Studies into the mechanism of arsenic-induced neurotoxicity

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Chapter 5: Arsenic neurotoxicity III

Arsenic Metabolites Affect Expression of the Neurofilament and Tau genes: an In-vitro Study into the Mechanism of Arsenic Neurotoxicity

Arsenic Metabolites Affect Expression of the Neurofilament and Tau genes: an In-vitro Study into the Mechanism of Arsenic Neurotoxicity

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Abstract

Neurological studies indicate that the central (CNS) and peripheral nervous system (PNS) may be affected by arsenic (As). As-exposed patients show significantly lower nerve conduction velocities (NCVs) in their peripheral nerves in comparison to healthy subjects. As may play a role in the disruption of neuroskeletal integrity, but the mechanisms by which it exerts a toxic effect on the peripheral and central nervous system are still unclear. In the present study, we examined the neurotoxic effects of various arsenic metabolites (iAs^{III}, iAs^{V}, MMA^{V} and DMA^{V}) on two different cell lines derived from the peripheral (ST-8814) and central (SK-N-SH) nervous system. The effects of the arsenic metabolites were examined on the relative quantification levels of the cytoskeletal genes, neurofilament-light (NEFL), neurofilament-medium (NEF3), neurofilament-heavy (NEFH) and microtubule associated protein-tau (MAPT), using Real-Time PCR. Our results show that iAs^{III} and iAs^{V} have no significant effects on either cell lines. On the other hand, MMA^{V} and DMA^{V} cause significant changes in expression levels of NEF3 and NEFL genes, while the expression level of the NEFH gene is significantly increased in both cell lines.

Keywords: Arsenic metabolites; Cytoskeletal genes; Gene expression; Neurotoxicity; SK-N-SH cells; ST-8814 cells**.**

1. Introduction

Arsenic is a naturally occurring toxic element, widely distributed in the earth's crust. In large parts of the world such as South East Asia (Rahman et al., 2001) and South America inorganic Arsenic (iAs) poisoning is a major health concern (Blanco et al., 2006; Ferreccio and Sancha 2006). This is mostly due to water pollution from natural sources and soil contamination. Arsenic compounds also have a long history of use in medicine, and have shown a re-emergence of late with the recent introduction of As trioxide treatment for acute promyelocytic leukaemia (Jing et al., 2001).

Inorganic arsenic is thought to be the most toxic, while most organic forms of arsenic are considered relatively less toxic (Styblo et al., 2000). In many species arsenic metabolism is characterized by two main types of reactions: (1) reduction reactions of pentavalent to trivalent arsenic, and (2) oxidative methylation reactions in which trivalent forms of As are sequentially methylated to form monomethyl- (MM-) and dimethyl- (DM) products: $iAs^{V} \rightarrow iAs^{III} \rightarrow MMA^{V} \rightarrow MMA^{III} \rightarrow DMA^{V} \rightarrow DMA^{III}$ (Aposhian et al., 1997).

The neurological manifestations of arsenic exposure are varied. Both the peripheral (PNS) and the central (CNS) nervous system can be damaged by acute or chronic exposure. Although PNS impairment is common in As-exposed populations (Hafeman et al., 2005), its mechanism has hardly been studied. The period of time between start of exposure to As and onset of neuropathy symptoms may range from 10 days to 3 weeks after a single dose and often resembles the Guillian-Barré syndrome (Winship, 1984). The mechanism by which As exerts an effect on the nervous system is unclear, but As neuropathy is thought to be a distal axonopathy (De Wolff and Edelbroek, 1994), which may result from derangement of central and peripheral nervous system neurons. Axonal degeneration is the probable cause of disturbance in nerve conduction velocity (Goebel et al., 1990; Greenberg, 1996).

Axonal integrity is maintained by three categories of cytoskeletal proteins: neurofilaments, which are found in high concentrations along the axons, the microtubules and the actin filaments (microfilaments). Neurofilaments are found in high concentrations along the axons of vertebrate neurons. Three types of neurofilament proteins exist, which co-assemble in-vivo, forming a heteropolymer that contains Neurofilament-Light (NF-L) plus either Neurofilament-Heavy (NF-H) or Neurofilament-Medium (NF-M) proteins. During axonal growth, new neurofilament subunits are incorporated all along the axons. The level of neurofilament gene expression seems to directly control axonal diameter (Hoffman et al., 1985), which in turn controls how fast electrical signals travel down the axon.

Microtubules consist of tubulin subunits. Their stability is regulated by their interaction with microtubule-associated proteins (MAPs). MAP function includes both stabilizing and destabilizing microtubules, guiding microtubules towards specific cellular locations, cross-linking microtubules and mediating the interactions of microtubules with other proteins in the cell. MAP-Tau is a microtubule-associated protein, which is primarily expressed in neurons.

It is hypothesized that a disruption in neurofilament structure, or in the cross-linking proteins that attach the neurofilaments to the microtubules and actin filaments distributed along the axon, can result in axonal disorganization and eventually axonal degeneration. In our previous study with rats, single exposure to iAs^{III} (i.v.) demonstrated that axonal cytoskeletal protein composition changes, namely that NF-L decreases in a time/dose dependent manner (Vahidnia et al., 2006).

In order to further characterize the effect of As compounds on the neuronal cytoskeleton, we examined the effects of As metabolites (iAs^{III}, iAs^{V}, MMA^{V} and DMA^{V}) on the expression of genes for NEFH, NEF3, NEFL and MAPT in two different cell lines originating respectively from the peripheral (ST-8814) and central (SK-N-SH) nervous system.

2. Materials and Methods

Chemicals. The following arsenic metabolites and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): (catalogue no.: PS-281), Sodium meta-arsenite (As^{III}) (product no. 228699-100G), Arsenic acid sodium (As^{V}) (product ref. A6756-50G), Trypsin-EDTA solution 1x (catalogue no. T3924), and retinoic acid 98% (all-*trans* $C_{20}H_{28}O_2$, Vitamin A, product no.: R2625). Dimethyl arsenic acid (DMA^{V}) (catalogue no.: PS-51) and Disodium methyl arsenate (MMA^{V}) were obtained from Chem Service (West Chester, USA). AlamarBlue dye was purchased from BioSource International, Inc. (catalogue no.: DAL1100. Camarillo, CA). RNeasy Mini Kit 250 was purchased from Qiagen Inc (catalogue no.74106, Valencia, CA, USA). Deoxyribonucleotide triphosphate (dNTPs) 5 mM, 0.1 M dithiothreitol (DTT), 5x buffers, reverse transcriptase (RT) and RNA inhibitor 40 units/µl (RNA sin) were purchased from Promega (Benelux BV, Netherlands). T-25 flasks with air-filter cap for cell growth were obtained from Greiner bio-one GmbH (catalogue no. 690175. Frickenhausen, Germany). Mouse anti-NF-90 antibody to all three neurofilament proteins (NF-H, NF-M and NF-L) was a gift from Prof. E. Marani of the Department of Neurosurgery at the Leiden University Medical Center, Netherlands (Oudega et al., 1996). Mouse anti-tau monoclonal antibody, Clone Tau 46 (catalog no. Ab24747) was obtained from Abcam (Cambridge, United Kingdom). Kodak Biomax XAR film (catalog no. 165 1454) was obtained from Kodak (Shelton, CT, USA). ECL plus™ western blotting detection reagent was bought from Amersham Biosciences (Piscataway, NJ, USA).

SYBRGreen PCR-mastermix 2x (P/N: 4309155, Foster City, CA, USA) was purchased from Applied Biosystems. Phosphate-buffered saline (PBS, NaCl 145 mmol/l, phosphate 1.4 mmol/l and pH 7.5) was prepared from analytical grade reagents by the Department of Pharmacy. The used primers were all obtained from Isogen Bioscience B.V (IJsselstein, the Netherlands) (Table 1) with an end concentration of 10pmol/µl in Tris/EDTA-buffer.

Cell cultures. Most of the materials for cell culture were purchased from Invitrogen (Breda, the Netherlands); otherwise more specific details are followed in brackets. A human neuroblastoma cell line and SK-N-SH cells were purchased from the American Type Culture Collection. The SK-N-SH cell line was maintained in Advanced Minimum Essential Medium (Advanced MEM) (catalogue no. 12492-013) supplemented with 10% heat-inactivated foetal bovine serum obtained from Greiner bio-one GmbH (catalogue no. 758093) (Frickenhausen, Germany), 2% sodium bicarbonate (catalogue no. 25080-094), 1 % L-glutamine (catalogue no. 25030-081) and 100 µg/ml penicillin/streptomycin (catalogue no. 15140-122) at 37˚C in a 5% CO_2 incubator. The Schwannoma cell line ST-8814 cell line was established from malignant schwannomas (neurofibrosacromas) from patients with neurofibromatosis type 1 (Ryan et al., 1994). The ST-8814 was maintained in Iscove's Modified Dulbecco's Media (IMDM) (Cambrex, catalogue no. BE12-722F), supplemented with 10% heat-inactivated foetal bovine serum obtained from Greiner bio-one GmbH (catalogue no. 758093) (Frickenhausen, Germany), 100 µg/ml penicillin/streptomycin (catalogue no. 15140-122) at 37˚C in a 5% CO_2 incubator.

Cytoskeletal protein expression. The three neurofilament proteins (NF's) and MAP-tau expression were analyzed by using the western blot technique. Both cell lines were grown in T-25 flasks. After trypsinization, the cells were centrifuged at 350xg and washed twice with PBS for 10 minutes at 4ºC. Afterwards, the cells were collected in 100µl of PBS and lysed by freeze-thawing procedure in liquid nitrogen (-80ºC) and water bath (37ºC), 3 times for 3 minutes each. The lysed cells were centrifuged at 10000xg for 1 min at room temperature and the supernatant was transferred in to a new 1.5ml tube. Protein concentrations of the homogenates were measured by the BCA™ Protein Assay Kit. To obtain the same protein concentrations prior to analysis by SDS-PAGE, the protein samples were standardized with

PBS. The protein samples were analyzed on an 8% acrylamide separation gel (Laemmli, 1970). The separation gels were used for immunoblotting on 0.2 µm nitrocellulose membranes in conjunction with monoclonal neurofilament antibodies to all 3 neurofilament proteins: NF-H, NF-M, NF-L, and MAP-tau. Using ECL plusTM western blotting detection reagent and exposing to Kodak Biomax XAR film for 30 seconds to 3 minutes concluded the western blot analysis. The band intensities on the Kodak biomax XAR film were analyzed by Quantity One – Densitometer GS-710 from Bio-Rad (Veenendaal, The Netherlands).

Experimental design. Cells were cultivated in triplicate for each As metabolite and concentration. From each culture, PCR was performed in duplicate, so each PCR was performed six times in total. First the growth rate of the cell lines ST-8814 and SK-N-SH and the effect of arsenic metabolites iAs^{III}, iAs^{V}, MMA^{V} and DMA^{V} on the growth rate were determined to provide a measure for the sensitivity of the cells and to give an overall picture of the toxicity of these metabolites.

The growth rates of the cell lines were determined by using the AlamarBlue assay to establish the growth rate and cytotoxicity of arsenic metabolites (Fields et al., 1993). The AlamarBlue assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. This assay incorporates an oxidation-reduction indicator that both fluoresces and changes color in response to the chemical reduction of growth medium resulting from cell growth (Page et al., 1993). The linearity of this assay (cell titration) was tested by making a trend line of both cell lines in a 24-wells plate and incubated overnight at 37˚C in a 5% CO_2 incubator. The used cell amounts were 0, 20, 40, 60, 80 and 100 (x1000) cells per well in triplicate. Subsequently, the cells were washed with phosphate-buffered saline (PBS), and medium containing 10% AlamarBlue dye was then added to the cells and incubated at 37˚C in a 5 % CO_2 incubator. Samples were taken after 24 hours. The absorbance of the cells was then measured in a 96-wells plate with the SpectraMAX 250 Micro plate Spectrophotometer from Molecular Devices (Wokingham, UK) at 540/630 nm (Fig. 2A). Use of AlamarBlue each time for the same duration of 24 hrs, provides the means to calculate the number of cells present in a well in conjunction with the trendline equation shown in Fig 2A.

The concentration that kills 50% of the cells in culture (LC_{50}) was also measured for each metabolite by using AlamarBlue dye. AlamarBlue dye was used as an indicator for the number of cells that survived in comparison to the controls, and as a means to standardize the used concentrations for both cell lines. The same amount of cells - $2x10^4$ - was added in 1 ml medium into each well on a 24-wells plate and incubated for 24-hours at 37˚C in a 5% CO_2 incubator. After 24-hours the cells were treated with various concentrations (0, 1, 3, 10 and 30 µM) of the arsenic compounds iAs^{III}, iAs^{V}, MMA^{V} and DMA^{V}, and incubated for another 24-hours at 37˚C in a 5% CO_2 incubator.

Subsequently, the cells were washed with PBS, and medium containing 10% AlamarBlue dye was then added to the cells and incubated for 24-hours at 37˚C in a 5 % CO_2 incubator. The absorbance of the cells was then measured in a 96-wells plate with the SpectraMAX 250 Microplate Spectrophotometer at 540/630 nm, where LC_{50} values were calculated by. The LC_{50} assay was performed in triplicate (Fig. 3A and B).

For the actual gene expression experiment, 1.10^6 cells were grown per Tissue Culture Flasks (T25) $25cm^2$, from Greiner bio-one GmbH (catalogue no. 690 175) (Frickenhausen, Germany). Various As metabolites (iAs^{III}, iAs^{V}, MMA^{V} and DMA^{V}) and concentrations of 0, 0.3, 1.0 and 3.0 µM dissolved in their appropriate cell culture medium were incubated for 24- and 48-hours. Afterwards, cells in T25 flasks were washed twice with PBS and RNA was isolated and purified from the cells by using Qiagen RNeasy mini columns (for more details, see RNeasy mini protocol for isolation of total RNA from animal cells). Ultimately cDNAs with an end concentration of 25 ng/µl were synthesized from the isolated RNAs and the samples were analyzed with the Applied Biosystems 7300/7500 Real Time PCR System

(Nieuwerkerek a/d Ijssel, the Netherlands). The used software, Relative Quantification (RQ), determines the change in expression of a nucleic acid sequence (target) in a test sample relative to the same sequence in a calibrator sample, after all the measurements are standardized through a chosen household gene (Livak and Schmittgen et al., 2001). The relative expression of each gene for the various doses and time indices are compared to their control groups for each time indices and 0 μM.

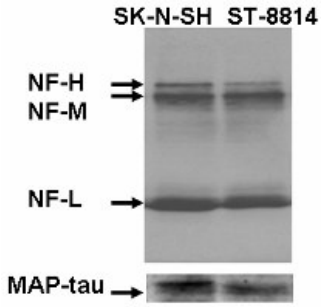
The data for each gene were statistically evaluated for each dose and time using the SPSS 14.0 for Windows. The t-Test for independent samples was used to evaluate the data for a single gene in both cell cultures (Fig. 1B). Chi-Squared for non-parametric tests (Kruskal-Wallis test) was performed to evaluate the data for dose and time and As metabolites as obtained for each gene. A level of $p<0.05$ was accepted as statistically significant.

Table 1. Primer sequences used for the various cytoskeletal genes.

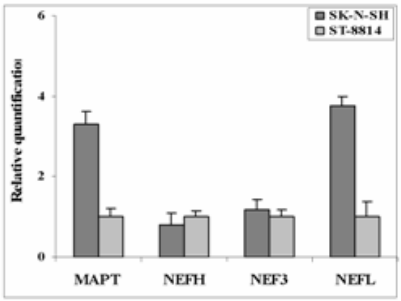
Name	**Sequence (5'-->3')**	**Gene**
ß2-Microglobuline-Sense	GATGCTGCTTACATGTCTCG	Household
ß2-Microglobuline-Anti-sense	CCAGCAGAGAATGGAAAGTC	Household
MAPT-Sense	TCACTTTTACAGCAACAGTCAGTG	Target
MAPT-Anti-sense	TGCCATGTTGAGCAGGACTA	Target
NEFH-Sense	GCCGAATGCCACACGTAAACACTT	Target
NEFH-Anti-sense	AAAGTGCGCCCTGGCATAATTCAG	Target
NEF3-Sense	AGAATATGCACCAGGCCGAAGAGT	Target
NEF3-Anti-sense	GCAAATGACGAGCCATTTCCCACT	Target
NEFL-Sense	AAGCATAACCAGTGGCTACTCCCA	Target
NEFL-Anti-sense	TCCTTGGCAGCTTCTTCCTCTTCA	Target

3. Results

The presence of the cytoskeletal intermediate filament proteins in the used cell lines is shown in Fig.1A. MAP-tau, NF-H, NF-M and NF-L are present in both SK-N-SH and ST-8814 cell lines. Fig 1A and 1B compares the neurofilament expression on the protein and mRNA levels in both cell lines. The SK-N-SH cell line shows a higher RNA expression for MAPT and NEFL by 3 to 4 fold (t-Test, $p<0.05$) (Fig. 1B). The expression levels of NEFH and NEF3 in both cell lines are similar.



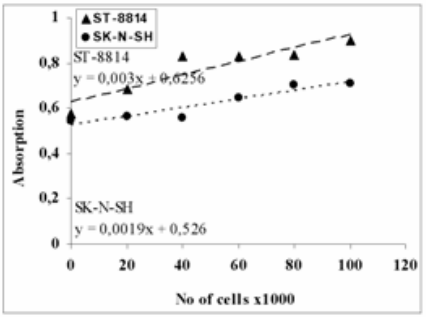
1A.



1B.

Fig. 1. 1A and 1B show the comparison of the different cytoskeletal protein and their gene expressions of various NFs and MAP-tau proteins in the SK-N-SH and ST-8814 cell lines. The t-Test for independent samples for each measured value per gene group revealed significant between-group differences as follows: MAPT and NEFL $p<0.05$ and no significant difference between the NEFH and the NEF3 of both cell lines. Fig 1B shows the relative mean values (± SD; n=6).

Both cell cultures were studied in conjunction with AlamarBlue dye to establish their cell amounts versus their proportional absorption levels (Fig. 2A). As can be seen from Fig. 2A, the ST-8814 cells have higher absorption for the same number of cells in comparison to SK-N-SH cells, namely 0.68 and 0.95 units for $2x10^4$ and $1x10^5$ ST-8814 cells respectively, in comparison to 0.57 and 0.71 units for $2x10^4$ and $1x10^5$ SK-N-SH cells. The growth rates of both cell lines were determined during 72 hours, using AlamarBlue dye as a measuring tool for the number of cells present at a certain time (Fig. 2.B).



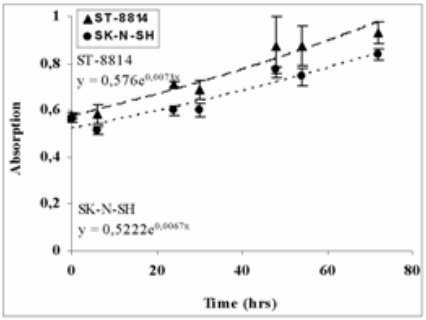
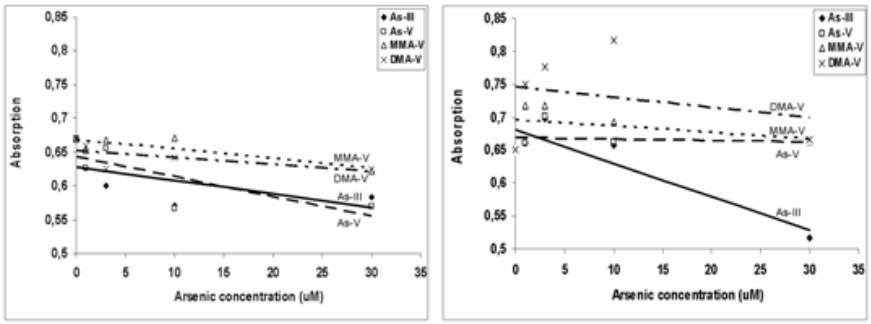


Fig. 2A. Linearity measurements (cell titration) of ST-8814 ($R^2 = 0.8422$) and SK-NSH ($R^2 = 0.887$) cell lines with AlamarBlue. These trend lines of indicate that the higher the amount of cells the greater the absorbance. The overall measured absorbances of the ST-8814 cells are higher than those of the SK-N-SH cells.
Fig. 2B. The growth rates of the two cell lines were measured during 72 hours. Mean values ± SD, n=3.

The LC_{50} for each cell line and for each As metabolite was determined by using AlamarBlue dye. Both ST-8814 and SK-N-SH responded differently to As metabolites, except for iAs^{III} (Fig. 3A and 3B). In both cultures, iAs^{III} was most lethal (Chi square test, $p<0.05$). The LC_{50} for ST-8814 is 12.82 μM and for SK-N-SH 17.63 μM.



3A. ST8814 3B. SK-N-SH

Fig. 3. LC_{50} measurements of various arsenic metabolites. Absorbance of A. ST-8814 and B. SK-N-SH cells after treatment with 0, 1, 3, 10 and 30 μM of the arsenic metabolites As^{III}, As^{V}, MMA^{V} and DMA^{V}. In both cell lines As^{III} is the most toxic form ($P<0.05$). From these trend lines, the LC_{50} for the four arsenic metabolites per cell line was calculated. Mean value; n=3.

The performed RT-PCR on both cell cultures was carried out by using the following concentration series; 0, 0.3, 1.0 and 3.0 μM of each arsenic compound, well below the LC_{50} of both cell lines. For the RT-PCR, relative quantification was used as a measure for the changes in expressions of the nucleic acid sequences of the targets (MAPT, NEFH, NEF3 and NEFL), relative to the same sequence in a standard, namely ß2-Microglobuline household gene (see table 1).

3.1. Effects of As on the gene expression of cytoskeletal proteins in Schwannoma cell line, ST-8814.

3.1.1. Effects of inorganic trivalent arsenic (iAs^{III}) in ST-8814 (Fig. 4A).

MAPT shows an increase after 24 hours for all 3 doses ($p<0.05$), and there were no significant changes after 48 hours. NEFH, NEF3 and NEFL show no significant changes after 24 or 48 hours.

3.1.2. Effects of inorganic pentavalent arsenic (iAs^{V}) in ST-8814 (Fig. 4B).

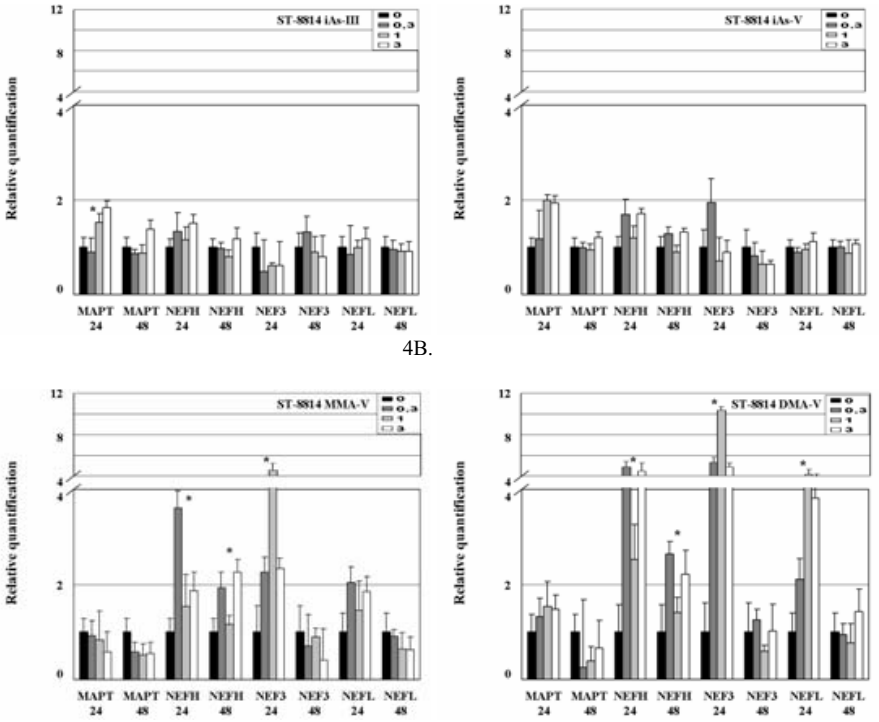
There are no significant changes on any of the MAPT, NEFH, NEF3 and NEFL genes after 24 or 48 hours.

3.1.3. Effects of organic monomethyl pentavalent arsenic (MMA^{V}) in ST-8814 (Fig. 4C).

MAPT shows no significant changes for all 3 doses after 24 or 48 hours. NEFH increases in the first 24-hours at all 3 concentrations, almost fourfold at 0.3 μM and twofold at 1.0 and 3.0 μM ($p<0.05$). After 48 hours, the increase is not as high as in the first 24 hours for the 0.3 μM, and it remains unchanged for the 1.0 and 3.0 μM during the second day ($p<0.05$). NEF3 has the most significant increase, especially at the 1.0 μM dose after 24 hours ($p<0.05$), but after 48-hours all the doses result in a lower expression level in comparison to their control. NEFL shows no significant changes after 24 or 48 hours.

3.1.4. Effects of organic dimethyl pentavalent arsenic (DMA^V) in ST-8814 (Fig. 4D).

MAPT shows no significant changes for the 3 doses after 24 or 48 hours. NEFH shows almost the same expression pattern with DMA^V as with MMA^V; an increase both after 24 and 48 hours ($p<0.05$), although the increase after 48 hours is slightly lower than the first 24 hours. NEF3 also shows a similar expression pattern for DMA^V as in MMA^V, a significant increase after 24 hours ($p<0.05$) and leveling off to the control level after 48 hours. NEFL shows a significant increase in the first 24 hours ($p<0.05$) and leveling off to the control level after 48 hours.



4A. 4B.

4C. 4D.

Fig. 4. Effects of various arsenic metabolites and concentrations on the various cytoskeletal gene expressions of Schwannoma cell line ST-8814 (*, $p<0.05$). Relative mean values ± SD; n=6.

3.2. Effects of As on the gene expression of cytoskeletal proteins in neuroblastoma cell line, SK-N-SH.

3.2.1. Effects of inorganic trivalent arsenic (iAs^{III}) in SK-N-SH (Fig. 5A).

MAPT shows no significant changes for the 3 doses after 24 or 48 hours. NEFH is unchanged after 24 hours. The expression levels for NEFH is increased after 48 hours for all three concentrations ($p<0.05$). NEF3 and NEFL are unchanged for the 3 doses after 24 or 48 hours.

3.2.2. Effects of inorganic pentavalent arsenic (iAs^V) in SK-N-SH (Fig. 5B).

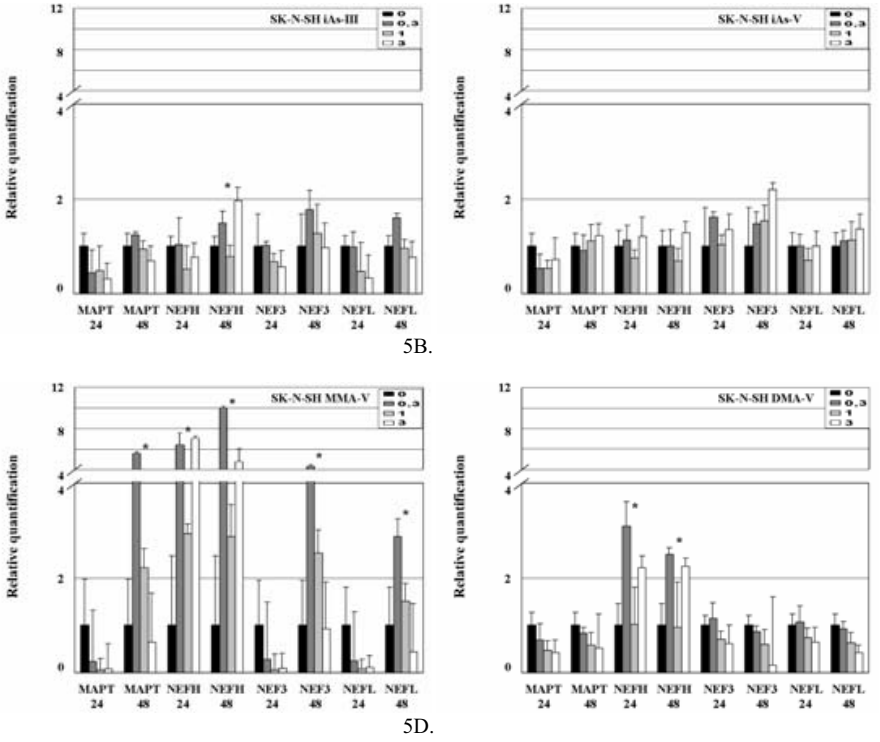
There are no significant changes on any of the MAPT, NEFH, NEF3 and NEFL genes after 24 or 48 hours.

3.2.3. Effects of organic monomethyl pentavalent arsenic (MMA^V) in SK-N-SH (Fig. 5C).

MMA^V shows the most profound effects on the cytoskeletal genes. MAPT expression is not significantly changed after 24 hours, but it is significantly increased after 48 hours ($p<0.05$). NEFH expression increased significantly for all three concentrations after 24 and 48 hours ($p<0.05$). NEF3 and NEFL have a similar expression pattern to its MAPT with no significant changes after 24 hours but they are increased after 48 hours ($p<0.05$).

3.2.4. Effects of organic dimethyl pentavalent arsenic (DMA^V) in SK-N-SH (Fig. 5D).

MAPT shows no significant changes for the 3 doses after 24 or 48 hours. NEFH is significantly increased after 24 and 48 hours ($p<0.05$). NEF3 and NEFL are unchanged for all 3 doses after 24 or 48 hours.



5A. 5B.

5C. 5D.

Fig. 5. Effects of various arsenic metabolites and concentrations on the various cytoskeletal gene expressions of Neuroblastoma cell line SK-N-SH (*, $p<0.05$). Relative mean values ± SD; n=6.

4. Discussion

The used cell lines were first characterized for the presence and comparison of the different cytoskeletal proteins and their gene expressions of various NFs and MAP-tau proteins in the SK-N-SH and ST-8814 cell lines (Fig. 1A and B). On this basis, we compare these cell lines and considered them to be representative for the CNS (SK-N-SH) and the PNS (ST-8814). SK-N-SH (neuroblastoma) cells are derived from the CNS; ST-8814 cells are cultivated from Schwannoma cells, which are derived from the PNS.

In most mammalian species As is metabolized from inorganic As to methylarsonic acid (MMA) and dimethylarsinic (DMA) by alternating reduction of pentavalent arsenic to trivalent and addition of a methyl group. This biotransformation of inorganic to organic arsenic is considered to be a detoxification step, due to the fact that methylated arsenics are reportedly less cytotoxic (Vahter and Concha, 2001). This is confirmed in this study as well by the effects of iAs on the LC_{50} of the studied cell lines (Fig. 3). This study was, however, also designed to clarify the effects of As-metabolites on the genes encoding for the cytoskeletal proteins NFs and MAPT. Both cell lines show linear properties (cell titration) for the increasing number of cells (Fig. 2A), with the difference that ST-8814 cells have higher absorption for the same number of cells in comparison to the SK-N-SH cells. This higher ratio is achieved by the higher slope coefficient of 0.0030 in ST-8814 than the slope coefficient of 0.0019 in SK-N-SH cells (Fig. 2A) in conjunction with their growth rate (Fig. 2B). This higher ratio shows that the ST-8814 cells have much higher redox reactions. The difference in absorption for blank wells (background) is caused by the use of two different cell culture mediums. ST-8814 has a doubling time of 20.44 hrs and SK-N SH has a doubling time of 17.74 hrs. The doubling time (growth rate) of the cells was calculated by using the absorption levels of each cell between 80 and 40 x1000 cells in Fig 2A, and subsequently using the curve equations of each cell (Fig. 2B) to calculate the doubling time.

AlamarBlue dye was also used to determine the LC_{50} of various arsenic metabolites for both cell lines (Fig. 2). As expected, iAs^{III} turns out to be the most lethal metabolite for both cell lines. Microscopic observations showed that at the highest concentration levels (30 μM), complete lethality was achieved. The LC_{50} for iAs^{V}, MMA^{V} and DMA^{V} could not be calculated in both cell lines. At the highest concentration levels (30 μM) of these three metabolites, complete lethality was not achieved.

Neurofilament and microtubule-associated protein-tau disorganization is a mark of various diseases (Pant et al., 1995). Tau regulates microtubule assembly, and stability is also involved in the establishment and maintenance of neuronal polarity. Dubois et al. (2005) concluded that NF-L protein plays an essential part in motor function, since mice with the deleted NEFL gene showed impaired motor functions (Dubois et al., 2005). In our previous work we showed that single exposure to iAs^{III} (i.v.) in rats cause changes in axonal cytoskeletal protein composition, namely that NF-L diminishes dependent on the dose and exposure time (Vahidnia et al., 2006). NF-L protein seems to play the most important role in neurofilament assembly in a number of neurodegenerative diseases, since it is the only neurofilament protein capable of organizing filaments on its own (Carpenter et al., 1996).

In agreement with this view, we show here that expression of the NEFL gene changes by DMA^{V} in the ST-8814 cell line and MMA^{V} in the SK-N-SH cell line. Furthermore, NEF3 and MAPT genes show to some extent the same expression patterns as NEFL gene under the influence of various As metabolites. However, NEFH expression is in the majority of the observations increased for both MMA^{V} and DMA^{V} metabolites in both cell lines (Fig. 4 and 5). A decrease in mRNA expression may also result in decrease in its translation to the corresponding protein. These findings suggest that in order to keep their cytoskeletal integrity, both cell types compensate the decrease in MAPT, NEF3 and NEFL by a significant increase

in NEFH (Fig. 4C, 4D, 5C and 5D). This explanation is supported by the fact that iAs^{III} and iAs^{V} metabolites do not cause an overall significant increase or decrease in the expression of MAPT, NEF3 and NEFL genes (Fig. 4A, 4B, 5A and 5B) and, as a result, there is no significant increase of NEFH evident in cells incubated with iAs^{III} and iAs^{V}.

Studies performed by Breen and Anderton (1991) in NEURO-3A neuroblastoma cells reveal that mRNA expression of NEFL and NEF3 are co-regulated while the NEFH gene is expressed independently. NEFL and NEF3 are expressed days before the appearance of NEFH (Breen and Anderton, 1991). Our results on NEFL and NEF3 expression are in agreement with their findings. In both cell lines, NEFL and NEF3 expressions are co-regulated, especially when MMA^{V} and DMA^{V} metabolites are used (Fig. 4C, 4D, 5C and 5D). The expression of these two genes could be co-regulated since both genes can be found on the same chromosome, namely: 8p21. At the same time, NEFH with the same arsenic metabolites does not follow the same expression pattern. NEFH increases in both cell lines and with both MMA^{V} and DMA^{V} metabolites. Furthermore, although MAPT in our study shows no significant changes, its expression pattern is similar to as expression patterns in NEF3 and NEFL. This may suggest that MAPT expression could also be co-regulated with the expression of NEFL and NEF3. An obvious explanation for the probable co-regulation of NEFL and NEF3 on the one hand, and MAPT on the other, is not readily available, as the MAPT gene is located on chromosome 17q21.1.

In conclusion, it can be said that although iAs^{III} and iAs^{V} metabolites are more lethal for both cell lines, the MMA^{V} and DMA^{V} metabolites are more toxic to the genes regulating the neurofilament proteins under study. MMA^{V} and DMA^{V} metabolites show large variations in expression of the cytoskeletal genes. Although iAs^{III} is most lethal but not genotoxic, it contributes to (hyper) phosphorylation of neurofilament and tau proteins (Giasson et al., 2002; Shea et al., 2003). Phosphorylation of neurofilament proteins change cytoskeletal protein composition, phosphorylated or hyperphosphorylated neurofilament proteins tend to accumulate in the cell body in vivo (Shea et al., 2003). The same is also true for arsenite induced hyperphosphorylated tau proteins (Giasson et al., 2002). As a result, hyperphosphorylated tau proteins are not able to organize microtubules in cells. The altered cytoskeletal gene expressions combined with the hyperphosphorylation of cytoskeletal proteins gives insight into the effects and neurotoxic mechanism of various arsenic metabolites.

In this study, we have been able to demonstrate altered cytoskeletal gene expression by various arsenic compounds. The significant increase of NEFH and simultaneous change of NEFL, NEF3 and MAPT may lead to changes in cytoskeletal composition, which in turn can influence axonal transport and result in diminished nerve conduction velocity (Le Quesne et al., 1977; Lagerkvist et al., 1994). This is in agreement with the observation that As intoxication may cause neuropathy (Hafeman et al., 2005).

In a next study in preparation, we will describe how changes in the cytoskeletal proteins may occur as a result of exposure to As metabolites.

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Chapter 5

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