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Comparison of the cytotoxicity induced by different exposure to sodium arsenite in two fish cell lines

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Abstract

Arsenic, a common environmental pollutant, is toxic to many mammalian cells. However, the arsenic-induced toxicity to aquatic animal species is unclear. This study attempted to compare the arsenic-induced cytotoxicity in various fish cells. Two fish cell lines, JF (fin cells of *Therapon jarbua*) and TO-2 cells (ovary cells of *Tilapia*), were treated with sodium arsenite in two ways to mimic acute and subacute exposure. The distinguishable alterations of cell morphology and microtubule network were observed in the cells treated by two arsenite exposure protocols. By the colony-forming assay, we demonstrated that the survival of both cell lines, treated with the high concentrations of arsenite (20–160 μM) for 2 h or with the low concentrations (0.125–10 μM) for 24 h, was decreased in a dose-dependent manner. The difference between the susceptibility of JF and TO-2 cells to arsenite was revealed by the factorial ANOVA to compare the survival rates of the arsenite-treated cells; JF cells were more sensitive than TO-2 cells ($P = 0.008$ and 0.013 for the high-concentration and the low-concentration treatment, respectively). The possible mechanisms to provoke the cytotoxicity of arsenite in two cell lines were also addressed. Antioxidants, *N*-acetyl-cysteine and dithiothreitol, significantly prevented JF cells, but not TO-2 cells, from the arsenite-induced inhibition of survival. Additionally, apparent apoptosis of JF cells and a mitotic arrest of TO-2 cells in response to the treatment of arsenite were also demonstrated by the DNA-fragmentation analysis and the flow cytometric analysis of cell-cycle progression. The results indicate that sodium arsenite induces apoptosis in JF cells probably by causing oxidative stress and disturbs the cell cycle of TO-2 cells. These two fish cell lines can serve as the potential tools to in detail study the toxicity and the hazards of arsenic compounds to aquatic animals at molecular level in the future.

Keywords: Cytotoxicity; Arsenite; Cell-cycle arrest; Apoptosis; Fish cell line

1. Introduction

Arsenic, a widely distributed metalloid in nature, is a common environmental pollutant released through industrial and agricultural processes into the aquatic environment (Ishinishi et al., 1986). Epidemiological studies have indicated that chronic exposure to inorganic arsenic is strongly associated with high risks of skin, lung, liver, and prostate cancers in the human population (IARC, 1980; Chen et al., 1985, 1992; Chen and Wang, 1990; Chiou et al., 1995). Inorganic arsenic compounds also induce many cytotoxic and

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genotoxic effects in a variety of cultured cells (Rein et al., 1979; Lee et al., 1985a,b, 1988, 1989; Seymour and Mothersill, 1988; Li and Chou, 1992; Ramirez et al., 1997; Yih et al., 1997; Li and Broome, 1999).

Most studies to understand the toxicity of arsenic compounds were performed in mammalian cells. However, the study of the arsenic toxicity to the aquatic animal species, including fish, is limited. In vivo bioassays are often administered when studying the hazards of environmental chemicals to fish, but the whole-fish are inconvenient, time consuming, difficult to reproduce, and require sacrificing the organisms. For several decades, the permanent fish cell lines have been widely used in laboratory test systems to measure the cytotoxicity and the genotoxicity of single compounds or environmental samples (Castaño et al., 2003). Therefore, fish cell lines seem to be the potential surrogates for entire fish to serve as the tools for studying the toxicity of the water-soluble arsenic compounds.

Arsenite, an inorganic and trivalent compound of arsenic (As^{3+}) with potent toxicity (Maitani et al., 1987; Hopenhayn-Rich et al., 1993), has a direct affinity to the sulfhydryl group (Sunderman, 1979; Scott et al., 1993) and also induces considerable accumulation of the reactive oxygen species in many animal cells (Liu and Huang, 1997; Wang et al., 1997). Cytoskeletal proteins, especially tubulin, contain abundant cysteine residues (Mellon and Rebhum, 1976), and certain sulfhydryl groups of the cysteine residues are important for microtubule polymerization (Kuriyama and Sakai, 1974). Therefore, microtubules could be the ideal targets for the damage induced by arsenite. Some studies have also shown that the treatment of arsenite can disturb the organization of microtubules in the cultured cells (Li and Chou, 1992; Ramirez et al., 1997). Many toxicants have unique toxic effects when they are applied to the cells with different doses and for different periods. Yih and Lee (1999) have also demonstrated that arsenite can induce various ratios of the kinetochore-positive and the kinetochore-negative micronuclei in the normal human fibroblasts treated according to the different exposure protocols. Their results further suggest that arsenite perhaps acts differently on animal cells under acute and subacute exposures.

Here, we compared the toxic effects induced by the acute and the subacute treatment of arsenite in two fish cell lines via monitoring the cell-survival rates and the alterations of general and cytoskeletal morphology. The possible mechanisms of the arsenite-induced cytotoxicity were also addressed through analyzing the progression of cell cycle and the DNA fragmentation of apoptosis. The results of our study reveal that the unique cytotoxic responses in the JF (fin cells of *Therapon jarbua*) and TO-2 (ovary cells of *Tilapia*) fish cell lines can be induced by the particular ways of arsenite treatment.

2. Materials and methods

2.1. Materials

Sodium *m*-arsenite ($NaAsO_2$), *N*-acetyl-cysteine (NAC), dithiothreitol (DTT), mouse anti-β-tubulin first antibody, and FITC-conjugated anti-mouse IgG secondary antibody were purchased from Sigma (St. Louis, MO). All tissue-culture products were from Gibco (Grand Island, NY).

2.2. Cell culture

JF cell line which mainly consists of fibroblast-like cells was established from the pectoral fins of *Therapon jarbua*, and the population doubling time of JF cells was about 72 h (Wen et al., 1990). TO-2 cell line which mainly consists of epithelial-like cells was established from the ovary of *Tilapia*, a hybrid of *T. mossambica* and *T. nilotica*, and the population doubling time of TO-2 cells was about 28 h (Chen et al., 1983). All cells, kindly provided by Prof. Shiu-Nan Chen (National Taiwan University), were maintained in the L-15 medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin; cells grown on Falcon dishes (Becton Dickinson, USA) were cultured in an incubator with saturated humidity in air at 28 °C. Stock cultures were passaged every 3–4 days.

2.3. Protocols of arsenite treatment

Two protocols were used to treat the exponentially growing JF and TO-2 cells. In protocol A, cells were treated with arsenite at high concentrations between 20 and 160 μM for 2 h (acute exposure); in protocol

B, cells were treated with arsenite at low concentrations between 1.25 and 10.0 μM for 24 h (subacute exposure). Sodium arsenite was dissolved in deionized water as stock solutions of 800 times the indicated concentrations prior to use. Cells were treated with the stock solution diluted in 5 ml serum-free L-15 medium, and all experiments were carried out in 3–5 replicates.

2.4. Colony-forming assay

After pretreated with or without an anti-oxidative modulator (5 mM NAC or 80 μM DTT) in 5 ml serum-free L-15 medium for 2 h, 5×10^6 fish cells in exponential growth were treated with arsenite at various concentrations according to two protocols described above. At the end of the treatment, colony-forming efficiency of cells was determined by the colony-forming assay as description of Lee et al. (1989) with slight modifications. Briefly, the arsenite-treated cells were washed twice with phosphate buffered saline (PBS) to completely remove arsenite, and then were trypsinized. Three hundred cells of each treatment were inoculated into a 100-mm culture dish and re-incubated for 14 days. The colonies of survival cells were stained with 1% crystal violet in the PBS containing 30% ethanol to calculate the colony-forming efficiency. The colony-forming efficiency of the treated cells was adjusted by the corresponding efficiency of the untreated cells to obtain the cell-survival rate.

2.5. Observation of morphological alterations of cells

Cells were treated with or without arsenite according to two protocols. The cell morphology was observed and photographed under an inverted microscope (TMS, Nikon).

2.6. Immunofluorescence microscopy

After the treatment of arsenite and methanol/acetone (1:1 v/v) fixation, the cells were soaked in PBS, perforated by 0.1% Triton X-100 in PBS, and incubated with the anti-β-tubulin first antibody (1:200 diluted in PBS containing 0.1% Tween 20, PBST) at 37 °C for 1 h. Careful washing with PBST was followed by applying the FITC-conjugated secondary antibody for 1 h to label the microtubules of cells. After thoroughly rinsed with PBST, the cells were mounted with anti-fading mounting medium for fluorescence (Vector Lab. Inc., Burlingame, CA). The results of staining were photographed under a fluorescence microscope (Labor lux S, Leica).

2.7. DNA fragmentation assay

Cells (5×10^6) treated according to protocol A were re-incubated in the fresh serum-free medium for 24 h before being scraped off, and those treated according to protocol B were immediately scraped off after treatment. Because apoptosis may not be progressed so soon to fragment the DNA within 2 h of drug treatment, the 24-h re-incubation of the cells treated by protocol A was to prevent a false-negative result from being detected. The collected cells were re-suspended in the lysis buffer solution (50 mM NaCl, 20 mM Tris–HCl, 20 mM EDTA, 0.5% sodium lauroylsarcosinate, 50 μg/ml RNase A, and 100 μg/ml proteinase K). Total genomic DNA of the cells was isolated using the QIAamp DNA isolation kit (Qiagen, Chatsworth, CA) according to the instructions of the manufacturer. The aliquots containing 6.5 μg genomic DNA of each sample were separated by electrophoresis in 1.5% agarose gels, and then stained with ethidium bromide. The bands of DNA fragments (DNA laddering pattern) were checked under UV light.

2.8. Flow cytometric analysis of cell-cycle progression

Cells (5×10^6) were treated with arsenite according to two exposure protocols, subsequently washed twice with PBS and then supplied with the fresh serum-free medium for re-incubation, which was the same as the previously described DNA fragmentation assay except its duration longer. The cells were harvested nine times at 4-h intervals for total 32 h after the treatment beginning, and the progression through cell cycle was monitored with a DNA flow cytometer. In brief, trypsinized cells were washed twice with PBS, fixed with 40% ethanol in PBS at 4 °C for 24 h, and then stained with 5 μg/ml propidium iodide (PI) in PBS containing 50 μg/ml RNase A for 30 min. The DNA content of 10,000 individual cells was analyzed

with a fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson Immunocytometry Systems) according to the procedure described by Lee et al. (1989). PI-fluorescence data were collected by the CellQuest software, and the percentages of the cells in the sub-G_1, G_1, S, and G_2/M phases of cell cycle were determined using the Modfit software.

2.9. Statistical analysis

The values of IC_{50} of arsenite to JF and TO-2 cell survival were calculated by the simple nonlinear regression. The difference between the susceptibility to arsenite of JF cells and that of TO-2 cells was analyzed by the two-factor ANOVA to compare the survival rates of the arsenite-treated cells. The effects of modulators on the survival rates of the arsenite-treated cells and the proportion of the cells in each phase of cell cycle at the indicated time points were analyzed by the two-tailed Student's *t*-test. The condition for statistical significance was $P < 0.05$.

3. Results

3.1. Effects of arsenite on general and cytoskeletal morphology of fish cells

For general morphology, the control JF and TO-2 cells attached firmly to the substratum with random orientations (Fig. 1A and B). Following the treatment of 80 μM arsenite for 2 h, JF cells retained their normal shapes and hardly had morphological alteration (Fig. 1C); they apparently retracted and detached from the substratum after the treatment of 5 μM arsenite for 24 h (Fig. 1E). In contrast, TO-2 cells showed severe retraction and more rounding following the treatment of 80 μM arsenite for 2 h (Fig. 1D); multinuclei and apparently swollen cells appeared in the TO-2 cells treated with 5 μM arsenite for 24 h (Fig. 1F).

For cytoskeletal morphology, the control JF and TO-2 cells contained the intricate networks of microtubules, which emanated from a brightly stained organizing center localized in the perinuclear area to the cell periphery (Fig. 2A and B). The characteristic density and curviness of microtubules were uniform among the examined population of control cells. The microtubules of JF cells were relatively undisturbed by the 2-h treatment with 80 μM arsenite (Fig. 2C). TO-2 cells, similarly exposed, exhibited severe aggregation of the microtubules and most lost their network morphology (Fig. 2D). However, the treatment of 5 μM arsenite for 24 h caused pronounced loss of the microtubules throughout the cytoplasm except the organizing center at the perinuclear area in both of JF and TO-2 cells (Fig. 2E and F).

3.2. Effects of arsenite on survival of fish cells

The average colony-forming efficiencies for the untreated JF and TO-2 cells were 20.8±4.4% and 23.8± 5.7%, respectively. Arsenite treatment, at 20–160 μM for 2 h (protocol A) or 1.25–10 μM for 24 h (protocol B), dose-dependently inhibited the survival of JF and TO-2 cells (Fig. 3). The values of IC_{50} of arsenite for JF and TO-2 cells under protocol A were 80.2 and 131.0 μM, respectively, and under protocol B were 4.6 and 9.3 μM, respectively. Examination of the cell-survival rates by the factorial ANOVA revealed that JF cells were significantly more susceptible to the toxicity of arsenite than TO-2 cells under both exposure protocols ($F_{0.05\,(1),\,1,\,31} = 8.062$ and $P = 0.008$ in protocol A; $F_{0.05\,(1),\,1,\,35} = 6.896$ and $P = 0.013$ in protocol B).

3.3. Effects of NAC and DTT on survival of arsenite-treated cells

Cells were pretreated with the antioxidant, 5 mM NAC or 80 μM DTT, to examine whether the oxidative stress could be involved in the arsenite-induced cytotoxicity of fish cells. After the cells treated with arsenite according to two protocols, the colony-forming assay was performed to determine the survival rates of cells. As presented in Table 1, both NAC and DTT pretreatment for 2 h significantly increased the survival rates of the JF cells treated with 80 and 160 μM arsenite for 2 h or with 5 μM arsenite for 24 h (the survival rate of the JF cells under 10 μM arsenite/24 h treatment was also increased with borderline significance by NAC pretreatment, $P = 0.055$). However, only NAC pretreatment significantly increased the survival rate of the TO-2 cells treated by 80 μM arsenite for 2 h ($P = 0.03$). DTT pretreatment did not significantly affect the survival rates of the arsenite-treated TO-2 cells.

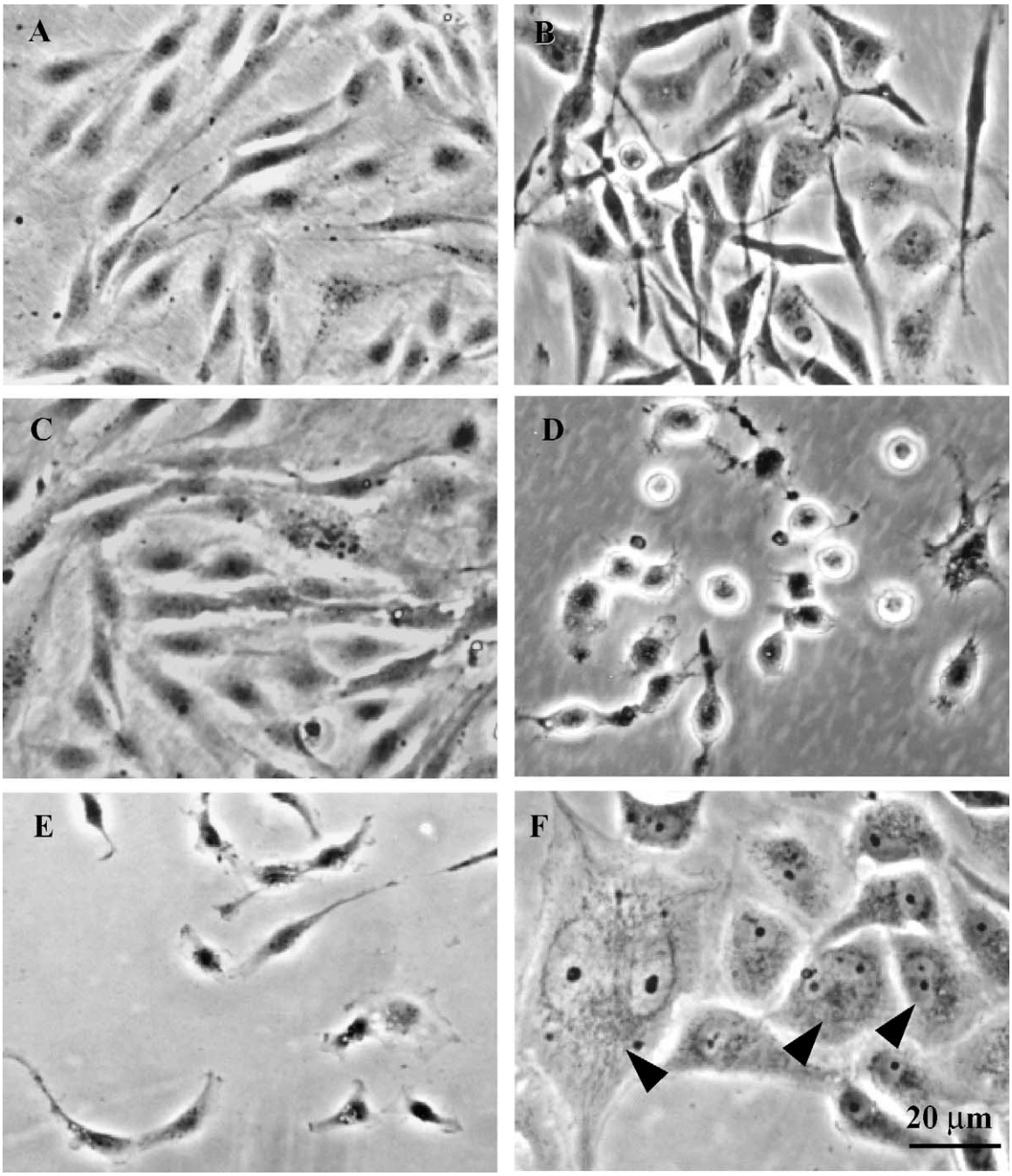

Fig. 1. The arsenite-induced alterations in the morphology of JF (A, C, E) and TO-2 (B, D, F) cells. The control cells (A, B) and the cells treated with 80 μM arsenite for 2 h (C, D) and 5 μM arsenite for 24 h (E, F) were observed with an inverted microscope. The arrowheads indicate the swollen and multinucleated TO-2 cells.

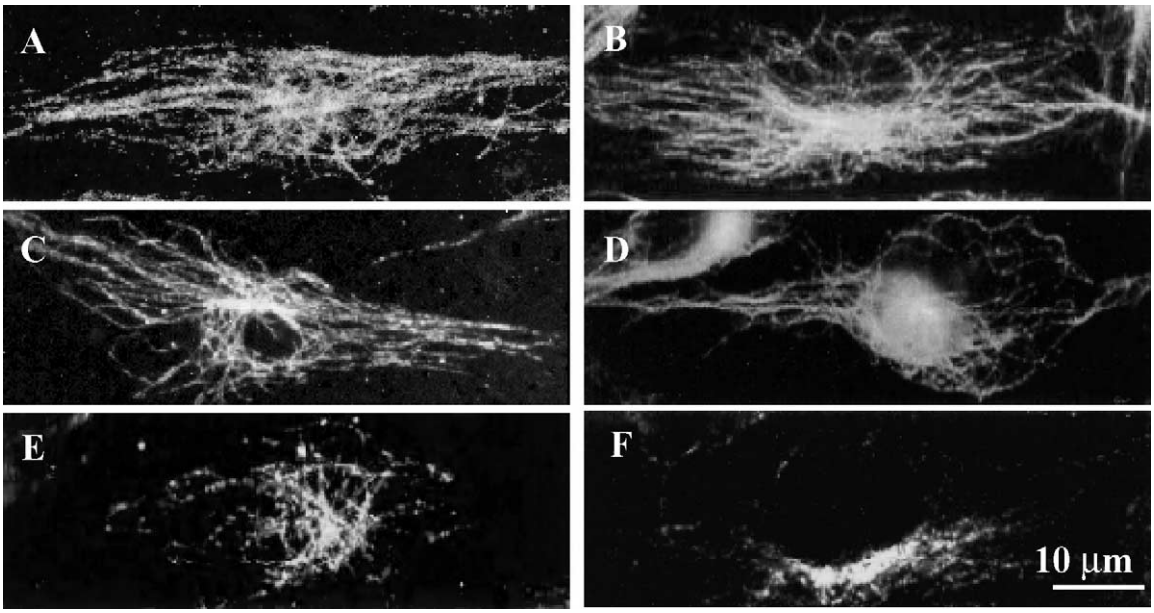

Fig. 2. The arsenite-induced alterations in the microtubule organization of JF (A, C, E) and TO-2 (B, D, F) cells. The photomicrographs show the microtubules in the control cells (A, B) and those in the cells treated with 80 μM arsenite for 2 h (C, D) and 5 μM arsenite for 24 h (E, F).

3.4. DNA fragmentation induced by aresenite in JF cells

The arsenite-treated JF cells exhibited the dose-dependent DNA laddering, a hallmark of apoptosis. Subacute exposure of arsenite induced more severe DNA laddering than acute exposure did in JF cells. However, no DNA laddering was observed in the TO-2 cells treated with arsenite according to protocol A or B (Fig. 4). JF apoptotic cells with a lower DNA

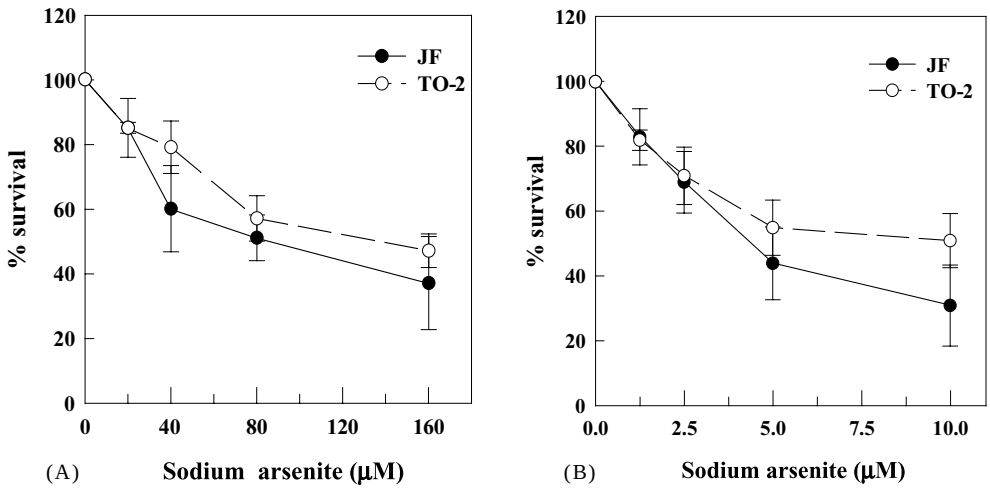

Fig. 3. Correlation between arsenite concentration and cytotoxicity in JF and TO-2 cells. Cells were treated with the indicated concentration of arsenite for 2 h (A) or 24 h (B), and cell-survival rates were determined using the colony-forming assay. The results were expressed as the percentages of the survival rate of control cells. All values are represented as means ± S.D. of at least four independent experimental results.

Table 1
The cell-survival rates (%) of two fish cell lines treated with arsenite following the pretreatment with (+) or without (−) anti-oxidative modulators[a]

Arsenite-treatment protocol	JF			TO-2		
	−	+NAC	+DTT	−	+NAC	+DTT
80 μM As^{3+}/2 h	50.63 ± 7.10	76.22 ± 9.81**	78.76 ± 8.93**	56.59 ± 7.03	72.25 ± 10.04*	54.15 ± 7.96
160 μM As^{3+}/2 h	37.21 ± 14.39	67.14 ± 10.75*	69.06 ± 7.90*	47.04 ± 5.18	52.93 ± 8.02	50.22 ± 5.33
5 μM As^{3+}/24 h	43.50 ± 11.22	70.63 ± 7.71**	60.60 ± 11.03*	55.49 ± 8.51	68.74 ± 13.14	51.32 ± 5.14
10 μM As^{3+}/24 h	30.52 ± 12.48	55.06 ± 7.36[b]	57.39 ± 10.60*	51.03 ± 8.35	60.23 ± 5.59	43.63 ± 7.31

The significance of the difference between the survival rates of the modulator-pretreated cells and the cells pretreated without any modulator was revealed by the two-tailed Student's *t*-test.

[a] Cells were pretreated with 5 mM NAC or 80 μM DTT for 2 h before the arsenite exposure. All values were represented as means ± S.D. of at least three independently experimental results.

[b] The difference between the survival rates of the NAC-pretreated JF cells and the cells pretreated without any modulator had borderline significance ($P = 0.055$).

* $P < 0.05$.

** $P < 0.01$.

content than that of the G_1 cells were also detected by flow cytometry (Fig. 5A, C and E).

3.5. Effects of arsenite on cell-cycle regulation

The cell proportion in each phase of cell cycle was stable in the controls of two cell lines (Fig. 5A and B). Of the control JF cells, 79.2 ± 0.6% were in the G_1 phase, 4.6 ± 0.2% were in the S phase, 14.9 ± 0.5% were in the G_2/M phase, and 2.2 ± 0.5% were in the sub-G_1 phase throughout 32 h of monitoring (mean ± S.D.) (Fig. 5A). After the treatment of 80 μM arsenite for 2 h, no various ratios of the apoptotic JF cells with sub-G_1 DNA content were detected during 10 h of the post-treated incubation. The proportion of JF cells in the sub-G_1 phase increased significantly after 14 h of the post-treated incubation and was remained in the next 16 h. The increase of

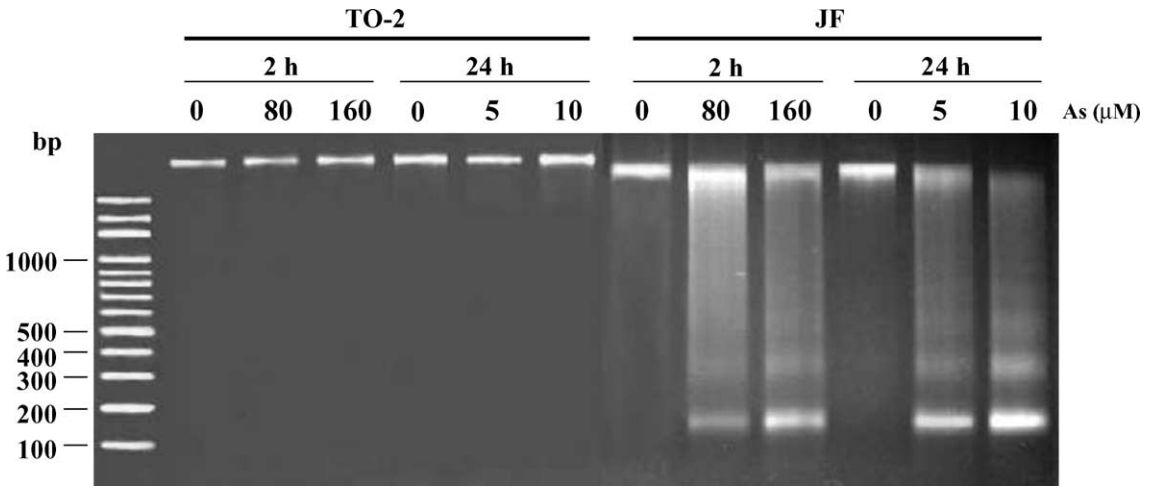

Fig. 4. The arsenite-induced apoptotic cell death signified by the DNA ladder. Agarose gel electrophoresis of the genomic DNA isolated from the JF cells treated with arsenite at the indicated concentrations and exposure time revealed the typical oligosomal-sized chromatin fragmentation and laddering pattern, a characteristic of apoptosis. In contrast, DNA laddering was not detected in the arsenite-treated TO-2 cells.

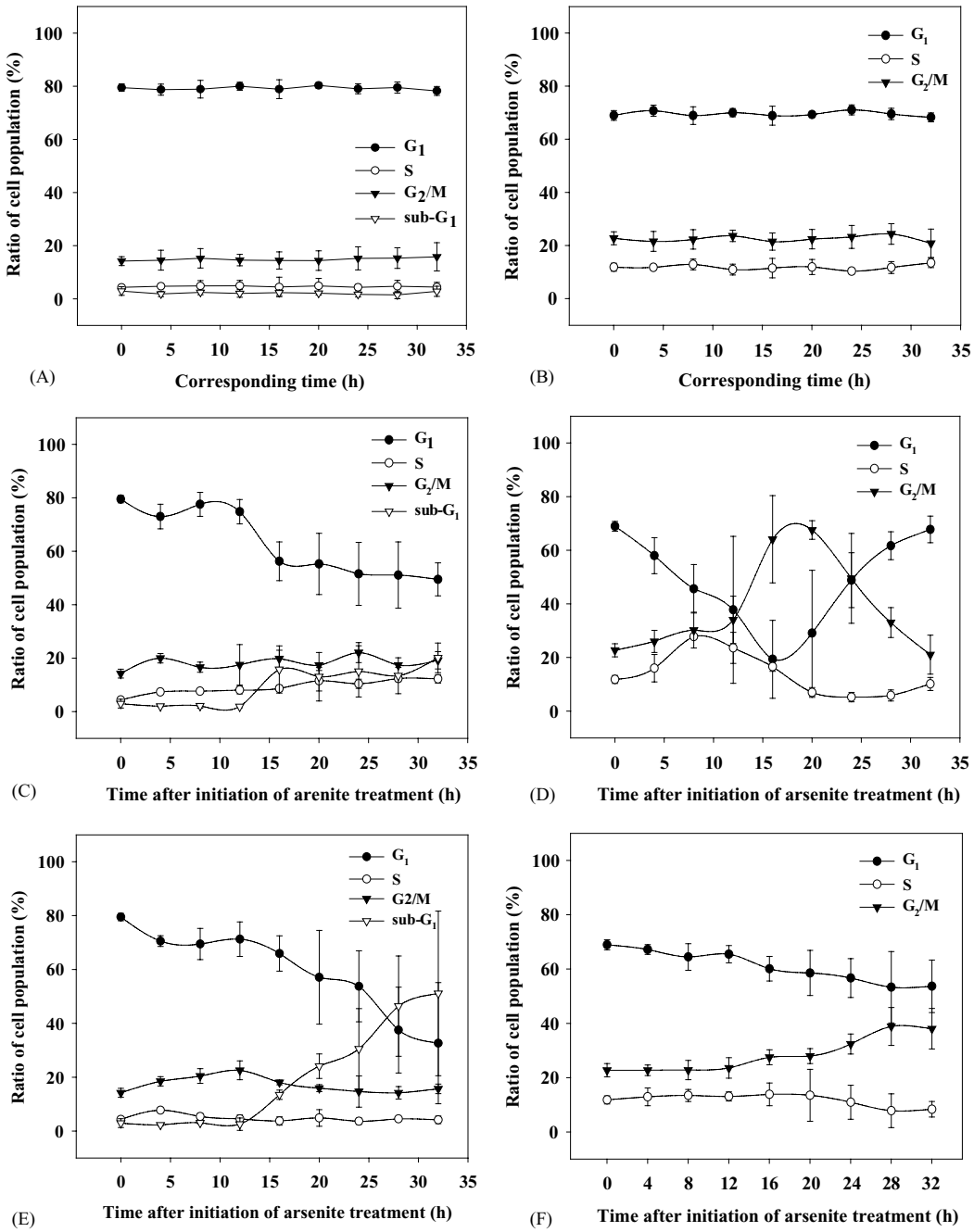

Fig. 5. The effects of arsenite treatment on the cell-cycle progression of JF (A, C, E) and TO-2 cells (B, D, F). JF and TO-2 cells were treated with arsenite, as described in Materials and methods. At the indicated time points, cells were harvested and their DNA content was examined by the flow cytometric analysis. The cell proportions in each phase of cell cycle at the indicated time points of the control cells (A, B), the cells treated according to 80 μM/2 h protocol (C, D), and the cells treated according to 5 μM/24 h protocol (E, F) were plotted. All values are means ± S.D. obtained from three to five independent experiments.

the sub-G_1 cells was matched with the decrease of the G_1 cells (Fig. 5C). Under the treatment of 5 μM arsenite for 24 h, the percentage of the JF cells in the sub-G_1 phase significantly increased to 13.5 ± 1.7% at 16 h after the arsenite treatment beginning. It kept increasing to 51.1 ± 30.6% at the end of monitoring (32 h after the arsenite treatment beginning) (Fig. 5E). JF cells exhibited apoptotic DNA content, regardless of whether they were treated according to protocol A or B. However, higher percentage of the sub-G_1 cells can be detected in the JF cells treated according to protocol B at the end of monitoring.

Of the control TO-2 cells, 69.5 ± 0.9% were in the G_1 phase, 11.8 ± 0.9% were in the S phase, and 22.5 ± 1.1% were in the G_2/M phase throughout 32 h of monitoring (mean±S.D.) (Fig. 5B). No appreciable cells with sub-G_1 DNA content were detected in the control or the arsenite-treated TO-2 cells. Following the treatment of 80 μM arsenite for 2 h, the percentage of the TO-2 cells in the G_1 phase significantly decreased to 19.3 ± 14.5% after 14 h of the post-treated incubation and returned to 67.8 ± 4.9% at the end of monitoring. Accompanying the proportion of the G_1 cells fluctuated, the percentage of the G_2/M cells significantly increased to 34.0±8.8%, 64.1±16.3%, and 67.5 ± 3.5% after 10, 14, and 18 h of the post-treated incubation, respectively, and returned to 21.0 ± 7.3% at the end of monitoring (Fig. 5D). With statistical significance, the percentage of the G_1 cells in the TO-2 cells treated with 5 μM arsenite for 24 h gradually decreased to 53.6 ± 9.7%, and the percentage of the G_2/M phase increased to 38.0 ± 7.5%, at the end of monitoring (Fig. 5F).

4. Discussion

In the present study, the apparent and particular cytotoxicity of arsenite to two permanent fish cell lines was reported. Morphological alterations are considered to be the primary indications of cytotoxicity and its underlying mechanisms. Li and Chou (1992) have noted that the microtubule organization in Swiss 3T3 cells is markedly damaged by arsenite in high doses (≧20 μM), but not by arsenite in low doses (2.5–10 μM). Moreover, Yih and Lee (1999) have also determined that arsenite has clastogenic effects at high dose exposure (5–80 μM/4 h) and aneugenic effects at low dose exposure (1.25–10 μM/24 h) by observing the formation of different types of micronuclei in the human fibroblasts. Therefore, the morphological alterations presented here suggest that arsenite may induce different cytotoxicity by different mechanisms in two fish cell lines under two exposure protocols.

Revealed by the colony-forming assay, protocols A and B of arsenite treatments resulted in similar death responses of JF and TO-2 cells. Table 2 shows the comparison of the IC_{50} values of 24-h arsenite exposure to JF and TO-2 cells with the midpoint toxicity doses of 24-h arsenite exposure, previously reported for other animal cell lines. The arsenite susceptibility of JF and TO-2 cells is similar with that of human fibroblasts and higher than that of the cell lines derived from bluegill sunfish, BALB/c mouse, and Chinese hamster tissue. The IC_{50} value of arsenite for TO-2 cells were estimated to be 1.6- and 2-fold higher than that for JF cells under protocols A and B, respectively. JF cells thus had significantly higher susceptibility to arsenite

Table 2
The midpoint toxicity doses of 24-h arsenite exposure to different mammalian and fish cell lines

Cell line[a]	Midpoint toxicity dose of arsenite (Mm)	Assay	Reference
HF	0.002	Colony-forming	(Lee et al., 1989)
CHO	0.028	Colony-forming	(Lee et al., 1989)
BALB/c 3T3	0.05	Neutral red uptake	(Borenfreund and Puerner, 1986)
BF-2	0.05	Neutral red uptake	(Babich et al., 1986)
BG/F	0.05	Neutral red uptake	(Babich et al., 1989)
BG/G	0.03	Neutral red uptake	(Babich et al., 1989)
JF	0.004	Colony-forming	This study
TO-2	0.01	Colony-forming	This study

[a] HF: human skin fibroblasts, CHO: Chinese hamster ovary cells, BALB/c 3T3: fibroblasts derived from the BALB/c mouse embryo, BF-2: fibroblastic cells derived from the caudal fin of bluegill sunfish (*Lepomis macrochirus*) fry, BG/F: epithelioid cells derived from the fin tissue of bluegill sunfish fingerlings, BG/G: fibroblastic cells derived from the gill tissue of bluegill sunfish fingerlings.

than TO-2 cells did. The result of examination of the cell-survival rates by the factorial ANOVA also supported that. One possible explanation is that the cells from animal skin tend to be highly sensitive to arsenic compounds. Liou et al. (1999) have reported that skin cancer is the dominant cancer in the Taiwanese population exposed to arsenic. Lee and Ho (1994) have also showed that normal human skin fibroblasts are 10-fold more susceptible than Chinese hamster ovary (CHO-K1) cells to sodium arsenite. All this evidence implies that animal skin cells may be more susceptible to, and easily affected by, arsenic than most visceral cells. However, the possibility that the difference between the susceptibility to arsenite of two cell lines resulted from the individual properties in the species of cell origin or from differences during the development and routine maintenance of the cell lines cannot be excluded.

Arsenic, generally considered as an anti-sulfhydryl reagent, can interact with the sulfhydryl groups of molecules, so it may denature or functionally inhibit the sulfhydryl-rich proteins such as tubulin. A recent study has showed that arsenic trioxide (As_2O_3) prevents monomeric tubulin from polymerization, causing apoptosis in the human leukemia cells (Li and Broome, 1999). Under the treatment of 5 μM arsenite for 24 h, the organization of microtubule networks in JF and TO-2 cells was seriously disturbed (Fig. 2), but apparent apoptosis was only observed in JF cells. Moreover, under the treatment of 80 μM arsenite for 2 h, JF cells showed apoptotic features within 16 h but the microtubules in the cells showed barely any alteration following the exposure. The results presented in this paper indicate that disorganization of the microtubules may follow from apoptosis, but microtubular disorganization may not be the major pathway to apoptosis induced by sodium arsenite in fish cells. These findings do not apply to the apoptosis induced by the dynamic instability of microtubules in the As_2O_3-treated human leukemia cells.

Numerous studies have reported that arsenic compounds may damage cells by producing the oxygen radicals (Burdon et al., 1987; Yamanaka et al., 1989; Blair et al., 1990). Our study also provides the evidence of the oxidative damage caused by arsenite in fish cells. Antioxidants, NAC and DTT, can protect JF cells from the arsenite-induced inhibition of survival under both exposure protocols. However, hardly any protection of NAC and DTT to TO-2 cells implicates the disassociation of oxidative stress with the arsenite-induced reduction of TO-2 cell survival. The difference between two cell lines in responds to antioxidant modulation may result from TO-2 cells probably having higher endogenous anti-oxidative ability, which sufficiently prevents cells from accumulating the arsenite-induced reactive oxygen species (ROS) and suffering oxidative damage, to attenuate the protective effects of exogenous antioxidants. Combined with the result of apoptosis analysis, it can be inferred that the oxidative damage may be strongly associated with the arsenite-induced apoptosis of JF cells. Notably, the apoptosis caused by the arsenite-induced oxidative damage has also been reported in the rat and the human neural cells (Chattopadhyay et al., 2002) and the human colon cancer cell lines (Nakagawa et al., 2002). The amount of oxidative damage and genetic mechanisms of apoptosis induced by arsenite in JF cells are currently clarified by our further work.

This research demonstrates that arsenite in TO-2 cells induces accumulation of the G_2/M cells accompanied by a decrease in the proportion of G_1 cells. We suppose that arsenite result in the significant arrest at the G_2/M phase in TO-2 cells by disturbing the functions of the mitosis-promoting factor (cyclin B1/cdc2 protein complex). This supposition is partly supported by the arsenite-induced cyclin B1 accumulation and cdc2 hyperphosphorylation causing the G_2/M arrest and mitotic prolongation in the human fibroblasts (Yih et al., 1997). Since arsenite can apparently disrupt the progression of cell cycle, the reason why the arsenite-treated TO-2 cells do not exhibit apoptosis is of interest. Unlike in JF cells, some important factors must counteract the apoptotic effects of arsenite in TO-2 cells. According to the report of Chen et al. (1988), a group of 27 kDa heat-shock proteins with fish and tissue specificity is expressed when TO-2 cells suffered the environmental stress, such as high temperature. Additionally, the human heat-shock protein 27 (hsp27) is implicated in resistance to the apoptosis induced by several cytotoxic drugs, such as etoposide and doxorubicin (Garrido et al., 1999; Hansen et al., 1999). Therefore, arsenite inducing TO-2 cells to express the 27 kDa heat-shock proteins, which can prevent apoptosis, may be one possible explanation for the interference of cell cycle without apoptosis in the arsenite-treated TO-2 cells.

Although arsenite can't induce the apoptosis of TO-2 cells, it still dramatically reduces their survival rate as revealed by the colony-forming assay. That may result from cells going through necrosis after cell cycle disturbed by arsenite. A similar result of necrosis, not apoptosis, following the G_2/M arrest in colorectal cancer cells treated with aspirin (Subbegowda and Frommel, 1998) partly supports our supposition. Nevertheless, colony-forming assay measuring more than survival but also the capacity to recover and proliferate, the decline of TO-2 cell survival determined by this assay may be only due to a significant inhibition of cell proliferation rather than cell death. The experiments to confirm necrotic responses in the TO-2 cells arrested by arsenite will be performed in the future.

Many kinds of entire fish have been used to investigate the in vivo toxicity of arsenic compounds. The 96-h LC_{50} of sodium arsenite for 4.5 g rainbow trout (*Oncorhynchus mykiss*) is 21.0 mg/l (about 162 μM), and all fish exposed to arsenite of 9.64 mg/l (about 74.2 μM) over 17 weeks show inflammation of gallbladder wall, a lesion absent at lower exposure concentrations (Rankin and Dixon, 1994). Suhendrayatna et al. (2002a) have also revealed the 7-day LC_{50} of sodium arsenite for Japanese medaka (*Oryzias latipes*) is 15 mg/l (about 115.5 μM). The toxic concentration of sodium arsenite affecting accumulation of arsenic in *Tilapia mossambica* under 7-day exposure is 10 mg/l (about 77 μM) (Suhendrayatna et al., 2002b). Comparing with previous whole-fish assays, sodium arsenite induces dramatic and distinct toxicity with lower doses and shorter exposure time in JF and TO-2 cells. That indicates the potential of two cell lines to serve as more sensitive and easily accessible materials for study of arsenic toxicity to aquatic animals. Clarifying the association of arsenic toxicity in two cell lines with that in the corresponding whole fish is essential to develop JF and TO-2 cells into mature research tools.

In summary, our investigation exhibits that arsenite can induce apoptosis probably because of induction of the oxidative stress in JF cells and disturb the cell cycle without induction of apoptosis in TO-2 cells; fish cell lines may serve as the sensitive surrogates for whole fish to study the cytotoxicity of other arsenic compounds. However, the mechanisms by which arsenite toxically affects fish cells were not analyzed in detail. Further studies at molecular level of the arsenite cytotoxicity in fish cell lines are warranted.

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