoptosis or necrosis or not. The test described discriminates intact cells (FITC-/PI-), "early" apoptotic cells with membrane integrity (FITC+/PI-) and "terminal" apoptotic cells without membrane integrity and dead cells (FITC+/PI+).

Exponentially growing suspension cultures of

Vero cells (1×10^5 cells/mL) were incubated in six wells with TCP (1, 5, 20, and 50 mg/L) for 24 h and 48 h. Cells in the six wells were digested and placed in polyethylene tubes with different labels one by one. Cells were centrifuged at 1 000 rpm for 10 min, and the supernatant carefully removed. The cell pellet was resuspended again in cold PBS and centrifuged as described above, and the process repeated. Binding buffer (200 μL) was added to these cell suspensions; the final concentration of cells was 1×10^6/mL. Cells were stained for 15 min with 5 μL of annexin-V-FITC at 4-8 ℃ in the dark (Annexin-V-FITC Apoptosis Assays kits were from Bipec Biopharma Company, Cambridge, MA, USA). PI (10 μL) was added to cells for 5 min at 4-8 ℃ without light. Samples were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA) within 1 h[20-21].

Cell Rejuvenation

After culturing in medium with a high concentration of agent (50 mg/L TCP, 800 mg/L $ZnSO_4$) for 24 h and 48 h, the medium was removed and cells rinsed once in PBS. Cells were placed in culture medium with 7% serum for 24 h. The observation was carried out in white light by microscopy (×40). Images were taken for morphological changes before and after cell rejuvenation.

Measurement of Total Organic Carbon (TOC)

TOC measurement of the leachate was carried out using a TOC analyzer (TOCVCPH, Shimadzu, Japan).

RESULTS AND DISCUSSION

Sensitivity of Different Cytotoxic Responses of Vero Cells Exposed to TCP

Preliminary results showed that cells were observed most readily if the density was 1×10^4 cells/mL (24-well plate). After five repeated exposed experiments, similar results were obtained and Fig. 1 shows one of them. Fig. 1 shows the morphological change of Vero cells inoculated at 1×10^4 cells/mL and exposed to different concentrations of TCP. Cells grew and adhered well with a triangular shape in the negative control (Fig. 1a). A very small part of cells rounded off at low TCP concentration (0.25 mg/L; Fig. 1b). This was nearly identical to the control group (Fig. 1a) because there were also a few rounded-off or dead cells in the negative control. More cells were rounded-off or floating on the medium when TCP concentration was >0.5 mg/L (Fig. 1c). With increasing TCP, the number of rounded-off and suspended cells increased, and the number of attached cells decreased (Fig. 1d and g). In the positive control, all of the cells were rounded-off (Fig. 1h). $ZnSO_4$ caused sero-flocullation and microscopy observation was difficult, so images were taken after the medium was removed and PBS added. The number of cells in Fig. 1h was less than actuality because suspended cells were discarded.

At different seeding densities, after 24 h exposure to TCP at selected concentrations (1, 5, 10, 20, and 50 mg/L, six parallel samples at each concentration), optical absorption was measured by MTT assay for determining inhibition of cell proliferation.

In general, the higher the optical density (OD), the higher the number of living cells. The relative growth rate (RGR) of cells represents cell viability. In this study, RGR was converted to the relative inhibition rate (RIR) to directly reflect TCP cytotoxicity. The RIR of the control group without TCP and blank control group without cells was 0% and 100%, respectively. The RIR of chemical pollutants was obtained by Equation 1:

$$\text{RIR}=[(\text{Non-treatment control}-\text{blank control})-(\text{treatment}-\text{blank control})]/(\text{Non-treatment control}-\text{blank control})\times100\% \quad (1)$$

Fig. 2 shows the RIR of proliferation of Vero cells exposed to TCP. When TCP concentration was >5 mg/L, the RIR increased with increasing TCP at different densities of cell seeding; it was nearly a linear relationship. Their relationship was not obvious when the TCP concentration was between 1 mg/L and 5 mg/L. At medium and high seeding density, the RIR was negative at a lower concentration of TCP (<1 mg/L), and a non-inhibitory effect on Vero cells was observed. The T-test result indicated that a positive correlation ($P<0.05$) existed between concentration and the inhibition rate only at >5 mg/L.

The density of cell seeding had an obvious effect on the RIR of cells exposed to TCP (Fig. 2). The curve developed gently at high seeding density, and the RIR was lower than that of other groups at identical TCP concentration. With the decrease of cell seeding density, the inhibition rate of cell proliferation increased. Owing to the relative errors of absorbance measurement being relatively less when OD was between 0.2 and 0.8, the cell seeding density had to be between 6×10^4 cells/mL and 1×10^5 cells/mL.

To further understand the inhibitory regulation of cells exposed to a lower TCP concentration, absorbance measurement was carried out at a seeding

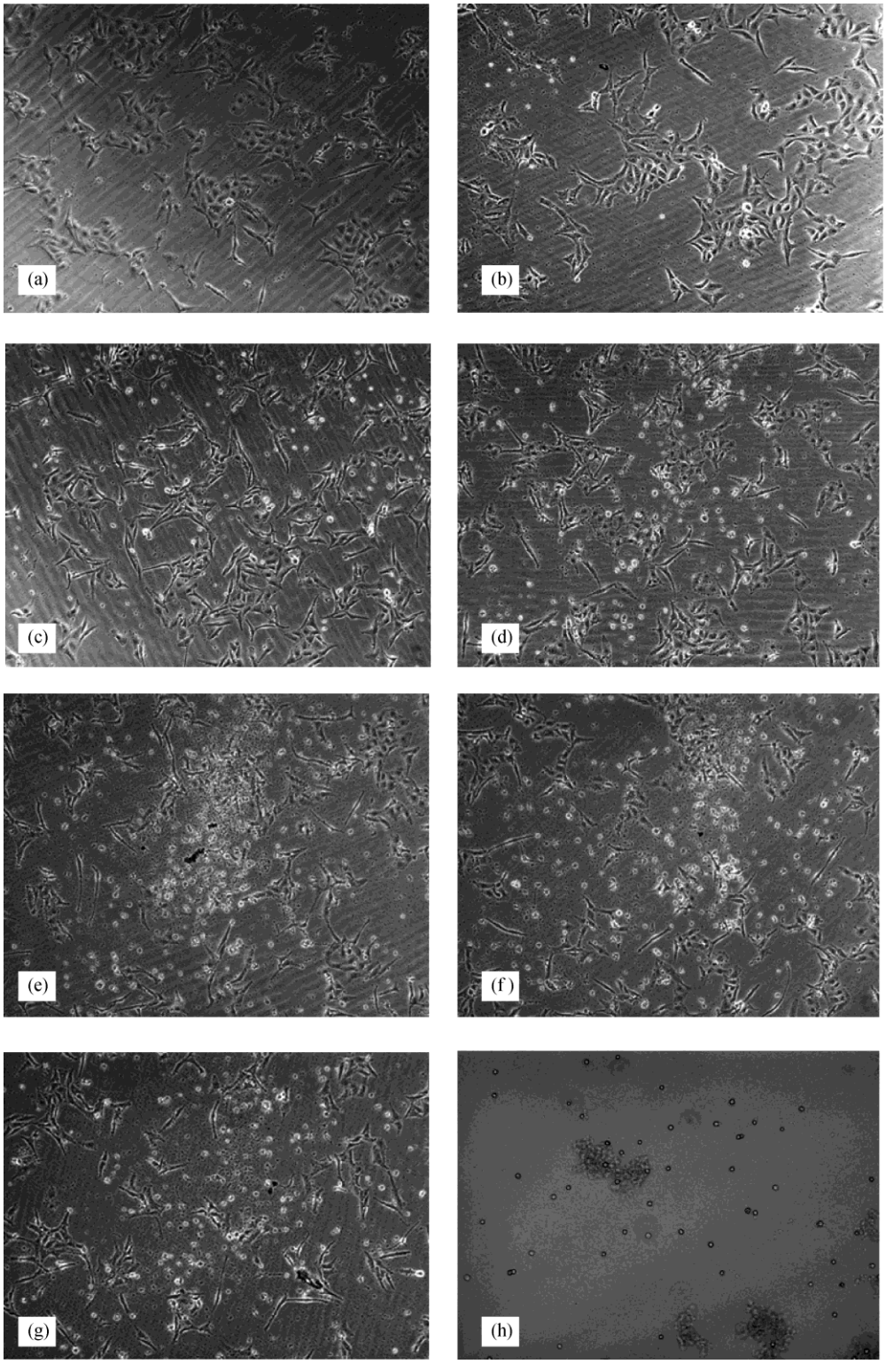

FIG. 1. Morphology change of Vero cells exposed to different concentration of TCP (1×10^4 cells/mL). a) negative control; b)-g) TCP in content of 0.25, 0.5, 1, 5, 10, 20 mg/L in sequence; h) positive control. The white points in above photos refer to suspending or "rounding off" cells.

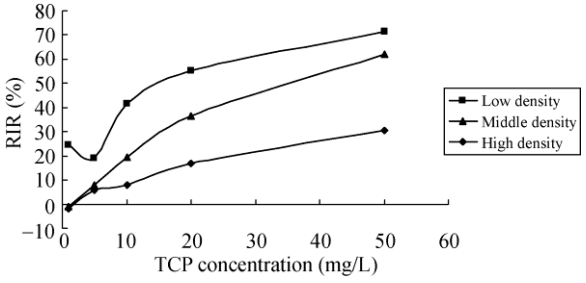

FIG. 2. Inhibitory effect to proliferation of Vero cells exposed to TCP.

density of 6×10^4 cells/mL at low TCP concentration (0.25, 0.5, 1, 2, and 5 mg/L) by MTT assay. The results of two parallel experiments are shown in Table 1. Measurement results were not stable when the concentration was between 1 mg/L and 5 mg/L. When TCP concentration was <1 mg/L, the inhibition rate was negative (i.e., non-cytotoxic). This indicated that MTT assay could not accurately reflect the correlation between the RIR and TCP concentration at low TCP concentrations.

Compared with proliferation inhibition, TCP >0.25 mg/L could cause part of Vero cells to round-off, and the percentage of rounded-off cells increased with increasing TCP concentration. Better correlation between TCP concentration and morphological change in Vero cells was therefore noted, and 0.25-0.5 mg/L could be regarded as the cytotoxicity response concentration for TCP for Vero cells.

As another control, bioluminescence method, which is based on the measurement of the reduction in light emission of the luminescent bacteria *Vibrio fischeri Q67* after contact with a toxicant, was also used to test the toxicity of TCP.

The toxicity of TCP to *Q67* was expressed as an inhibition ratio (E or x) as follows:

$$E(\%) = \frac{I_0 - I}{I_0} \times 100 \qquad (2)$$

where I_0 was an average of the RLU of Q67 exposed to the controls and I an average of the RLU to the TCP (three parallels) in one microplate.

The concentration-response curve (CRC) is shown in Fig. 3. It is found that the Logit can excellently describe the positive relationship between chemical concentration and percent inhibition on bioluminescence, that is the inhibition increased with the increasement of TCP concentration. However, their linear relativity is more obvious when the inhibition ratio is from 30% to 80%[22], and the inhibition ratio in this experiment was below 30%.

Although the sensitivity of bioluminescence method seems to be similar to morphological change in Vero cells, the bacterial in this method is not homologous enough with human body cells to reflect the toxicity to human body.

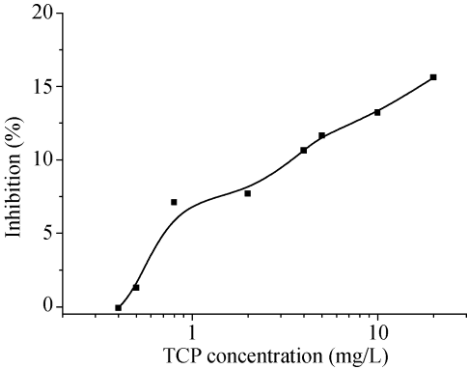

FIG. 3. Inhibition on bioluminescence of Q67 exposed to TCP.

These experimental results indicated that the cytotoxic response represented by change in cell morphology is more sensitive and reliable than that of inhibition of cell proliferation and bioluminescence method for testing the bio-toxicity of chemical pollutants.

Reliability of Using Change in Cell Morphology for Testing the Bio-toxicity of PRDW

The method based on morphological change in Vero cells is more sensitive than proliferation inhibition, so the former may be used to test the cytotoxity of trace compounds in the environment. For verifying reliability, the former method was applied to test the cytotoxicity of leachate from PRDW (which normally has low toxicity) and the latter method used as the reference.

TOC is an important parameter to reflect the content of organic compounds in the leachate of PRDW extracted by water. At extracting temperatures of 4 ℃, 37 ℃, and 60 ℃, for PVC tube, the TOC content of extracts from it was 1.256, 4.385, and 5.395 mg/L, and for PP cup, the TOC content was 1.010, 2.650, 4.454 mg/L, respectively. As extracting temperature increases, the extracts increase. At the same extracting temparature, the TOC content of PP was less than that of PVC, and it indicates that the extracts from PP was less than that of PVC.

Fig. 4 shows the microscopy images for Vero cells exposed to leachates extracted at different temperatures. The ratio of morphologically changed cells (which was caused by chemicals in the leachate) was obtained by counting and calculating cells according to the results in Fig. 4. The ratio in the negative control was 5.45%. At 4 ℃, 37 ℃, and 60 ℃, the ratio was 4.7%, 17.9%, and 23.1% for PVC, and 10.3%, 19.1%, and 26.8% for

a PP cup, respectively.

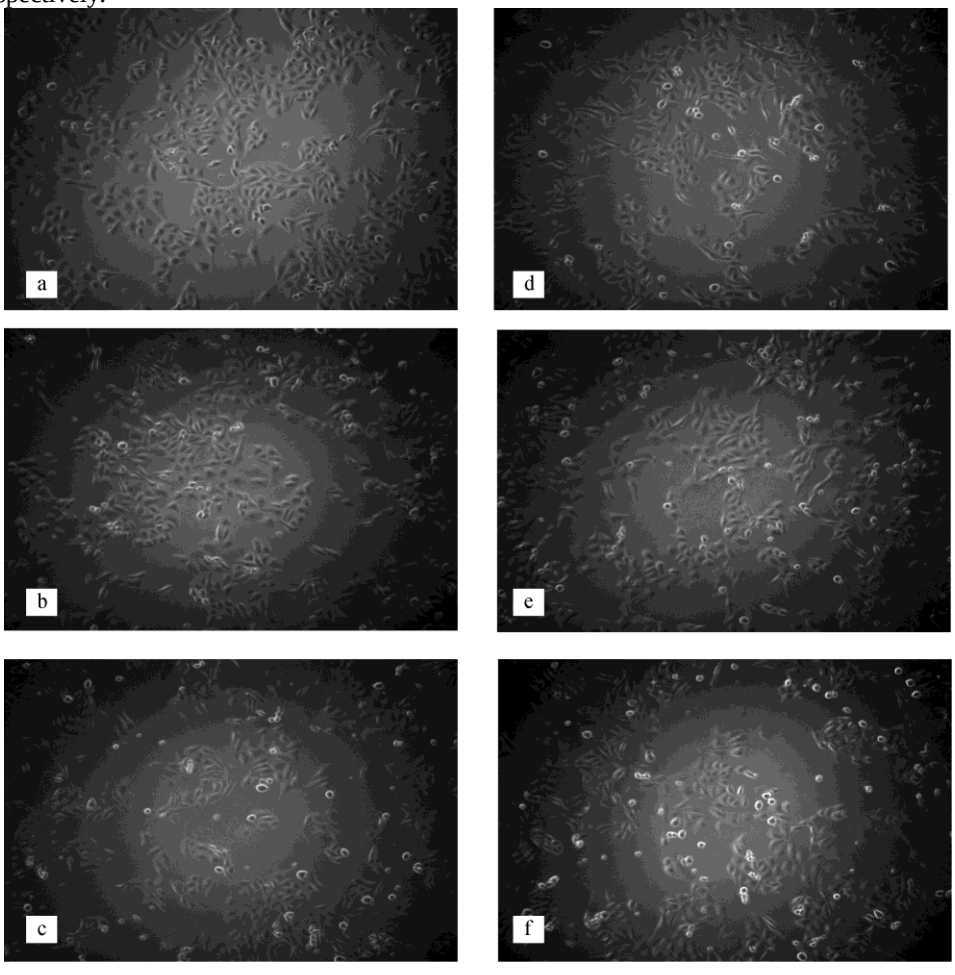

FIG. 4. Morphological change of Vero cells exposed to leachate. a), b), c) extracted from PVC at 4 ℃, 37 ℃, and 60 ℃; d), e), f) extracted from single-use plastic cup at 4 ℃, 37 ℃, and 60 ℃; negative control is Fig. 1a.

With increase in extracting temperature, the ratio of morphologically changed cells increased accordingly. Because TOC also increased with increasing extracting temperature, there was a fine correlation between the liquid concentration of extracts and the ratio of morphologically changed cells. The morphological change in cells caused by single-use PP cups was more distinct than that caused by the PVC tube under identical conditions, which means the Vero cells are more sensitive to PP. But it is too early to draw a conclusion that more morphology changed cells is equal to bigger cytotoxicity, since the low TOC content was far from the level of acute toxicity. The determination of cytotoxicity requires further studies.

Inhibition of cell proliferation was also carried out at 6×10^4 cells/mL seeding density by MTT assay. When the extracting temperature was 4 ℃, 37 ℃, and 60 ℃, the RIR was 6.80%, 5.00%, and 2.20% for PVC, and 7.70%, 11.30%, and 5.60% for PP, respectively. The extract liquid of the two products had an inhibiting effect on cell proliferation, but MTT assay failed to reflect the correlation between extracting temperature and proliferation inhibition of Vero cells. As extracting temperature increased, the inhibiting effect from PVC gradually decreased, whereas that from the single-use PP cup initially decreased and then increased.

From TOC measurements, we know that as the extracting temperature increased, the compounds in the leachate increased accordingly. The results of the MTT assay did not agree with this model. So the method based on the morphological change in Vero cells is more sensitive and reliable than the method based on inhibition of cell proliferation.

Quantifying the cytotoxicity of trace chemicals is

difficult because of the subjective judgments of morphological change with microscopic observation. The mechanism of morphological change in Vero cells therefore should be studied to find the specific cell components related with morphological change. This may provide a theoretical basis for establishing quantitative analyses of morphological change ratio, except for microscopic observation.

Relationship between Damage to the Cell Membrane and Morphological Change

Vero cells exposed to TCP for 24 h and 48 h were analyzed by annexin-V-FITC / PI-flow cytometry to clarify the mechanisms of morphological change. The test can discriminate intact cells (lower left (LL) quadrant, FITC-/PI-), "early" apoptotic cells with membrane integrity (lower right (LR) quadrant, FITC+/PI-) and "terminal" apoptotic cells without membrane integrity and dead cells (upper right (UR) quadrant, FITC+/PI+). Intensive study of the fourth quadrant (upper left (UL) quadrant, FITC-/PI+) is lacking. In general, it is believed that the cells in this quadrant are damaged by mechanical action[23]. Cells in this quadrant were analyzed in this contribution.

Fig. 5 is a scatter diagram of stained cells by annexin-V-FITC/PI after 24 h TCP treatment. In Fig. 5, with increasing TCP concentration, the proportion of viable cells in the LL quadrant gradually decreased from 93.68% to 43.75%, and that of dead cells (including latter apoptotic and necrotic cells) in the UR quadrant gradually increased from 4.98% to 27.70%. The number of "early" apoptotic cells did not visibly increase in the LR quadrant. As TCP

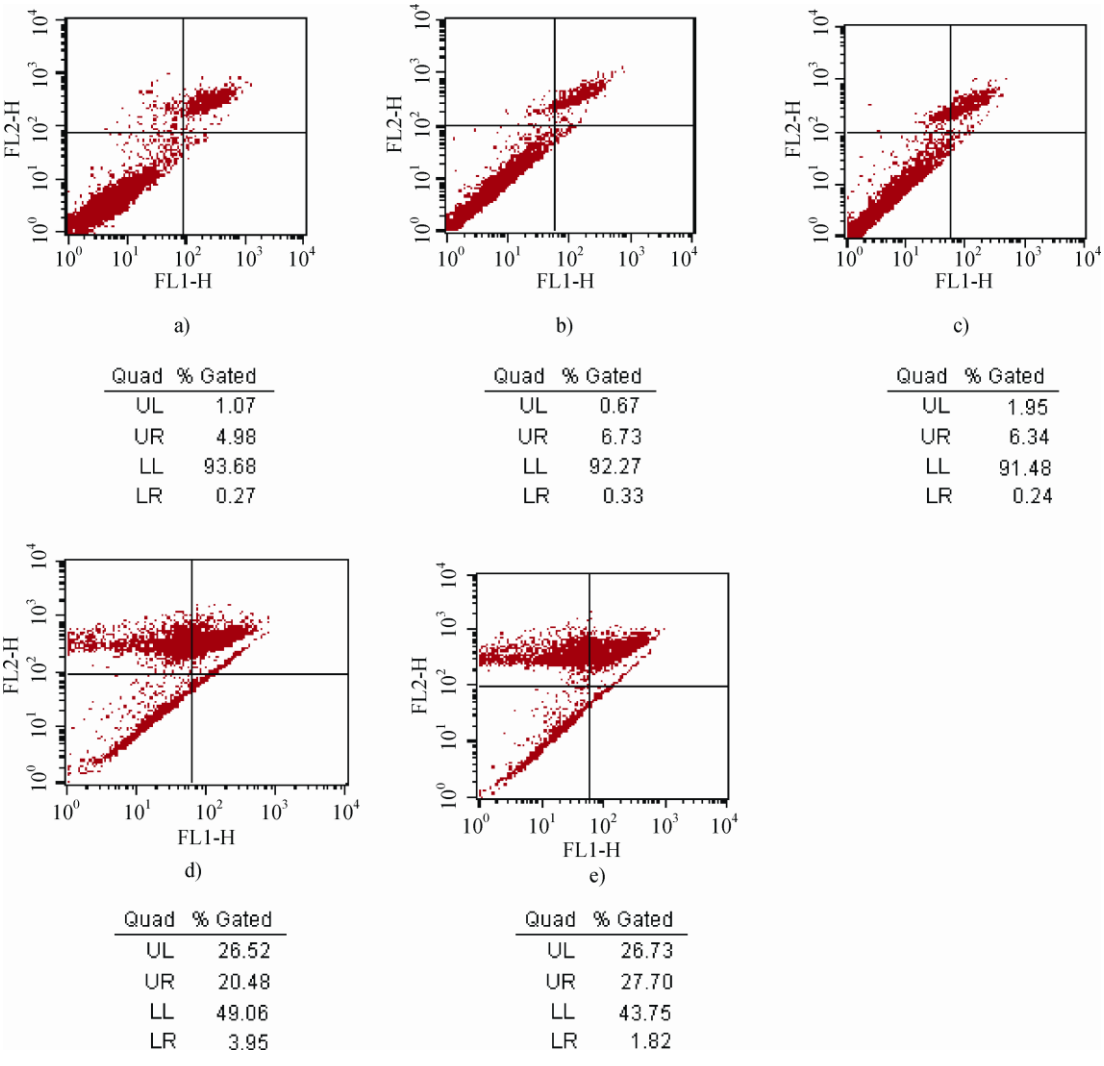

FIG. 5. Double parameter scatter plots of Vero cells exposed to TCP after 24 h. Quad-quadrant, UL-upper left, UR-upper right, LL- lower left, LR-lower right. a) negative control, b)-e) TCP in content of 1, 5, 20,

50 mg/L in sequence.

concentration increased from 1 mg/L to 50 mg/L (particularly over 20 mg/L), the proportion in the UL quadrant increased and exceeded the total number of dead cells (including apoptotic and necrotic cells). It is generally acknowledged that cells in the UL quadrant are damaged by mechanical disturbances during the testing process, so cells in this quadrant should stay at a background level. That is, the proportion of cells with mechanical damage in the UL quadrant should be almost identical at different concentrations of TCP if the mechanical damage was caused only by mechanical disturbances. However, in the 24 h experiment, the proportion of cells in this quadrant clearly increased with increasing TCP concentration. In theory, "PI-stained" means that the cell membrane has been not integrated (membrane damage), whereas "FITC-unstained" means that PS does not translocate to the external cell surface, i.e., apoptosis has not occurred yet. Compared with the small portion of cell apoptosis, damage to the integrity of the cell membrane may be the main form of Vero cytotoxic response after 24 h exposure to TCP, and cells in the UL quadrant may be membrane-damaged, not apoptotic or necrotic. Ana *et al.*[24] and Murata *et al.*[25] also found that the change in the cell membrane skeleton happened before apoptosis and necrosis.

In the scatter diagram of cells exposed to TCP for 48 h (Fig. 6), the proportion of viable cells (LL) decreased and that of dead cells (including apoptotic and necrotic cells) increased as TCP concentration increased from 1 mg/L to 50 mg/L. (When TCP concentration was 1 mg/L, the proportion of dead cells (UR) was slightly smaller than that of blank control group.) The proportion of "early" apoptotic cells did not visibly increase (LR). The percentage of cells without membrane integrity, which were stained by PI rather than FITC, increased from 0.81% to 8.02% in the UL quadrant, but this percentage was much smaller compared with that after 24 h treatment. The proportion of apoptotic and necrotic cells in the UR quadrant was bigger than that of cells in the UL quadrant. These results indicated that under this condition, apoptosis and necrosis are the main forms of cytotoxic response of TCP exposed to Vero cells. According to these results, our preliminary conclusion was that membrane damage is the "early damage phase" after TCP treatment. That is, TCP causes membrane damage of Vero cells before it causes apoptosis and necrosis. Membrane damage is therefore an earlier and more sensitive measurement of cytotoxicity than apoptosis and necrosis.

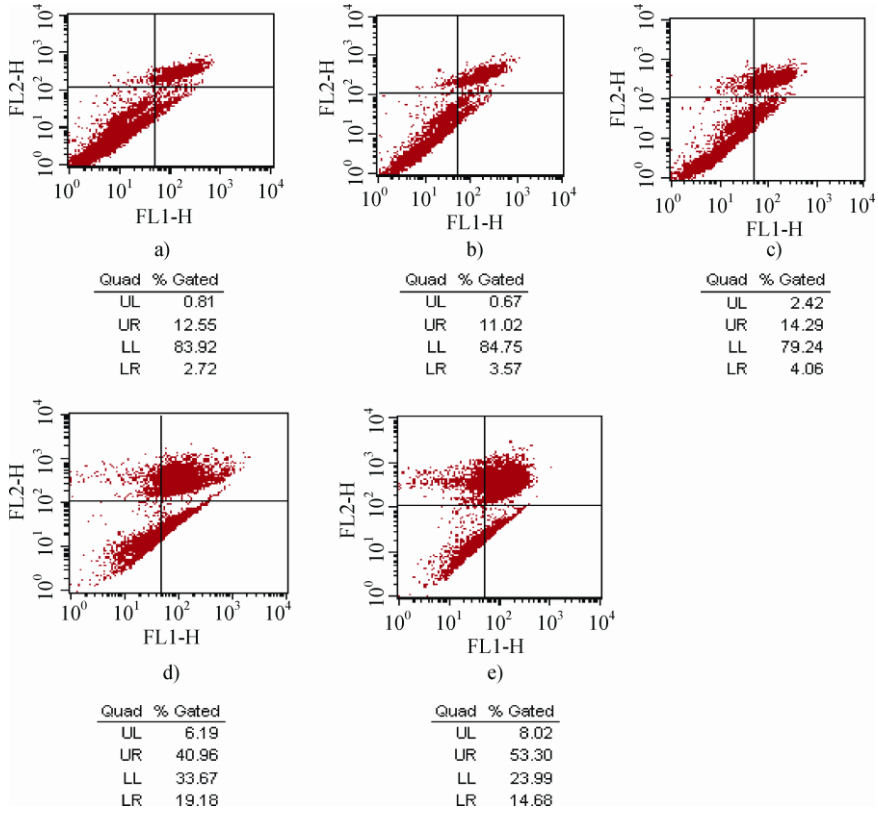

FIG. 6. Double parameter scatter plots of Vero cells exposed to TCP after 48 h. Quad-quadrant, UL-upper left,

UR-upper right, LL-lower left, LR-lower right. a) negative control, b)-e) TCP in content of 1, 5, 20, 50 mg/L in sequence.

Cell rejuvenation studies further confirmed that most morphologically changed cells within 24 h were not dead (including those in the latter stages of apoptosis and necrosis). After treating for 24 h by a high concentration of TCP or $ZnSO_4$, morphologically changed cells could return to normal shape and continue to grow after they were transferred to normal medium for rejuvenation, but cells treated for 48 h could not be rejuvenated. This indicated that necrosis or apoptosis did not happen in morphologically changed cells in 24 h.

Considering the results of cell morphology, flow cytometry and cell rejuvenation, it can be postulated that morphological change of a cell may be related to damage of the integrity of the cell membrane, and the morphological change in cells may be one of the macroscopic reflections of membrane damage.

The skeleton of the cell membrane has an important role in maintenance of cell shape, so morphological change may be caused by variation or release of some components related to the rigid structure of cells. There are two explanations on the reason for the components variation: one is the variation at DNA level leads to the expression change of membrane protein, and then the phospholipids; the other is no variation at DNA level happens, but the final assembly process of membrane protein and phospholipids may be interfered by chemicals.

Since the present results show that membrane damage of Vero cell by organic chemical pollutant is in an earlier cytotoxic phase than apoptosis and necrosis is, it may be assumed that the corresponding gene on membrane damage might be unchanged at DNA level, but the assembly process of membrane protein and phospholipid may be interfered by chemicals, and the components of cell membrane may change, thus leading to cell morphological change on a macroscopic level.

The cell morphological change is probably a more sensitive parameter to indicate the cytotoxicity of chemicals compared with proliferation inhibition, apoptosis and necrosis. Clarifying and quantifying the variation or release of some components in the plasma membrane of morphologically changed cells could establish the foundation for quantifying the morphological change in Vero cells.

CONCLUSION

The cytotoxicity of Vero cells exposed to a low dose of chemicals in the early phase may be a morphological change which is led by membrane damage; cell apoptosis and necrosis may occur at a high dose of chemical or after a longer period of treatment. The membrane damage related with morphological change is therefore a more sensitive method to test the composite bio-toxicity of trace chemicals compared with the inhibition of cell proliferation, apoptosis and necrosis.

The test of the morphological change in Vero cells is dependent upon on qualitative assessment, such as microscopic observation. This produces several problems: (1) discriminating the difference in morphological change of cells exposed to different concentrations of chemicals is difficult; (2) precisely defining and counting morphologically changed cells is difficult; and (3) observational results are readily disturbed if cells are in a poor condition. These problems are barriers to establishing a quantitative functional relationship between the toxicity of chemicals and the proportion of morphologically changed cells. This contribution has given a preliminary deduction that the morphological change in Vero cells is relevant to the damage to the integrity of the cell membrane. Future work will focus on the discovery and quantitative analysis of specific membrane components related with morphological change of cells. This may provide the theoretical foundation for establishing a quantitative relationship between the cell deformation ratio and these specific cell membrane components. Thereby, the weakness of the method based on the morphological change in Vero cells will be overcome through quantifying the cell deformation ratio.

ACKNOWLEDGEMENTS

The authors thank the National Natural Science Foundation in China for supporting to this work (No. 20677043 and 40871217).

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(*Received August* 10, 2009 *Accepted March* 21, 2010)