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## **Studies into the mechanism of arsenic-induced neurotoxicity**

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**Chapter 4: Arsenic neurotoxicity II**

**Arsenic-induced neurotoxicity in relation to toxicokinetics:  
effects on sciatic nerve proteins**

*Food and Chemical Toxicology  
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Chapter 4

## **Arsenic-induced neurotoxicity in relation to toxicokinetics: effects on sciatic nerve proteins**

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### **Abstract**

In our previous study in rats acutely exposed to As, we observed an effect of As on neurofilaments in the sciatic nerve. This study deals with the effects of inorganic As in Wistar rats on the cytoskeletal protein composition of the sciatic nerve after subchronic intoxication. Sodium meta-arsenite dissolved in phosphate-buffered saline (PBS) was administered daily in doses of 0, 3 and 10 mg/kg body weight/day (n = 9 rats/group) by intragastric route for 4, 8 and 12 week periods. Toxicokinetic measurements revealed a saturation of blood As in the 3- and 10-mg/kg dose groups at approximately 14 µg/ml, with an increase in renal clearance of As at increasing doses. After exsanguination, sciatic nerves were excised and the protein composition was analyzed. Analysis of the sciatic nerves showed compositional changes in their proteins. Protein expression of neurofilament Medium (NF-M) and High (NF-H) was unchanged. Neurofilament protein Low (NF-L) expression was reduced, while µ- and m-calpain protein expression was increased, both in a dose/time pattern. Furthermore, NF-H protein was hypophosphorylated, while NF-L and microtubule associated protein tau (MAP-tau) proteins were (hyper)-phosphorylated. In conclusion, we show that expression of µ- and m-calpain protein is increased by exposure to As, resulting in increased NF-L degradation. In addition, hyperphosphorylation of NF-L and MAP-tau by As also contribute to destabilization and disruption of the cytoskeletal framework, which eventually may lead to axonal degeneration.

**Keywords:** arsenite, calpain, neurofilament, microtubule associated protein tau, neurotoxicity, phosphorylation

## 1. Introduction

As is a semi-metalloid element that occurs naturally in our environment. Throughout history, As has been used as a pesticide, herbicide, homicide or suicide agent in Chinese medicine and as make-up in eye shadows in the Roman era. Nowadays, millions of people in South East Asia (Rahman et al., 2001) and several South American countries are daily exposed to As due to contaminated drinking water with considerable adverse public health effects.

Worldwide, chronic intoxication with As is a much bigger problem in comparison to acute intoxication (Centeno et al., 2002). As intoxication, whether acute (Greenberg, 1996) or chronic, leads to peripheral neurotoxicity, which to date has only been demonstrated clinically and in controlled electrophysiological studies (Le Quesne and McLeod 1977; Goebel et al., 1990). Neurotoxic effects have been reported in many cases, although PNS impairment is common in As-exposed populations (Hafeman et al., 2005). Peripheral neuropathy, manifested by decreased sensibility, ataxia, pain and paresis are common neurological symptoms of As intoxication, which often begins in the lower extremities. Although PNS impairment is common in As-exposed populations (Goebel et al., 1990; Greenberg, 1996; Lagerkvist and Zetterlund, 1994), its mechanism has hardly been studied.

Nerve cytoskeletal proteins form a flexible framework for the cell, provide attachment points for organelles and formed bodies, and enable the possibility of communication between cell parts. The major protein constituents of the cytoskeleton are microtubule and microtubule-associated proteins (MAPs), intermediate filaments and microfilaments. The intermediate filaments are subdivided into three types of neurofilaments that are specific to the nervous system: NF-H, NF-M and NF-L, according to their molecular weight of 200, 150 and 68 kDa, respectively. Neurofilaments are the major component in large myelinated neurons. Several studies have shown that changes in neurofilament proteins and MAP-tau are related to many neurodegenerative diseases, such as Alzheimer's (Dowjat et al., 2001; Trojanowski et al., 1993), diabetes (Schechter et al., 2005), amyotrophic lateral sclerosis (Lariviere and Julien, 2004) and the Guillian-Barré syndrome (Winship, 1984).

MAP-tau promotes tubulin assembly in microtubules; although tau proteins are found in all cells, they form the major components of neurons and are predominantly associated with microtubules of the axon. In the brains of patients with Alzheimer's disease the neuronal cytoskeleton is progressively disrupted and replaced by tangles of paired helical filaments (PHFs), which are composed mainly of hyperphosphorylated forms of tau proteins (Alonso et al., 1996). NF-L has been shown to be essential in filament assembly (Zhu et al., 1997); furthermore, mice with the deleted *Nefl* gene demonstrated impaired motor functions (Dubois et al., 2005). In a previous study we showed that after a single acute exposure to As, the protein composition of the sciatic nerve in rats, especially NF-L, was affected immediately (Vahidnia et al., 2006), while also delayed effects occurred, whereby NF-M and NF-L proteins decreased dose dependently over time after a single exposure to arsenite.

Neurodegenerative diseases result from deterioration of neurons, caused by functional disturbances and/or pathological changes in the peripheral nervous system (Miller et al., 2002). NF and tau proteins compete for the same microtubule binding site. Phosphorylated NFs have diminished affinity to the microtubule. Hyperphosphorylation of tau and neurofilament may underlie the cytoskeletal abnormalities and neuronal death seen in several neurodegenerative diseases including Alzheimer's disease (AD) (Alonso et al., 1996). Tau protein, in its hyperphosphorylated form, is the major component of PHF and neurofibrillary tangles and in plaques in Alzheimer's disease brain.

In this study, we study in Wistar rats the effects of inorganic As on the cytoskeletal protein composition of the sciatic nerve after subchronic intoxication. Sodium meta-arsenite solution

dissolved in PBS was administered daily by gavage to the rats at dose levels of 0, 3 and 10-mg/kg body weight/day (n = 9 rats/group) for 4, 8 and 12 weeks. The present research, which is a follow-up to a previous study (Vahidnia et al., 2006) aims at investigating the semi-chronic effects of arsenite on rat sciatic nerve proteins after repeated exposure in order to introduce a better comparison model for the human situation as regards the peripheral axonopathy and the pathological changes in the peripheral nervous system. It is the purpose of this study to investigate the effects of As on the peripheral nervous system as a step forward to understanding the mechanism of As neurotoxicity.

## 2. Materials and Methods

### 2.1. Chemicals.

Sodium meta-arsenite ( $\text{NaAsO}_2 \cdot \text{As}^{\text{III}}$ ) (product no. 22,869-9, 98% pure) and acrylamide/bis-acrylamide (product no. A 3699, Mix ratio 37.5:1, T30%, C2.6%) and color markers high range (HMW color marker) (product no. C 3312) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). TEMED (catalog no. 161-0800) and sodium dodecyl sulphate (catalog no. 161-0301) were purchased from Bio-Rad (Veenendaal, Netherlands). Tris-HCl (catalog no. 108219) and EDTA (catalog no. 108418) were purchased from Merck (Darmstadt, Germany). BCA<sup>TM</sup> Protein Assay Kit (Pierce, product no. 23225, Rockford, IL USA) was obtained from Pierce (Rockford, USA). 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. Kodak biomax XAR film (catalog no. 165 1454) was obtained from Kodak (Shelton, CT, USA). ECL plus<sup>TM</sup> Western blotting detection reagent was obtained from Amersham Biosciences (Piscataway, NJ, USA). 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) to the six isoforms and mouse monoclonal antibody to Phosphoserine/threonine/tyrosine (catalog no. Ab15556) and monoclonal antibody to GAPDH (catalog no. Ab8245) were obtained from Abcam (Cambridge, United Kingdom). Rabbit polyclonal anti-Calpain (H-60) (catalog no. sc-30065) was purchased from Santa Cruz biotechnology, inc. (California, USA).

### 2.2. Animals and housing.

Male Wistar rats (225-250g) were obtained from Charles River, Maastricht, Netherlands. The rats were acclimatized for 7 days and housed in 9 groups of 3 in plastic cages on sawdust in a 12/12 h light/dark cycle. The rats were fed a standard diet and tap water *ad libitum* in their plastic and metabolic cages. The protocol for this study was submitted to and agreed by the Animal Ethical Committee of the Leiden University Medical Center.

### 2.3. Experimental design.

The rats received sodium arsenite solution in PBS once daily by gavage. 0, 3 and 10 mg/kg  $\text{As}^{\text{III}}$  ( $\text{NaAsO}_2$ ) levels of dose were used for n=3 rats per time index of 4, 8 and 12 weeks per dose (n=9 rats/dose group). The highest dose, 10 mg/kg, is four times lower in dosage intensity than the LD<sub>50</sub> of 41 mg/kg that is indicated by the manufacturer for sodium meta-arsenite. The administered volumes ranged from 0.25 to 0.45 ml depending on body weight. The control groups were intubated with PBS 1 $\mu$ l/g body weight, so a rat with 250 gram body weight received 0.25 ml of PBS without added arsenite. This semi-chronic experiment was divided in 3 periods of 4, 8 and 12 weeks. Urine samples were weekly collected in plastic containers at the same time and during the individual housing of each rat in a metabolic cage for 6, 8 and 10 hours for the 0-, 3- and 10-mg/kg dose groups, respectively. The As-treated rats were kept longer in the metabolic cages in order to collect sufficient urine for analysis. After the intended duration of 4, 8 or 12 weeks, the rats in a plastic sealed cage with in- and out-flow tubes were anesthetized individually with isoflurane. Subsequently, they were exsanguinated by means of arterial blood withdrawal from the inferior mesenteric artery. *Nervus ischiadicus* (sciatic nerve) arises from sacral plexus and passes about halfway down the thigh where it divides into the common peroneal and tibial nerves. The nerve is dissected

on one end from the sacral plexus as close as possible to the spinal column and on the other end just before its division. The nerve of each leg is dissected by about 2cm. In these experiments the sciatic nerve from the right leg is used for protein analysis and the one from the left leg is used for As measurement.

#### 2.4. Arsenic analysis.

Urine samples for the measurement of As elimination were collected weekly at the same time for 6, 8 and 10 hours for the 0-, 3- and 10-mg/kg dose groups, respectively, by placing the rats in metabolic cages.

The blood samples for As analysis obtained at the end of the exposure period were first digested in an acid mixture comprising one part perchloric acid and one part nitric acid (1:1) (digestive acid). Fifty  $\mu$ l bloods were added to 950  $\mu$ l of digestive acid and incubated for 1 hr at 70 °C. The sciatic nerve samples were weighed and digested in 1 ml digestive acid as in blood and incubated for 1 hr at 70 °C. The urine samples of the highest dose groups were first diluted 200 times with Milli-Q water (MQ-H<sub>2</sub>O). From the digested blood or nerve or the diluted urine samples 0.5 ml was added to 4.5 ml of reduction acid consisting of 216 ml MQ-H<sub>2</sub>O, 27 ml 37% hydrochloric acid (1M HCl), 6 g sodium iodide (NaI) and 3 g L-(+)ascorbic acid (C<sub>6</sub>H<sub>8</sub>O<sub>6</sub>) and incubated for 1 hr at 70 °C.

The As content of urine, digested blood and digested sciatic nerve was measured with atomic absorption spectrometry (Perkin-Elmer FIAS-3100 AAS). The technique for measuring total As content is hydride generation coupled to AAS (HG-AAS). An As electrodeless discharge lamp of 7W was used; As absorption was measured at 193.7 nm. The matrix of the calibration solution for urine consisted of 4.5 ml 1M HCl and 0.5 ml As standard solution dissolved in MQ-H<sub>2</sub>O ranging from 0  $\mu$ g/l to 200  $\mu$ g/l end concentration. Where the digested blood and nerves are concerned, the matrix of the calibration solution comprised 4.5 ml 1M HCl and 0.5 ml of As standard solution dissolved in digestive acid ranging from 0  $\mu$ g/l to 200  $\mu$ g/l end concentration.

#### 2.5. Protein sample processing and analysis.

The three neurofilament proteins (NFs) and MAP-tau expression and phosphorylation were analyzed by using Western blot technique. Excised sciatic nerves weighing 12.5 mg to 18.5 mg were homogenized with a blender in a 1.5 ml Tris-HCl buffer (200 mM, pH 8.0), also containing EDTA 3 mM and sodium dodecyl sulphate (SDS) 1% w/v. SDS was added to inactivate enzymes by disrupting the non-covalent bonds. The nerve tissues were homogenized on ice for 2 minutes and subsequently heated for 5 minutes at 100°C. The homogenization and heating procedure was repeated twice. The homogenized samples were centrifuged for 1 min at 10,000xg and the supernatant was transferred to a new 1.5 ml eppendorf tube. Protein concentrations of the homogenates were measured by the BCA<sup>TM</sup> Protein Assay Kit. To obtain the same protein concentrations prior to analysis by SDS-PAGE, the nerve homogenates were standardized with Tris-HCl buffer. Nerve homogenates were analyzed on an 8% acrylamide separation gel (Laemmli, 1970). The separation gels were used for immunoblotting on 0.2  $\mu$ m nitrocellulose membranes in conjunction with polyclonal neurofilament antibodies to all 3 neurofilament proteins: NF-H, NF-M and NF-L, monoclonal antibody against MAP-tau and monoclonal antibody against phosphoserine/threonine/tyrosine and monoclonal antibody against GAPDH as loading control for SDS-PAGE. Application of the ECL plus<sup>TM</sup> Western blotting detection reagent and the 30-seconds to 3-minutes exposure to Kodak biomax XAR film concluded the Western blot analysis. The western blot analysis of the same samples was performed in triplicate. The band intensities on the Kodak

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biomax XAR film were analyzed by Quantity One – Densitometer GS-710 from Bio-Rad (Veenendaal, Netherlands).

Although monoclonal antibody against phosphoserine/threonine/tyrosine is not specific for NF and tau proteins, the positions of NF's and tau proteins were determined through side-by-side comparison, digitally. The nitrocellulose blots for NFs and tau were aligned with those in the anti-phospho blot. High molecular weight Color marker proteins on all blots were used as reference points to align the phosphorylated protein bands, for comparison with protein expression bands.

#### 2.6. Statistical analysis.

The arsenic biokinetics in body fluids, nerve tissue data and western blot analysis were statistically evaluated for each dose group using the SPSS 14.0 for Windows. Analysis of variance (ANOVA) for means was carried out to evaluate the data in. A level of  $p < 0.05$  was accepted as statistically significant.

### 3. Results

#### 3.1. General health

Increase in the As<sup>III</sup> dose results in a decrease in body weight gain (Fig. 1). The difference between weight gain in control rats and the reduced weight gain in rats treated with 10 mg/kg As<sup>III</sup> becomes evident after 2 weeks ( $p < 0.001$ ). Furthermore, in both dose groups, diarrhea, which is also a symptom in acute As poisoning, was observed. The 3 mg/kg As<sup>III</sup> doses also resulted in diminished appetite and reduced general alertness. Body weight in the 3-mg/kg dose group increased to such extent that there was no significant difference between treated and control animals.

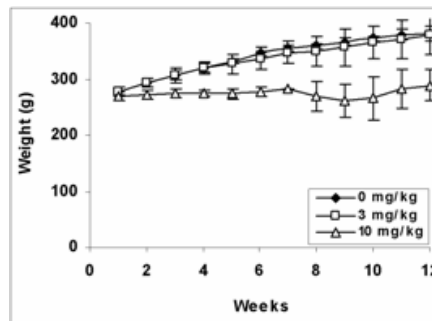


Fig. 1. Body weight increase in three different dose groups of the 12-week exposure group. Each dose group of 0, 3 and 10 mg/kg consists of 3 rats, which were exposed to As and monitored over 12 weeks. The highest dose group of 10 mg/kg lags behind. ANOVA for mean value per dose group showed significant difference of  $p < 0.001$ .

## 3.2. Arsenic distribution and elimination

The total amount of As excreted in urine was measured and expressed as  $\mu\text{g}/\text{ml}$  (Table 1). The As concentration in urine of 3-mg/kg dose group increased from  $8.57 \pm 1.52 \mu\text{g}/\text{ml}$  to  $13.65 \pm 0.95 \mu\text{g}/\text{ml}$  after 4 weeks and 12 weeks, respectively. In the 10-mg/kg dose group, the As concentration in urine increased from  $36.6 \pm 10.73 \mu\text{g}/\text{ml}$  to  $55.8 \pm 59.45 \mu\text{g}/\text{ml}$  after 4 weeks and 12 weeks, respectively. The As excretion in urine was also calculated in relation to the creatinine content (Fig. 2). The 3-mg/kg dose group showed a parallel progress to the control group, with only a slight elevation. However, the 10 mg/kg dose group showed a proportional increase from the start up to the last week.

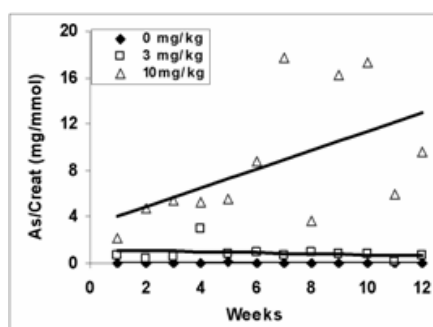


Fig. 2. As excretion, measured weekly as the amount of As (mg) excreted in urine expressed per mmol creatinine. ANOVA for mean value per dose group showed significant difference of  $p < 0.001$ .

Table 1 shows the As concentration in blood ( $\mu\text{g}/\text{ml}$ ). The As content of blood was saturated by approximately  $14 \mu\text{g}/\text{ml}$  after 4 weeks in both 3- and 10-mg/kg group.

The As content of the sciatic nerve was measured and expressed in  $\mu\text{g}/\text{g}$ . In the control groups, the As content was between  $0.02 \pm 0.01$  and  $0.08 \pm 0.02 \mu\text{g}/\text{g}$  tissue. The 3-mg/kg dose group showed a decrease in As content from  $0.43 \pm 0.25 \mu\text{g}/\text{g}$  tissue to  $0.35 \pm 0.24 \mu\text{g}/\text{g}$  tissue after 4 and 8 weeks, respectively and a decrease to  $0.11 \pm 0.01 \mu\text{g}/\text{g}$  tissue after 12 weeks, whereas the 10-mg/kg dose group showed an As decrease from  $1.17 \pm 0.23 \mu\text{g}/\text{g}$  tissue to  $0.53 \pm 0.20 \mu\text{g}/\text{g}$  tissue after 4 and 8 weeks, respectively, and a decrease to  $0.89 \pm 0.15 \mu\text{g}/\text{g}$  tissue after 12 weeks.

The volume of distribution ( $V_D$ ) ( $\text{l}/\text{kg}$ ) was calculated from the As content measured in the nerve tissue ( $\mu\text{g}/\text{g}$ ) divided by the As content of the blood ( $\mu\text{g}/\text{ml}$ ). The  $V_D$  in 3-mg/kg dose group decreased from  $0.043 \pm 0.0264$  to  $0.024 \pm 0.0156$  after 8 weeks and to  $0.008 \pm 0.0005$  ml/g after 12 weeks of treatment. The 10-mg/kg dose group also showed a decrease from  $0.106 \pm 0.0233$  to  $0.039 \pm 0.0161$  after 4 and 8 weeks, respectively, and a decrease to  $0.067 \pm 0.0114$  ml/g after 12 weeks.

Table 1. Arsenic biokinetics in body fluids and nerve tissue (mean  $\pm$  S.D.).

Dosage (n)	Weeks	Diuresis <sup>a</sup> (ml/h)	As in urine <sup>*b</sup> ( $\mu$ g/ml)	As in blood <sup>c</sup> ( $\mu$ g/ml)	As in <sup>d</sup> sciatic nerve ( $\mu$ g/g) (W/W)	Total body <sup>e,**</sup> clearance (Cl) (ml/h)	Relative distribution volume ( $V_D$ ) <sup>***</sup> (nerve tissue) (ml/g)
0 mg/kg (9)	4	0.45 $\pm$ 0.22	0.01 $\pm$ 0.004	0.07 $\pm$ 0.016	0.08 $\pm$ 0.02	0.05 $\pm$ 0.012	---
	8	0.91 $\pm$ 0.30	0.01 $\pm$ 0.001	1.20 $\pm$ 0.300	0.02 $\pm$ 0.01	0.01 $\pm$ 0.002	---
	12	0.48 $\pm$ 0.17	0.01 $\pm$ 0.002	0.30 $\pm$ 0.200	0.05 $\pm$ 0.03	0.02 $\pm$ 0.006	---
3 mg/kg (9)	4	0.30 $\pm$ 0.06	8.57 $\pm$ 1.52	10.19 $\pm$ 0.72	0.43 $\pm$ 0.25	0.25 $\pm$ 0.019	0.043 $\pm$ 0.0264
	8	0.21 $\pm$ 0.04	9.04 $\pm$ 0.98	14.78 $\pm$ 0.53	0.35 $\pm$ 0.24	0.13 $\pm$ 0.011	0.024 $\pm$ 0.0156
	12	0.36 $\pm$ 0.15	13.65 $\pm$ 0.95	14.59 $\pm$ 1.19	0.11 $\pm$ 0.01	0.34 $\pm$ 0.127	0.008 $\pm$ 0.0005
10 mg/kg (9)	4	0.35 $\pm$ 0.10	36.55 $\pm$ 10.73	11.07 $\pm$ 0.32	1.17 $\pm$ 0.23	1.18 $\pm$ 0.496	0.106 $\pm$ 0.0233
	8	0.31 $\pm$ 0.12	24.81 $\pm$ 18.66	13.83 $\pm$ 0.85	0.53 $\pm$ 0.20	0.46 $\pm$ 0.139	0.039 $\pm$ 0.0161
	12	0.29 $\pm$ 0.22	55.76 $\pm$ 59.45	13.34 $\pm$ 0.03	0.89 $\pm$ 0.15	0.74 $\pm$ 0.399	0.067 $\pm$ 0.0114

Mean values ( $\pm$  SD) for the number of rats per dose group (N) is presented in this table. ANOVA for mean value per dose group showed significant between-group differences as follows:

<sup>a</sup> For diuresis: 0- and 3- and 10-mg/kg dose groups  $p < 0.01$ .

<sup>b</sup> For excreted As in urine: 0- and 3- and 10-mg/kg  $p < 0.001$ .

<sup>c</sup> For blood: 0- and 3- and 10-mg/kg  $p < 0.001$

<sup>d</sup> For sciatic nerve: 0- and 3- and 10-mg/kg  $p < 0.001$

<sup>e</sup> For total body clearance: : 0- and 3- and 10-mg/kg  $p < 0.001$

\* Last urine collected before sacrifice. The urine was collected for 6, 8 and 10 hours for the 0, 3 and 10 mg/kg dose groups, respectively.

\*\* Clearance (Cl) is calculated by dividing urinary elimination of As in  $\mu$ g per h by the blood concentration in  $\mu$ g/ml. Urine and blood collection took place just before sacrifice

\*\*\* The relative volume of distribution ( $V_D = \text{ml/g}$ ) was calculated by using As content measured in nerve tissue ( $\mu$ g/g) divided by the As content measured in blood ( $\mu$ g/ml).

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3.3. Expression of the cytoskeletal proteins

The protein expression analysis showed As-induced changes in the protein composition of the sciatic nerve. Western blot analysis showed no apparent changes in the NF-H and NF-M during the 12-week time window and increased dosage. However, the NF-L protein content was significantly decreased in the 3- ( $p<0.05$ ) and even more so in the 10-mg/kg dose group ( $p<0.01$ ) (Fig. 3). However, the microtubule-associated protein tau showed no significant changes in its expression.

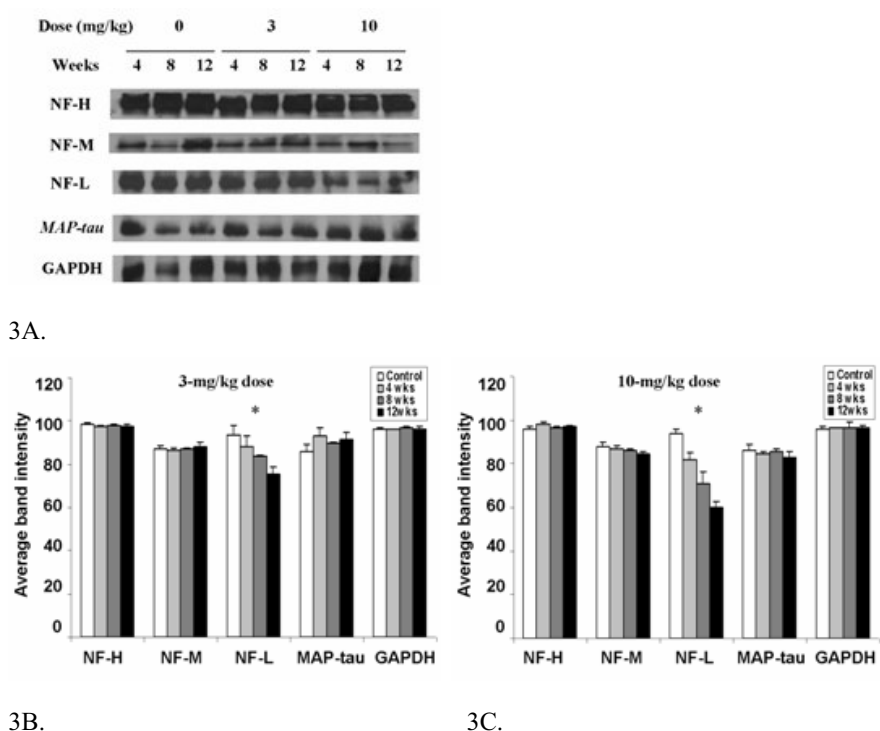
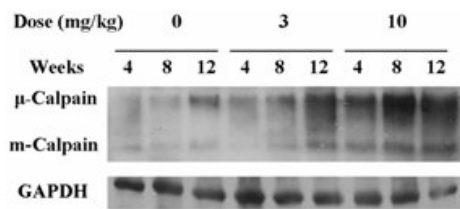


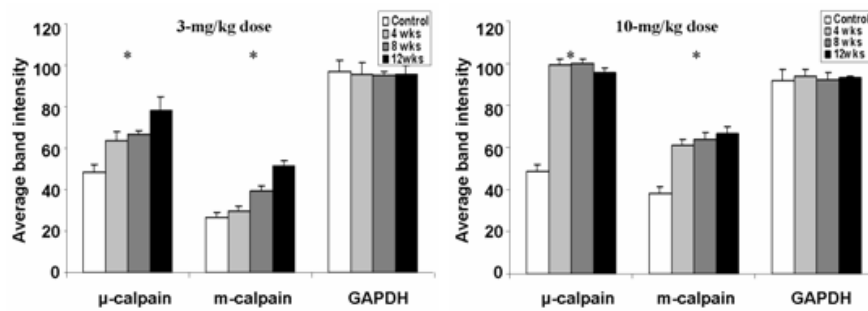
Fig. 3. 3A. Western blot analysis of the expression levels of the three-neurofilament proteins and total-tau of the sciatic nerve in the three dose groups in relation to time after dosing. Figure 3B and C. show the densitometry measurements in relative mean values ( $\pm$  SD) after 4, 8 and 12 weeks exposure for the number of performed western blots ( $n=3$ ). ANOVA showed a significance of  $p<0.01$  (\*) for decrease in NF-L expression.

### 3.4. Calpain protein expression in sciatic nerves

The analysis of  $\mu$ - and m-calpain protein expression in As-treated rats showed significant increase with time for  $\mu$ -calpain ( $p < 0.01$ ) and m-calpain ( $p < 0.05$ ) in both the 3- and the 10-mg/kg dose group (Fig. 4).



4A.



4B.

4C.

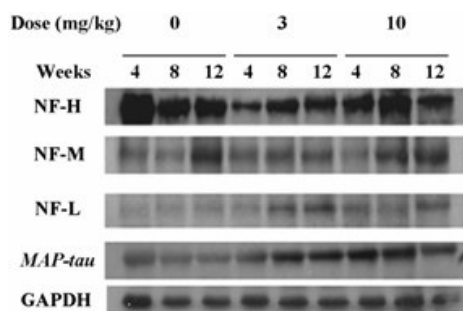
Fig. 4. 4A. Western blot analysis of the expression levels of the  $\mu$ -calpain and m-calpain proteins of the sciatic nerve in the three dose groups in relation to time after dosing. Figure 4B and C. show the densitometry measurements in relative mean values ( $\pm$  SD) after 4, 8 and 12 weeks exposure for the number of performed western blots ( $n=3$ ). ANOVA showed a significance of  $p < 0.01$  (\*) for increased  $\mu$ -calpain expression and  $p < 0.05$  (\*) for m-calpain expression.

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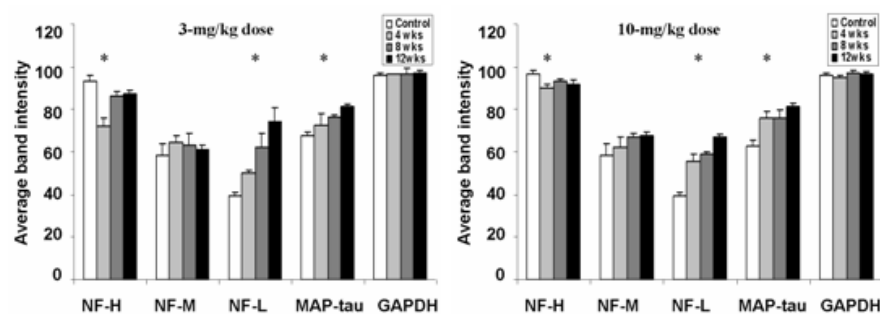
3.5. Phosphorylation of cytoskeletal proteins

Phosphorylation of various proteins was made visible by using antibodies against the hydroxy amino acids in proteins (Fig. 5). Phosphorylation of NF-H shows for the 0-mg/kg dose group a decrease in time from 4 to 12 weeks. For the 3-mg/kg dose group, the total phosphorylation level is lower than that of the control groups; however, in the dose group itself, phosphorylation level is increased from 4 to 12 weeks ( $p < 0.05$ ). The 10-mg/kg dose group shows the same phosphorylation pattern as the 3-mg/kg dose group but with a higher intensity.

The NF-M protein shows no significant phosphorylation on their serine, threonine or tyrosine amino acid groups, with or without arsenite. However, the NF-L and the MAP-tau proteins are more phosphorylated in the 3- and 10-mg/kg dose groups. The NF-L protein is hyperphosphorylated in both the 3- ( $p < 0.05$ ) and the 10-mg/kg dose group ( $p < 0.001$ ). The MAP-tau protein is also hyperphosphorylated in both the 3- ( $p < 0.01$ ) and the 10-mg/kg dose groups ( $p < 0.001$ ).



5A.



5B.

5C.

Fig. 5. 5A. Western blot analysis of four sciatic nerve proteins visualized with antibody against phosphoserine/threonine/tyrosine. Figure 5B and C. show the densitometry measurements in relative mean values ( $\pm$  SD) after 4, 8 and 12 weeks exposure for the number of performed western blots ( $n=3$ ). ANOVA showed a significance of  $p < 0.05$  (\*) for decrease in NF-H and  $p < 0.001$  (\*) for increase in NF-L and MAP-tau phosphorylation.

#### 4. Discussion

Reduction in body weight gain is used as a parameter for the decrease in the rats' general state of health. Rats treated with 3 mg/kg arsenite show no changes in body weight gain, unlike the control groups treated with PBS only. However, after the first week and significant lagging behind over the course of time, the 10-mg/kg dose group shows a decrease in body weight gain in comparison to the 0- and 3-mg/kg dose groups (Fig. 1). The effects of As on body weight are most probably a result of reduced food intake as a result of decrease in general health and of the observed gastrointestinal tract toxicity (diarrhea) rather than a direct effect of As on hypothalamic function.

Urine samples for the measurement of As elimination were collected as described in 2.3., Experimental design (Table 1). The 3-mg/kg dose group showed a gradual increase in As excretion from 8.57 (4 weeks) to 13.65 (12 weeks) mg As/l. The As excretion in the 10-mg/kg dose group was much higher, starting from 36.6 mg As/l after 4 weeks to 55.8 mg As/l after 12 weeks of treatment. The high As concentration in the 10-mg/kg dose group is the result of a significantly decreased diuresis in the 3-mg/kg ( $p < 0.01$ ) and the 10-mg/kg dose group ( $p < 0.05$ ). This drop in diuresis is most probably the result of diarrhea in both dose groups. As can be seen from Table 1, renal clearance of As is increased in time and also clearance is higher in the 10-mg/kg dose group than the 3-mg/kg dose group, in spite of reduced diuresis. This is also emphasized in Fig. 2, where renal excretion is shown as mg As excreted per mmol creatinine, whereby the 10-mg/kg dose group shows the highest increase ( $p < 0.01$ ) in comparison to the 0- and the 3-mg/kg dose groups.

The blood As concentration in both 3- and 10-mg/kg dose groups increased from 4 to 8 weeks ( $p < 0.001$ ) and leveled off as from the 8<sup>th</sup> week due to approximately 14  $\mu\text{g/ml}$  As concentration. The 3- and 10-mg/kg dose groups show no difference in As levels at all three times. From the renal clearance of As in both dose groups, it can be seen that the As blood concentration is maintained at a maximum level by enhanced renal excretion. The mechanism of this apparent homeostasis is not clear as yet.

The amount of As measured in the sciatic nerve tissues of the 3-mg/kg dose group is higher in comparison to the controls ( $p < 0.001$ ). However, the measured concentrations diminished from  $0.43 \pm 0.25 \mu\text{g/g}$  As after four weeks exposure to  $0.11 \pm 0.01 \mu\text{g/g}$  As ( $p < 0.001$ ) in the group exposed for twelve weeks. A decrease pattern is also present in the 10-mg/kg dose group, but with a higher concentration of  $1.17 \pm 0.23 \mu\text{g/g}$  As in the group exposed for four weeks, followed by a decrease to  $0.53 \pm 0.20 \mu\text{g/g}$  tissue after 8 weeks and a decrease to  $0.89 \pm 0.15 \mu\text{g/g}$  tissue after 12 weeks ( $p < 0.001$ ). A possible explanation for this decrease may be that on the one hand a decrease in expression levels of NF-L results in less available sulphhydryl (-SH) groups for As to undergo a covalent binding (Fig. 3). On the other hand, the phosphorylation of various proteins by As in the sciatic nerves also results in less available binding sites for As (Fig. 5) (Shea et al., 2003).

The relative volume of distribution ( $V_D$ ) of As in sciatic nerve was calculated from the As concentrations in blood and nerve tissue. In comparison with the total body  $V_D$  (5.62-6.15 ml/g) (Vahidnia et al., 2006); the  $V_D$  in nerve tissue is very low, namely 0.043-0.008 ml/g for the 3-mg/kg dose group, and 0.106-0.039 for the 10-mg/kg dose group. It should be noted that in our previous work, As was administered intravenously and in this study orally. A possible explanation of the difference between total body and nerve tissue  $V_D$ s is that trivalent As has a high affinity for reduced sulphhydryl (-SH) groups. Orally administered arsenite is metabolized in the liver before entering the circulation, whereas intravenously administered arsenite is more readily available to interact with reduced sulphhydryl (-SH) groups.

This study demonstrates the effects of orally administered arsenite on the composition of sciatic nerve proteins, namely MAP-tau and NFs. As shown in Fig. 3, there are no apparent changes in the expression levels of MAP-tau, NF-H and NF-M proteins. However, NF-L expression is gradually decreased in the 3-mg/kg dose group from 4 to 8 and to 12 weeks. The decrease is more apparent in the 10-mg/kg dose group ( $p < 0.01$ ), which demonstrates that the NF-L decrease depends on the dose and exposure time. The decrease of NF-L expression found in this study may play an important role in As-induced pathological changes of the peripheral nervous system, since NF-L is the only NF protein capable of organizing and co-assembling filaments on its own in vivo. NF-H and NF-M each need an NF-L protein to form a heteropolymer (Carpenter and Ip, 1996). Thus far, it can be concluded that the decrease in NF-L expression caused by arsenite results in less available NF-L for the formation of heteropolymer in the cytoskeletal framework. This decrease in NF-L expression is possibly a post-translational activity as a result of a proteolytic process, since in vitro studies with  $iAs^{III}$  in neuroblastoma (SK-N-SH) and Schwannoma (ST-8814) cell lines show no effect on the mRNA expression level of cytoskeletal genes (Vahidnia et al., 2007). Calpain is calcium-activated cytoplasmic protease that is responsible for NF-L degradation, since neuroblastoma cells (SY-5Y) treated with arsenic trioxide (trivalent As) show an increase in intracellular calcium (Florea et al., 2007). Figure 4 shows an increase of  $\mu$ -calpain ( $p < 0.01$ ; activated by  $\mu M$  amount of calcium) and increase of  $m$ -calpain ( $p < 0.05$ ; activated by  $mM$  amount of calcium) in both the 3- and 10-mg/kg dose groups. Studies in PC12 cells under oxidative stress circumstances have shown an increase in calcium within the cells and up-regulation of calpain leading to degradation of NF-L protein (Ray et al., 2000). Furthermore, inactivation of calpain by calpain inhibitor (MDL-28170) prevents NF-L breakdown (Kunz et al., 2004; Lopez-Picon et al., 2006). These results suggest that As-induced destabilization and disruption of the cytoskeletal framework is partly due to increased expression of calpain, which in turn is responsible for NF-L degradation in a calcium-induced proteolytic process.

Neurofilament proteins are synthesized exclusively in the cell body of neurons and are transported along nerve fibers as part of the slow component of axonal transport (Hoffman and Lasek, 1975). Phosphorylation of proteins causes conformational changes in protein leading to an altered function or pathological changes in most cases. Neurofilament accumulation in the cell body due to hyperphosphorylation is seen in several neurological diseases and this phosphorylation is believed to be a regulatory mechanism that controls the speed of movement (Miller et al., 2002). Hyperphosphorylated NF-H tends to accumulate in the cell body, which would result in disruption of neurofilament transport. As shown in Fig. 5, the cytoskeletal proteins in this study have also been affected in various ways by As-induced toxicity. It shows the various phosphorylation states of the NF and MAP-tau proteins. As mentioned earlier, the NF-H protein expression in the axon remains constant, independent of the used dose or exposure time (Fig. 3). However, after exposure to As, the NF-H shows an initial reduction in its phosphorylation, which is increased again depending on exposure time and increased dose (Fig. 5A). A possible explanation may be that the initial decrease of NF-H in axons in the 3-mg/kg dose group is caused by reduced transport of hyperphosphorylated NF-H from cell body to axon (Schlaepfer, 1987; Shea et al., 2003). The subsequent increase of NF-H in the axon may be explained by combination of minimum transport of phosphorylated NF-H in the axons and increased phosphorylation caused by As in the axon itself. After administration of 3 and 10 mg/kg As, NF-L and MAP-tau are phosphorylated as well. The NF-M protein shows no significant changes in its phosphorylation state. The absence of significant NF-M phosphorylation is remarkable, particularly given the similar serine, tyrosine and threonine content in the other NF proteins.

Hence, it can be concluded that the hyperphosphorylated NF-H proteins in the cell body are transported to a lesser extent into the axons. NF-L and MAP-tau are both phosphorylated in a

dose/time dependant manner in the 3- and 10-mg/kg dose groups, which is not the case with the control group. The increase in NF-L phosphorylation (Fig. 5) shows that NF-L is strongly phosphorylated, although its total protein expression has decreased at the highest As dose (Fig. 3). NFs and MAP-tau proteins can be phosphorylated by a number of serine/threonine kinases, such as: glycogen synthase kinase 3 beta (GSK3 $\beta$ ), which is involved in energy metabolism, neuronal cell development, and body pattern formation (Plyte et al., 1992), cyclin-dependent kinase 5 (Cdk5), and various kinases such as protein kinase A (PKA). In conclusion, we have shown that As interacts with the cytoskeletal structure and the sciatic nerve function by affecting the NF phosphorylation state and MAP-tau proteins.

## Chapter 4

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