Studies on toxicity of multi-wall carbon nanotubes on suspension rice cells

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Abstract

Possible toxic effects of multi-walled carbon nanotubes (MWCNTs) on plant cells were investigated. Suspension rice cells (Oryza sativa L) were cultured with MWCNTs; reactive oxygen species (ROS) increased and cell viability decreased were observed. When ascorbic acid, a primary antioxidant, was introduced into the culture suspension, the ROS content decreased and cell viability increased. Transmission electron microscopy revealed individual tubes in contact with the cell walls. The suspension rice cells with individual MWCNTs at their cell wall seemed to undergo a hypersensitive response, namely the ROS defense response cascade, which is sufficient to prevent microbial pathogens from completing their life cycle.

1. Introduction

Carbon nanotubes (CNTs) are a carbon allotrope that was relatively recently identified. The CNTs are classified as single-walled carbon nanotubes tubes (SWCNTs) (one graphene cylinder) and multi-walled carbon nanotubes (MWCNTs) (2-50 graphene cylinders with a common long axis). The CNTs are one of the most promising nanomaterials in nanotechnology due to their unique physico-chemical, electronic, and mechanical properties and the large number of potential applications. The possible applications for CNTs range from biomedicine to nanoelectronics and mechanical engineering. To improve the quality of life, nanotechnology has been used in consumer products ranging from health care to agriculture, and concerns about the possible side effects of this technology in ecosystems, human health, and agricultural industries has been addressed [1]. The global demand for CNTs is predicted to be more than many thousands of tons in a single year, and MWCNTs have already been appearing in the market in industrial quantities. Many studies have assessed the results of CNT exposure in animal and human cell lines and the toxicity in cells, bacteria, and organisms [2-7], but the exact toxic mechanism of CNTs in humans and animals is still unclear. In the currently hypothesized toxicity mechanisms, reactive oxygen species (ROS) generation and oxidative stress is the best paradigm for explaining the toxic cellular effects of CNTs. For example, CNTs can fuse with the plasma membrane, and they have been shown to cause cell damage through lipid peroxidation and oxidative stress [5, 8].

Plants and plant communities are very important to humans and their environment (i.e. ecosystem), but very few studies have been conducted with ecological terrestrial test species (plants, wildlife, soil invertebrates, or soil microorganisms) to assess the potential

toxicity of nanoparticles [9-11]. The number of studies reporting the toxicity of MWCNTs is far less compared to SWCNTs; only a limited number of studies have been done on the phytotoxicity of MWCNTs. There have been contradictory results on CNT toxicity in plants. Canas et al. [10] found the toxic effect of SWCNTs on the root elongation of cucumber; scanning electron microscope (SEM) examination confirmed the interaction of chemically unmodified CNTs and modified CNTs with the root surface. The CNTs formed nanotube sheets on the surface of cucumber but did not enter the roots. Canas et al. [10] also examined the toxicity of SWCNTs on root elongation in six crop species: cucumber, cabbage, carrot, lettuce, tomato, and onion. Of these six crop species, four (cucumber, lettuce, tomato, and onion) had affected root elongation. On the other hand, Lin and Xing [11] reported insignificant MWCNT toxicity on seed germination and root growth in six plant species, but only one concentration was examined, which indicates the need for further study. The effects of alumina nanoparticles on root elongation were also demonstrated by Yang and Watts [12] in five crop species: corn, cucumber, soybean, cabbage, and carrot. Our previous study found that MWCNTs interact directly with rice cells and may have a detrimental effect on rice growth [9].

A possible method of introducing nanoparticles into the soil environment is the use of nanoparticles in agriculture. Thus, it is important to know the potential toxicity of manufactured nanoparticles in various crop species. The objective of this study was to investigate the toxic effects and mechanism of MWCNTs in rice cells. The study was carried out by culturing rice cell suspensions in the presence of MWCNTs; new insights into the toxic mechanisms were obtained.

2. Experimental Procedures

2.1. *Carbon nanomaterials*

The MWCNTs were purchased from Sheuzhen Nanotech Port Co., Ltd. (China). The physical dimensions, elemental components, and surface areas are summarized in Table 1. Carbon blacks (CBs), partial-like carbon nanomaterials that have been the industry utilized structural or conductive filler in plastics, were chosen as the reference samples.

TABLE 1 here

2.2. *Cell culture*

The rice cell line Oriza sativa (C5928) was obtained from RIKEN Bioresource Center (Tsukuba, Japan). Cells were cultured in Murashige and Skoog Basal Medium (MS). The cells were subcultured once a week and maintained at 26°C in darkness on an orbital shaker at 120 rpm. For the MWCNT toxicity studies, cells in the exponential phase of growth (6-day-old cell culture) were used.

2.3. *MWCNT treatment*

The sonicated MWCNTs (S-MWCNTs) were dispersed using an ultrasonic bath with high operating frequencies (45 kHz) for 15 hours. Carbon blacks (CBs) used as the reference materials were also dispersed by sonication without adding of any kind of surfactants (note that very few individual tubes of the MWCNTs were observed by TEM, data not shown). These carbon nanomaterials were then added in to deionized water to

create the stock solutions. These stock solutions were sterilized and then use for preparing the media for the suspension cell cultivations. The as-received MWCNTs (A-MWCNTs) were put directly into the cell suspension after the sterilization and added to the medium to yield a final concentration of 20 mg/L. Control cells and treated cells were harvested for biochemical analysis after 6 days of cultivation. All experiments were repeated three times.

2.4. *Morphological observation by transmission electron microscope (TEM)*

The morphology was examined with a TEM (Hitachi H-800) using an accelerating voltage (75 kV). The cells obtained from 5-day-old subculture were observed in the following procedure. The cells were washed twice with PBS and then fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.2) at 4°C for 4 h. The cells were then washed with PBS and post-fixed in 1% osmium tetraoxide at room temperature for another 2 h. After dehydration with gradient ethanol and Epon812 resin and acetone embedding, the ultrathin sections were made for TEM examination.

2.5. *TTC assay*

Cell viability was tested using 2, 3, 5 triphenyltetrazolium chloride (TTC) reduction to red formazon [13]. Percent viability was calculated considering the optical density (OD) values at 485 nm of rice cell suspensions.

2.6. *Determination of ROS*

Intracellular ROS were detected using the fluorescent probe 2', 7'-dichlorofluorescin diacetate (DCFH-DA) according to Yin et al. [14] with slight modifications. Briefly, DCFH-DA was added at a final concentration of 5 μM to cells suspended in 3 ml of 0.1 M PBS (pH 7.8), and the mixture was placed in an incubator at 25°C in the dark for 1 h. The cells were then washed three times with PBS (0.1 M, pH 7.8) and finally suspended in 3 ml PBS (0.1 M, pH 7.8). The fluorescence intensity was monitored using a spectrofluorometer (Hitachi F-4500) with the excitation wavelength at 485 nm and emission wavelength at 522 nm. The fluorescence intensity at 522 nm normalized to the protein content which was used to estimate the relative ROS production. Bovine serum albumin was used as a standard to calculate the soluble proteins.

3. Results

3.1. *Morphological observations*

The TEM imaging can help increase the understanding of how MWCNTs enter cells, where they migrate, and their fate after uptake. To study MWCNT uptake and the toxic effects on rice cells with the TEM, we used a concentration of 20 mg/L. After 2 days of MWCNT exposure, the MWCNTs were associated with the outer layer of the rice cell wall (Fig. 1). Detachment of the plasma membrane from the cell wall was observed, indicating that the cell was damaged. We found that the MWCNTs can interact with the cell in a manner in which the MWCNTs only associate with the cell wall; no nanotubes were found intracellularly, suggesting that the cell wall can hold back MWCNTs from entering the cytoplasm. To the best of our knowledge, this is the first demonstration that individual MWCNTs can interact with the surface of the rice cell wall and prevent the

MWCNTs from entering the cytoplasm. The cell wall only exists in prokaryotic cells, such as plant cells, and all cells have cell membranes. The cell wall is located outside the cell membrane and is made of cellulose in plants, providing structural support, protection, and acting as a filtering mechanism. The cell membrane is made of a lipid bilayer with proteins and cholesterol, regulating what enters and exits the cell. Because of the cell wall and its structural properties, it is more difficult for CNTs to cross the cell wall than the cell membrane. With the two structural barriers (cell wall and membrane), the plant cell more easily holds back the CNTs from becoming intracellular compared to the animal cell. This observation is the first evidence that the defense mechanism of plant cells for CNTs is different from animal cells. Note that we have not observed the intracellular MWCNTs in the samples of rice cells co-treated with MWCNTs even at the highest concentration of 80mg/l.

FIGURE 1 here

Dose-dependent changes in rice cell morphology were observed in the presence of MWCNTs. After 2 days of MWCNT (20 mg/L) exposure, the majority of cells appeared to have altered morphology; chromatin began to condense inside the cytoplasm (Fig. 2b, yellow arrow) and caused cell death. In comparison, after 5 days of exposure to 20 mg/L MWCNTs, we observed features characteristic of apoptosis: plasma membrane detachment from the cell wall (Fig. 2c, e, white arrows) and cell shrinkage (Fig. 2c, blue arrow). Exposure to a high concentration (80 mg/L) of MWCNTs resulted in cytoplasm leakage and membrane disruption (Fig. 2d, red arrows), indicating that the cells had

undergone necrosis. In untreated cells, the cytoplasm exhibited the typical ultra structure of a nucleus that contained a nucleolus and numerous normal organelles, including vacuoles (Fig. 2a). Cell death occurred via two processes: apoptosis and necrosis. Apoptotic cells appeared shrunken, with much chromatin condensation and, in the case of necrotic cells, the cytoplasmic content leaked out and membrane disruption was visible. Using the TEM, MWCNTs were seen to localize only on the cell wall, not intracellularly, implying that they may interact with the cell wall, which would greatly enhance their toxic potential. The mechanism of neither penetration nor effect is fully understood, and could be either to traverse the cell membrane or diffuse through the lipid bilayer. The cell membrane is permeable to different substances that can diffuse through it. A recent report demonstrated that the interaction of various polypeptides with individual carbon nanotubes, both MWCNTs and SWCNTs, investigated by atomic force microscopy (AFM) [15]. Another study used spectroscopic techniques to examine a composite formed by SWCNTs and rice starch, and identified non-covalent interactions between the CNTs and starch [16].

FIGURE 2 here

3.2. *Cell viability assay*

To further study the toxic effect of MWCNTs on viability, the TTC assay was used. Cells were exposed to different concentrations of MWCNTs (0, 10, 20, 40and 80 mg/L), and the percentage of viability was calculated considering the OD values at 485 nm after 1-10 days of incubation. Fig. 3 shows the typical data for the cells exposed to 20, and 40

mg/L of S-MWCNTs. Data for the control cells and the cells exposed to 20 mg/L S-CBs are also given in Fig. 3, for comparison. The number of viable cells showed a MWCNT concentration-dependent decrease, and as the concentration of S-MWCNTs increased, cell viability decreased. Also, the cell viability of the CB-treated group was the same as in the control group.

FIGURE 3 here

The toxic effect appeared after 4 days of exposure, and differences between control and the S-MWCNT-treated group became more prominent after culturing the cells for 5-7 days. An analysis of the dose-dependent toxicity after 4 days of S-MWCNT exposure revealed that the number of viable cells decreased as a function of the S-MWCNT dose. After 2 days' co-culture with 80mg/L of the S-MWCNTs, the cell proliferation almost stopped. Taken together, the results provide additional evidence that CNTs may have a toxic influence on plants.

3.3. *ROS accumulation induced by MWCNTs and its effects on cell viability*

To assess the accumulation of ROS, rice cells were exposed to MWCNTs at a concentration of 20 mg/L. The generation of ROS was monitored through increases in the fluorescence intensity of the oxidant-sensitive dye DCFH-DA. In the presence of MWCNTs, the ROS content significantly increased in a time-dependent manner with prolonged stress compared to control cells, indicating the induction of ROS by MWCNTs in rice cells. Significant differences from the controls were observed in cells after 1 day of exposure to 20 mg/L of the S-MWCNTs, whereas cell viability in the A-MWCNT

treated group did not exhibit obvious differences compared to the control group (Fig. 4). The ROS level in the S-MWCNT treated group increased very quickly. The highest level of ROS was found in the presence of S-MWCNTs and was almost 3.5-times higher than in the control. The overproduction of ROS is known to induce signals that lead to cell death.

FIGURE 4 here

To assess the role of ROS in rice cells in the presence of MWCNTs and antioxidant, we treated the MWCNT-exposed rice cells with AsA, a scavenger of ROS. Rice cells were exposed to S-MWCNTs or A-MWCNTs at a concentration of 20 mg/L for 6 days in the presence or absence of AsA, and the generation of ROS was monitored. We found that pretreatment with AsA decreased the content of the S-MWCNT-induced ROS in a time-dependent manner (Fig. 5). A significant decrease was seen, especially on the third day, after treatment with AsA and S-MWCNTs compared to only S-MWCNTs for 1-6 days of exposure. However, the ROS content of the AsA-treated group and AsA co-treated with A-MWCNTs increased slightly compared to control, and the ROS levels were at very low levels for all treated groups (Fig. 6). It is known that a variety of abiotic stresses, such as heavy metals, drought, salinity, extreme temperatures, high irradiance, and UV light, cause oxidative damage to plants either directly or indirectly through the formation of ROS [17-22]. Our present work showed that MWCNTs result in a significant induction of intracellular ROS formation in plant cells after treatment with 20 mg/L S-MWCNTs (Fig. 5). The drastically increased ROS content suggests that the cells

are under oxidative stress, and it may possibly lead to a decrease in cell proliferation or even lead to cell death via the apoptotic pathway or necrosis.

FIGURE 5, 6 here

To further assess the role of ROS in MWCNT-induced cell proliferation in the presence of AsA (2 mM), rice cells were exposed to S-MWCNTs or A-MWCNTs (20 mg/L) for 6 days in the presence or absence of AsA and the percentage of viable cells was calculated considering the OD values at 485 nm (Figs. 7 and 8). We also found that pretreatment with AsA increased the S-MWCNT-induced cell viability compared to the S-MWCNT only group in a time-dependent manner (Fig. 7). No significant change was found between the cell viability in the A-MWCNT treatment groups (Fig. 8). This finding correlates well with the observation of increased ROS and loss of cell viability.

FIGURE 7, 8 here

The ROS levels from rice cells co-treated with AsA and S-MWCNTs were significantly inhibited as early as 1 day, indicating that AsA pretreatment is able to effectively inhibit the ROS generation induced by MWCNTs, but no significant change was measured in the AsA treated group compared to control (Fig. 5). Pretreatment with AsA also clearly increased the percentage of viable cells in S-MWCNT-treated suspensions (Fig. 7), corresponding to the inhibition of ROS generation by AsA. These observations suggest that AsA increases cell viability in the S-MWCNT-treated

suspensions via reductions in ROS production, which implies that ROS are strongly associated with the MWCNT inhibition of rice cell proliferation. The antioxidants protect the cell membrane from oxidative damage. AsA is a major water-soluble antioxidant and powerful electron donor that reacts with superoxide, peroxide, and hydroxyl radicals, the typical species of the ROS to form dehydro-ascorbic acid [23], which is found in chloroplasts and other cellular compartments. Such antioxidants are crucial for plant defense against oxidative stress damage and ROS [24]. Nel et al. [25] demonstrated that the induction of intracellular oxidative stress is a key component of the biological effects of many particle types.

4. Discussion

The TEM observation demonstrated that MWCNTs can interact with the cell in a manner in which the MWCNTs only associate with the cell wall. The ROS determinations suggest that the MWCNTs can increase ROS, leading to their accumulation in rice cells, and these changes lead to oxidant stress. Ascorbic acid prevented the increase in ROS generation and clearly increased cell viability, suggesting that oxidative stress is the main reason for reduced cell viability when MWCNTs are introduced in to the rice cell suspensions.

We hypothesize a hypersensitive response (HR) mode to explain the mechanism of ROS induction. One of the first symptoms of MWCNT infection is the appearance of brown and necrotic lesions resembling a HR upon TEM visualization. The HR is a positive plant defense reaction that can produce phytoalexins and pathogenesis-related proteins, ion fluxes across the plasma membrane, and ROS [26]. To examine whether

plants inoculated with MWCNTs produce ROS, which are an important component of the HR [27], we detected ROS production in rice cell suspensions treated with MWCNTs using DCFH-DA. This assay clearly showed that ROS content increased in a time-dependant manner, that MWCNTs induced ROS generation, and that the ROS induction was diminished in the presence of AsA. These results are in agreement with higher ROS levels and HR reported in cowpea infected with cucumber mosaic virus (CMV) [28]. ROS induction by elicitors often occurs in two distinct phases. Phase I is a very rapid response (within minutes or hours) and is not always correlated with plant disease resistance, whereas phase II ROS production (hours or days) correlates with the resistance/susceptibility of the plant to the pathogen [29, 30]. Our present results correlate well with phase II ROS production. Masaki et al. [31] demonstrated that oxidative phase II responses correlate with the HR and cell death, in contrast to the phase I response. Masaki et al. [31] also reported that HR induced cell death is recognized as a part of apoptotic cell death, and the direct toxic effect of ROS might dominantly lead to necrotic cell death. These reports are in agreement with our morphological observation of rice cells treated with MWCNTs under TEM.

As we know, one significant event in the plant defense reaction is an oxidative burst, a common early response of host plant cells to a pathogen attack and elicitor treatment. Generally, plant respiratory burst oxidase homologues located in the mitochondria are the major source of ROS generation in aerobic cells, but another four possible mechanisms have been proposed to explain how ROS production occurs in host plant cells. One mechanism is at the level of the external face of the plasma membrane and is mediated by NADPH oxidases, whereas three mechanisms are at the level of the

cell wall matrix, which would involve the action of Class III peroxidases, poly(di)amine oxidases, and oxalate oxidases [26]. Under MWCNT stress, the interaction between the nanotubes and cell wall possibly activates these enzymes, leading to the production of ROS. When rice cells are exposed to MWCNTs, there are two possible explanations of how an individual nanotube interacts with the cell wall. One possibility is adhesion forces, such as hydrogen bonding, between components (polysaccharides or proteins) of the rice cell walls and MWCNTs. Li et al. [15] reported that, between oxidized MWCNTs and polytryptophan (a class of polypeptides with aromatic structures), the adhesion forces are strong due to a π-π stacking interaction, in addition to hydrogen bonding between the amine on polytryptophan and the carboxylate groups. Another possibility is physical wrapping, which allows the MWCNTs to penetrate into the space among the residues of the polysaccharides and proteins. MWCNTs used for our studies are the pristine ones; this implies that the physical wrapping would be the dominated driving forces. After the components of the cell wall renew in the course of metabolism, some of the MWCNTs that contacted the polysaccharide and protein residues will be embedded by new polysaccharides or proteins. The interaction between the components of the cell wall and MWCNTs possibly offer the opportunity of making nanotubes change the dimensional structure of the ambient signal molecules (including proteins or polysaccharides), and the change in structure will lead to potentially inducing a signaling cascade of the HR, resulting in the induction of ROS. The mechanism of the signaling pathway inducing ROS requires further study.

Finally note here that the MWCNTs used for our study also contain a very small amount of metallic impurities (La and Ni). The influence of metallic impurity in CNTs of

their toxic responses, however, remained as debate so far; this is mostly because no reliable quantification method of analyzing the metallic impurities. Recently, Hurt and the co-workers have established a standard method for quantification of Ni in CNTs [32]. This method will help end the long-time debate on this issue and in our further works, the metallic impurities will be measured using this standard method and their possible synergetic impacts on the plants will be studied.

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Table 1 Dimensions, element contents, and surface area of MWCNTs

Items	Test methods	Nominal	Results
Appearance		Black powder	Black powder
Diameter range	TEM	10-30 nm	10-30 nm
CNT content (% vol)	TEM, TPO	> 95	95
Inner diameter		< 10 nm	< 10 nm
Length	TEM	5-15 μm	5-15 μm
Amorphous carbon	TEM	< 3%	< 3%
Ash (La, Ni, etc.)	EDS, TGA	Ni	0.12%
		La	0.06%
		Silicate	0.02%
SSA	BET	40-300 m^2/g	86 m^2/g

This table is provided by Shenzhen Nanotech Port Co., Ltd

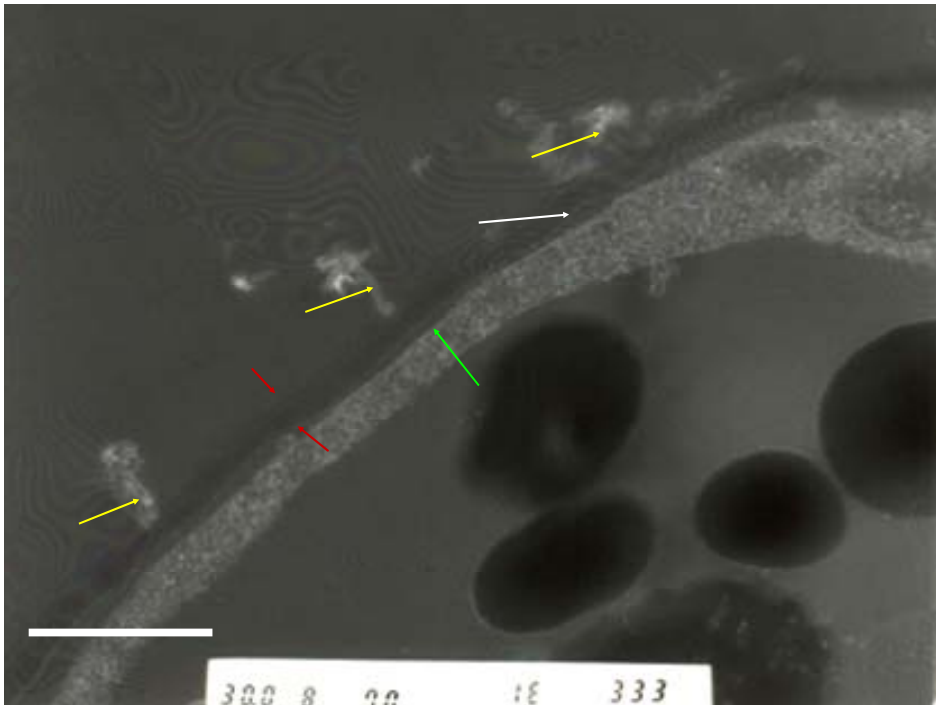

Fig.1 TEM image of rice cells exposed to 20 mg/L MWCNTs for 2 days. Red arrows indicate the cell wall; yellow arrows indicate MWCNTs; the green arrow indicates the membrane; and the white arrow indicates plasma membrane detachment from the cell wall. Scale bar, 0.5 μm.

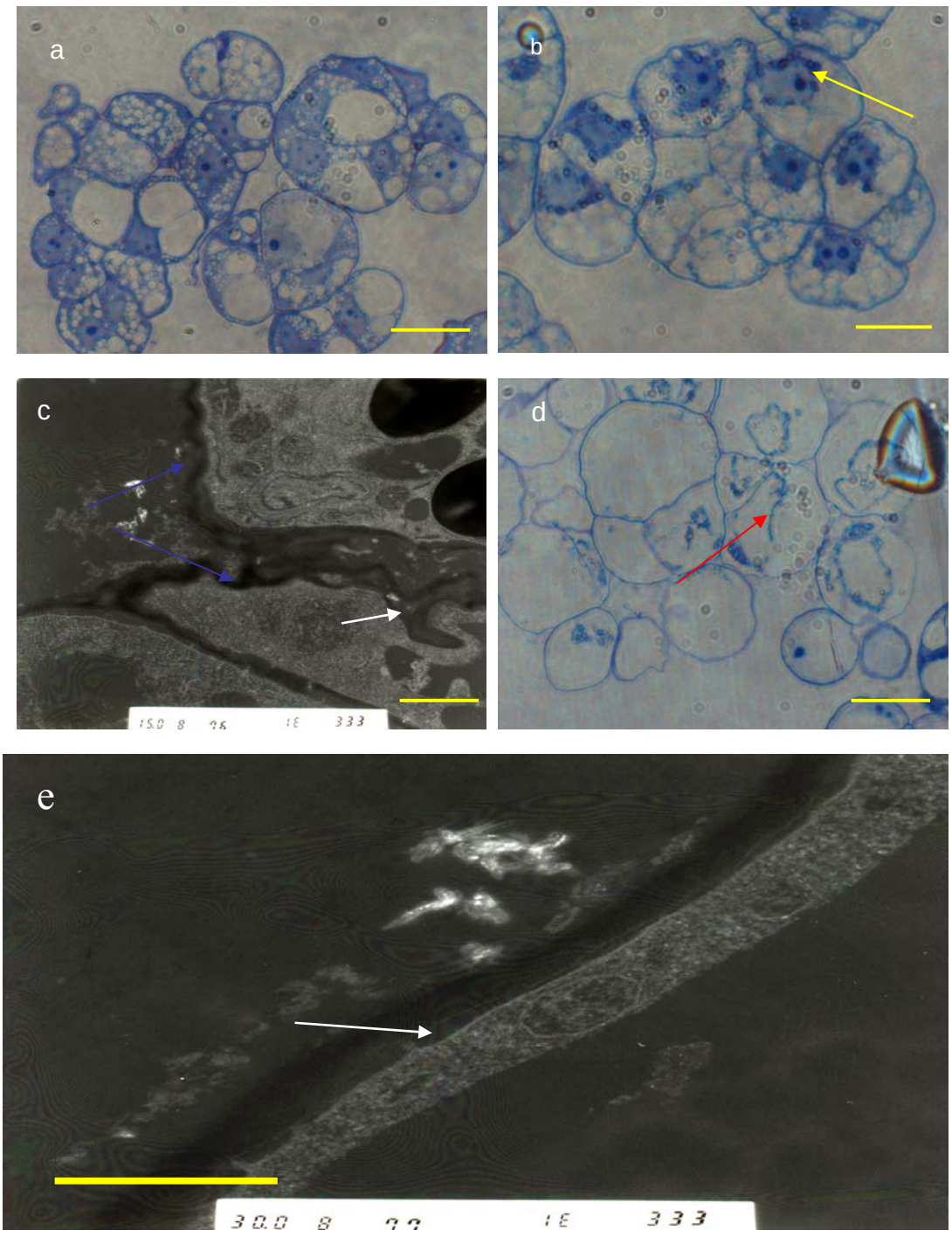

Fig.2 Photo-microscopic images of control cells (a), rice cells treated with 20 mg/L MWCNTs (b, c) and 80 mg/l MWCNTs (d) for 2 days. Yellow arrow (b) shows chromatin condensation. Blue arrows show cell shrinkage. TEM images; white arrows (c, e) show plasma membrane detachment from the

cell wall. Red arrow shows cytoplasm leaked out of cells and cell membrane disruption. Scale bars are 40μm in a, b, d; 1μm in c; 0.5μm in e, respectively.

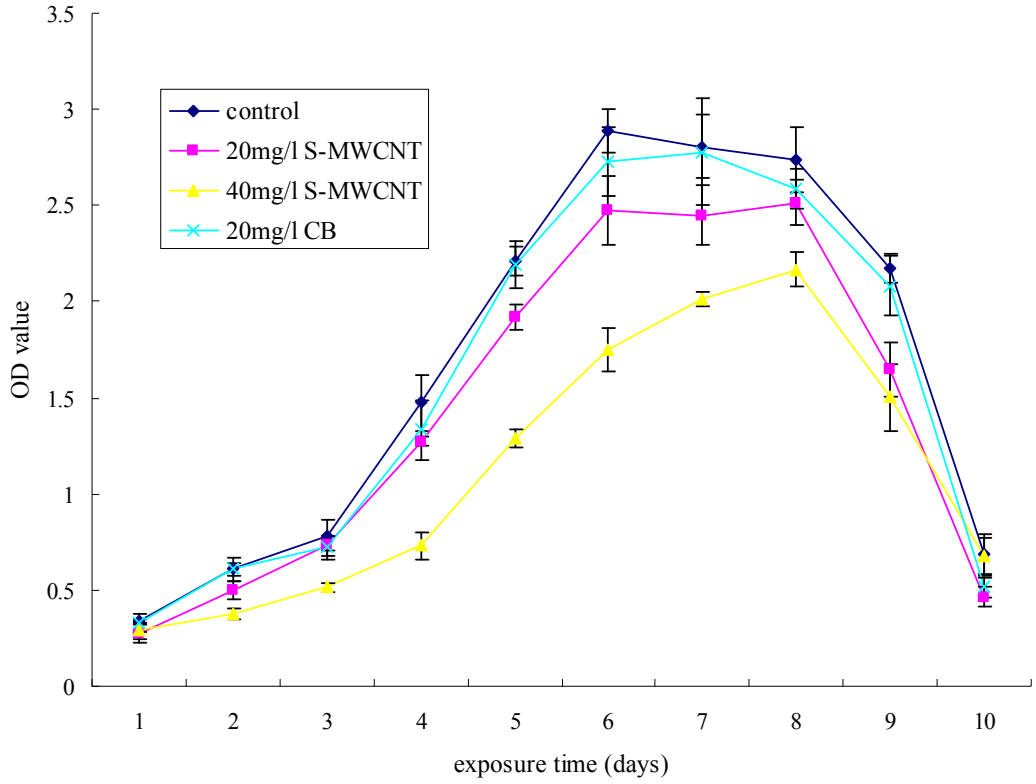

Fig.3 Rice cell viability after 10 days exposure to 20 mg/L and 40 mg/L of S-MWCNTs. The cell viability of treated cells was significantly different from the controls. Viabilities for cells treated with carbon blacks (BC) are also shown, for comparison.

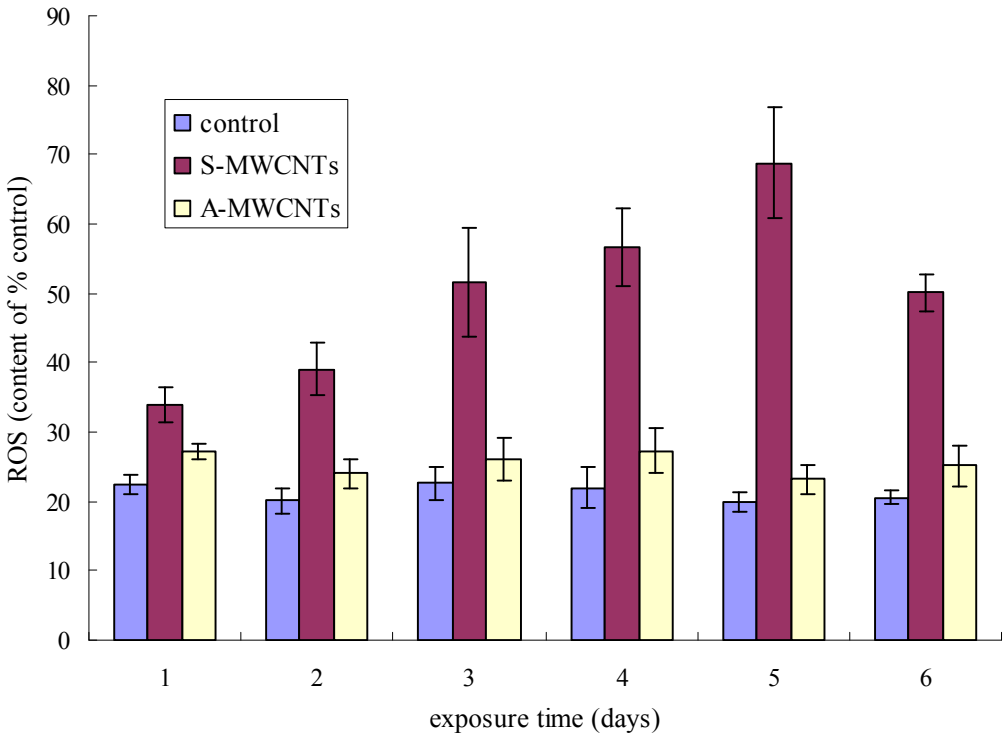

Fig.4 Time-dependent effects of 20 g/ml S-MWCNTs on ROS generation in rice cells. ROS levels in the S-MWCNT treated cell were significantly different from the controls. A-MWCNTs gave similar results to the controls. For ROS detection, stock cell suspensions were diluted 5 times.

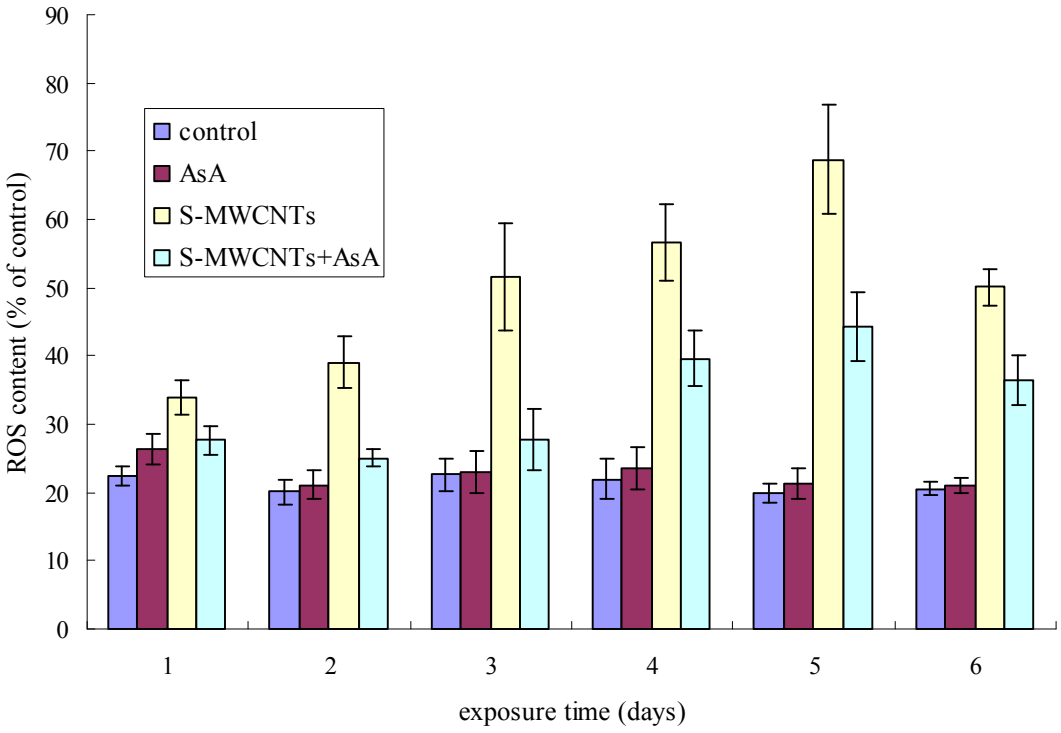

Fig.5 Time-dependant cell viability of rice cells co-treated with 2.0 mM AsA and 20 mg/L S-MWCNTs. The ROS levels decreased significantly after AsA was introduced.

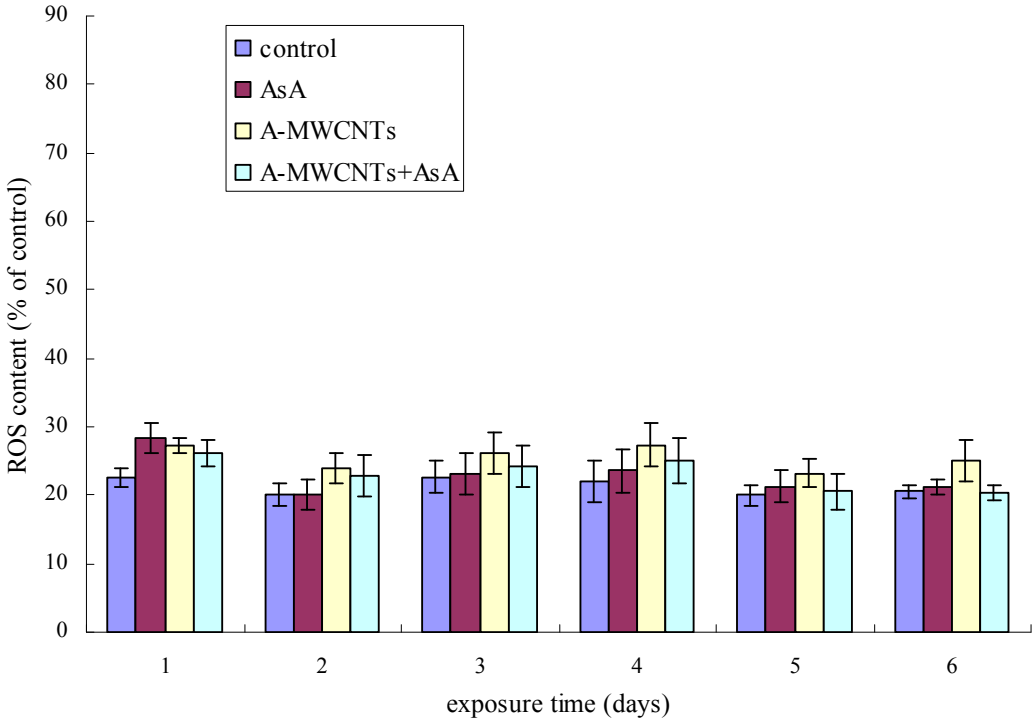

Fig.6 Time-dependant cell viability of rice cells co-treated with 2.0 mM AsA and 20 mg/L A-MWCNTs. The ROS levels of the four groups showed no significant difference.

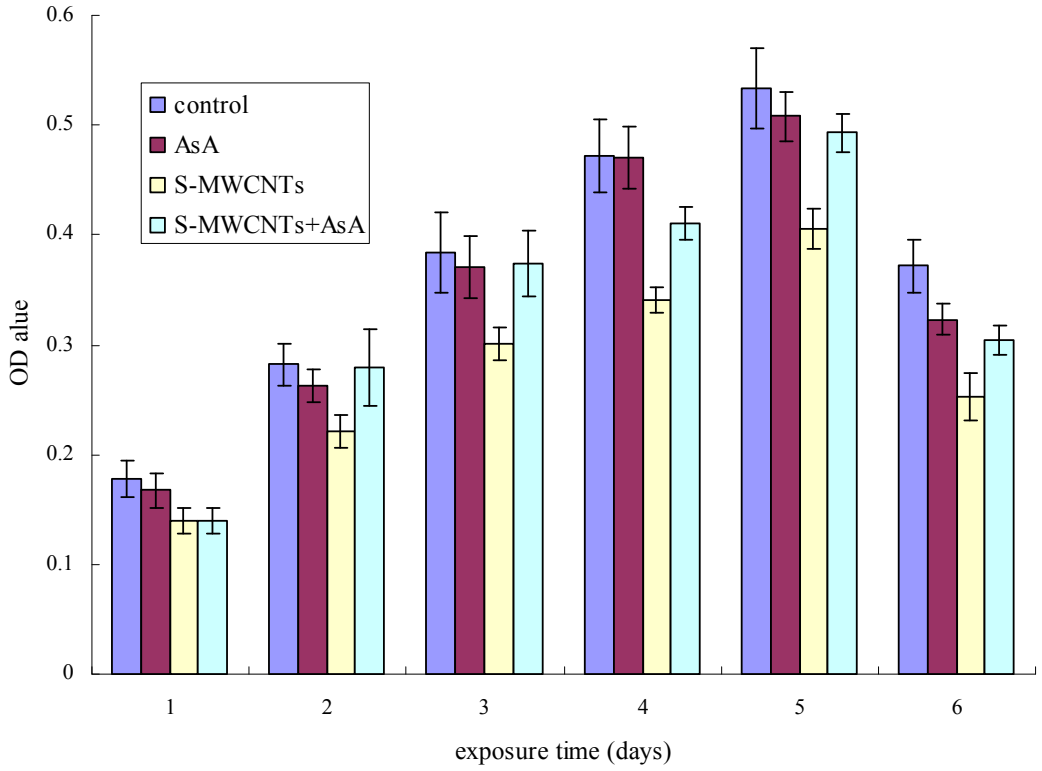

Fig.7 Time-dependant cell viability of rice cells co-treated with 2.0 mM AsA and 20 mg/L S-MWCNTs. Pretreatment with AsA significantly increased the cell viability in S-MWCNT-treated suspensions.

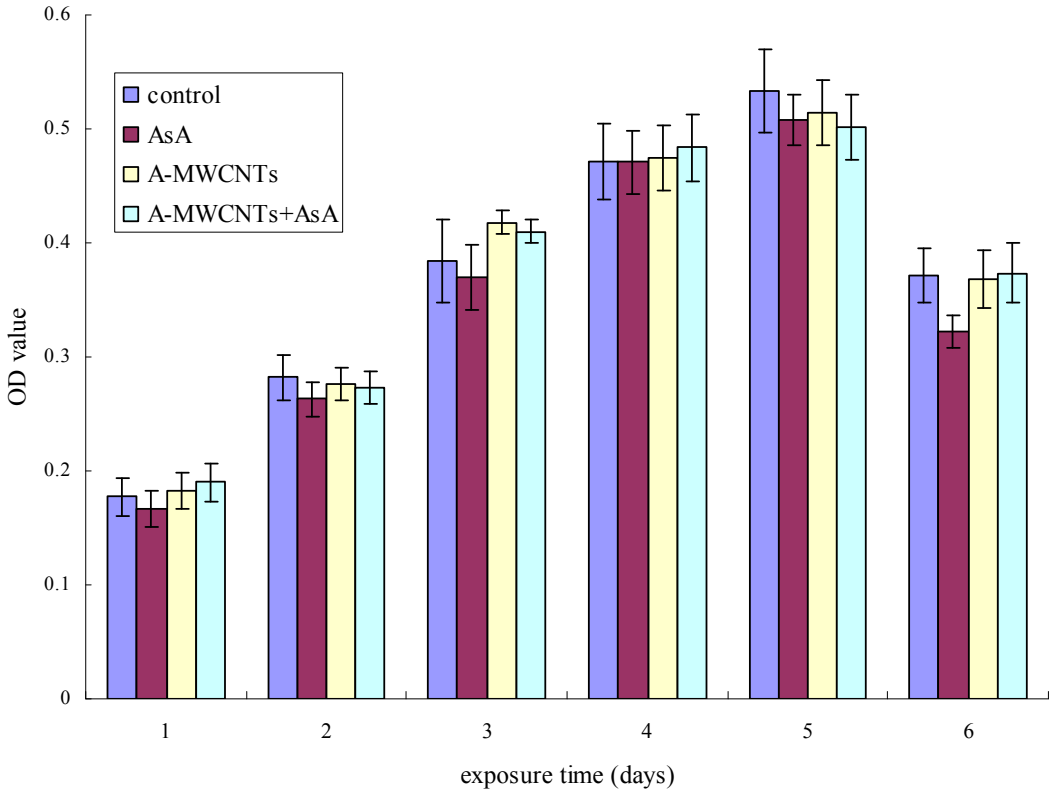

Fig.8 Time-dependant cell viability of rice cells co-treated with 2.0 mM AsA and 20 mg/L A-MWCNTs. The cell viability in the four groups showed no significant difference.