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Studies into the mechanism of arsenic-induced neurotoxicity

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Citation

Vahidnia, A. (2008, February 14). *Studies into the mechanism of arsenic-induced neurotoxicity*. Retrieved from <https://hdl.handle.net/1887/12605>

Version: Corrected Publisher's Version

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Chapter 3: Arsenic neurotoxicity I

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in Sciatic Nerve**

Human & Experimental Toxicology
2006 (25): 667-674

Chapter 3

Arsenic-Induced Toxicity: Effect on Protein Composition in Sciatic Nerve

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Abstract

Exposure to arsenic (As) compounds may lead to skin and lung cancer and various disorders such as vascular disease and peripheral neuropathy in humans. Peripheral arsenic neurotoxicity has been demonstrated clinically and in electrophysiological studies. Patients intoxicated with arsenic show neurological symptoms in their feet and hands. These patients show significantly lower nerve conduction velocities (NCVs) in their peripheral nerves in comparison with controls. The mechanism of As PNS toxicity, however, has never been described before. This is the first study to investigate the toxicity of As on the peripheral nervous system (PNS). Male Wistar rats were exposed to arsenite given as a single dose *i.v.* After sacrifice, sciatic nerves were excised and the protein composition was analyzed. Protein analysis of sciatic nerves showed disappearance of neurofilament and fibroblast proteins in rats treated with arsenite doses of 15 and 20 mg/kg in comparison with the control groups. Some fibroblast protein bands had disappeared in the 20-mg/kg dose group. The analyzed neurofilament-M and -L proteins decreased dose-dependent over time. Arsenic affects the composition of proteins in the rat sciatic nerve, especially the neurofilaments. The reduction of signals in western blot analysis reveals changes in cytoskeletal composition, which may well lead to neurotoxic effects *in vivo*.

Keywords: arsenic, arsenite, fibroblast, neurofilament, neurotoxicity, *Nervus ischiadicus*

Introduction

Arsenic is a toxic, natural contaminant in soil, water and air, depending on geography. Inorganic arsenic (As) poisoning is a major health issue in large parts of the world, such as in India and Bangladesh¹ and several South American countries, and is mostly due to environmental exposure through water, air pollution² and soil contamination. Contaminated drinking water in a country such as Bangladesh is an important source of arsenic intake.³ A famous example is the arsenical poisoning of drinkers of contaminated beer in England toward the end of the 19th century.⁴⁻⁶ Patients showed neurological symptoms in their feet and hands, for example, a burning sensation in the soles of the feet and tingling sensations in fingers and toes.

The rat is a species often chosen to study toxicity in order to predict potential effects in humans.⁷ It has been established that rats methylate inorganic arsenic analogous to humans, although this process occurs in rats at a faster rate. Although the toxicokinetics of arsenic in rats is different to humans, their toxicodynamics are similar.^{8,9} We, therefore, chose rats as our experimental model to investigate neurotoxicity caused by arsenic. It was not the purpose of this study to investigate the side effects of therapeutic applications of arsenic.

Exposure to As by inhalation,¹⁰ oral ingestion,⁵ or injection¹¹ induces neuropathy. Arsenic has also been known for its therapeutic properties, as it is used for the treatment of acute promyelocytic leukemia (APL). Many APL patients develop symptoms of neuropathy after injection with trivalent arsenic as a side effect¹² (As₂O₃) (up to 0.15 µg/kg daily as indicated in the manufacturer's information accompanying the drug Trisenox). It has been suggested that trivalent arsenicals inhibit many enzymes by reacting with the sulfur groups present and that, as a rule, the trivalent forms of arsenic are the major source of poisoning.¹³

A single exposure to arsenic in an acutely toxic dose leads to severe clinical reactions such as diarrhea and vomiting, often leading to death through dehydration.^{14,15} For example, Duenaslaita *et al.* described a case of non-fatal acute arsenic poisoning where a very high arsenic concentration of 67.5 mg/l was measured in the urine.¹⁵ In cases where patients survive, recovery occurs weeks after intoxication. However, delayed effects may appear in the form of peripheral neurotoxicity both after acute and chronic exposure. These delayed effects of arsenic have been demonstrated clinically^{4,16} and in electrophysiological studies.¹⁷ Patients show significantly lower nerve conduction velocities (NCVs) in their peripheral nerves in comparison to healthy subjects. It is also worth mentioning that in suicidal/accidental cases, where a single dose of As was taken, the dose was exceptionally high in comparison to chronic exposure or therapeutic dose.^{15,17,18}

In contrast to the bulk of information on carcinogenicity, the mechanism of As-induced PNS toxicity is not yet understood. Our hypothesis is that diminished NCV is probably caused by arsenic-induced axonal degeneration, which in turn could change the myelin composition. This study presents the first results of a project that are aimed toward elucidating the mechanism of the peripheral neurotoxicity of arsenic. As a first step in this investigation, the short- and long-term effects of arsenite on rat sciatic nerve proteins were studied as a model for peripheral axonopathy.

Materials and Methods

Chemicals. Sodium meta-arsenite ($\text{NaAsO}_2 : \text{As}^{\text{III}}$) (product no. 22,869-9, 98% pure) and acrylamide/bis-acrylamide (product no. A 3699, Mix ratio 37.5:1, T30%, C2.6%) 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. Tris-HCl (catalog no. 108219) (Veenendaal, Netherlands) and EDTA (catalog no. 108418) were purchased from Merck (Darmstadt, Germany). A micro BCA protein assay kit (product no. 23235) was purchased 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 purchased from Kodak (Shelton, CT, USA). Coomassie brilliant blue R250 (product no. 27816) was obtained from Fluka (Buchs SG, Switzerland). ECL plusTM western blotting detection reagent was purchased from Amersham Biosciences (Piscataway, NJ, USA). 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.¹⁹

Animals. Male Wistar rats (225-250g) were obtained from Charles River, Maastricht, Netherlands. The rats were acclimatized for 7 days and housed in groups of three 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 agreed upon by the Animal Ethical Committee of the Leiden University Medical Center.

Experimental design. Rats were injected with arsenite dissolved in PBS as a single dose (between 0- to 20-mg/kg) in a tail vein. The doses used for short-term single arsenic exposure were 0-, 15- and 20-mg/kg As^{III} . The long-term single exposures were 0-, 3- and 10-mg/kg As^{III} . The injected volumes varied from 0.30 to 0.35 ml depending on body weight. The control groups were injected with 0.30 ml PBS without any As^{III} . Urine samples were collected after injection by individual housing of each rat in a metabolic cage. In the short-term section of the experiment, rats were kept in metabolic cages for the intended duration of 3, 6 and 9 hours. In the long-term section of the experiment, rats were kept in metabolic cages 24 hours after injection. Afterwards, rats that received the same dose were combined into one group and housed for 2, 3 and 4 weeks in plastic cages on sawdust. After the intended duration, rats were individually anesthetized with isoflurane in a plastic sealed cage with in- and out-flow tubes. Subsequently, they were exsanguinated by means of arterial blood withdrawal from the inferior mesenteric artery after 3, 6 and 9 hours (single-dose, short-term exposure) or 2, 3 and 4 weeks (single-dose, long-term exposure), respectively.

For the short-term, single-dose exposure, one rat was used for each dose and duration in accordance with the guidelines of the Animal Ethical Committee. For the long-term, single-dose exposure, three rats were used for each dose and duration in accordance with the guidelines of the Animal Ethical Committee.

Nervus ischiadicus arises from the sacral plexus and passes about halfway down the thigh where it divides into the common peroneal and tibial nerves. The nerve was 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 was dissected from both legs and in each case was approximately 2 cm in length. In these experiments, the sciatic nerve from the right leg was used for protein analysis and the one from the left leg was used for arsenic measurement.

The blood samples for arsenic analysis were first digested in an acid mixture (digestive acid) comprised of one part perchloric acid and one part nitric acid (1:1). Fifty μl of blood sample was added to 950 μl of digestive acid and incubated for 1 hr at 70 °C. The sciatic nerve

samples were first weighed and digested in 1 ml digestive acid as in blood and incubated for 1 hr at 70 °C. The urine samples were first diluted with Milli-Q water (MQ-H₂O) with a factor of 200 to 250. 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-H₂O, 27 ml 37% hydrochloric acid (1M HCl), 6 g sodium iodide (NaI) and 3 g L-(+) ascorbic acid (C₆H₈O₆) and incubated for 1 hour at 70 °C.

The arsenic content of urine, digested blood and digested sciatic nerve were measured with atomic absorption spectrometry (Perkin-Elmer FIAS-3100 AAS). The technique for measuring total arsenic content is hydride generation coupled to AAS (HG-AAS).²⁰ An As electrodeless discharge lamp of 7W was used; arsenic 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 arsenic standard solution dissolved in MQ-H₂O ranging from 0.0 µg/l to 200 µg/l end concentration. For the digested blood and nerves, the matrix of the calibration solution consisted of 4.5 ml 1M HCl, and 0.5 ml of arsenic standard solution dissolved in digestive acid ranging from 0.0 µg/l to 200 µg/l end concentration.

Neurofilament Characterization. Excised sciatic nerves weighing 12.5 mg to 18.5 mg were homogenized with a blender in 1.5 ml Tris-HCl buffer (200 mM, pH 8.0), also containing EDTA 3 mM and sodium dodecyl sulphate 1% w/v. The nerve homogenates were analyzed on a 6% acrylamide separation gel.²¹ The samples were run non-reduced without the use of dithiothreitol (DTT). The protein gels were stained for 3 min with 0.4 g/L Coomassie Blue in a fixing solution also containing 40% v/v methanol and 7% acetic acid v/v. Gels were de-stained in 2 steps with discoloring solution containing 40% methanol and 7% acetic acid. Further, the separation gels were also used for immunoblotting on 0.2 µm nitrocellulose membrane²² in conjunction with NF-90 monoclonal antibody to all 3 neurofilament proteins: NF-H; NF-M and NF-L. Using ECL plusTM western blotting detection reagent and exposing the nitrocellulose membranes to Kodak Biomax XAR film for 30 seconds concluded the western blot analysis. The band intensities on the Kodak films were measured and compared to each other by Quantity One – Densitometer GS-710 from Bio-Rad.

Fibroblast Characterization. The fibroblast portion of the sciatic nerve was determined by separating fibroblasts from Schwann cells by means of magnetic-activated cell separation (MACS).²³ The sciatic nerve is covered with epineurium consisting of collagen. Inside this cover, the nerve harbors nerve cells, Schwann cells and fibroblasts. Excision of the sciatic nerve in rats results in the tearing of the motor rootlet; as a result the nerves cannot be cultured *in vitro*. The remaining Schwann cells and fibroblasts can be grown in culture. As described previously by Vroemen and Weidner²³ the Schwann cells in the Schwann/fibroblast culture are incubated with anti-p75LNGFr monoclonal antibodies for 10 min at room temp. This antibody is directed at cell surface antigen to select Schwann cells in the p75 low affinity nerve growth factor receptor (p75LNGFr). Thereafter, the labeled mixture is washed and incubated with secondary antibody, namely: microbead-linked rat anti-mouse IgG1 for 15 min at 4°C. After 2 to 4 wash steps, cells were resuspended in MACS buffer (included in the kit) and added to a MS column (also included in the kit) held in a magnet, as the secondary antibodies have magnetic properties. Cells were passed through the column and washed 4 times with MACS buffer (500 µL). Labeled cells were retained in the column, the column was removed from the magnet, and cells were eluted into 1 ml MACS buffer. The isolation procedure was repeated to increase the purity of the isolated cells. At this stage the majority of the fibroblasts are separated from the labeled Schwann cells. The content of the fibroblast-eluted portion can be tested next to the homogenized sciatic nerve on a separation gel. First the separated fibroblasts are lysed in a Tris-HCl buffer, the same buffer that is used in homogenated sciatic nerves. Subsequently, the 2 homogenates were loaded on a 6% separation gel and stained with Coomassie Blue.

This comparison enabled us to identify the fibroblast protein bands in the whole sciatic nerve tissue.

Results

General health

During the single-exposure, long-term effect study, the gain in body weight was used as a parameter for general health. Increasing the As^{III} dose results in a decrease in body weight gain (Figure 1). The difference between weight gain of control rats and the reduced weight gain of rats treated with 10 mg/kg As^{III} becomes evident after 2 weeks. Furthermore, in the dose groups exceeding 10 mg/kg, diarrhea was observed. The 3-mg/kg As^{III} doses also resulted in diminished appetite and decreased overall alertness levels of the injected rats. The body weight in this dose group 2 weeks after exposure increased to such extent that there was no significant difference between treated and control animals.

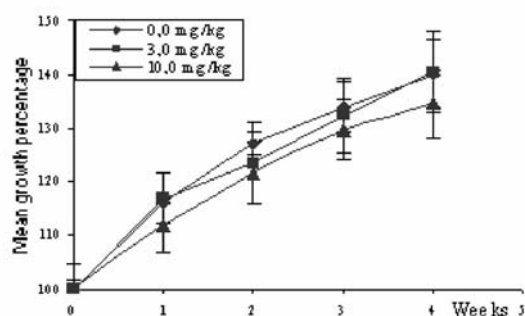


Figure 1. Body weight gain in control rats and in arsenite-treated rats during 4 weeks. Rats treated with 10-mg/kg arsenite show significant reduced growth in comparison to the control. The group of 3-mg/kg dosage shows recovery in their growth.

Arsenic elimination

For the long- and short-term single dose effect, the total amount of arsenic excreted in urine was measured and expressed as mg As/mmol creatinine (Table 1). The arsenic content in the short-term experiment was expressed as excreted total arsenic in the excreted urine volume. This is done due to their short survival time of up to 9 hours after injection. The content in urine varied from 18.06 to 31.87 mg As/mmol creatinine after 15-mg/kg dose. In the 20-mg/kg dose group, urinary arsenic varied between 22.56 and 44.13 mg As/mmol creatinine. In the long-term exposure group, samples of 24-h urine were collected after injection. In this case, the arsenic content in urine varied from 0.34 mg As/mmol creatinine to 2.05 mg As/mmol creatinine for the 3-mg/kg dose group. The 10-mg/kg dose group showed arsenic concentrations varying between 2.06 to 2.40 mg As/mmol creatinine.

Table 1 also shows the arsenic content in digested blood (mg/l), which could only be measured in the short-term exposure group. The 15-mg/kg group showed arsenic content between 1.58 and 2.30 mg/l, while the 20-mg/kg group showed between 2.34 and 2.90 mg/l arsenic content in blood. The blood arsenic content of long-term, single exposure rats was below the detection limit (As < 5 µg/l).

The arsenic content in the *N. ischiadicus* was expressed in µg/l (w/v) and was measured in both short- and long-term groups. Digested sciatic nerve was used to study the interaction of arsenic with peripheral nerve proteins for various dose groups and duration. In the short-term, single exposure group, arsenic content of 0.96 to 2.24 µg/g tissue was measured for the 15-mg/kg dose group and 0.30 to 1.08 µg/g for the 20-mg/kg dose group, both up to 9 hours.

The As content in the long-term, single exposure group 3-mg/kg decreased from 0.508 μg after 2 weeks to 0.302 μg arsenic per gram tissue after 4 weeks. The sciatic nerve arsenic content in the 10 mg/kg dose group also decreased from 0.367 μg arsenic per gram tissue after 2 weeks to 0.237 μg arsenic per gram tissue after 4 weeks.

Table 1. Total arsenic content in the short-term, long-term, single exposure was measured in urine, blood and sciatic nerves.

	Number of rats per dose group*			Total Arsenic in urine (mg As/ mmol creatinine)	Blood 3 to 9 hrs (mg/l)	<i>Nervus ischiadicus</i> ($\mu\text{g/g}$) (ww)
Short-term single exposure**	3	Control	0 hrs	0.0 $\mu\text{g/L}$	0.0	0.0
	3	15 mg/kg	3 hrs	18.97	2.30	2.24
			6 hrs	31.87	1.97	0.96
			9 hrs	18.06	1.58	2.14
	3		20 mg/kg	3 hrs	44.13	2.90
		6 hrs		26.27	2.84	1.08
		9 hrs		22.56	2.34	0.30
Long-term single exposure***	9	Control	wks	0.0 $\mu\text{g/L}$	n.d.	0.0
	9	3 mg/kg	2 wks	0.60 \pm 0.66		0.508
			3 wks	2.05 \pm 1.88		0.496
			4 wks	0.34 \pm 0.03		0.302
	9	10 mg/kg	2 wks	2.06 \pm 0.17		0.367
			3 wks	2.40 \pm 0.71		0.367
			4 wks	2.07 \pm 0.85		0.237

*Number of animals per group per dose was 3. The number of animals was prescribed by the Animal Ethical Committee.

**Urine samples were collected at 3, 6 and 9 hours after injection. For this reason total arsenic was measured as total arsenic excreted in the urine portion.

***Total arsenic was measured in 24-hour urine

n.d. = not detectable (As <5 $\mu\text{g/l}$)

Protein degradation

The protein analysis showed changes in the protein composition of the sciatic nerve. Two proteins in sciatic nerve fibroblasts (40 and 140 kDa) disappeared in rats treated with the highest dose of 20-mg/kg arsenite in comparison to the 15-mg/kg dose group and the controls, as can be seen in SDS-PAGE (Figure 2). However, the conclusive evidence of protein composition changes was obtained from western blot analysis. This showed the gradual disappearance of NF-L and NF-M after short-term exposure (Figure 3). As described in the Materials and Methods section, fibroblast protein bands are determined through side-by-side comparison in SDS-PAGE (data not shown) of primary fibroblasts in culture and homogenized sciatic nerves from the rat. Furthermore, the same separation gels of fibroblast and sciatic nerve homogenates were checked with Western blot analysis for presence of neurofilament proteins. It was apparent that fibroblasts grown in culture do not contain any neurofilament proteins (data not shown).

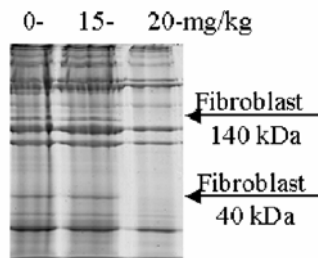
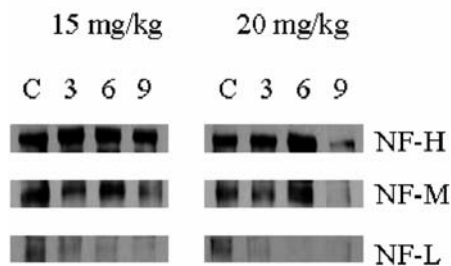
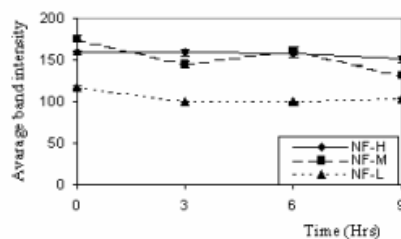


Figure 2. Coomassie staining of three sciatic nerve protein homogenates with various arsenic doses. A high dose of 20-mg/kg arsenite results in disappearance of fibroblast band sizes of 40 and 140 kDa as indicated by the arrows. However these bands are still present in lower dose group and the controls.

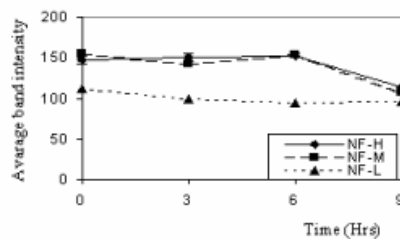
Neurofilament proteins gradually disappear after *i.v.* injection of As^{III} , as is shown in Figure 3A. NF-L has almost totally disappeared after 9 hours in both dose groups treated with 15- and 20-mg/kg arsenite. Figures 3B and 3C show the intensity of the Western blot signals. After 3 hours NF-L reaches almost the same signal as its background in both dose groups. NF-M shows a decrease in intensity in both cases after 6 hours. NF-H appears to be unaffected in the 15-mg/kg-dose group; however, it diminishes in the 20-mg/kg-dose group after 9 hours.



(A)



(B)



(C)

Figure 3 (A) Western blot analysis of NF-H, -M and -L of rats sciatic nerves. The changes in cytoskeletal proteins are followed in time, measured up to 9 hours and compared to their control (C). The short-term effect of 15- and 20-mg/kg arsenic results in gradual disappearance of primarily NF-L. The effect is more profound in the 20-mg/kg dosage than the 15-mg/kg dosage. (B and C) The mean intensity signals of various neurofilament protein bands derived from fig 3A. Fig 3B shows the mean intensity of NF-H, -M and -L belonging to 15-mg/kg doses. Signal reduction in NF-L can be seen as a result of exposure duration.

The long-term effect of arsenite on sciatic nerve proteins in lower doses of 3 and 10 mg/kg is shown in Figure 4. No significant changes appear in the protein composition in the Western blot analysis of neurofilament proteins. The signal intensity of NF-H remains constant at all times in both dose groups. This applies also for neurofilament-M and -L (NF-M and NF-L). The same is also true in the long-term 15-mg/kg As^{III} dose group (data not shown), in which neurofilaments did not show any sign of degradation.

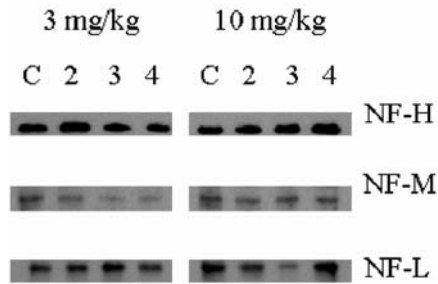


Figure 4. Western blot analysis of NF-H, -M and -L of rats sciatic nerves. The changes in cytoskeletal proteins are followed in time, measured up to 4 weeks and compared to their control (C) The long-term effect with lower doses namely 3- and 10-mg/kg arsenic show no significant effect.

Discussion

The gain in body weight is used as a parameter for general health; a decrease in body weight gain is considered a toxic effect of arsenite in the long term, single exposure part of the study. Reduced body weight gain is the result of arsenite administration, whereby the 10-mg/kg dose group lags behind in their growth in comparison to the 3-mg/kg dose group and the control group. After 3 weeks, however, the body weight in the 3-mg/kg single dose group was the same as in the control group. The effects of arsenic on body weight are most probably a result of reduced food intake due to a decrease in overall health and to the observed gastrointestinal tract toxicity (diarrhea) rather than a direct effect of arsenic on hypothalamic function.

Urine samples for measurement of As elimination in the long-term, single exposure group were collected during 24 hours (Table 1). The 3-mg/kg dose group excreted 0.60 to 2.05 mg As/mmol creatinine and the 10-mg/kg dose group showed an arsenic excretion between 2.06 and 2.40 mg As/mmol creatinine. The high concentration of 2.05 mg As/ mmol creatinine in the long-term, single exposure group is the result of a small urine volume with high arsenic content. In contrast to the long-term single exposure, the short-term single exposure group shows a much higher excretion, ranging between 18.06 and 31.87 mg As/mmol creatinine for the 15-mg/kg dose and 22.56 to 44.13 mg As/mmol creatinine for the 20-mg/kg dose. The difference between the long- and short-term studies is the result of the variation in dose range and the duration of urine collection. Rats in the short-term experiment received a much higher dose of 15- and 20-mg/kg arsenite as compared with 3- and 10-mg/kg arsenite in the long-term group. It is likely that in the first few hours after injection, rats excrete most of the arsenic dose, either methylated or not, in the urine. This suggests that the arsenic concentration in urine toward the end of the 24-hour period of urine collection is diminished. This is also observed in the 20-mg/kg dose group. In the 15-mg/kg dose group, the amount of excreted arsenic after 3 hours is not as high as in the 20-mg/kg dose group.

The blood arsenic concentration in the 15-mg/kg dose group drops from 2.30 at 3 hours to 1.58 mg/l at 9 hours; in the 20-mg/kg dose group a decrease of 2.90 at 3 hours to 2.34 mg/l at 9 hours is observed. The calculated half-life time ($T_{1/2}$) in both dose groups is 11 and 17 hours, respectively. Furthermore, the calculated relative volume of distribution (V_D) in both dose groups is 5.62 and 6.15 L/kg, respectively, indicating a high degree of tissue binding.

The amount of arsenic retrieved in the sciatic nerve tissue is inversely related to its corresponding dose increase: 2.24 $\mu\text{g/g}$ tissue at 15-mg/kg dose group after 9 hours in comparison to 0.30 $\mu\text{g/g}$ tissue at 20-mg/kg dose group. The increase in arsenic doses leads to the disappearance of NF-M and NF-L proteins in a time-dependent way. Parallel to this remarkable observation, all 3 neurofilament proteins in the sciatic nerves disappear in the 20-mg/kg dose group after 9 hours. A possible explanation for this observation may be that NF-M/L proteins encapsulate most of the arsenic through covalent binding with their sulfhydryl groups. This would suggest that the dramatic reduction of the arsenic content measured in sciatic nerves is the result of the arsenic-induced degradation of NF-M/L proteins and the subsequent release of arsenic from sciatic nerves into the circulation and other (much larger) body compartments.

This study demonstrates the effects of arsenite on the composition of peripheral nerve proteins, namely neurofilament and fibroblast proteins in sciatic nerve. Fibroblast proteins with approximate band sizes of 40 and 140 kDa degraded after a high dosage, as shown in Figure 2. Interestingly, this indicates an all-or-none effect, which occurs between 15 and 20 mg/kg. In the lower dose of 15-mg/kg and the control protein homogenate no changes are visible in the Coomassie Brilliant Blue stained gels. It is clear that a higher dose of arsenite resulted in a stronger effect on NF-L and some effect on NF-M. An effect of arsenite on NF-

H is only visible after 9 hours in the highest dose group, 20-mg/kg. In this experiment, it became clear that the Wistar rats do not survive a 20-mg/kg dose after 24 hours. The fact that NF-H begins to disappear 9 hours after injection could be the result of metabolism and body failure just before dying rather than a direct effect of sodium arsenite.

Our data may indicate intraspecies variation in susceptibility. In our experiment, all rats (Wistar strain) died within 24 hours after iv arsenite injection. This is in contrast to the findings from a previous study using Sprague-Dawley rats, which seem to be more resistant to arsenic.⁸ This has also been found by Todorov (oral presentation on 21.05.2004). However, this difference is most likely due to differences in administration route, as in the latter study arsenic was administered orally and thus the dose (and dose rate) may have been attenuated.

Our results demonstrate the capacity of arsenic as a neurotoxin, but do not allow conclusions on its molecular mechanism. It has been established that arsenite has a high affinity for sulfhydryl groups, and it may substitute for phosphate and disrupt oxidative phosphorylation by replacing phosphoryl with less stable arsenyl compounds.⁸ Sulfhydryl (-SH) groups are abundantly present in cysteine-containing proteins. Arsenic interacts with -SH groups in their reduced form through covalent binding. Since a sufficiently high arsenic dose resulted in gradual disappearance of NF-M and NF-L (Figure 3A) and assuming that arsenite is the direct cause of protein degradation, it may be concluded that the NF-L is one of the main proteins involved in arsenic interaction with its reduced -SH groups. Confirmation of this hypothesis can be deduced from the arsenic content of sciatic nerves. The inverse relationship between dose and effect in the case of the 20 mg/kg arsenite group in comparison to the other groups can be explained through the fact that the arsenic decrease is due to degradation of NF-L/arsenic complex leading to disappearance of arsenic from the sciatic nerve. NF-L has been reported to act as a linking protein in conjunction with NF-H/M and its role in assembly of NFs and maintenance of axonal caliber.²⁴ NF-H and NF-M contain 5 and 2 cysteine groups, respectively, whereas NF-L contains only one. NF-L is the most predominant protein in neurofilaments. One potential mechanism of NF-L binding to other protein molecules is through disulfide bridge formation. Arsenic may uncouple disulfide bridges in NFs, leading to a complex of As with two cysteine groups in the same molecule or in two neighboring proteins.

If axonal degeneration of protein is not directly related to interaction of arsenite with sulfhydryls, another probable cause could be the calcium-mediated degradation of neurofilament proteins.²⁵ It is possible that arsenic affects the calcium movement and distribution, which in turn could result in protein degradation. The question would be why proteins like NF-L are more susceptible to degradation by calcium-mediated protein degradation than others such as NF-H.

Phosphorylation of NFs proteins may be another target for arsenic in addition to sulfhydryl groups. Changes in cytoskeletal protein composition are related to NF phosphorylation.²⁶ All 3 phosphorylated NFs proteins tend to accumulate in the cell body.^{24,27} This possible effect of arsenic on phosphorylation may play an additional role in neuropathy.

There are different causes of peripheral neurotoxic injury involving metals as neurotoxins: neuronopathy, axonopathy and myelinopathy.¹³ The mechanism suggested above is in agreement with axonopathy. In addition to this possible mechanism, other possibilities are feasible instead of, or in addition to, our proposed mechanism. Arsenic may affect cell body function, which could lead either to accumulation of NF fragments in the cell body, or to inhibited production. Both possibilities would lead to a decrease of cytoskeletal proteins in the axons. However, if this were true, one would expect a much slower onset of NF-L reduction than the actual results after only 3-9 hours dosing. Neuronopathy as basis for

Chapter 3

arsenic-induced peripheral neuropathy is probably unlikely. There is more research needed to definitively exclude neuronopathy as the basis for As-induced peripheral neuropathy.

The present set of experiments does not allow the conclusion that arsenic is not a myelinotoxin. However, myelinopathy as cause of arsenic-induced peripheral neuropathy is not likely, given the rapid response of NF-proteins to arsenic exposure.

In arsenic-exposed patients neurophysiological abnormalities have been described extensively.⁸ Both sensory and motor conduction velocity can be decreased. Recently, Dubois *et al.*²⁸ demonstrated that motor functions in mice with the deleted *Nefl* gene were impaired. These authors concluded that NF-L plays an essential part in motor function. These findings corroborate our conclusions that decrease in NF-L is a sensitive parameter of the peripheral nervous system for arsenic toxicity.

We have been able to demonstrate a neurotoxic effect of arsenic in rats. Future studies to elucidate the mechanism of arsenic neurotoxicity are in progress.

Acknowledgements

Dr. Egbert Lakke and Marga Deenen assisted with the purification and isolation of fibroblast cells. Jolien Visser contributed to the measurement of arsenic.

Reference List

1. Rahman MM, Chowdhury UK, Mukherjee SC, Mondal BK, Paul K, Lodh D et al. Chronic arsenic toxicity in Bangladesh and West Bengal, India--a review and commentary. *J Toxicol Clin Toxicol* 2001; 39(7):683-700.
2. Lagerkvist BJ, Zetterlund B. Assessment of exposure to arsenic among smelter workers: a five-year follow-up. *Am J Ind Med* 1994; 25(4):477-488.
3. Hafeman DM, Ahsan H, Louis ED, Siddique AB, Slavkovich V, Cheng Z et al. Association between arsenic exposure and a measure of subclinical sensory neuropathy in Bangladesh. *J Occup Environ Med* 2005; 47(8):778-784.
4. Buchanan RJM, Lond MRCD. Cases of arsenical peripheral neuritis. *The Lancet* 1901;170-172.
5. Kelynack TNV, Lond MRCD. Arsenical poisoning from beer drinking. *The Lancet* 1900;1600-1603.
6. Reynolds ES, Lond MRCD. An account of the epidemic outbreak of arsenicals poisoning occurring in beer drinkers in the north of engeland and the midland counties in 1900. *The Lancet* 1901;166-170.
7. Rowland IR, Davies MJ. Reduction and methylation of sodium arsenate in the rat. *J Appl Toxicol* 1982; 2(6):294-299.
8. De Wolff FA, Edelbroek PM. Neurotoxicity of arsenic and its compounds. In: Vinken & Bruyn's, editor. *Handbook of Clinical Neurology*. Elsevier Science B.V., 1994: 283-291.
9. Aposhian HV. Enzymatic methylation of arsenic species and other new approaches to arsenic toxicity. *Annu Rev Pharmacol Toxicol* 1997; 37:397-419.
10. Otha M. Ultrastructure of Sural Nerve in a Case of Arsenical Neuropathy. *Acta Neuropath* 1970; 16:233-242.
11. Soignet SL, Frankel SR, Douer D, Tallman MS, Kantarjian H, Calleja E et al. United States multicenter study of arsenic trioxide in relapsed acute promyelocytic leukemia. *J Clin Oncol* 2001; 19(18):3852-3860.
12. Yip SF, Yeung YM, Tsui EY. Severe neurotoxicity following arsenic therapy for acute promyelocytic leukemia: potentiation by thiamine deficiency. *Blood* 2002; 99(9):3481-3482.
13. De Wolff FA, Treijtel N, Vermeulen M. Mechanism of peripheral neurotoxicity. In: Lester DS, Slikker.W., Lazarovici P, editors. *Site-Selective Neurotoxicity* . London; New York: Taylor & Francis, 2002: 282-303.
14. Kishi Y, Sasaki H, Yamasaki H, Ogawa K, Nishi M, Nanjo K. An epidemic of arsenic neuropathy from a spiked curry. *Neurology* 2001; 56(10):1417-1418.
15. Duenas-Laita A, Perez-Miranda M, Gonzalez-Lopez MA, Martin-Escudero JC, Ruiz-Mambrilla M, Blanco-Varela J. Acute arsenic poisoning. *Lancet* 2005; 365(9475):1982.
16. Jha S, Dhanuka AK, Singh MN. Arsenic poisoning in a family. *Neurol India* 2002; 50(3):364-365.
17. Le Quesne PM, McLeod JG. Peripheral neuropathy following a single exposure to arsenic. Clinical course in four patients with electrophysiological and histological studies. *J Neurol Sci* 1977; 32(3):437-451.
18. Shen Y, Shen ZX, Yan H, Chen J, Zeng XY, Li JM et al. Studies on the clinical efficacy and pharmacokinetics of low-dose arsenic trioxide in the treatment of relapsed acute promyelocytic leukemia: a comparison with conventional dosage. *Leukemia* 2001; 15(5):735-741.

Chapter 3

19. Oudega M, Voormolen JH, Usunoff KG, Marani E, Thomeer RT. Immunocytochemical localization of neurofilaments in the fibre systems of the developing rat spinal cord white matter. *Eur J Morphol* 1996; 34(5):329-346.
20. Buchet JP, Lauwerys R, Roels H. Comparison of several methods for the determination of arsenic compounds in water and in urine. Their application for the study of arsenic metabolism and for the monitoring of workers exposed to arsenic. *Int Arch Occup Environ Health* 1980; 46(1):11-29.
21. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227(5259):680-685.
22. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 1979; 76(9):4350-4354.
23. Vroemen M, Weidner N. Purification of Schwann cells by selection of p75 low affinity nerve growth factor receptor expressing cells from adult peripheral nerve. *J Neurosci Methods* 2003; 124(2):135-143.
24. Al Chalabi A, Miller CC. Neurofilaments and neurological disease. *Bioessays* 2003; 25(4):346-355.
25. Van Ginkel MF, Heijink E, Dekker PB, Miralem T, van der Voet GB, de Wolff FA. Effect of aluminum chloride, -citrate, and -maltol on the calcium-mediated degradation of neurofilament proteins. *Neurotoxicology* 1993; 14(1):13-18.
26. Shea TB, Jung C, Pant HC. Does neurofilament phosphorylation regulate axonal transport? *Trends Neurosci* 2003; 26(8):397-400.
27. Schlaepfer WW, Micko S. Chemical and structural changes of neurofilaments in transected rat sciatic nerve. *J Cell Biol* 1978; 78(2):369-378.
28. Dubois M, Strazielle C, Julien JP, Lalonde R. Mice with the deleted neurofilament of low molecular weight (Nefl) gene: 2. Effects on motor functions and spatial orientation. *J Neurosci Res* 2005; 80(6):751-758.