Studies into the mechanism of arsenic-induced neurotoxicity

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STUDIES INTO THE MECHANISM OF ARSENIC-INDUCED NEUROTOXICITY

Proefschrift

ter verkrijgen van de graad van Doctor
aan de Universiteit Leiden,
op gezag van de Rector Magnificus
Prof. Mr. P.F. van der Heijden,
volgens besluit van
het college voor promoties
te verdedigenop 14 februari 2008
klokke 13.45 uur door

Ali Vahidnia
geboren te Amman, Jordanïe
in 1972

Promotiecommissie

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The research presented in this thesis was performed in the Toxicology Laboratory of department of Clinical Pharmacy and Toxicology and in the Department of Clinical Chemistry at the Leiden University Medical Center, Leiden, The Netherlands.

The publication of this thesis was financially supported by the AZL Onderzoeks- en Ontwikkelingskrediet Apotheek and the J.E. Juriaanse Stichting.

Printed by: Gildeprint drukkerijen

ISBN: 978-90-9022722-1

Voor mijn ouders/for my parents

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Arsenic Neurotoxicity - A Review

Arsenic Neurotoxicity - A Review

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Abstract

Arsenic (As) is one of the oldest poisons known to men. Its applications throughout history are wide and varied: murder, make-up, paint and even as a pesticide. Chronic As toxicity is a global environmental health problem affecting millions of people in the Unites States and Germany to Bangladesh and Taiwan. Worldwide, As is released into the environment by melting of various metals, combustion of fossil fuels, as herbicides and fungicides in agricultural products. The drinking water in many countries, which is tapped from natural geological resources, is also contaminated as a result of the high level of arsenic in groundwater. The environmental fate of As is contamination of surface and groundwater with a contaminant level higher than 10 particles per billion (ppb) as set by World Health Organization (WHO). Arsenic exists in both organic and inorganic species and either form can also exist in a trivalent or pentavalent oxidation state. Long-term health effects of exposure to these As metabolites are severe and highly variable: skin and lung cancer, neurological effects, hypertension and cardiovascular diseases. Neurological effects of As may develop within a few hours after ingestion but usually is seen in 2-8 weeks after exposure. It is usually a symmetrical sensori-motor neuropathy, often resembling the Guillain-Barré syndrome. The predominant clinical features of neuropathy are paresthesias, numbness and pain, particularly in the soles of the feet. Electrophysiological studies performed on patients with As neuropathy have revealed a reduced nerve conducting velocity (NCV), typical of those seen in axonal degeneration. Most of the adverse effects of As are caused by inactivated enzymes in the cellular energy pathway, whereby As reacts with the thiol groups of proteins and enzymes and inhibits their catalytic activity. Furthermore, As-induced neurotoxicity, like many other neurodegenerative diseases, causes changes in cytoskeletal protein composition and hyperphosphorylation. These changes may lead to disorganization of the cytoskeletal framework, which is a potential mechanism of As-induced neurotoxicity.

Introduction

The word *arsenic* is derived from the Persian *zarnikh:* زرنیخ, meaning 'yellow orpiment'.[1] In Aramaic (זרניך) and Arabic (ألزرذيخ), the word was borrowed from the Persian. It came to the Western languages through the Greek rendering of *zarnikh:* 'αρσενικόν: *arsenikon,* which, in Greek, also means 'masculine'. Arsenic sulphide also occurs in a red form: *realgar* or *sandarach.* Long known and used in Persia and elsewhere since ancient times, As was also used in traditional Chinese and Indian medicine and as a cosmetic product in eye shadow in the Roman era. Given that the symptoms of acute As poisoning are easily confused with acute diarrhea associated with cholera, it quickly became a favorite homicidal agent. In 1832, a certain John Bodle was brought to trial for poisoning his grandfather by putting As in his coffee. James Marsh, a chemist working at the Royal Arsenal in Woolwich was called by the prosecution to detect its presence.[2] He performed the standard test by passing hydrogen sulfide through the suspect fluid. While Marsh was able to detect As, the yellow precipitate did not keep very well, and by the time it was presented to the jury it deteriorated. The jury was not convinced, and John Bodle was acquitted. Angered and frustrated by this, especially when John Bodle confessed later that he had indeed killed his grandfather, Marsh decided to devise a better test to demonstrate the presence of As. Taking Scheele's method as basis, he constructed a simple glass apparatus capable of not only detecting minute traces of As but also of measuring its quantity. While the Scheele test used nitric acid, in Marsh's case the suspect fluid would be mixed with sulfuric acid (H_2SO_4) and passed through a U-shaped tube with a piece of arsenic-free zinc at the end (Fig 1). Even a slight trace of As would cause arsine gas to form. When he ignited this gas, it decomposed into As and hydrogen and when he held a cold ceramic bowl against the flame, the As formed a silvery-black deposit mirror on the bowl. Not only could minute amounts of As be detected (as little as 0.02 mg), the test was very specific for As. Although antimony (Sb) could give a false-positive test by forming a similar black deposit, it would not react with sodium hypochlorite (NaOCl), while As would.[2,3]

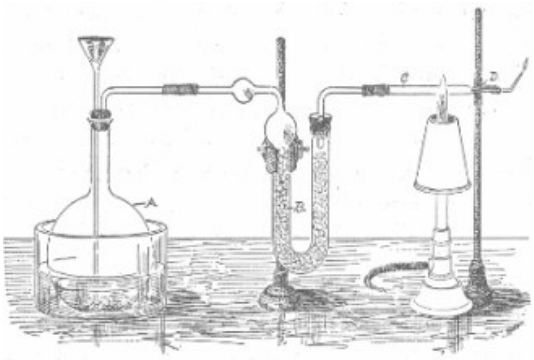

Fig 1. Schematic representation of the Marsh apparatus. (A) hydrogen generator, (B) chloride of calcium drying tube, (C) hard glass-tube and (D) Arsenic mirror.

Due to its use by the ruling class for killing one another and the incredible potency and discreetness, As has been called the Poison of Kings and the King of Poisons. The idea of using As as a murder weapon can even be seen in early movies and theater shows such as

Frank Capra's 'Arsenic and old lace', in which two elderly ladies used As in wine to kill their male suitors.

Arsenic has a long history of use as an intentional poison. Perhaps the most famous victim of As poisoning was Napoleon Bonaparte. He seems to have been exposed chronically during 1816, as appeared from neutron activation analysis of a hair sample.[4] A much lower concentration was found, however, in another hair sample attributed to Napoleon,[5] which may indicate that the identity of at least one of these samples should be questioned. One of the most prolific As poisoners in history was Goeie Mie ('Good Mary') of Leiden, The Netherlands, who lived in the 19th century. She poisoned at least 102 friends and relatives between 1867 and 1884, distributing arsenic-trioxide in hot milk to her victims after opening life insurance policies in their names. Of the 102 people poisoned, 45 persons became seriously ill, often with neurological symptoms, and 27 persons died; 16 of whom were her own relatives.[6]

Arsenic is used in folk medicine and in pesticides in many countries and also in modern western medicine for the treatment of leukemia.[7,8] In traditional Chinese medicine, preparations can be obtained in the form of coated or uncoated pills, powder or syrups. Different studies have shown that the majority of traditional Chinese medicines, such as Chinese herbal balls, show high doses of As varying between 0.1 and 36.6 mg per tablet, causing patients to get intoxicated by the high As dose, and Indian ayurvedic herbal medicine products are also known to cause lead, mercury and As intoxication.[9-12] Nowadays, the therapeutic use of As is making a comeback in modern medicine. Arsenic trioxide (ATO), for instance, is used to treat patients with relapsed acute promyelocytic leukemia (APL).[13-16] But ATO is also known for its less favorable side, as in causing temporary cardiac and neurotoxic side effects in APL patients.[8] Arsenic was one of the primary ingredients in pesticides before synthetic organic pesticides were available; its long-term application in agricultural pesticides has resulted in high levels of arsenic in the body of workers, who are exposed by inhalation during the spraying. Arsenic-containing rodent pesticides used for pest and insect control were banned due to human health concerns in production, use, and accidental poisoning and possible abuse in intentional poisoning. Various case reports and studies have revealed that exposure to As has resulted in various forms of cancer and peripheral neuropathy.[7,17,18]

Acute and chronic exposure to arsenic

Acute. A single exposure, to a high dose may lead to severe reactions such as diarrhea, vomiting, pain, dehydration and weakness. Nowadays, acute intoxication rarely occurs in western European countries; if it occurs, it is usually the result of intentional (suicide or homicide) or accidental poisoning. Occupational exposure to As is rare and usually occurs in the form of arsine gas, which causes symptoms different to those caused by As ingestion.[6] Exposure often occurs when arsine gas escapes during transport or when it is generated while arsenic-containing ores or metals are treated with acid.[19,20] Acute oral exposure to As is associated with gastrointestinal symptoms such as nausea, vomiting, abdominal pain and severe diarrhea. Cardiovascular and respiratory symptoms include hypotension, shock, pulmonary edema and heart failure. If survived, acute poisoning with As is also combined with neurological symptoms like light-headedness, weakness, delirium, encephalopathy and peripheral neuropathy, which have been reported.[21] Peripheral neuropathy as a result of As intoxication may be delayed several weeks after the initial toxic insult.[22] After a few weeks the patients show signs of recovery, however, when tested with electrophysiological studies 6 to 8 years after exposure, the patients still showed reduced motor conduction velocity.[22] Biopsies on the sural nerves 10 weeks after exposure showed axonal degeneration, which was at an early stage in some fibers. These findings suggest that diminished nerve velocity

conduction is a severe and latent effect of As intoxication, which contributes to As-induced neuropathy.
Chronic. Environmental and occupational As exposure is not only caused by contaminated drinking water due to the leaching of natural geological resources, but may also occur from mining and other industrial processes.[23]
Chronic ingestion of low concentration of As levels can occur through industrial accidents, work and environment, which eventually may cause a higher tolerance for As. An example of such an industrial accident can be found in the early 20th century, in reports on As intoxication caused by contaminated beer in Salford, UK.[24] The subject of these reports, the patients, had a few symptoms in common, they all suffered from 'peripheral neuritis' characterized by weakness in the limbs that made it difficult for them to walk[25]. In several cases some of the patients suffered from rashes and itching, sometimes these complaints were accompanied by darkening of skin texture. The presence of As in the beer was due to the use of contaminated glucose and inverted sugar by the brewers. Furthermore, the contamination of this sugar is caused by the use of very impure sulphuric acid. The source of As contamination was traced back to invert sugar, which was caused by the action of sulphuric acid on various forms of starch. The original source was the Spanish pyrites from which the sulphuric acid was made and often contained large percentages of As.
Chronic As poisoning represents a global, serious health concern, if As can be found in high concentrated levels in the environment from natural or industrial processes. Arsenic contamination of groundwater has occurred in various parts of the world such as in the Americas, Bangladesh, India, Taiwan and many other Asian countries. In Bangladesh, a country of 125 million, between 35-77 million people are currently exposed to As through drinking water, which is also the major cause of death, especially among children.[26,27] As a quick and inexpensive solution for the lack of sufficient and clean drinking water, UNICEF and the World Bank advocated to tap into deeper groundwater. Millions of wells were constructed; infant mortality and gastrointestinal illness were reduced by half. However, later studies revealed that over 40% of these wells are contaminated with arsenic. As a result, due to the daily exposure to As in their drinking water, the population in south east Asia has to endure various ailments caused by As. Chronic symptoms of As exposure are pigmentation changes, gastrointestinal symptoms, anemia, liver disease, a metallic taste and Mees' lines on the nails,[28] blackfoot disease and diabetes mellitus.[29] Apart from pigmentation changes, arsenic-induced skin pathology caused by chronic intoxication also causes hyperkeratosis, Bowen's disease, squamous cell carcinoma and basal cell carcinomas.[30,31] Hafemann *et al*. (2005) have shown an association between arsenic exposure and peripheral neuropathy in the Bangladesh population that has been chronically exposed to arsenic in drinking water. They showed that increased As exposure, as measured by both cumulative and urinary measures, was associated with evidence of sub-clinical sensory neuropathy. The correlation between As exposure and neuropathy was shown with the increased vibrotactile threshold, a sign of subclinical sensory neuropathy, on the one hand and sub-clinical loss of vibratory sensation in the lower extremities, on the other hand.[32]

Diagnosis and treatment of As poisoning

Arsenic concentration measurements for diagnostic purpose are usually carried out in urine. Acute As toxicity is usually diagnosed by increased urinary As in excess of 50 µg/l urine sample or 100 µg in 24-hour urine, and a shorter time span before examination, if no seafood has been ingested. The urine is collected in metal-free containers. Other biological samples, such as blood, and even hair and nails in chronic cases, are also used in the clinical laboratory.

For treatment of acute As poisoning, the primary concern is to correct the dehydration caused by As and restore vital bodily functions. In order to increase elimination, physicians prescribe gastric lavage and activated charcoal, but haemodialysis may also be considered. The efficacy of these detoxification methods, however, has not been well recorded. Although the metalloid As cannot be categorized as a metal, it shows some metal properties. Based on this fact, chelators can be used to remove As ions from the body. For treatment of acute As poisoning, the chelator 2.3-dimercapto-1-propanol (British Anti-lewisite, BAL) has been used with successful results.[33,34] Patients who were administered this drug showed elevated As excretion in their urine. At follow-up, their urinary As concentration was decreased to the background level. At the moment of admittance, neurological examination demonstrated no signs of nervous system depression. However, these patients survived the high dose of ingested As with only latent neuropathy symptoms. Neurological complications such as distal, symmetrical, sensory, axonal neuropathy are late effects of acute As poisoning. These neurological effects are non-responsive to chelation.[35] In clinical cases with chronically poisoned patients, trials with 4 As chelators such as BAL, meso-2.3-dimercaptosuccinic acid (DMSA), D-penicillamine and sodium 2.3-dimercapto-1-propamesulfonate (DMPS) did not provide any clinical, biochemical or histopathological benefits.[36,37] On the other hand studies done in rats with an As chelator such as BAL showed depletion of tissue As and its excretion via urine and faeces.[38] Although the binding affinity of a chelating agent for the metal is greater than for endogenous ligands, chelating is generally ineffective for treating established arsenical peripheral neuropathy.[39]

Kinetics of various arsenic metabolites and their molecular mechanism of toxicity

Kinetics. Arsenic absorption takes place mainly in the small intestines; also a minimal absorption occurs from skin contact and inhalation.[31,40,41] After ingestion, As is metabolized from inorganic to organic compounds with varying degrees of toxicity and the behavior of the different As species vary markedly. Arsenic speciation of inorganic and organic forms of As is often as important as total quantification, because of their different toxicity and mobility.[42] As speciation in biological samples is an essential tool to gain insight into its distribution in tissues and its specific toxicity to target organs.

Arsenic metabolites exist both in organic and inorganic forms and both types can exist in either trivalent or pentavalent oxidation states. The bioavailability of inorganic As is up to 60%. Inorganic As such as arsenate (iAs^{V}), the pentavalent form, and arsenite (iAs^{III}), the trivalent form, are the most aggressive single-substance toxicants, specially the trivalent form. A redox reaction reduces the pentavalent As to its trivalent state. This reduction step from pentavalent to trivalent, releases a more toxic compound, which in fact results in bio-activation (Fig. 2).

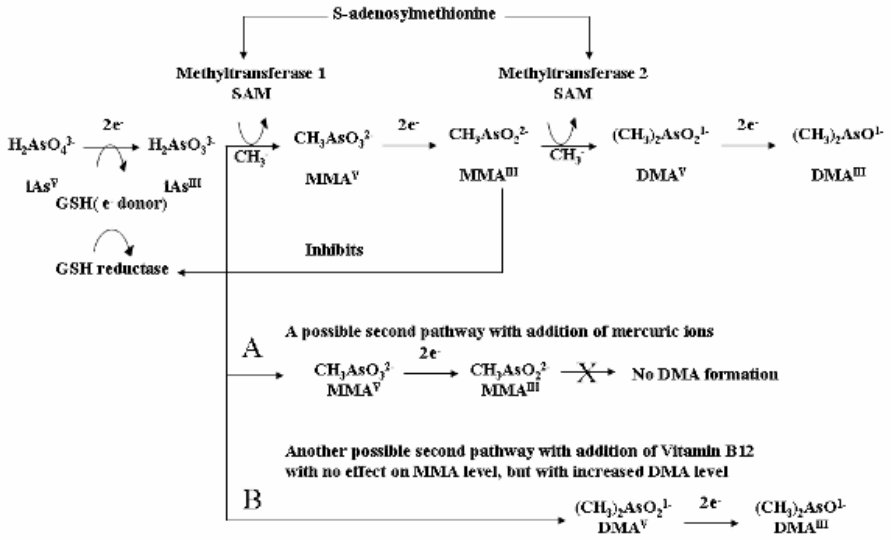

Fig. 2. Two possible pathways are shown for arsenate methylation. The main pathway is a straightforward bio-activation (metabolic activation) of arsenate in MMA, DMA and the theoretical conversion into TMA (not shown).[46,53,59] A possible second pathway is direct metabolism of iAs (A) to either MMA without further conversion to DMA or (B) increase in DMA levels, while the MMA levels stay intact.[44]

Arsenic metabolism shows a common route of absorption, distribution and excretion both in humans and various animals with subtle interspecies differences. Metabolism of inorganic As starts with intake and absorption. Distribution of As varies per species. This is a result of various factors such as species, bodyweight, route of intake and duration of exposure. After its absorption, As can be found in different organs, especially in the liver.[43,44] Arsenic undergoes hepatic biomethylation to form monomethyl arsenic (MMA) and dimethyl arsenic (DMA). Various *in vitro* studies on human and animal livers reveal the next step in iAs detoxification (Fig. 2). Detoxification starts with transformation of inorganic As to organic As and reduction of pentavalent arsenate to trivalent arsenite, which results in a more toxic component. The reduction of arsenate to arsenite is catalyzed by gluthatione (GSH) and other thiols, which are reducing agents.[45] Arsenate at physiological pH is ionized and as such is not able to pass cellular membranes. Conversion to arsenite at physiological pH facilitates passage through the cellular membrane.

Metabolism of As continues by using arsenite as its substrate for methylation, which is distributed in tissues and cytosol. However, methylation does not appear to be the primary detoxification pathway for arsenite. Protein binding has been suggested as the initial protective mechanism. Arsenite binding to protein serves as a reservoir and takes place after initial increase in arsenite concentration. When methylation enzymes start to become effective, the reservoir may slowly release small amounts of arsenite for methylation.[46] The methylation of arsenite is catalyzed by a specific methyltransferase using s-adenosylmethionine as a methyl donating cofactor (SAM).[43,47] Addition of a methyl group to arsenite leads to synthesis of pentavalent monomethylarsonic (MMA^V). The MMA^V in turn is reduced to trivalent MMA (MMA^{III}) by GSH, the reducing agent. Another round of methyltransferase activity with MMA^{III} results in production of dimethylarsenic acid (DMA).

However, it is not clear whether the same SAM is used for methylation of MMA and DMA or that two separate SAMs are being used for these two methylation steps. Theoretically, conversion of arsenate to DMA could be extended by one more round to achieve trimethylarsenic acid (TMA).[43,48] A second possible conversion route to MMA and DMA was introduced by the *in vitro* studies of Buchet and Lauwerys, by adding mercuric ions; they prevented the formation of DMA without affecting MMA formation.[44, 45] Furthermore, it was evident that the addition of cyanocobalamin or methylcyanocobalamin (vitamin B12) and coenzyme B12 in combination with SAM resulted in a significant increase of DMA and no additional increase in MMA level. This experiment revealed two different enzymatic activities involved in methytransferase to either MMA or DMA (Fig 2).

The methylation process has been thought to be the detoxification mechanism for As. This is true when this line of reasoning is applied to parameters of acute toxicity of As species, such as LC_{50} or LD_{50}. As a rule, the trivalent As compounds are more cytotoxic then their pentavalent forms. However, various studies in animals and cell cultures have shown the adverse effects of methylated As, such as DMA^V as a tumor promoter[49,50] or direct genotoxic action of MMA^{III} and DMA^{III} *in vitro*.[43,51]

The main excretion route of As is through the urine and bile. However, the various As metabolites do not excrete in the same fashion in different animals and humans. For example, studies in rats have shown that pentavalent metabolites such as iAs^V, MMA^V and DMA^V are exclusively excreted into urine, MMA^{III} only into bile and iAs^{III} into both bile and urine.[52] In contrast, human studies revealed the presence of MMA^{III} in urine.[53,54] Arsenic studies carried out by Csanaky and Gregus in rats, mice, hamsters, rabbits and guinea pigs revealed some similarities. All species injected with iAs^V excreted various As metabolites into urine, in contrast to injection with iAs^{III}, which showed higher excretion into bile rather than urine.[52]

Mechanism. These metabolites exert their toxicity by inactivating many enzymes, especially those involved in the cellular energy pathway and DNA synthesis and repair. Arsenic is substituted for phosphate in high-energy compounds such as ATP. Arsenic binds covalently with sulfhydryl groups in their reduced form. These interactions also take place with certain enzymes necessary for cellular metabolism.[17] Various As carcinogenesis studies have revealed that As may alter one or more DNA repair processes. Andrew *et al*. have shown that patients exposed to As have altered nucleotide excision repair mainly on the excision repair cross-complementing 1 (ERCC1) component. Arsenic exposure was associated with decreased expression of ERCC1 in isolated lymphocytes at the mRNA and protein levels.[55] In short, As exposure results not only in general toxicity but also in neuronal diseases and carcinogenesis.

Tri-valent arsenic (iAs^{III}, MMA^{III} and DMA^{III}) compounds are thought to interact with thiol groups of proteins and enzymes in their reduced state. This is believed to inhibit the catalytic activity of enzymes.[54] The common thinking is that tri-valent arsenic metabolites inhibit pyruvate dehydrogenase (PDH), which leads to disruption of the energy system of the cell,[56] which in turn may release an apoptosis-inducing factor (AIF) resulting in cell damage and death. AIF is released from the mitochondrial intermembrane space from where it translocates to the cell nucleus.[57] Apoptosis is associated with early formation of ring-like perinuclear condensed chromatin co-localized with AIF, DNA fragmentation and finally cell death. Pentavalent As (iAs^V, MMA^V and DMA^V) is substituted for phosphorus in many biochemical reactions. Replacing the stable phosphorus anion in phosphate with the less stable As^V anion leads to rapid hydrolysis of high-energy bonds in compounds such as ATP. At the level of the citric acid cycle, As inhibits succinate dehydrogenase and by competing with phosphate it uncouples oxidative phosphorylation, thus inhibiting energy-linked reduction of NAD+, mitochondrial respiration, and ATP synthesis. This leads to loss of high-energy phosphate bonds and effectively uncouples oxidative phosphorylation.[58,59]

Another approach is that trivalent As inhibits enzyme complexes by reactive oxygen species (ROS), indicating that ROS production by trivalent As causes pyruvate dehydrogenase (PDH) inactivation through oxidation.[60] Inactivation through oxidation causes impaired gluconeogenesis and reduced oxidative phosphorylation.[61] Production of ROS by As was determined by use of a nonfluorescent dye 5',6'-chlomethyl-2',7'-dichlorodihydrofluorscein (CM-H_2DCFDA), which is a non-specific radical detector to identify the radical species.[62] Cells pretreated with CM-H_2DCFDA and subsequently exposed to arsenite exhibit a dose-dependent increase in fluorescence levels within minutes of treatment when compared to controls.[63] Using DMSO in these experiments as radical scavenger in the reaction mix reduced the fluorescence signal to a near-background level.

Arsenic-induced neurotoxicity

Arsenic effects manifest themselves weeks after first exposure as both central and peripheral neuropathy. Central neuropathy due to As poisoning has been reported to cause impairment to neurological functions such as learning, short-term memory and concentration.[64] People chronically poisoned by arsenic occurring naturally in groundwater may suffer from toxic delirium and encephalopathy.[39] Neuropsychological tests showed mildly impaired psychomotor speed and attentive processes, whereas verbal learning and memory were severely impaired.
The most frequent neurological manifestation by As is peripheral neuropathy that may last for several years or even life-long. The peripheral neuropathy may lead to rapid severe ascending weakness, similar to the Guillan-Barré syndrome, requiring mechanical ventilation. Peripheral neuropathy is common in persons chronically exposed to As-contaminated drinking water.[65]
From human clinical cases studied by Le Quesne and McLeod it has become clear that As exposure results in a latent reaction to the nervous system, which was established through their reduced Nerve Conduction Velocities (NCVs) measurements.[22] These patients showed some recovery in the years following exposure to As; however, a full NCV regain was not achieved. It is doubtful whether PNS symptoms will ever disappear completely.
Patients exposed to As show significantly lower NCVs in their peripheral nerves in comparison to their referents.[21,34,66] Perhaps, changes in cytoskeletal composition may be the major reason of As poisoning leading to axonal degeneration, which in turn could lead to axonopathy.
An actual mechanistic model for arsenic neurotoxicity is as yet not easy to hypothesize, although interference with cytoskeletal proteins is a primary consideration. Therefore, one may look for such mechanistic parallels in other neurotoxins, especially neurotoxic metals, but also in other neurodegenerative diseases.

Metals as environmental pollutants such as lead and mercury have been associated with neurodegenerative diseases.[67] Exposure to aluminum, lead and mercury are known to have caused abnormalities in the nervous system related to interference with the cytoskeleton. Clinically, symptoms may occur as peripheral and central neuropathies. Aluminum has been known to cause dialysis encephalopathy in some individuals with renal failure. Furthermore, it has been suggested that aluminum might be implicated in Alzheimer's disease (AD), because of some similarities in pathological changes. The pathological changes in both cases can be summarized as presence of neurofibrillary tangles as diagnostic hallmarks AD.[68] However, further examination of these tangles has shown differences in their tangles between AD and the aluminum-exposed patients, in AD patients tangles consist of paired helical filaments, whereas those induced by aluminum are single.[69] Aluminum exposure in animal studies has shown induction of neurofibrillary degeneration. Phosphorylation of cytoskeletal proteins appears to modulate their interactions with one another and with other cellular

proteins. Disruption of the phosphorylation of cytoskeletal proteins results in disorganization of the cytoskeletal structure.[70] Other metals as neurotoxicants such as lead have also been indicated in the etiology of amyotrophic lateral sclerosis (ALS), whereas manganese has been reported to be involved in Parkinson's disease (PD) or a similar syndrome, Parkinsonism. Exposure to lead has been shown to be related to ALS in a case control study conducted in New England from 1993 to 1996.[71,72] Generally, ALS is divided into two forms: (1) the classic sporadic form; (2) the familial, presumably hereditary form. The cause of the sporadic form is unknown. The cause of the familial form is believed to be genetic, attributable to a mutation in Cu–Zn superoxide dismutase.[73] Studies in occupational exposure have suggested an association between PD and elevated exposure to manganese.[74] Exposure to manganese can cause neurotoxicity and a neurological syndrome that resembles PD.[75]

Neurofilament proteins are major constitutes of neurons and they control axonal caliber, transport and signal.[76] In neurodegenerative diseases such as AD it appears that the metabolism of neurofilaments is disturbed, as indicated by the presence of neurofilament epitopes in the neurofibrillary tangles, as well as by the severe reduction of the expression of the gene for the light neurofilament subunit of the neurofilament triplet (neurofilament High, -Middle and –Light) in brains of AD patients.[77] Accumulation of neurofilaments in the proximal cell body and the perikaryon of motor neurons is a hallmark of ALS and PD.[73,76] Disruption and disorganization of neurofilament transport and neuron cytoskeletal network is a pathological feature seen in all of these neurodegenerative diseases.

In rats exposed to As , decrease of the neurofilament Light subunit (NF-L) in sciatic nerve is evident.[78] Arsenic-induced decrease of NF-L may play an important role in the pathological changes of the nervous system, since NF-L is the only NF protein capable of independently organizing and co-assembling filaments *in vivo*. Both NF-H and NF-M need NF-L protein to form a heteropolymer in the cytoskeletal framework.[79] However, in vitro studies with iAs^{III} in neuroblastoma (SK-N-SH) and Schwannoma (ST-8814) cell lines show no effect on their mRNA expression level of cytoskeletal genes.[80] Thus, it can be suggested that the decrease in NF-L expression is a post-translational activity as a result of a proteolytic process. Calpain (calcium-activated cytoplasmic protease) could be responsible for NF-L degradation, since neuroblastoma cells (SY-5Y) treated with arsenic trioxide (trivalent As) show an increase in intracellular calcium.[81] Studies in PC12 cells under oxidative stress circumstances have shown an increase of calcium in the cells and up-regulation of calpain leading to degradation of NF-L protein.[82] Furthermore, inactivation of calpain by calpain inhibitor (MDL-28170) prevents NF-L breakdown.[83,84] These results suggest that As-induced destabilization and disruption of the cytoskeletal framework is partly due to activation of calpain, through influx of Ca^{2+}, which in turn is responsible for NF-L degradation in a calcium-induced proteolytic process.

Another important cytoskeletal protein in neurodegenerative diseases is the tau protein (MAP-tau), which is a member of the microtubule protein family transcripted by alternative splicing of a single gene. It has tandem repeats of a tubulin binding domain and promotes tubulin assembly. Although tau proteins are found in all cells, they are major components of neurons where they are predominantly associated with microtubules of the axon. Changes in tau-protein may play a role in the pathogenesis of neurodegenerative diseases. In AD patients, MAP-tau becomes abnormally hyperphosphorylated and accumulates as tangles of paired helical filaments in neurons undergoing degeneration. Hyperphosphorylated MAP-tau disorganizes microtubules assembly from normal tau and tubulin, which may lead to the formation of the neurofibrillary tangles and the degeneration of the affected neurons in AD patients. Arsenic may affect the phosphorylation of tau-proteins as well. Giasson *et al.* demonstrated hyperphosphorylation of tau-proteins in Chinese hamster ovary (CHO) cells *in vitro* after treatment with iAs^{III}.[85] They also showed that iAs^{III} causes a significant increase in

the phosphorylation of several amino acid residues in tau. This is in line with experiments in rats with iAs^{III}, which have also shown that the rats' MAP-tau was hyperphosphorylated after dosing them with iAs^{III} {unpublished data}. These results indicate that As may be involved in the cascade leading to deregulation of tau function associated with neurodegeneration.

Conclusion

Arsenic compounds are toxic substances with very profound effects on human health. Metabolism of As involves reduction of pentavalent As to a trivalent state and subsequent oxidative methylation. The adverse effects caused by As metabolites vary. The molecular mechanism of action can be summarized as involvement in cellular energy pathway, disruption of oxidative phosphorylation, hyperphosphorylation and altering the DNA synthesis and repair. Acute As intoxication has only been treated successfully with BAL. Furthermore, none of the chelators have ever been successful in treating chronic As intoxication. Arsenic neuropathy occurs weeks or even months after initial exposure in the acute cases, and it is also present in chronically exposed patients. Reduced nerve conducting velocity in PNS is a hallmark of As neuropathy. The neuropathy is primarily due to destruction of axonal cylinders and compositional changes, leading to axonopathy. The cytoskeletal changes are caused by disruption of the neurofilament and microtubule network in the nerve cells, namely through gradual degradation of NF-L by calpain, since iAs^{III} does not affect expression on gene level and hyperphosphorylation of NF-L and MAP-tau. The probable functional disturbances of these proteins such as transport and signaling and pathological changes such as reduction in caliber of axons may lead to axonopathy. These functional and pathological changes manifest themselves in clinical signs of neuropathy.

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Aims and objectives of the present investigation

Studies into the mechanism of arsenic-induced neurotoxicity

Aims and objectives of the present investigation

Ali Vahidnia

The aim of this study is to investigate the mechanism by which arsenic (As) induces its neurotoxic effects.

Background

The term 'neurotoxic' is used to describe a substance, condition or state that damages the nervous system and/or brain, usually by killing neurons. The term is generally used to describe a condition or substance that has been shown to result in observable physical damage.
Arsenic is a semi-metalloid and exposure to As is a world wide health problem causing various disorders and diseases in millions of people around the world. Arsenic causes various diseases such as numerous organ cancers and also patients show severe effects on their nervous system. The effects on the nervous system may be assessed with the use of clinical studies like nerve conduction velocities (NCV). In As exposed patients, their NCVs are diminished in comparison to healthy unexposed subjects. However, the mechanisms of As neurotoxicity remain somewhat obscure while the instance of As exposure, especially at chronic levels, remains a prevalent human health concern.
To start our investigation we had to ask the pertinent questions that comes with any investigations; why? How? And what kind of strategies must be taken?

Objectives and investigation of this thesis

As the molecular mechanism of arsenic neurotoxicity has not been described before, it was our aim to elucidate this mechanism. To start this investigation, we had to see whether the As effect that can be measured by reduced NCV, can be shown on molecular level in nerves. We devised studies in rats (*in vivo*) to determine the possible effects of As on the nervous system (Chapter 3 & 4). These studies were designed to answer whether As effects could be found on the nervous system and whether these effects could be established by different rout of exposure, dose and duration.
We studied the acute short effects of high As doses intravenously after a single exposure (Chapter 3) and the subchronic effects of intragastric administration of As for a longer exposure (Chapter 4). These studies deal with the effects of inorganic As in Wistar rats on the cytoskeletal protein composition of the sciatic nerve after acute and subchronic intoxication. The strategy in these two studies were to chart the pharmacokinetics in blood and urine and concentration in sciatic nerve and correlated them with effects that could arise on PNS with help of western blot technique on various cytoskeletal proteins.

After establishing the As effect *in vivo*, we started further investigation on the possible mechanisms of As neurotoxicity *in vitro*. *In vitro* studies were designed to study various As metabolites and compare their effects on genetic level for various cell cultures derived from neuroblastoma (SK-N-SH) or Schwannoma (ST-8814) representatives for nervous system, as well as non-neuronal derived cells such as, HeLA and Chinese Hamster Ovaries (CHO). These cell lines were chosen as a model for neurons as they harbor the mentioned neuro-cytoskeletal proteins like NFs and MAP-tau. We examined the (neuro-) toxic effects of various arsenic metabolites on these cells. The DNA expression levels of cytoskeletal proteins and genes involved in phosphorylation were studied after exposure to various arsenic metabolites and concentrations. The effects were examined on the relative quantification levels of the cytoskeletal genes, using Real-Time PCR (Chapter 5 & 6). To conclude these studies, the mechanism of NFs and MAP-tau phosphorylation was studied with the use of a p35 construct in HeLa cell line (Chapter 6). CHO cell lines such as AA8, UV20 and UV5 were also used to get additional information, especially to investigate effects of various As metabolites on the DNA repair mechanism namely nucleotide excision repair genes such as Ercc1 and Ercc2 (Chapter 7).

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

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 Bangladesh[1] and several South American countries, and is mostly due to environmental exposure through water, air pollution[2] 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 injection[11] 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 effect[12] (As_2O_3) (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, Duenas-laita *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 ($NaAsO_2$: As^{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 plus™ 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-H_2O) 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-H_2O, 27 ml 37% hydrochloric acid (1M HCl), 6 g sodium iodide (NaI) and 3 g L-(+) ascorbic acid ($C_6H_8O_6$) 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-H_2O 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 membrane[22] 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 Weidner[23] 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.

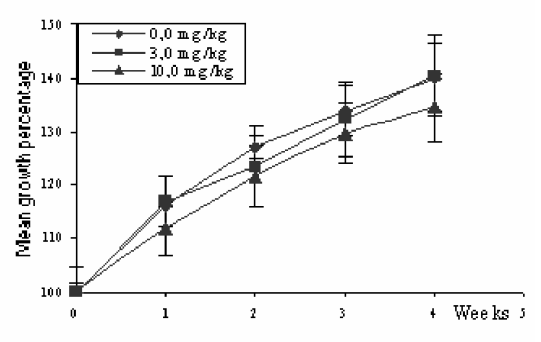


Figure 1. Body weight gain in control rats and in arsenite-treated rats during 4 weeks. Rats treated with 10-mg/kg arsenite show significant reduced growth in comparison to the control. The group of 3-mg/kg dosage shows recovery in their growth.

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)	*Nervus ischiadicus* (µg /g) (ww)
Short-term	3	Control	0 hrs	0.0 µg/L	0.0	0.0
single	3	15 mg/kg	3 hrs	18.97	2.30	2.24
exposure**			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	1.02
			6 hrs	26.27	2.84	1.08
			9 hrs	22.56	2.34	0.30
Long-term	9	Control	wks	0.0 µg/L	n.d.	0.0
single	9	3 mg/kg	2 wks	0.60 ± 0.66		0.508
exposure***			3 wks	2.05 ± 1.88		0.496
			4 wks	0.34 ± 0.03		0.302
	9	10 mg/kg	2 wks	2.06 ± 0.17		0.367
			3 wks	2.40 ± 0.71		0.367
			4 wks	2.07 ± 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 µg /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).

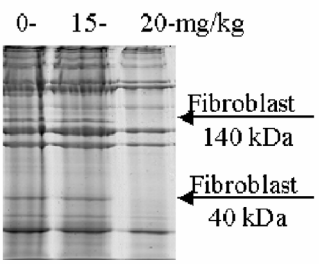
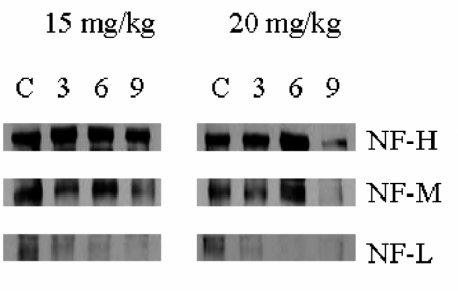
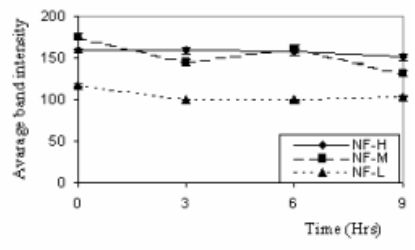


Figure 2. Coomassie staining of three sciatic nerve protein homogenates with various arsenic doses. A high dose of 20-mg/kg arsenite results in disappearance of fibroblast band sizes of 40 and 140 kDa as indicated by the arrows. However these bands are still present in lower dose group and the controls.

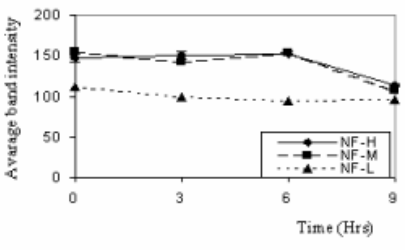
Neurofilament proteins gradually disappear after *i.v.* injection of As^{III}, as is shown in Figure 3A. NF-L has almost totally disappeared after 9 hours in both dose groups treated with 15- and 20-mg/kg arsenite. Figures 3B and 3C show the intensity of the Western blot signals. After 3 hours NF-L reaches 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.

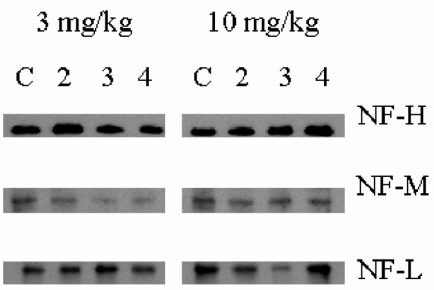


Figure 4. Western blot analysis of NF-H, -M and –L of rats sciatic nerves. The changes in cytoskeletal proteins are followed in time, measured up to 4 weeks and compared to their control (C) The long-term effect with lower doses namely 3- and 10-mg/kg arsenic show no significant effect.

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 µg/g tissue at 15-mg/kg dose group after 9 hours in comparison to 0.30 µg/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

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.

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

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 ($NaAsO_2$; As^{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™ 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™ 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. (Californina, 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 As^{III} ($NaAsO_2$) 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_{50} 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 1µl/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 µl bloods were added to 950 µl 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_2O). 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_2O, 27 ml 37% hydrochloric acid (1M HCl), 6 g sodium iodide (NaI) and 3 g L-(+)ascorbic acid ($C_6H_8O_6$) 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_2O ranging from 0 µg/l to 200 µg/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 µg/l to 200 µg/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 100ºC. 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™ 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 µm 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 plusTM 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

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^{III} 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^{III} 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^{III} 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.

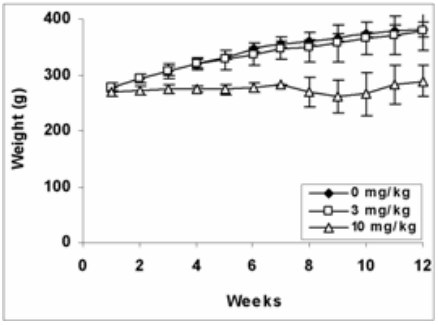


Fig. 1. Body weight increase in three different dose groups of the 12-week exposure group. Each dose group of 0, 3 and 10 mg/kg consists of 3 rats, which were exposed to As and monitored over 12 weeks. The highest dose group of 10 mg/kg lags behind. ANOVA for mean value per dose group showed significant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

3.2. Arsenic distribution and elimination

The total amount of As excreted in urine was measured and expressed as µg/ml (Table 1). The As concentration in urine of 3-mg/kg dose group increased from 8.57 ± 1.52 µg/ml to 13.65 ± 0.95 µg/ml after 4 weeks and 12 weeks, respectively. In the 10-mg/kg dose group, the As concentration in urine increased from 36.6 ± 10.73 µg/ml to 55.8 ± 59.45 µg/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.

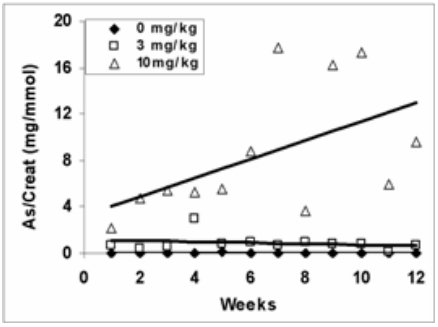


Fig. 2. As excretion, measured weekly as the amount of As (mg) excreted in urine expressed per mmol creatinine. ANOVA for mean value per dose group showed significant difference of $p<0.001$.

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

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

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

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

Dosage (n)	Weeks	Diuresis[a] (ml/h)	As in urine*[b] (µg/ml)	As in blood[c] (µg/ml)	As in[d] sciadic nerve (µg/g) (W/W)	Total body[e,**] clearance (Cl) (ml/h)	Relative distribution volume (V_D)*** (nerve tissue) (ml/g)
0 mg/kg (9)	4	0.45 ± 0.22	0.01 ± 0.004	0.07 ± 0.016	0.08 ± 0.02	0.05 ± 0.012	---
	8	0.91 ± 0.30	0.01 ± 0.001	1.20 ± 0.300	0.02 ± 0.01	0.01 ± 0.002	---
	12	0.48 ± 0.17	0.01 ± 0.002	0.30 ± 0.200	0.05 ± 0.03	0.02 ± 0.006	---
3 mg/kg (9)	4	0.30 ± 0.06	8.57 ± 1.52	10.19 ± 0.72	0.43 ± 0.25	0.25 ± 0.019	0.043 ± 0.0264
	8	0.21 ± 0.04	9.04 ± 0.98	14.78 ± 0.53	0.35 ± 0.24	0.13 ± 0.011	0.024 ± 0.0156
	12	0.36 ± 0.15	13.65 ± 0.95	14.59 ± 1.19	0.11 ± 0.01	0.34 ± 0.127	0,008 ± 0.0005
10 mg/kg (9)	4	0.35 ± 0.10	36.55 ± 10.73	11.07 ± 0.32	1.17 ± 0.23	1.18 ± 0.496	0.106 ± 0.0233
	8	0.31 ± 0.12	24.81 ± 18.66	13.83 ± 0.85	0.53 ± 0.20	0.46 ± 0.139	0.039 ± 0.0161
	12	0.29 ± 0.22	55.76 ± 59.45	13.34 ± 0.03	0.89 ± 0.15	0.74 ± 0.399	0.067 ± 0.0114

Mean values (± 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:

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

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

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

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

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

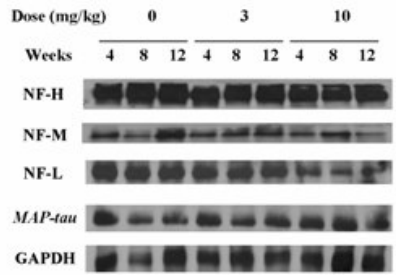
* Last urine collected before sacrifice. The urine was collected for 6, 8 and 10 hours for the 0, 3 and 10 mg/kg dose groups, respectively.

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

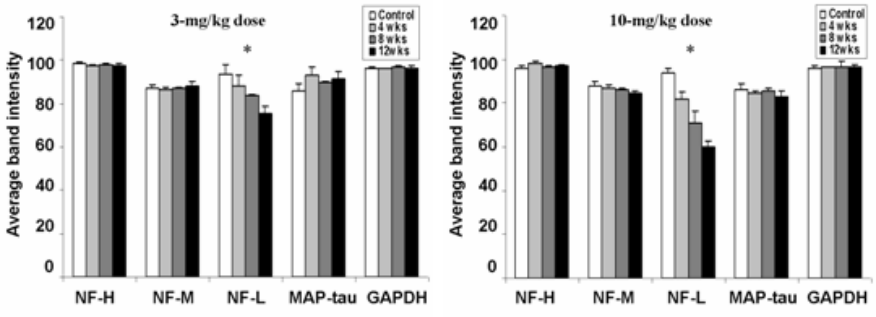
*** The relative volume of distribution (V_D = ml/g) was calculated by using As content measured in nerve tissue (µg/g) divided by the As content measured in blood (µg/ml).

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.



3A.

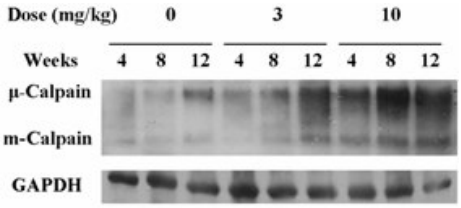


3B. 3C.

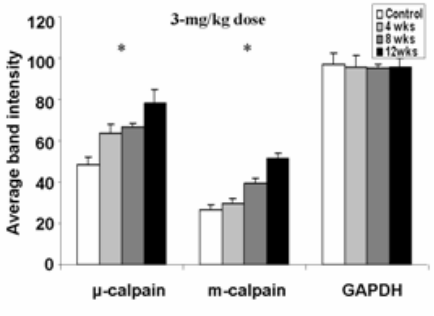
Fig. 3. 3A. Western blot analysis of the expression levels of the three-neurofilament proteins and total-tau of the sciatic nerve in the three dose groups in relation to time after dosing. Figure 3B and C. show the densitometry measurements in relative mean values (± SD) after 4, 8 and 12 weeks exposure 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

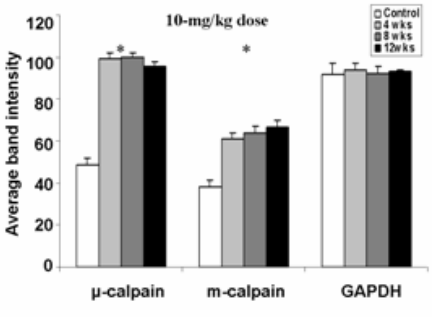
The analysis of μ- and m-calpain protein expression in As-treated rats showed significant increase with time for μ-calpain ($p<0.01$) and m-calpain ($p<0.05$) in both the 3- and the 10-mg/kg dose group (Fig. 4).



4A.



4B.

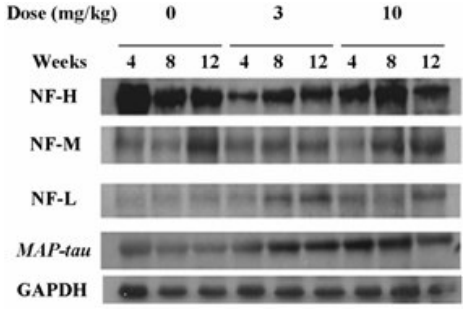


4C.

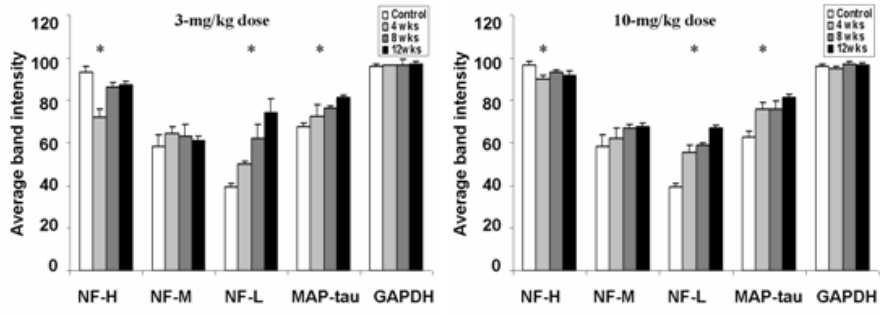
Fig. 4. 4A. Western blot analysis of the expression levels of the μ-calpain and m-calpain proteins of the sciatic nerve in the three dose groups in relation to time after dosing. Figure 4B and C. show the densitometry measurements in relative mean values (± SD) after 4, 8 and 12 weeks exposure for the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

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 (± 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 8th week due to approximately 14 µg/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 ± 0.25 µg/g As after four weeks exposure to 0.11 ± 0.01 µg/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 ± 0.23 µg/g As in the group exposed for four weeks, followed by a decrease to 0.53 ± 0.20 µg/g tissue after 8 weeks and a decrease to 0.89 ± 0.15 µg/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 sulphydryl (-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_Ds is that trivalent As has a high affinity for reduced sulphydryl (-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 sulphydryl (-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 μ-calpain ($p<0.01$; activated by μM 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 (GSK3ß), 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.

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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

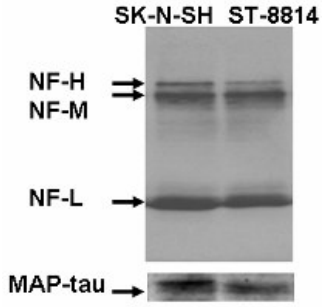
(Nieuwerkerek a/d Ijssel, the Netherlands). The used software, Relative Quantification (RQ), determines the change in expression of a nucleic acid sequence (target) in a test sample relative to the same sequence in a calibrator sample, after all the measurements are standardized through a chosen household 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.

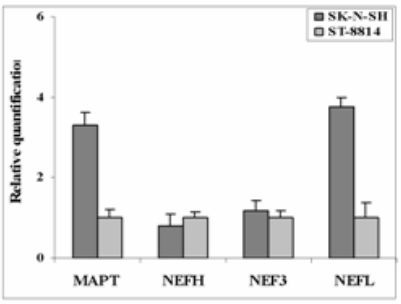
Name	**Sequence (5'-->3')**	**Gene**
ß2-Microglobuline-Sense	GATGCTGCTTACATGTCTCG	Household
ß2-Microglobuline-Anti-sense	CCAGCAGAGAATGGAAAGTC	Household
MAPT-Sense	TCACTTTTACAGCAACAGTCAGTG	Target
MAPT-Anti-sense	TGCCATGTTGAGCAGGACTA	Target
NEFH-Sense	GCCGAATGCCACACGTAAACACTT	Target
NEFH-Anti-sense	AAAGTGCGCCCTGGCATAATTCAG	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.



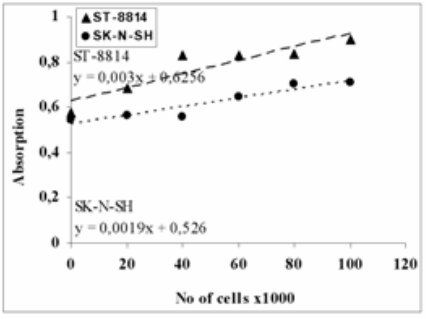
1A.



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).



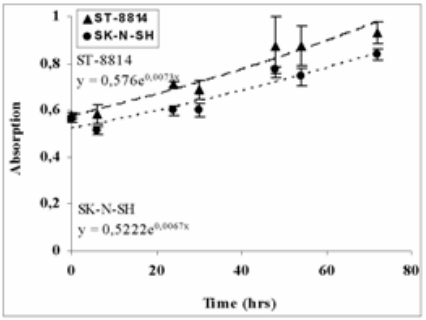
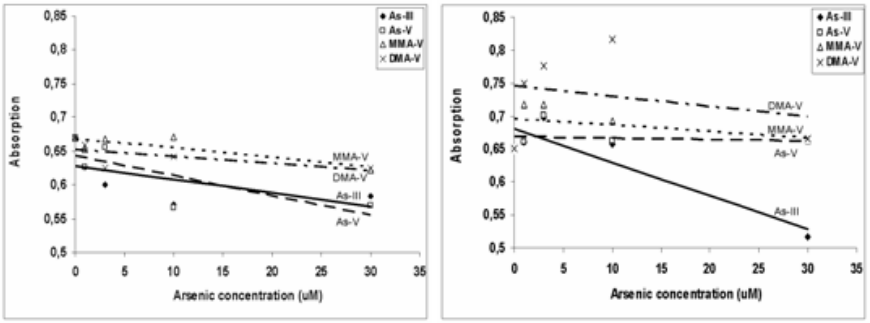


Fig. 2A. Linearity measurements (cell titration) of ST-8814 ($R^2 = 0.8422$) and SK-NSH ($R^2 = 0.887$) cell lines with AlamarBlue. These trend lines of indicate that the higher the amount of cells the greater the absorbance. The overall measured absorbances of the ST-8814 cells are higher than those 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).

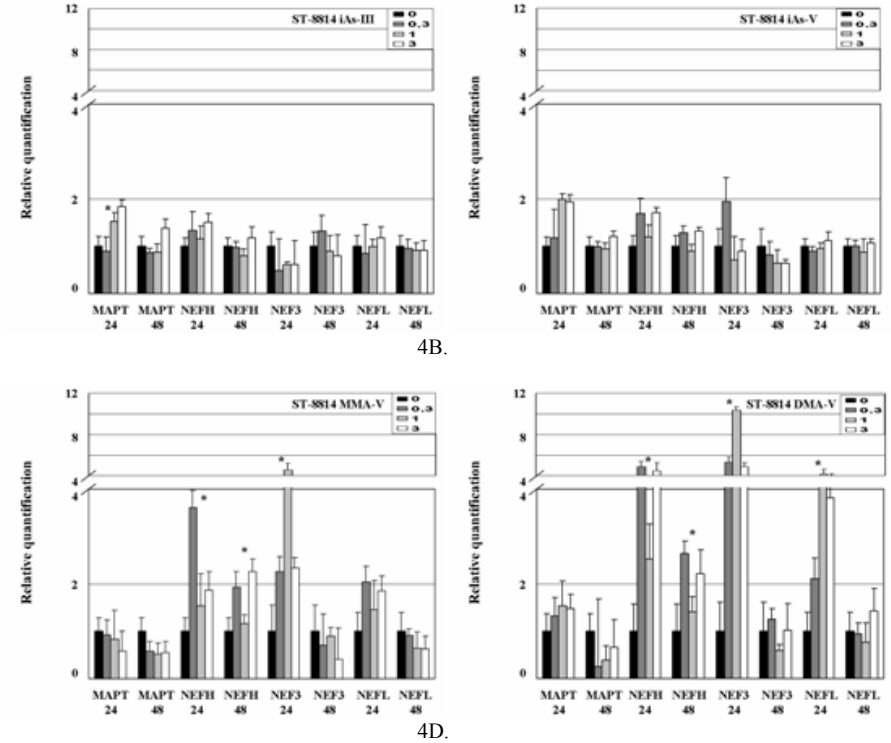
There are no significant changes on any of the MAPT, NEFH, NEF3 and NEFL genes after 24 or 48 hours.

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).

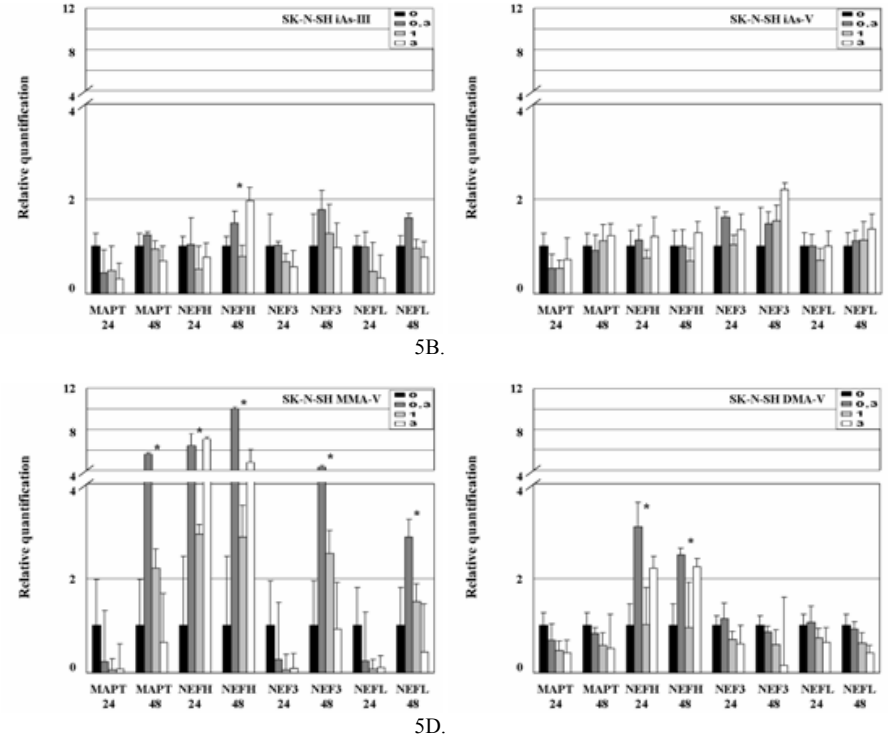
There are no significant changes on any of the MAPT, NEFH, NEF3 and NEFL genes after 24 or 48 hours.

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.

Acknowledgements

Janice Hernandez B.Sc. assisted with all laboratory work.

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Mechanism of Arsenic-Induced Neurotoxicity May Be Explained Through Cleavage of p35 to p25 by Calpain

Mechanism of Arsenic-Induced Neurotoxicity May Be Explained Through Cleavage of p35 to p25 by Calpain

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Abstract

In recent studies we have demonstrated that arsenic (As) metabolites change the composition of neuronal cytoskeletal proteins *in vivo* and *in vitro*. To further examine the mechanism of arsenic-induced neurotoxicity with various arsenate metabolites (iAs^V, MMA^V and DMA^V) and arsenite metabolites (iAs^{III}, MMA^{III} and DMA^{III}) we investigated the role of the proteolytic enzyme calpain and its involvement in the cleavage of p35 protein to p25, and also mRNA expression levels of calpain, cyclin-dependant kinase 5 (*cdk5*) and glycogen synthase kinase 3 beta (*gsk3ß*). A HeLa cell line transfected with a p35 construct (HeLa-p35) was used as a model, since all other proteins such as calpain, CDK5 and GSK3ß are already present in HeLa cells as they are in neuronal cells. HeLa-p35 cells were incubated with various As metabolites and concentrations of 0, 10 and 30 µM for duration of 4 hours. Subsequently the cells were either lysed to study their relative quantification levels of these genes or to be examined on their p35-protein expression. P35-RNA expression levels were significantly ($p<0.01$) increased by arsenite metabolites, while p35 protein was cleaved to p25 (and p10) after incubation with these metabolites. The cleavage of p35 is caused by calcium (Ca^{2+}) induced activation of calpain. Inhibition of calpain activity by calpeptin prevents cleavage of p35 to p25. These results suggest that cleavage of p35 to p25 by calpain, probably As-induced Ca^{2+}-influx may explain the mechanism by which arsenic induces its neurotoxic effects.

Keywords: Arsenate and arsenite metabolites; Calpain; HeLa cells; Neurotoxicity; p35**.**

1. Introduction

Long-term exposure to As causes serious effects to various organs such as bladder, kidney, liver, lung and skin cancer, cardiovascular disease and neurological effects.

Neurological effects of As may develop within a few hours after ingestion but are usually seen 2-8 weeks after exposure (Kishi et al. 2001; Jha et al. 2002). It is usually a symmetrical sensori-motor polyneuropathy, often resembling Guillain-Barré syndrome (Perriol et al. 2006). The predominant clinical features of neuropathy are paresthesias, numbness, and pain, particularly of the soles of the feet. Electrophysiological studies performed on patients with As neuropathy have revealed a reduced nerve conducting velocity (NCV), typical of those seen in axonal degeneration, which was confirmed by sural nerve biopsy (Hilmy et al. 1991). However, the mechanism of action of As-induced neurotoxicity is poorly understood.

Neurological disorders have been correlated with disorganization and changes in the expression levels of neuronal cytoskeleton proteins such as neurofilament-high (NF-H), neurofilament-middle (NF-M), neurofilament Low (NF-L) and microtubule associated protein tau (MAP-tau) (Trojanowski et al. 1993).

In specific neurodegenerative diseases such as Alzheimer's Disease (AD), the accumulation of amyloid beta peptide is believed to be an early and critical event leading to synapse and neuronal cell loss (Smith et al., 2006). Subsequently, neurofibrillary tangles are formed by tau hyperphosphorylation (Alonso et al. 1996). This hyperphosphorylation is caused by proteolytic cleavage of p35 to p25 by calpain at a specific site post-translationally, whereby p25 acts as hyperactivator of CDK5. P35 and also p25 are the neuron specific activator of CDK5. CDK5 is involved in terminal differentiation of neurons and muscle cells. The p35/CDK5 protein complex is required for proper development of the central nervous system and is this complex is required for neurite outgrowth (Nikolic et al. 1996). Calpain is a Ca^{2+} dependant kinase enzyme, which is activated by the internal increase of Ca^{2+} in the cells. Activated calpain is responsible for the cleavage of p35 to p25 (Lee et al. 2000). Hyperphosphorylation of cytoskeletal proteins is a potential mechanism of neurodegenerative disease, which is result of p25 overexpression, such as in AD-patients (Monaco 2004; Ahlijanian et al. 2000). A p25-mediated increase in CDK5 activity results in hyperphosphorylation of tau and neurofilaments (NFs). As a result microtubule (MT) network will destabilize leading to disruption of cytoskeleton (Trojanowski et al. 1993; Alonso et al. 1996). Glycogen synthase kinase 3 is involved in energy metabolism, neuronal cell development, and body pattern formation. GSK3ß also regulates the microtubule cytoskeleton during axon outgrowth (Goold & Gordon-Weeks 2001).

In our previous studies, we have shown that iAs^{III} causes compositional changes in sciatic nerve proteins, namely reduction in NF-L expression (Vahidnia et al. 2006). Furthermore, *in vitro* studies with various arsenic metabolites have shown that MMA^{V} and DMA^{V} affect expression of neurofilaments and tau genes, but not iAs^{III} (Vahidnia et al. 2007). Neurofilament-L decrease may play an important role in the pathological changes of nervous system, since NF-L is the only NF protein capable of organizing and co-assembling of filaments on its own *in vivo*. Both NF-H and NF-M need a NF-L protein to form a heteropolymer in the cytoskeletal framework (Carpenter & Ip 1996). Arsenite is also known to phosphorylate tau proteins (Giasson et al. 2002) and we have also seen similar results in sciatic nerves of rats exposed to iAs^{III}, whereby tau proteins were hyperphosphorylated (as yet unpublished). Hyperphosphorylated tau proteins have a reduced ability to bind to microtubules. Decrease in NF-L and hyperphosphorylation of tau proteins under As-induced toxicity.

Here we investigated a possible mechanism of As-induced neurotoxicity by which arsenite metabolites may be responsible for hyperphosphorylation and degradation of NF and tau proteins caused by cleavage of p35 to the p25 fragment. Transfected HeLa-p35 cells were

incubated with various As metabolites. Gene expression levels were monitored for p35, calpain, CDK5 and GSK3ß. Furthemore, the cleavage of p35 to p25 after incubation with arsenite metabolites was studied by western blot experiments.

2. Materials

2.1. 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). Dimethyl arsenic acid (DMA^{V}) (catalogue no. PS-51) and Disodium methyl arsenate (MMA^{V}) were obtained from Chem Service (West Chester, USA). Methylarsonous diiodide (MMA^{III}) and Dimethylarsinous iodide (DMA^{III}) were purchased from Dr. Cullen of the Department of Chemistry at the University of British Columbia, Vancouver, Canada. AlamarBlue dye was purchased from BioSource International, Inc. (catalogue no. DAL1100. Camarillo, CA). RNeasy Mini Kit 250 was supplied by 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). Monoclonal antibody to GAPDH (catalog no. Ab8245) and rabbit polyclonal anti-p35 (catalog no. Ab10570) were supplied by Abcam (Cambridge, United Kingdom). Rabbit polyclonal anti-Calpain (H-60) (catalog no. sc-30065), goat anti-rabbit IgG-HRP (catalog no. sc-2004) and goat anti-mouse IgG-HRP (catalog no. sc-2055) were purchased from Santa Cruz biotechnology, Inc. (Califorina, USA). Cyclin-dependant kinase 5, regulatory subunit 1 (CDK5R1, p35) construct (catalog no. IOH11747-pDEST26) was purchased from RZPD (Berlin, Germany) and Lipofectamine^{TM} 2000 (catalog no. 11668-019) from Invitrogen (Breda, The Netherlands). Calpeptin (catalog no. 03-34-0051) was purchased from Calbiochem (Darmstadt, Germany). Kodak Biomax XAR film (catalog no. 165 1454) was obtained from Kodak (Shelton, CT, USA). ECL plus^{TM} 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) with a final concentration of 10 pmol/µl in Tris/EDTA-buffer (Table 1).

Table 1. Primer sequences used for the RT-PCR.

Name	Sequence (5'-->3')	Gene
ß2-Microglobuline-Sense	GATGCTGCTTACATGTCTCG	Household
ß2-Microglobuline-Anti-sense	CCAGCAGAGAATGGAAAGTC	Household
Cdk5-Sense	GCACAAGAACATCGTCAGGCTTCA	Target
Cdk5-Anti-sense	GTGTAGCACATTGCGGCTATGACA	Target
Gsk3β-Sense	TGATTCAGGAGAACTGGTCGCCAT	Target
Gsk3β-Anti-sense	TTTCCGGAACATAGTCCAGCACCA	Target
µ-Calpain-Sense	ACGAGAACTTCAAGGCCCTCTTCA	Target
µ-Calpain-Anti-sense	TGCCATCACGATCCATGAGGTTCA	Target
m-Calpain-Sense	ACCGAGAAATCGACGTTGACAGGT	Target
m-Calpain-Anti-sense	CAGCCGAACCAAACACCGAACAAA	Target
p35-Sense	TGACATGCCTGTACCTCTCCTACT	Target
p35-Anti-sense	CGGAGAAGACCTGTGTGAAGTAGTGT	Target

2.2 Cell cultures.

Most of the materials for cell culture were purchased from Invitrogen (Breda, The Netherlands); otherwise more specific details are followed in brackets. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM, catalog no.) and were supplemented with 10% heat-inactivated foetal bovine serum obtained from Greiner bio-one GmbH (catalogue no. 758093) (Frickenhausen, Germany) and supplemented with 100 IU/ml penicillin/streptomycin (catalogue no. 15140-122), and maintained at 37 °C, 5% CO_2 in a humidified incubator.

2.3. Cell Transfection.

HeLa cells were seeded in T25 flasks for a transient transfection and grown to 70-80% confluence for 24 h. HeLa cells were exposed to a mixture of 30 µl of Lipofectamine Reagent™ (Invitrogen) and 8 µg of plasmid DNA for 5 h in serum-free medium, after which the medium was refreshed by a full culture medium. After 24 h, the transfected cells were trypsinized and seeded in a 6-wells plate and grown to a 80-90% confluence, ready for treatment.

2.4. Protein expression.

Calpain, CDK5, GAPDH, GSK3β and p35 expression was analyzed by the western blot technique in triplicate. Transfected HeLa cells were grown in 6-wells plates. After trypsinization, the cells were centrifuged at 350xg and washed twice with PBS for 6 minutes at 4ºC. Afterwards, the cells were collected in 100µl of lysis buffer containing 25mM Tris-base, 2.5% SDS (w/v) and 2.5 mM DTT at pH 6.8. After addition of lysis buffer, cells were incubated at 100ºC for 5 minutes before and after lysing by freeze-thawing in liquid nitrogen (-80ºC) and a water bath (37ºC), 3 to 4 times for 3 minutes each. 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 lysis buffer. The protein samples were analyzed on an 8 or 12% acrylamide separation gel. The western blot analysis of the same samples was performed in triplicate.

2.5. Experimental design.

2.5.1. Relative Quantification of various genes.

For RT-PCR, 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 (n=6). For the study of gene expressions, cells were grown in 6-wells plates, from Corning Incorporated Costar (catalogue no. 3506) (Frickenhausen, Germany) till 80-90% confluency was reached. Various As metabolites (iAs^{III}, iAs^{V}, MMA^{III}, MMA^{V}, DMA^{III} and DMA^{V}) in concentrations of 0, 10 and 30 µM dissolved in cell culture medium were incubated for 4-hours. Afterwards, cells in 6-wells plate 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 a final concentration of 20 ng/µl were synthesized from the isolated RNAs and the samples were analyzed with the Applied Biosystems 7300/7500 Real Time PCR System (Nieuwerkerk 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 et al. 2001). The relative expressions of each gene for the various concentrations are compared to their control.

2.5.2. Cleavage of p35 to p25 by calcium and arsenite metabolites.

The conversion of p35 to p25 was analyzed by western blot. The transfected HeLa-p35 cells were seeded and grown in 6-wells plates and incubated until 80-90% confluence was reached. To investigate cleavage of p35 to p25, cultures were stimulated with either $CaCl_2$ in a range of 0 to 2 mM or arsenite metabolites at end concentrations of 10 or 30 μM (without addition of any $CaCl_2$) for 4 hours in the incubator at 37˚C in a 5 % CO_2 atmosphere. For inhibition experiments, calpeptin, a cell-permeable calpain inhibitor, was used with an end concentration of 50 μM and incubated an hour prior to challenge with various calcium concentrations or arsenite metabolites. After treatment, cells were collected for further analysis with SDS-PAGE and western blot.

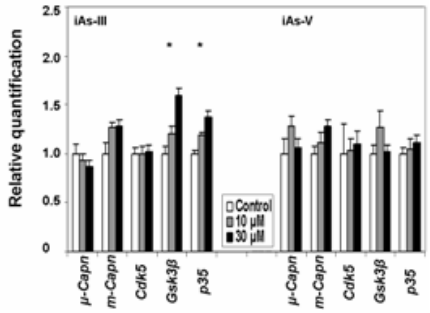
2.6. Statistical analysis.

The data for each gene were statistically evaluated for each concentration using SPSS 14.0 for Windows. Chi-Squared for non-parametric tests (Kruskal-Wallis test) was performed to evaluate the data for concentration and As metabolites as obtained for each gene. A level of $p<0.05$ was accepted as statistically significant.

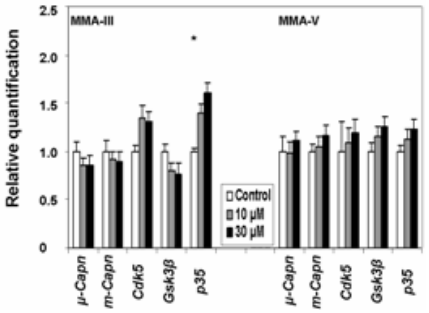
3. Results

3.1. Relative Quantification of various genes.

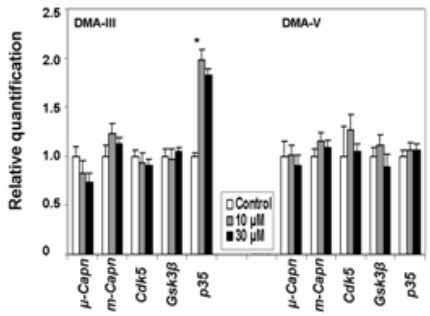
After treament of the transfected cells with various arsenic metabolites, relative quantification indicated that the significant changes on RNA levels were caused by arsenite metabolites on the increase of p35 gene expression ($p<0.05$) (Fig. 1A-C). *Gsk3ß* was significantly increased in its incubation only after incubation with iAs^{III} ($p<0.05$). Arsenate metabolites did not cause significant changes in the expression levels of any of the genes. The presence of the genes coding for calpain (76 and 80 kDa), CDK5, GSK3ß and the transfected p35 proteins was confirmed using the appropriate antibodies (Fig. 1D).



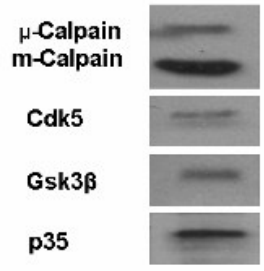
1A.



1B.



1C.

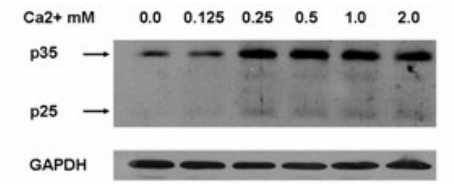


1D.

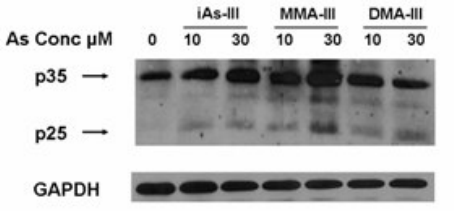
Fig. 1. Effect of various arsenic metabolites, iAs (1A), MMA (1B) and DMA (1C) and concentrations on the various genes involved in phosphorylation of cytoskeletal proteins (*, $p<0.05$). Relative mean values ± SD; n=6. Fig. 1D shows the presence of the µ- and m-calpain, CDK5, GSK3β and the transfected p35 proteins in HeLa cell line.

3.2. Cleavage of p35 to p25 by calcium and arsenite metabolites.

Calcium induced proteolytic cleavage of p35 from 0.25 mM upwards (Fig. 2A). The arsenite metabolites caused a cleavage of p35 to p25 at concentrations of 10 and 30 µM of each metabolite.



2A.

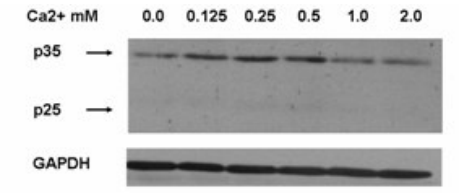


2B.

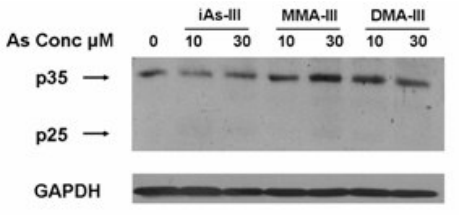
Fig 2. Cleavage of p35 to p25. A titration series of Ca^{2+} ranging from 0 to 2 mM shows that a minimum concentration of 0.25 mM of Ca^{2+} is needed for the cleavage (2A). All three arsenite metabolites (without addition of any $CaCl_2$) cleave p35 to p25 at concentrations of 10 and 30 µM (2B).

3.3. Inhibition of p35 cleavage to p25 by calpeptin.

Pretreatment of the transfected cells with 50 µM calpeptin inhibited the cleavage of p35 to p25 after incubation of calpeptin with various Ca^{2+} concentrations (Fig. 3A) and 10 and 30 µM concentrations of arsenite metabolites (Fig. 3B).



3A.



3B.

Fig 3. Cleavage of p35 to p25 is inhibited by pretreatment with 50 µM of calpeptin. Titration series of Ca^{2+} ranging from 0 to 2 mM shows no cleavage to p25 is visible after Ca^{2+} treatment (3A). The arsenite metabolites (without addition of any $CaCl_2$) are not able to cleave p35 to p25 at concentrations of 10 and 30 µM after pre-incubation with calpeptin (3B).

4. Discussion

A number of metal compounds, such as lead and mercury are known to induce neurotoxicity. The organic mercury compound methylmercury has been shown to induce the cleavage of p35 to p25 after treatment of neuronal cells through calpain activation (Sakaue et al.; 2005).
In this *in vitro* study, we first evaluated RNA expression profiles of μ-calpain (calpain-I), m-calpain (calpain-II), *cdk5*, *gsk*3β and *p35* after treatment with various arsenic metabolites. These genes are involved in hyperphosphorylation and degradation of some of cytoskeletal proteins in the nerves. The p35 RNA expression levels were significantly ($p<0.05$) increased after incubation with all the arsenite metabolites at 10 and 30 μM, while GSK3β expression was only significantly ($p<0.05$) induced by iAs^{III} (Fig. 1A-C). However, the arsenate metabolites had no significant effects on any of the gene expression levels. The transfected HeLa cells were also used to characterize their protein expression of the used genes as basis of our model (Fig. 1D).
In our previous study in rats, we showed that NF-L protein expression was decreased after treatment with iAs^{III} (Vahidnia et al. 2006), while its expression on RNA level was unchanged after treatment with iAs^{III} *in vitro* (Vahidnia et al. 2007). This suggests that the reduced expression is a post-translational activity. Calpain could be 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). Furthermore, cleavage of p35 to p25 is a Ca^{2+}-induced conversion by calpain, which is seen in AD patients (Lee et al. 2000). Here we have shown that Ca^{2+} induces cleavage of p35 to p25 (Fig. 2A) and p25 overexpression after incubation with various arsenite metabolites and 10 and 30 μM concentrations (Fig. 2B). This is in agreement with other studies that show the breakdown of cdk5/p35 into cdk5/p25 increases its kinase activity and neurotoxicity (Camins et al., 2006)
Other studies with PC12 cells under oxidative stress circumstances have shown an increase of calcium in 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 activation of calpain, through influx of Ca^{2+}, which in turn is responsible for NF-L degradation in a calcium-induced proteolytic process. Here we have shown that inhibition of calpain by 50 μM calpeptin prevented both Ca^{2+}- and arsenite-induced cleavage of p35 to p25 (Fig. 3A and 3B).
From these results can be concluded that arsenite treated cells show cleavage of p35 to p25, which is probably mediated by an increase of Ca^{2+} in the cells. An increase in internal Ca^{2+} levels and subsequent calpain activation may be the primary step in the mechanism of arsenite-induced their neurotoxicity. In addition, calpain splits p35 into p25, which in turn is a hyper-activator of CDK5. This p25/CDK5 complex is responsible for the hyperphosphorylation of MAP-tau and NF proteins. Although RNA expression levels of *cdk5* are unaffected with the various arsenic metabolites (Fig. 1A-C), a complex of p25/CDK5 has shown to be responsible for hyperphosphorylayion of tau proteins (Lee et al. 2000; Town et al. 2002). Hyperphosphorylation of tau reduces its ability to associate with microtubuli proteins as their organizer as it has been seen in AD patients (Kesavapany et al. 2003).
Degradation and hyperphosphorylation of neuronal proteins may lead to disruption of neurofilament organization, resulting in pathophysiological changes in the axon. In conclusion, we have proposed a mechanism of As^{III}-induced neurotoxicity. Future studies will show how Ca^{2+}-influx is affected by arsenite metabolites.

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ERCC2 Deficient Cells React Differently Than *ERCC1* Deficient and Wild Type Cells after Incubation with Arsenite Metabolites

ERCC2 Deficient Cells React Differently than *ERCC1* Deficient and Wild Type Cells after Incubation with Arsenite Metabolites

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Abstract

In this study, we investigated the cytotoxic effects of inorganic As (arsenate, iAs^{V}; arsenite, iAs^{III}) and the methylated As metabolites monomethylarsonic acid (MMA^{V}), monomethylarsonous acid (MMA^{III}) dimethylarsenic acid (DMA^{V}) dimethylarsinous acid (DMA^{III}) in three different Chinese hamster ovary (CHO) cell types: AA8 (wild type), UV20 (*ERCC1* deficient) and UV5 (*ERCC2* deficient). The As metabolites were applied at different concentrations (0 to 100 μM) for 24 hours (n=6). Cytotoxic effects were measured with sulphorhodamine B as an indicator for the number of cells that survived in comparison to the controls. Our results show that in the selected concentration range pentavalent As metabolites; iAs^{V}, MMA^{V} and DMA^{V} are not cytotoxic, unlike the trivalent As metabolites; iAs^{III}, MMA^{III} and DMA^{III}. The measured LC_{50} demonstrated a significant difference ($p<0.001$) for each trivalent metabolite between the three cell lines. UV5 cells also showed a higher resistance to MMA^{III} and DMA^{III} in comparison to AA8 and UV20 cells, whereas iAs^{III} demonstrated a higher level of cytotoxicity than MMA^{III} and DMA^{III} in comparison to AA8 and UV20 cells. This might be explained through the generation of hydrogen peroxide (H_2O_2), which is generated by increase of interacellular Ca^{2+} level. Generation of H_2O_2 after incubation with MMA^{III} and DMA^{III} is significantly lower than iAs^{III} ($p<0.01$). In conclusion, absence of *ERCC2* leads to a reduced generation of H_2O_2 by DMA^{III} and increased H_2O_2 generation by iAs^{III} in UV5 cells, which is in contrast to AA8 and UV20 cells.

Keywords: arsenite and arsenate metabolites, *ERCC1*, *ERCC2*, hydrogen peroxide.

1. Introduction

The very elements in the environment - food, water and air - that are a source of life for man also expose him to arsenic (As) and its derivatives. Arsenic exposure is associated with hypertension, cancer and neurological impairment. Its effect on DNA is indirect, through inhibition of DNA repair mechanisms. Nucleotide excision repair (NER) is a major DNA repair pathway that removes DNA lesions. Arsenic (As) is an environmental chemical of high concern for human health (ATSDR, 2005) and it is a potent toxicant that may exist in trivalent (arsenites) or pentavalent (arsenates) oxidation states and in a number of inorganic and organic forms. Arsenic cannot be destroyed in the environment. It can only change its form, or become attached to or separated from particles. It may change its form by reacting with oxygen or other molecules present in air, water, or soil, or by the action of bacteria that live in soil or sediment. 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: $iAs^{V} \rightarrow iAs^{III} \rightarrow MMA^{V} \rightarrow MMA^{III} \rightarrow DMA^{V} \rightarrow DMA^{III}$. Although there may be differences in the potency of different chemical forms, it is generally considered that arsenites tend to be more toxic than arsenates, and inorganic is more toxic than the organic forms (Aposhian, 1997). Many studies have shown that As can cause DNA damage both *in vitro and in vivo* (Nesnow et al., 2002; Schwerdtle et al., 2003; Palus et al., 2005). Trivalent inorganic As can promote mutagenicity and carcinogenicity of other carcinogens and trivalent As species have high affinity for thiol-groups in proteins. Considerable attention has focused on its interference with DNA repair, especially the nucleotide excision repair (NER) pathway (de Laat et al., 1999; Andrew et al., 2003), whereas less is known about the effect of arsenic on the induction of DNA damage by other agents.

Trivalent arsenic metabolites are a human carcinogen reported to inhibit DNA repair (Walter et al., 2007). The binding of arsenites to functional thiol groups of DNA repair enzymes has in the past been suggested as a possible mechanism for the effect of arsenites on DNA repair (Snyder and Lachmann, 1989).

The focus of the present study is to evaluate the importance of excision repair cross-complementing 1 and 2 (*ERCC1* and *ERCC2*) in the cytotoxic effects of various As metabolites (iAs^{III}, iAs^{V}, MMA^{III}, MMA^{V}, DMA^{III} and DMA^{V}). To that end, *ERCC1* and *ERCC2* deficient cell types were compared to wild type after incubation with various As metabolites and concentrations.

2. Material and methods

2.1 Chemicals

The following arsenic metabolites and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): Sodium meta-arsenite (As^{III}) (catalog no. 228699-100G), Arsenic acid sodium (As^{V}) (catalog no. A6756-50G), Trypsin-EDTA solution 1x (catalog no. T3924). Dimethyl arsenic acid (DMA^{V}) (catalog no. PS-51) and Disodium methyl arsenate (MMA^{V}) (catalog no.: PS-281) were obtained from Chem Service (West Chester, PA, USA). Methylarsinous diiodide (MMA^{III}) and Dimethylarsinous iodide (DMA^{III}) were purchased from Dr. Cullen of the Department of Chemistry at the University of British Columbia, Vancouver, Canada.

2.2. Cell cultures

Three different Chinese hamster ovary (CHO) cell lines; AA8 (wild type; ATCC no. CRL-1859^{TM}), UV20 (*ercc1* deficient; ATCC no. CRL-1862^{TM}) and UV5 (*ercc2* deficient; ATCC no. CRL-1865^{TM}) were purchased from American Type Culture Collection-ATCC (Manassas, VA, USA). Most of the materials for cell culture were purchased from Invitrogen (Breda, Netherlands); other more specific details are enclosed in brackets. The cells were grown in Dulbecco's modified Eagle's medium (DMEM, catalog no. 41966) and supplemented with 10% heat-inactivated fetal bovine serum obtained from Greiner bio-one GmbH (catalog no. 758093) (Frickenhausen, Germany), in turn supplemented with 100 IU/ml penicillin/streptomycin (catalog no. 15140-122), and maintained at 37 °C, 5% CO_2 in a humidified incubator.

2.3. Growth inhibition assay

Cytotoxicity to As metabolites were assessed using the sulphorhodamine B (SRB) growth inhibition assay for each As metabolites. Cytotoxicity was determined by assessing their median lethal concentration (LC_{50}). SRB was used as an indicator for the number of cells that survived in comparison to the controls. On two different occasions, cells were cultivated in triplicate for each As metabolite and concentration in a 96-wells plate (n=6). A total of 1.2×10^3 cells (AA8, UV20, UV5) were seeded into each well of 96-well plates in a volume of 150 µl and incubated at 37°C overnight. Arsenic concentrations of 0, 1, 3, 10, 30, and 100 µM were prepared in culture medium immediately before use and 50µl of drug-medium mixture was added to the appropriate wells. Plates were incubated for 24 h at 37°C. Following drug treatment, the medium was replaced with 200 µl of fresh complete medium, and the plates were incubated for 3 days at 37°C. The growth medium in the wells was removed, and 50 µl of ice-cold 50% (w/v) trichloroacetic acid was added to fix the cells for 1 hour at 4°C. The cells were subsequently washed six times with MQ-H_2O and stained with 50 µl of 0.4% (w/v) SRB-1% acetic acid for 20 min. at room temperature. Unbound dye was removed by washing 6 times with 1% acetic acid, and plates were dried. The dye was dissolved by the addition of 150 µl of 10 mM Tris-base into each well. Plates were incubated for 20 min. at room temperature, and the optical density (OD) at 570 nm was measured with the SpectraMAX 250 Microplate Spectrophotometer from Molecular Devices (Wokingham, UK) (Fig. 1A-C).

2.4. Detection of peroxide generation

Peroxide generation by As metabolites was assessed using dihydrorhodamine 123 (DHR123) as an indicator for H_2O_2 generation through its conversion to rhodamine 123 (R123) (Sakurada et al. 1992; Smit et al. 2006). From each cell line, the same amount of cells $–1.10^4$ – was added in 0.2 ml medium into each well on a 96-wells plate and incubated for 24 h at 37 ºC in a 5% CO_2 incubator. Two 96-wells plate in triplicate (n=6) were incubated with the various trivalent As metabolites at a concentration of 3 µM and 1 µM DHR123 for duration of 4 hours. The fluorescence absorbance caused by conversion of DHR123 to R123, was analyzed by PerkinElmer model Victor^2 1420-012 Multilable Counter (Groningen, Netherlands) in the 96-wells plates at 485/535 nm (Fig. 2).

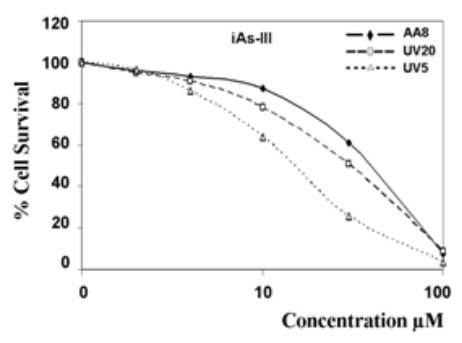
2.5. Statistical analysis.

The LC_{50} and the H_2O_2 generation in the three cell lines were statistically evaluated for each metabolite using the SPSS 14.0 for Windows. Chi-square for non-parametric tests (Kruskal-Wallis test) was performed to evaluate the data. A level of $p<0.05$ was accepted as statistically significant.

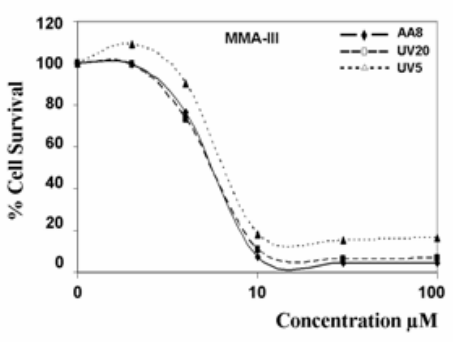
3. Results

3.1. Growth inhibition assay

The LC_{50} was determined only for trivalent As metabolites, since pentavalent As metabolites did not reach complete lethality at the highest concentration of 100 µM. The LC_{50} estimation was carried out by plotting the measured absorbance associated with the different concentrations (Fig 1A-C). The LC_{50} values for each trivalent As metabolite are shown in Table 1.



1A.



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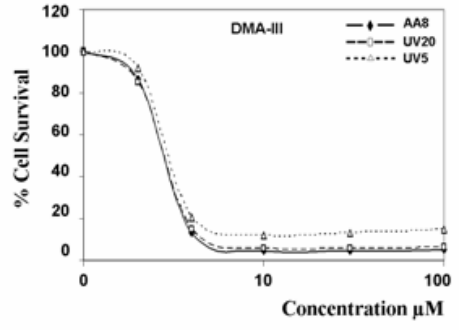

1C.

Fig 1. The increasing concentrations of the three trivalent arsenic metabolites iAs^{III} (A), MMA^{III} (B) and DMA^{III} (C) results in decreased sulphorhodamine B (n=6 for each concentration). The data obtained from the growth inhibition assay are used to determine the LC_{50}.

Table 1. LC_{50} of various metabolites in three different CHO cell types.

Metabolite (n=6)	AA8	UV20	UV5
iAs^{III}	38.2 ± 5.6	30.9 ± 3.4	14.9 ± 1.4
MMA^{III}	4.9 ± 0.3	4.8 ± 0.2	6.9 ± 0.3
DMA^{III}	1.5 ± 0.1	1.5 ± 0.1	2.1 ± 0.1

Each arsenite metabolite (iAs^{III}, MMA^{III}, and DMA^{III}) showed significant differences between the three CHO cell types ($p<0.001$). Mean values (± SD) for the number of determined LC_{50} (n=6).
At 100μM, no significant cell death was observed for iAs^{V}, MMA^{V}, and DMA^{V}. Therefore, their LC_{50} could not be determined.

3.2. H_2O_2 generation by arsenite metabolites

Hydrogen peroxide generation, as a result of incubation with 3 μM arsenite metabolites, was measured and expressed as mean fold increase (MFI). They demonstrated a significantly ($p<0.01$) higher H_2O_2 generation by iAs^{III} in UV5 cells was increased 3.45 MFI), while generation by iAs^{III} was only increased by 2.31 and 2.33 MFI in AA8 and UV20, respectively. The H_2O_2 generation by MMA^{III} showed no significant increase between the three cell lines, 2.83, 2.69 and 2.81 MFI for AA8, UV20 and UV5, respectively. The H_2O_2 generation by DMA^{III} was significantly ($p<0.01$) increased for the three cell lines in comparison to control, 3.87, 2.97 and 1.41 MFI for AA8, UV20 and UV5, respectively (Fig. 2).

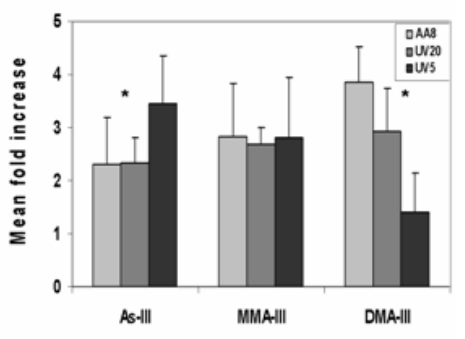

Fig 2. Mean fold increase of hydrogen peroxide generation by various As metabolites (± SD; n=6). Arsenite metabolites iAs^{III} DMA^{III}, showed a significant difference ($p<0.01$, *) between the various cell types (AA8, UV20 and UV5) at 3 μM concentration and control groups. However, incubation with MMA^{III} showed no significant changes.

4. Discussion

Nucleotide excision repair is a major DNA repair pathway that removes DNA lesions including certain DNA crosslinks, UV photolesions and bulky chemical adducts (Mullenders et al., 2001). Arsenic affects expression of a variety of genes through modification of the transcription factor expression and activity as well as the methylation status of the cell (Hughes, 2002).

The LC_{50} results are evaluated in two different manners. On the one hand, we examined each As metabolite separately in the three cell types (Table 1, per row), and on the other, we checked the effects of the three metabolites per cell type (Table 1, per column). Our results showed that the absence of *ERCC1* did not contribute to the cytotoxic effects of the arsenite metabolites in comparison to wild type (Table 1). The mono- and dimethylated arsenites were significantly more cytotoxic to the AA8 and UV20 (*ERCC1* deficient) cells ($p<0.001$). The differences between the metabolites per cell type are also statistically significant ($p<0.001$). The UV5 cells, which are *ERCC2* deficient, showed more resistance to methylated arsenites in comparison to AA8 and UV20 cells. Results could be summarized as follows: in CHO cells, methylated arsenites are more cytotoxic than its inorganic form. However, iAs^{III} is more cytotoxic to UV5 cells than to AA8 and UV20 cells. Whereas, MMA^{III} and DMA^{III} are less cytotoxic to UV5 cells than AA8 and UV20 cells.

Production of reactive oxygen species (ROS), such as hydroxyl radicals ($OH^{\cdot}$), superoxide anions (O_2^{-}), and hydrogen peroxide (H_2O_2), are a normal byproduct of aerobic metabolism in all eukaryotic organisms. However, elevation of intracellular ROS may also occur through exposure to environmental toxicants, such as arsenic (Schwerdtle et al., 2003; Hei and Filipic, 2004).
The measured LC_{50} for AA8 (wild type) and the UV20 (*ERCC1* deficient) cells show more or less the same cytotoxicity pattern with the three arsenite metabolites in contrast to UV5 (*ERCC2* deficient) cell type. A possible explanation can be deduced from the levels of H_2O_2 generation in the CHO cells. At a concentration of 3 µM of arsenite metabolite, the measured H_2O_2 generation in MFI for iAs^{III} was somewhat higher in UV5 cells (3.45 MFI) in comparison to AA8 and UV20 cells (2.31 and 2.33, respectively). The H_2O_2 generation by MMA^{III} in all three cell lines was significantly increased in comparison to controls but not in comparison to each other. This correlates with the determined LC_{50}'s in all three cell lines. The measured H_2O_2 generation in MFI for DMA^{III} was somewhat lower in UV5 cells (1.41 MFI) in comparison to AA8 and UV20 cells (3.87 and 2.97, respectively).
As yet, it is not clear why the absence of *ERCC2* leads to a reduced generation of H_2O_2 by DMA^{III} and increased H_2O_2 generation by iAs^{III} in UV5 cells, which is in contrast to AA8 and UV20 cells. However, all eukaryotic organisms have multiple cellular mechanisms to prevent the excessive accumulation of ROS and protect against their harmful effects. Antioxidant defense mechanisms include those that involve nonenzymatic molecules such as glutathione and several vitamins, as well as ROS scavenger enzymes such as superoxide dismutase and catalase, and glutathione peroxidase (Anderson and Phillips 1999). Ercc2 protein is also a subunit of the basal transcription factor TFIIH, a large complex involved in the initiation of transcription, unlike ercc1 protein. It is possible that the absence of *ERCC2* in UV5 cells and its involvement in the initiation of transcription, could lead to a compensatory mechanism by which these antioxidants are more active. This possibility is currently under investigation.

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Chapter 8

General Discussion and Summary

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Arsenic

Arsenic is a metalloid with the symbol As and atomic number 33. It has an atomic mass of 74.92. It has been known since ancient times, though probably in compound form rather than in its pure state. Arsenic has a long history of medical applications; before penicillin was developed an arsenic compound was used to treat syphilis. Arsenic has also been used in combination with other materials in pigments, poison gases and insecticides (such as Paris Green, calcium arsenate, lead arsenate and lewisite as chemical agent for warfare) and is well known from former use as a rat poison. In forensic science, it is well-known from a great number of homicidal cases over many centuries. Arsenic is known to be used as a preservative in tanning and taxidermy, as well as in Wolman's salt as a wood preservative and even playground materials. Other uses of As compounds are or have been used in ammunition manufacturing. Its use in combination with other metals helps to improve the hardness of bullets.

Arsenic is a notoriously poisonous metalloid with known hazardous effects to human health. The element exists in both organic and inorganic forms and either form can also exist in trivalent (-3, +3 or arsenite, As^{III}) or pentavalent oxidation state (+5 or arsenate, As^{V}). In many animal 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: $iAs^{V} \rightarrow iAs^{III} \rightarrow MMA^{V} \rightarrow MMA^{III} \rightarrow DMA^{V} \rightarrow DMA^{III}$. Although there may be differences in the potency of different chemical forms, it is generally considered that arsenites tend to be more toxic than arsenates and inorganic is more toxic than the organic form. Intoxication with As may occur in the *acute* (short-term) and *chronic* (long-term) forms, which are separate syndromes. Acute As exposure may cause nausea, vomiting, diarrhea, weakness, loss of appetite, shaking, cough, headache and neuropathy. Chronic exposure may lead to a variety of symptoms including skin pigmentation, numbness, and cardiovascular disease. It is also known to cause various forms of cancer such as skin (non-melanoma type), kidney, bladder, lung, prostate and liver cancer and, again, neuropathy.

The presence and levels of As can be analyzed in urine, blood and hair. Tests may either be devised to measure the total As content in tissues or bodyfluids, or the different types of arsenic species. For total As concentration measurement is carried out with atomic absorption spectrometry (AAS) in various samples. Atomic absorption spectroscopy is based on the absorption of light to measure the concentration of gas-phase atoms. Since samples are usually liquids or solids, the analyte atoms or ions must be vaporized in a flame or graphite furnace. The atoms absorb ultraviolet or visible light and make transitions to higher electronic energy levels. The analyte concentration is determined from the amount of absorption. Applying the Lambert-Beer law directly in AAS is difficult due to variations in atomization efficiency from the sample matrix, and non-uniformity of concentration and path length of analyte atoms (in graphite furnace AAS). Concentration measurements are usually determined from a calibration curve after calibrating the instrument with standards of known concentration. Prior to quantification of the analyte by AAS it is usually necessary to destroy the organic matrix and bring the element into inorganic solution. In many cases, As samples are usually predigested with a digestive acid such as a mixture of perchloric and nitric acid (Sakamoto et al. 2001). The samples for As analysis prior to measurements are added to the

calibration matrix consisting of 1M HCl (reduction acid) in order to convert all the pentavalent to trivalent As.

When studying the hazardous effects of various As metabolites, determination of total arsenic concentration needs to be completed with the analysis of individual arsenic species and compounds. Various techniques such as high pressure liquid chromatography (HPLC) coupled with ICP-MS, ICP-AES or AAS are most suitable for the quantification of arsenic species (Londesborough et al. 1999).

Arsenic and neurotoxicity

Exposure to As may affect both the central and peripheral nervous systems; symptoms include tremors, headaches, numbness, irritability, muscular weakness, convulsions and coma. Although peripheral nervous system (PNS) impairment is common in As-exposed populations, its mechanism is poorly understood. Arsenic effects manifest themselves weeks after first exposure as both central and peripheral neuropathy. The most frequent neurological manifestation by As is peripheral neuropathy that may last for several years, if not life-long. Peripheral neuropathy may lead to rapid severe ascending weakness, similar to the Guillain-Barré syndrome, requiring mechanical ventilation (artificial respiration). From human clinical cases studied by Le Quesne and McLeod (1977) it has become clear that As exposure results in a late reaction of the nervous system, which was established by decrease in their nerve conducting velocity (NCV) measurements (Le Quesne & McLeod 1977). Patients exposed to As show significantly lower Nerve Conduction Velocities (NCVs) in their peripheral nerves in comparison to their referents (Greenberg 1996; Tseng et al. 2006; Otto et al. 2007). Although this reduction in NCV is a hallmark in As-induced neurotoxicity, its mechanism of action on the molecular level is not known. Therefore, the project described in this thesis was aimed at elucidating the probable mechanism of As-induced neurotoxicity in animals *in vivo* and in cell cultures *in vitro*.

The animal studies in this thesis were designed to answer questions about the effect of As on the peripheral nervous system after sub-acute and chronic intoxication of laboratory rats. Axonal integrity is maintained by three categories of cytoskeletal proteins: neurofilaments, which are found in high concentrations along the axons, microtubules and actin filaments (microfilaments). Neurofilaments are specific to neurons and 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, 68 kDa) plus either Neurofilament-Medium (NF-M, 150 kDa) or Neurofilament-Heavy (NF-H, 200 kDa) proteins. During axonal growth, new neurofilament subunits are incorporated all along the axons. In an intact nerve, the level of neurofilament proteins control how fast electrical signals travel down the axon. Microtubules consist of tubulin subunits. Their stability is regulated by the interaction with microtubule-associated proteins (MAPs). MAP function includes both stabilizing and destabilizing microtubules, guiding microtubules toward 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. They are required for tubulin assembly into the microtubules and stabilize the assembled microtubules.

Chapter 3 of this thesis describes the compositional changes in rat sciatic nerves after a single exposure to arsenite i.v. with various doses. As a first step in this study, the short- and long-term effects of arsenite on rat sciatic nerve proteins were studied as a model for peripheral axonopathy. Male Wistar rats were exposed to inorganic arsenite (iAs^{III}) given as a single dose i.v. dissolved in PBS (between 0 and 20 mg/kg) in a tail vein. The doses used for short-term single arsenic exposure were 0-, 15- and 20-mg/kg iAs^{III} (n=3). The long-term single exposures were 0-, 3- and 10-mg/kg iAs^{III} (n=9). 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. Afterward, rats that received the same dose were combined into one group and housed for 2, 3 and 4 weeks in plastic cages on sawdust. The difference between the long- and short-term studies is the result of the variation in dose range and the duration of urine collection. 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 to the control groups. Some fibroblast protein bands with band sizes of 40 and 140 kDa degraded after a high dosage in the 20-mg/kg dose group. The analyzed neurofilament-M and -L proteins decreased dose-dependently over time. Arsenic affects the composition of proteins in the rat sciatic nerve, especially the neurofilaments. The reduction of signals in western blot analysis revealed changes in cytoskeletal composition, which may well lead to neurotoxic effects *in vivo*.
Chapter 4 of this thesis describes studies on the compositional changes in rat sciatic nerves after semi-chronic exposure. In our previous study in rats exposed to As, we observed an effect of As on neurofilaments in the sciatic nerve. This study deals with the effects of iAs^{III} in Wistar rats on the cytoskeletal protein composition of the sciatic nerve after subchronic intoxication. Inorganic arsenite dissolved in phosphate-buffered saline (PBS) was orally 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. The control group received only PBS without added arsenite. Toxicokinetic measurements revealed a saturation of blood As levels 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 NF-H and NF-M remained 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 phosphorylated.

These two *in vivo* studies present direct evidence for the As effect on the neuronal skeleton, as a basis for neurotoxicity. The first real proof of As involvement was the elevated As measurements in the sciatic nerve tissues of the rats in sub-acute and semi-chronic induced-toxicity in these rats. The second evidence was achieved by the decrease in cytoskeletal NF-L protein. Several studies that were performed to elucidate the role of neurofilament proteins have concluded that both NF-H and NF-M each need a NF-L protein to form a heteropolymer (Carpenter & Ip 1996). The third evidence was achieved through the (hyper) phosphorylation of MAP-tau and NF-L. Phosphorylation of the MAP-tau and NF-L proteins leads to conformational changes leading to destabilization and disruption of the cytoskeletal framework. These results suggest that the mechanism of As induced-neurotoxicity lays in the cytoskeletal proteins that have been affected by As in various ways.

In the in vitro studies, effects of other As species were tested in various cell culture models (chapter 5) and the manner of their hyperphosphorylation was further studied (Chapter 6) for a better understanding of the disruption of neuroskeletal integrity by As.
Chapter 5 of this thesis describes the 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 As 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. 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 in triplicate. Afterward, cDNAs were synthesized from the isolated RNA to determine their relative quantification (RQ) and follow the changes in expression of these genes under influence of the various As metabolite and concentrations. Our results showed that iAs^{III} and iAs^{V} have no significant effects on either cell lines. Conversely, 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. Increase in NF-H may suggest a compensatory mechanism. However, this needs further study.

In chapters 3 to 5 we have demonstrated that arsenic metabolites change the composition of cytoskeletal proteins *in vivo* and *in vitro*. Furthermore, in chapter 4 we demonstrated that calpain expression is increased with the increase of As dose and exposure time. To further examine the mechanism of arsenic-induced neurotoxicity with various As metabolites (iAs^{III}, iAs^{V}, MMA^{III}, MMA^{V}, DMA^{III} and DMA^{V}), we studied the role of p35 and calpain enzyme (chapter 6) and its involvement in hyperphosphorylation of cytoskeletal proteins. Calpain is a calcium-activated cytoplasmic protease that seems to be involved in some neurodegenerative diseases such as Alzheimer disease (AD) (Lee et al. 2000). Alzheimer patients form neurofibrillary tangles through tau hyperphosphorylation. Calpain has also been shown to be 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). 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). Moreover, 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.

Another approach to destabilization and disruption of the cytoskeletal framework is through phosphorylation of cytoskeletal proteins. In a normal situation, p35 binds to cyclin-dependent kinase 5 (Cdk5), which is responsible for neurite-outgrowth. In diseased patients, p35 is cleaved to p25 by calpain, whereby p25 binds to Cdk5, resulting in hyper-activation of Cdk5 and hyperphosphorylation of tau and neurofilament proteins (Fig. 1)

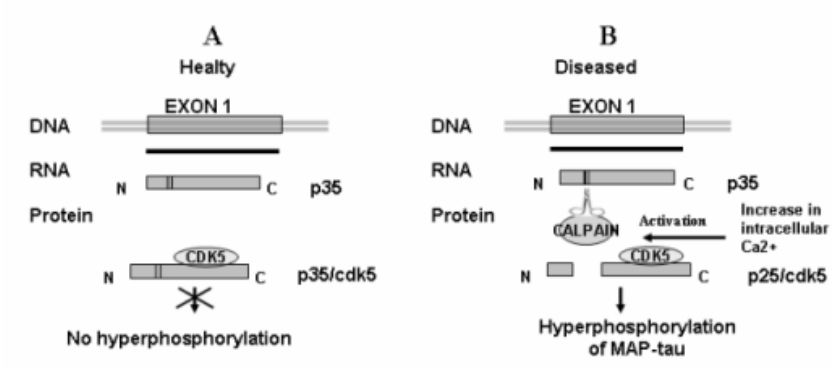

Fig.1. Cleavage of p35 to p25 by calpain. A. Healthy subjects where complex of p35/cdk5 is responsible for the neurite outgrowth. B. Influx of Ca^{2+} in patients with Alzheimer's disease, for example, results in activation of calpain protease. Calpain cleaves p35 to p25, whereby the p25/Cdk5 complex results in hyperphosphorylation of MAP-tau proteins.

In chapter 6 (Arsenic Neurotoxicity IV), we tried to examine whether the cleavage of p35 to p25 can be induced by As. Furthermore, we studied mRNA expression levels of calpain, cyclin-dependant kinase 5 (Cdk5) and glycogen synthase kinase 3 beta (Gsk3ß). A transfected HeLa cell line with a p35 construct (HeLa-p35) was used as a model, since all other necessary proteins such as calpain, Cdk5 and Gsk3ß are already present in HeLa cells. HeLa-p35 cells were incubated with various As metabolites and concentrations of 0, 10 and 30 μM for duration of 5 hours, after which the cells were either lysed to analyze p35 protein expression or examined on the relative quantification levels of the genes.
Calpain activation may contribute to As-induced neurotoxicity in two ways. As shown in chapter 4, incubation with As leads to hyperphosphorylation of MAP-tau and NF proteins, resulting in deregulation and disorganization of the cytoskeletal framework on the one hand. On the other, it causes calpain activation-induced degradation of NF-L protein (Kunz et al. 2004). These authors have shown that calpain inhibition prevents inflammation-induced NF-L breakdown in the spinal cord. This may suggest that inorganic arsenic causes degradation on the protein level *in vivo* by activating proteases such as calpain. In chapters 3 and 4, we showed that NF-L protein expression was decreased after treatment with iAs^{III}. It is reasonable to suggest that since As results in activation of calpain through influx of Ca^{2+} that calpain is responsible for NF-L degradation in a calcium-induced proteolytic process. The results found in our *in vivo* and *in vitro* experiments suggest that As exerts its toxic effects in two different manners depending on the As species. Rats treated with iAs^{III} showed a decrease in NF-L expression on their protein level, while the *in vitro* study with iAs^{III} (chapter 5) showed no changes in expression on the mRNA level after treatment with inorganic As.

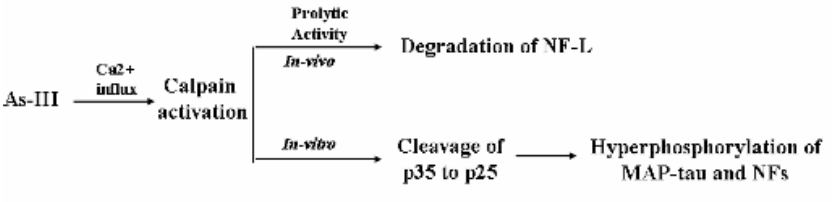

Fig. 2. Two probable mechanism of As^{III} induced neurotoxicity with involvement of calpain activity. As^{III} results in decrease of NF-L protein, which could be the result of a calpain-induced proteolytic process. As^{III} induces hyperphosphorylation of cytoskeletal proteins through calpain-induced activation of the cleavage of p35 to p25.

Effects of As on the DNA level is not reserved to cytoskeletal genes only. Arsenic is also known to be carcinogenic and to be involved in inhibition of DNA repair mechanisms (Andrew et al. 2006). However, the carcinogenic mechanism of As has yet to be fully understood. Many studies have shown the adverse effects of As on chromosomal and DNA level (Colognato et al. 2007; Yedjou & Tchounwou 2007) and to some extent on the effects of nucleotide excision repair and As. Andrew *et al.* (2006) have shown that people exposed to As in drinking water have decreased DNA repair abilities, namely the decrease of excision repair cross-complementing 1 (ERCC1) (Andrew et al. 2003; Andrew et al. 2006). In chapter 7 of this thesis, we describe the effect of various As metabolites (iAs^{III}, iAs^{V}, MMA^{III}, MMA^{V}, DMA^{III} and DMA^{V}) on three Chinese hamster ovary (CHO) cell lines, AA8 (wild type), UV20 (ERCC1 deficient) and UV5 (ERCC2 deficient). Cytotoxicity to the As metabolites was assessed by determining the concentration at which 50% of the cells in culture is killed (LC_{50}). The LC_{50} was only determined for trivalent As metabolites, since

pentavalent As metabolites did not reach complete lethality at the highest concentration. Our results showed that in CHO cells, methylated arsenites are more cytotoxic to AA8 and UV20 cell types than As^{III} in the inorganic form, while absence of *ERCC2* in UV5 cell types contributes to higher resistance to methylated arsenite species.

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Chapter 9

Nederlandse Samenvatting

Nederlandse Samenvatting

Achtergrond

Arsenicum of arseen is een scheikundig element met symbool As en atoomnummer 33. Het woord arseen is afgeleid van het Perzische woord zarnikh (زرنیخ), dat later via de Arabische taal (al-zarnikhu) door de Grieken als Arsenikon (‘αρσενικόν) geïntroduceerd werd in de westerse talen. De oude Grieken gebruikten arseen onder meer als make-up. Het element is door de eeuwen heen veelal gebruikt voor veel verschillende doeleinden. Arseen is een van de oudste giften die de mens bekend zijn. Het werd veelal gebruikt als bestanddeel van tonica in de geneeskunde, ook in de huidige traditionele Chinese en Indische geneeskunde. De therapeutische waarde van arseen in de vorm van Salvarsan (3.3'-Diamino-4.4'-dihydroxy-arsenobenzeen) bleek bij de behandeling van syfilis. Een andere toepassing is het gebruik als pesticide, dat later werd afgeschaft vanwege de schadelijke bijwerkingen voor de mens. Arseen is en wordt ook gebruikt als moordmiddel. Een recent voorbeeld hiervan is de zaak van de Indonesische mensenrechtenactivist Munir, die op weg van Jakarta naar Amsterdam op 7 september 2004 in het vliegtuig dodelijk vergiftigd werd. Misschien een van de meest bekende slachtoffers van arseen is Napoleon Bonaparte, die vermoedelijk chronisch vergiftigd werd, hetzij opzettelijk, hetzij accidenteel door arseen uit het behang van zijn verbaningsoord. Een ander bekend voorbeeld van arseenvergiftiging is het werk van de Leidse gifmengster; Maria Catharina van der Linden – Swanenburg, beter bekend als Goeie Mie. Door haar grote hulpvaardigheid, waaraan ze ook haar bijnaam "Goeie Mie" dankte, bouwde ze een grote kring aan buren, kennissen en vrienden op. Goeie Mie leefde in de 19de eeuw, was getrouwd en had twaalf kinderen, maar zes daarvan stierven al op jeugdige leeftijd, zoals in die tijd vaak voorkwam. Haar wens voor een minder armoedig bestaan en om aan haar alcohol behoefte te kunnen voldoen, leidde tot het stelselmatig vergiftigen van familieleden en buurtbewoners, maar niet nadat zij een verzekering op hun leven had afgesloten met zichzelf als begunstigde. Tussen 1867 en 1884 vergiftigde zij minstens 102 van de door haar verzekerden met arseen, dat ze in de koffie, melk of erwtensoep strooide. Van de vergiftigde mensen werden er 45 ernstig ziek met neurologische restverschijnselen en 27 mensen overleden als gevolg van arseenvergiftiging, waaronder 16 familieleden.

Arseen komt voor als anorganische en organische verbindingen. Organisch As onderscheidt zich van anorganisch As door de aanwezigheid van één of meer methylgroepen aan het arseenatoom. Bij één methylgroep spreekt men van monomethyl- (MM-) en bij twee van dimethyl- (DM) arseen. Verder kennen deze arseenverbindingen verschillende oxidatietoestanden, driewaardig (arseniet; As^{III}) en vijfwaardig (arsenaat; As^{V}). Een algemeen geldende regel is dat een anorganische arseenverbinding meer toxisch is dan de overeenkomstige gemethyleerde arseenverbinding, en voorts dat het driewaardige arseen toxischer is dan de vijfwaardige verbinding. Bij inname van arseen door mensen ondergaat As een reeks van metabole stappen waarbij anorganisch arseen wordt omgezet in organisch arseen als volgt:

$$aAs^{V} \rightarrow aAs^{III} \rightarrow MMA^{V} \rightarrow MMA^{III} \rightarrow DMA^{V} \rightarrow DMA^{III}$$

Deze arseenverbindingen komen in variabele hoeveelheden in de natuur voor. Arseen bevindt zich in de bodem, en het kan via stof en water in de lucht en op het land terecht komen.

Mensen kunnen via voedsel, water en door inademing blootgesteld worden aan arseen. Expositie kan ook plaats vinden via huidcontact met besmette grond of water. Bij langdurige blootstelling kunnen verschillende aandoeningen ontstaan, zoals ontwikkeling van verschillende soorten kanker, variërend van huidkanker tot maligniteiten van de organen en natuurlijk neurologische aandoeningen.

Chronische blootstelling aan arseen is een wereldwijd probleem. Miljoenen mensen in Aziatische landen als Bangladesh, India en Taiwan en zelfs in moderne industriële landen zoals Amerika en Duitsland hebben gezondheidsproblemen door blootstelling aan arseen. De herkomst van arseen kan herleid worden tot het drinkwater in vele derde-wereldlanden, waarbij hun drinkwater dat afkomstig is van natuurlijke geologische bronnen is verontreinigd met arseen. In het jaar 2000 sloeg de Wereld Gezondheid Organisatie (WHO) groot alarm: de helft van de bevolking van Bangladesh werd met ziekte bedreigd door arseenblootstelling. Oorzaak van de massavergiftiging waren de miljoenen waterputten die in het kader van een WHO-project daar in de afgelopen jaren waren geslagen, bedoeld als verbetering van de drinkwatervoorziening. Een concentratie kleiner dan 10 delen per miljard (parts per billion, ppb) is volgens de WHO een toelaatbare hoeveelheid arseen in grondwater dat gebruikt wordt als drinkwater; helaas komen in vele delen van Bangladesh concentraties hoger dan 50 ppb voor.

Arseen en Neurotoxiciteit

Blootstelling aan arseen beïnvloedt zowel het centrale als het perifere zenuwstelsel. Effecten op het zenuwstelsel manifesteren zich ondermeer als trillingen, stuiptrekkingen, hoofdpijn en gevoelloosheid, spierzwakte en uiteindelijk coma. De meest voorkomende neurologische manifestatie van arseenvergiftiging is op het perifere zenuwstelsel. Deze symptomen kunnen jarenlang aanhouden, als het niet levenslang is. Deze kenmerken zich vooral door effecten in de lagere extremiteiten. Klinische studies in met arseen vergiftigde populaties hebben aangetoond dat arseen leidt tot een verlate manifestatie in zenuwen die weken tot maanden later waarneembaar is. Bij deze patiënten is de geleidingssnelheid in de zenuwbanen aanzienlijk afgenomen in vergelijking tot gezonde mensen. Hoewel deze symptomen bij arseenvergiftiging goed beschreven zijn, is het mechanisme van arseen-neurotoxiciteit op moleculair niveau niet goed begrepen en onderzocht. Daarom wordt in dit proefschrift getracht de mogelijke mechanismen die hieraan ten grondslag liggen te vinden en verklaren.

De opzet van de studies die beschreven staan in dit proefschrift is tweeledig. Enerzijds hebben zij plaats gevonden in ratten (*in vivo*) als model voor het aantonen van effecten van anorganisch arseniet (aAs^{III}) op zenuwen op eiwitniveau (hoofdstuk 3 & 4). Anderzijds zijn studies uitgevoerd in neuronale en niet-neuronale cellen (*in vitro*) voor nader onderzoek van effecten van verschillende arseenverbindingen op moleculair niveau (Hoofdstukken 5, 6 & 7).

De studies in ratten waren ontworpen ter verduidelijking van acute en subchronische effecten van arseen op de axonen van het perifere zenuwstelsel. Veranderde axonale integriteit kan een mogelijke verklaring en benadering zijn ter verduidelijking van het mechanisme van arseen in de zenuwen. Axonen bevatten structuren die zijn samengesteld uit een aantal eiwitgroepen, deze zorgen voor de integriteit en het transport in de zenuwen. Dit zogenaamde cytoskelet is opgebouwd uit neurofilamenten, microtubuli en actine-filamenten (microfilamenten). Neurofilamenten zijn specifiek voor neuronen en ze zijn in grote hoeveelheden aanwezig in de neuronen. Er bestaan drie soorten neurofilament-eiwitten die samen een netwerk van heteropolymeren maken; neurofilament-laag (NF-L; 68 kDa), neurofilament-medium (NF-M;

150 kDa) en neurofilament-hoog (NF-H; 200 kDa). Bij een intact neuron controleert dit netwerk van neuronfilament-eiwitten het elektrische signaal en het axonale transport. Microtubuli bestaan uit tubuline-eiwitten. De stabiliteit van deze microtubuli wordt gereguleerd door interacties met microtubulus-geassocieerde eiwitten (MAPs), met MAP-tau als neuron-specifieke vorm.

In hoofdstukken 3 & 4 worden de resultaten weergegeven van onderzoek naar de effecten van arseen op deze eiwitten in ratten. Hoofdstuk 3 beschrijft het acute effect van aAs^{III} na intraveneuze toediening die leidt tot verandering van eiwitsamenstelling in de heupzenuw (*N. ischiadicus*). De hoogste doseringen van 15 en 20 mg/kg leiden tot afname van de NF-M- en NF-L-eiwitexpressie. Verlaging van NF-L is ook beschreven in hoofdstuk 4, waarbij ratten een lagere chronische dosering van 3 en 10 mg/kg oraal toegediend hebben gekregen. Verlaging van NF-L speelt mogelijk een belangrijke rol in de neurotoxiciteit van arseen, aangezien NF-H en NF-M beide een NF-L eiwit nodig hebben tot vorming van een heteropolymeer en het behouden van de integriteit van het cytoskelet. Verder onderzoek zoals in hoofdstuk 4 beschreven heeft ook laten blijken dat MAP-tau en NF-L beide sterker gefosforyleerd worden onder invloed van arseen in vergelijking tot de controlemonsters. Fosforylering van eiwitten kan leiden tot conformatieveranderingen, waarbij ze in mindere mate toegankelijk zijn voor interacties met andere eiwitten en dus bijdragen aan het destabiliseren van de zenuwintegriteit.

En mogelijke verklaring kan zijn de activatie van het enzym calpaine, dat een calcium-afhankelijk protease is. Verschillende studies door anderen hebben aangetoond dat arseen de interne calciumconcentratie in de cellen verhoogt, waardoor calpaine wordt geactiveerd, leidende tot NF-L afbraak en MAP-tau en NF-L hyperfosforylatie. Aan de hand van deze resultaten kan worden gesuggereerd dat NF-L afname een posttranslationele activiteit op eiwit niveau is en niet op dat van DNA/RNA. Deze conclusie wordt verder bevestigd door de verhoogde calpaine-expressie onder invloed van chronische aAs^{III} toediening, zoals is aangetoond in hoofdstuk 4. De afname van NF-L is een gevolg van verhoogde afbraak door calpaine, en niet door verlaagde NF-L expressie op RNA-niveau, zoals is aangetoond in hoofdstuk 5.

In hoofdstuk 6 wordt de mogelijke verklaring beschreven van hyperfosforylering van MAP-tau en NF-L onder invloed van arseen. Cycline-afhankelijke kinasen (Cyclin-dependent kinases; CDK) zijn verantwoordelijk voor de fosforylering van vele eiwitten op hun serine- en threonine-plaatsen. Een voorbeeld van een dergelijk kinase is CDK5 met zijn genactivator p35. In hoofdstuk 6 is beschreven dat onder invloed van verschillende arsenietmetabolieten, de p35 activator/regulator van CDK5 toeneemt in expressie. Een verhoogde expressie kan leiden tot verhoogde fosforylering van cytoskeleteiwitten, in het bijzonder MAP-tau en NF-L. Hieruit blijkt dat de verhoogde expressie *alleen* door driewaardig en *niet* door vijfwaardig arseen veroorzaakt wordt. Bovendien wordt p35 door calpaine gesplitst in p25 en p10. P25 is ook na splitsing in staat om complex te vormen met CDK5 dat leidt tot hyperactivatie en hyperfosforylering van cytoskeleteiwitten.

In hoofdstuk 7 wordt een zijstap gemaakt van de hoofdlijn arseen en neurotoxiciteit, en wordt er gefocust op de cytotoxiteit van arseenmetabolieten in relatie tot DNA-herstelmechanisme. Allerlei studies hebben gesuggereerd dat arseen indirect DNA-schade veroorzaakt, door het DNA-herstelmechanisme te verstoren. Helaas zijn er in dit verband geen volledige studies te vinden, waarbij alle arseenmetabolieten getest worden. In deze oriënterende studie hebben we gebruik gemaakt van een drietal celtypen afkomstig van Chinese-hamsterovaria, AA8 (wild type), UV20 (*ERCC1* deficiënt) en UV5 (*ERCC2* deficiënt) in combinatie met de zes arseenmetabolieten in een concentratiereeks van 0 tot 100 µM. Arsenaatmetabolieten laten zien dat ze bij de maximale concentratie van 100 µM niet cytotoxisch zijn.

Arsenietmetabolieten laten daarentegen een bijzonder beeld zien met deze drie type cellijnen. Bij AA8 en UV20 komen de groeiinhibitiecurven overeen voor de drie arsenietmetabolieten i.t.t. UV5 cellijn, waarbij een tegengesteld beeld te zien is. De gemethyleerde arseenmetabolieten zijn minder toxisch dan anorganisch arseen in de UV5 cellijn. Een mogelijke verklaring hiervoor is dat in de UV5 cellijn minder waterstofperoxide wordt gegeneerd door de gemethyleerde arsenietverbindingen dan door de anorganisch arseniet. Dit is tegengesteld aan de productie van waterstofperoxide in de beide andere cellijnen onder invloed van arseniet..

Samenvattend kan gesteld worden dat ons onderzoek een bijdrage heeft geleverd tot het begrijpen en verklaren van het mechanisme van arseenneurotoxiciteit, oftewel; het *wat* en het *hoe*. Door eerst het effect op eiwitniveau in de zenuwen te bevestigen werd het *wat* gedeelte van de vraag behandeld, vervolgens werd het *hoe* verklaard door de afbraak en fosforylering van de zenuweiwitten. Hopelijk kunnen deze studies een bijdrage leveren aan het verdere onderzoek naar de mogelijke behandeling van arseenneurotoxiciteit.

CURRICULUM VITAE

Ali Vahidnia werd op 13 oktober 1972 geboren te Amman, Jordanië. Als zoon van een diplomaat is hij samen met het hele gezin teruggekeerd naar zijn vaderland, Iran, na de revolutie van 1979. In 1986 is hij na 1,5 jaar verblijf in Turkije samen met een van zijn zussen, Farahnaz Vahidnia, in Nederland terechtgekomen. Vanaf augustus 1989 tot en met mei 1993 volgde hij het Voortgezet Wetenschappelijk Onderwijs (VWO) aan de Berlage Scholengemeenschap te Amsterdam. Na het behalen van zijn VWO-diploma is hij Medische Biologie gaan studeren aan de faculteit Biologie van de Universiteit van Amsterdam.

Zijn eerste wetenschappelijke stage van 9 maanden liep hij bij Solvay Pharmaceuticals in Weesp, bij de afdeling 'Drugs safety' onder begeleiding van Dr. H. Keizer. Hier heeft hij gewerkt aan een kunstmatig model voor passief geneesmiddelentransport *in vitro*. Voor zijn tweede wetenschappelijke stage koos hij voor het fundamentele onderzoek bij het Academisch Medisch Centrum in Amsterdam, afdeling biochemie. Hier heeft hij onderzoek gedaan naar de expressie van bacterieel glycogeensynthase.
In augustus 2002 heeft hij het doctorale examen voor Medische Biologie behaald en op 1 januari 2003 is hij begonnen als Toxicoloog in Opleiding bij de afdeling Klinische Farmacie en Toxicologie van het Leids Universitair Medisch Centrum, onder supervisie van Dr. G.B. van der Voet en Prof. Dr. F.A. de Wolff. Het onderzoek dat hij daar gedaan heeft, staat beschreven in dit proefschrift.
Naast genoemde werkzaamheden is hij sedert 1999 leraar karate aan het Universitair Sport Centrum Amsterdam. In het kader van zijn opleiding tot Toxicoloog heeft hij tijdens zijn opleidingsperiode de verplichte modules van de postdoctorale opleiding tot Toxicoloog gevolgd en met succes afgerond. Na voltooiing van deze dissertatie hoopt hij spoedig te kunnen worden geregistreerd als erkend Toxicoloog.